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Effects of echinomycin on endothelin-2 expression and ovulation in immature rats primed with gonadotropins

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Abbreviations: dsODN, oligodeoxynucleotide; ET-2, endothelin-2; HIF-1, hypoxia-inducible factor-1α; HRE, hypoxia-response element; OHSS, ovarian hyperstimulation syndrome; PCOS, polycystic ovarian syndrome

Abstract

Echinomycin is a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity, which plays a crucial role in ovarian ovulation in mammalians. The present study was designed to test the hypothesis that hypoxia-inducible factor (HIF)-1α-mediated endothelin (ET)-2 expressions contributed to ovarian ovulation in response to human chorionic gonadotropin (hCG) during gonadotropin-induced superuvulation. By real-time RT-PCR analysis, ET-2 mRNA level was found to significantly decrease in the ovaries after echinomycin treatment, while HIF-1α mRNA and protein expression was not obviously changed. Further analysis also showed that these changes of ET-2 mRNA were consistent with HIF-1 activity in the ovaires, which is similar with HIF-1α and ET-2 expression in the granulosa cells with gonadotropin and echinomycin treatments. The results of HIF-1α and ET-2 expression in the granulosa cells transfected with cis-element oligodeoxynucleotide (dsODN) under gonadotropin treatment further indicated HIF-1α directly mediated the transcriptional activation of ET-2 during gonadotropin-induced superuvulation. Taken together, these results demonstrated that HIF-1α-mediated ET-2 transcriptional activation is one of the important mechanisms regulating gonadotropin-induced mammalian ovulatory precess *in vivo*.

Keywords: echinomycin; endothelin-2; gene expression regulation; gonadotropins; granulosa cells; hypoxia-inducible factor 1, α subunit; ovary; ovulation

Introduction

In female rats, two pituitary gonadotropin hormones, FSH and LH, control reproductive cyclical events, including ovarian follicles mature, rupture, and release fertilizable oocytes during a 4-day reproductive cycle at puberty initial stage. During each cycle, FSH stimulates a pool of preantral follicles to develop into mature follicles, while the ensuing surge of LH induces ovulation, through LH-activated downstream signaling pathways in the preovulatory follicles and eventually triggering the release of oocytes from these follicles (Richards, 1994; Richards *et al*., 2002). At present, the regulatory mechanisms controlling the growth and final differentiation of a mammalian follicle has advanced exponentially, but our understanding of even the most fundamental pathways is still not complete during these dynamic processes. Until recently, Endothelin-2 (ET-2) was recently proposed as a granulosa cell-derived contractile signal that facilitates ovulation (Ko *et al*., 2006; Al-Alem *et al*., 2007; Na *et al*., 2008; Kim *et al*., 2009).

ET-2 is a small, 21 amino acid peptide that is produced by granulosa cells at the time of ovulation (Ko *et al*., 2006; Al-Alem *et al*., 2007; Kim *et al*., 2009). Expression of mRNA for *ET-2*, but not *ET-1* or *ET-3*, is dramatically increased in the periovulatory follicle for only a very brief period of time (1-2 h) around ovulation and blockade of endothelin receptor binding

delay/inhibit the process of follicular rupture (Ko *et al*., 2006; Al-Alem *et al*., 2007; Na *et al*., 2008; Kim *et al*., 2009). This rather unique spatial and temporal specific pattern of gene expression suggests the involvement of direct signaling pathways to accurately coordinate controlled production of this peptide (Ko *et al*., 2006; Al-Alem *et al*., 2007; Na *et al*., 2008; Kim *et al*., 2009). Therefore, understanding of the regulatory mechanism controlling production of endothelin-2 (ET-2) becomes more and more important for further knowing the precise regulatory mechanism of mammalian ovulation.

Given intrafollicular changes in oxygen concentration during follicular growth and development, intrafollicular microenvironment is considered as hypoxia, which stimulates hypoxia inducible factor-1a expression in granulosa cells (Fischer *et al*., 1992; Basini *et al*., 2004; Nishimura and Okuda, 2010). Indeed, oxygen partial pressure is reported to decrease with increasing follicular size in women (Fischer *et al*., 1992) and swine (Basini *et al*., 2004). Furthermore, hCG regulates expression of hypoxia inducible factor (HIF)-1α in mammalian granulosa-lutein cells and its responsive genes, which participates in different physiological processes in the ovary (Herr *et al*., 2004; Kazi *et al*., 2005; Kazi and Koos, 2007; Wang *et al*., 2010, 2011, 2012; Zhang *et al*., 2010, 2011a, b; Zhu *et al*., 2011, 2012). Overall, several lines of evidence are consistent with the induction of hypoxic stress within the periovulatory follicle and gonadotropin prior to ovulation (Nishimura and Okuda, 2010; Zhang *et al*., 2011a, 2011b). HIF-1, a helix-loop-helix transcriptional factor, which consists of HIF-1α and HIF-1β, has been cloned and characterized as a transcriptional activator of many oxygen-sensitive genes, such as erythropoietin, heme oxygenases, transferrin, and several glycolytic enzymes (Wang and Semenza, 1993a, 1993b, 1995; Wang *et al*., 1995, 2010, 2011; Wenger *et al*., 1996; Zhang *et al*., 2011a). It has been indicated that HIF-1α is an inducible protein by a decrease in tissue or cellular O_2. HIF-1β is not inducible, but it can be bound to HIF-1α to form a dimer to activate the transcription of many genes containing cis hypoxia-response element (HRE) in their promoter or enhancer regions and to regulate their transcriptional expression (Kazi *et al*., 2005; Kazi and Koos, 2007; Molitoris *et al*., 2009; Zhang *et al*., 2011a), which can be blocked by HIF-1α small molecular inhibitor echinomycin.

Hence, the present study was designed to test the hypothesis that HIF-1α-mediated ET-2 expression contributed to ovarian ovulation in response to hCG during gonadotropin-induced superuvulation. Using gonadotropin-induced immure rat ovarian follicular development model, we determined the effects of enchinomycin on ET-2 gene expression and ovulation, accompanying with animal experiment and granulosa cell culture experiment with enchinomycin and oligodeoxynucleotide treatment. These experiments will provide the direct evidences that ET-2 is transcriptionally activated by HIF-1-mediated mechanism in granulosa cells under hCG and the potential role of HIF-1α-ET-2 signaling in ovarian ovulatory process *in vivo*.

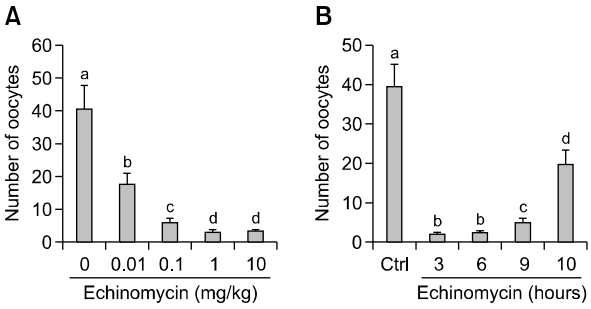

Figure 1. Effects of enchinomycin on ovarian ovulation in rats. After PMSG priming for 48 h, immature rats were treated with hCG to induce ovulation. (A) In a set of enchinomycin dose dependent experiments, rats were injected with either vehicle (dimethylsulfoxide) or various dose of enchinomycin (0.01, 0.1, 1 or 10 mg/kg body weight) at 6 h after hCG. (B) In another treatment time dependent experiments, rats were given either vehicle or a fixed dose of enchinomycin (1 mg/kg body weight) at 3, 6, 9 or 10 h after hCG. The ovulated eggs were counted at 20 h after hCG, and their numbers were compared between the enchinomycin-treated or untreated groups. Each value represents the means $\pm$ SE. One-way analysis of variance (ANOVA) was used to analyze the data. Different superscripts denote significant values ($P < 0.05$) by Tukey's multiple-range test.

Results

Effects of echinomycin on ovarian ovulation in rats induced by gonadotropin

Our result showed that echinomycin can block gonadotropin-induced superovulation in a dose-dependent manner (Figure 1A), while this blockage is also in a time-dependent manner (Figure 1B). The obvious increase of released oocytes in the rats with echinpmycin treatment at 10 h after hCG also indicated echinpmycin with time sensitivity and complex regulation.

Effects of echinomycin on ET-2 mRNA, HIF-1α mRNA and HIF-1α protein expression in rats induced by gonadotropin

According to the results of ovarian ovulation, immature rats given either vehicle or 1 mg/kg body weigh echinomycin at 6 after hCG treatment were used for further investigated. By real-time PCR

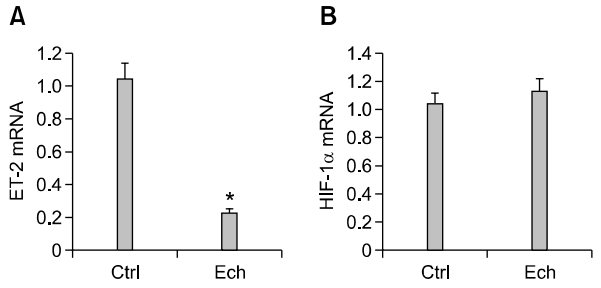

Figure 2. Effects of enchinomycin on ET-2 mRNA and HIF-1α mRNA levels in rat ovaries. After PMSG priming for 48 h, immature rats were treated with hCG to induce ovulation and ovaries were collected from each group at 11 h after hCG. (A) The relative mRNA levels of ET-2 by real-time RT-PCR analysis. (B) The relative mRNA levels of HIF-1α by real-time RT-PCR analysis. Each value represents the mean ± SE. Student's *t* test was used to evaluate statistical significance of differences between two groups. The asterisk denotes significant values ($P < 0.05$). Ech, enchinomycin.

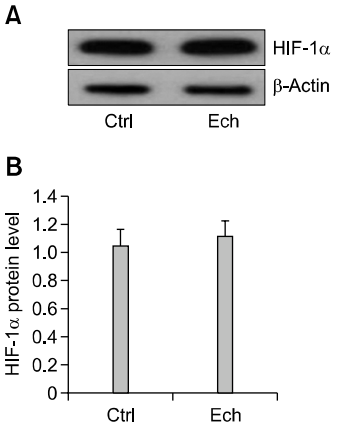

Figure 3. Effects of enchinomycin on HIF-1α protein expression in rat ovaries. After PMSG priming for 48 h, immature rats were treated with hCG to induce ovulation and ovaries were collected from each group at 11 h after hCG. (A) Representative ECL gel documents of Western blot analyses depicting the protein level of HIF-1α. (B) Summarized intensities of HIF-1α blot normalized to control. Different superscripts and asterisk denote significant values ($P < 0.05$) by Student's *t* test. Ech, enchinomycin.

analysis, ET-2 mRNA level was found to significantly decrease in the ovaries treated with echinomycin (Figure 2A), while HIF-1α mRNA (Figure 2B) and protein expresion (Figure 3) was no obviously changes, implying ET-2 may play a crucial role in ovarian ovulation in mammalians which consistent with previous results (Ko *et al*., 2006; Al-Alem *et al*., 2007; Na *et al*., 2008; Kim *et al*., 2009).

Effects of echinomycin on HIF-1 activity in rats induced by gonadotropin

In the present study, HIF-1 activity in the ovaries

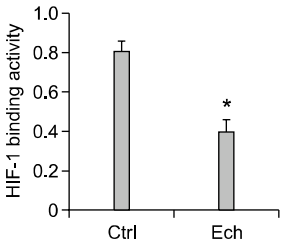

Figure 4. Effects of enchinomycin on HIF-1 binding activity in rat ovaries. After PMSG priming for 48 h, immature rats were treated with hCG to induce ovulation and ovaries were collected from each group at 11 h after hCG. HIF-1 binding assay in ovarian nuclear extracts from each experiment group, The asterisk denotes significant values ($P < 0.05$) by Student's t test. Ech, enchinomycin.

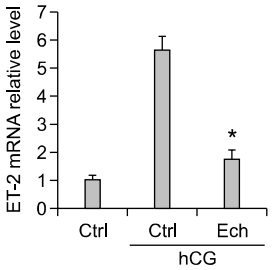

Figure 5. Effects of enchinomycin on ET-2 mRNA levels in ovarian granulosa cells. Animals were sacrificed after 48 h PMSG-treatment for collecting ovaries and granulosa cells were isolated for *in vitro* culture experiments with hCG and Ech. The relative mRNA levels of ET-2 by real-time RT-PCR analysis. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. The asterisk denotes significant values ($P < 0.05$) by Tukey's multiple-range test. $n = 6$ batches of cells. Ech, enchinomycin.

was also detected. The result showed echinomycin, as a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity, can significantly inhibit HIF-1 activity in the echinomycin group (Figure 4).

Effects of echinomycin on ET-2 mRNA Levels in ovarian granulosa cells

ET-2 mainly expressed in ovarian granulosa cells in mammalian. In order to further confirm our finding, we also performed the experiments to observe whether echinomycin blocks the level of ET-2 mRNA induced by gonadotropin in ovarian granulosa cells. The result showed that echinomycin treatment significantly blocked hCG-induced ET-2 mRNA increase (Figure 5), comparing with vehicle group.

Effects of HIF-1α decoy on ET-2 mRNA, HIF-1α protein, HIF-1 activity in response to hCG in ovarian granulosa cells

In order to further confirm our hypothesis that HIF-1α

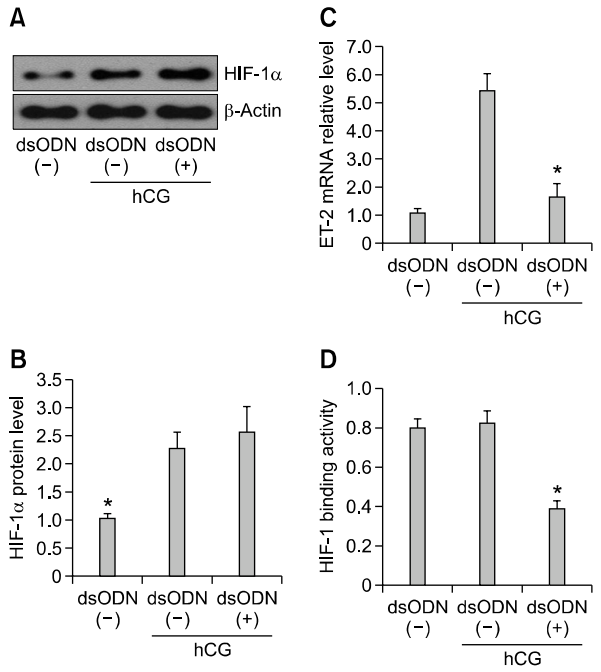

Figure 6. Effects of HIF-1α decoy ODNs transfection on ET-2 mRNA, HIF-1α expression and HIF-1 binding activity in response to hCG in granulosa cells. Animals were sacrificed after 48 h PMSG-treatment for collecting ovaries and granulosa cells were isolated for *in vitro* culture experiments with hCG and dsODN. (A) Representative ECL gel documents of Western blot analyses depicting the protein level of HIF-1α. (B) Summarized intensities of HIF-1α blot normalized to control. (C) The relative mRNA levels of ET-2 by real-time RT-PCR analysis. (D) HIF-1 binding assay in the nuclear extracts from different granulosa cell treatment groups. Each value represents the mean ± SE. One-way analysis of variance (ANOVA) was used to analyze the data. The asterisk denotes significant values ($P < 0.05$) by Tukey's multiple-range test. $n = 6$ batches of cells.

directly mediated ET-2 gene transcription, we used these cells transfected with HIF-1α decoy oligodeoxynucleotides to determine the effect of dsODNs on ET-2 gene expression in response to hCG. The results had shown that hCG induced ET-2 gene expression (Figure 6C), while this hCG-induced increase in ET-2 mRNA levels was depressed in the cells transfected with specific dsODN [dsODN(+)] containing 5'-*CGTG*-3' (Figure 6C). However, this decoy of HIF-1 had no effect on HIF-1α protein expression in response to hCG (Figures 6A and 6B), comparing with their inhibitory effects on HIF-1 binding activity (Figure 6D). As a control, scrambled dsODN [dsODN(-)] without 5'-*CGTG*-3' was used to transfect granulosa cells, but it had no effect on the increase in ET-2 mRNA levels induced by hCG (Figure 6C). Together, it was clearly demonstrated that the decoy of HIF-1α by its specific binding dsODN blocked the induction of ET-2 mRNA.

Discussion

The results of our present study clearly demonstrated that HIF-1α small molecular inhibitor enchinomycin can block gonadotropin-induced superovulation in a dose- and time-dependent manner. Furthermore, ET-2 mRNA level was decreased in enchinomycin-treated group by real-time RT-PCR analysis. These results together indicated ET-2 may be involved in mammal ovarian ovulatory process though HIF-1α signaling pathway.

In the present animal experiment, HIF-1α mRNA and protein levels were detected and no obvious expression changes of HIF-1α between enchinomycin treated and no-treated groups. But HIF-1 activity significantly decreased after enchinomycin treatment, which is consistent with ET-2 mRNA level, implying ET-2 transcriptional activation may be through HIF-1α signaling pathway during gonadotropin-induced superovulation *in vivo* (Ko *et al*., 2006; Kim *et al*., 2009; Nishimura and Okuda, 2010). HIF-1α is a member of the basic-Helix-Loop-Helix-PAS family of transcription factors, expressed in many tissues and serves as critical regulators of these tisues' response to changes in oxygen levels and gonadotropin stimulations (Wang *et al*., 2010, 2011; Zhang *et al*., 2010, 2011a, 2011b). Echinomycin is a sequence-specific DNA-binding agent, which hinders the binding of a HIF-1 heterdimer to HRE, inhibiting the expression of the targets. In rats undergoing gonadotropin-induced ovulation, blockage of HIF activity by this small molecular inhibitor enchinomycin profoundly impaired the rupture of the preovulatory follicles, thereby drastically reducing the number of released eggs during ovulation. In order to confirm our finding in the present animal experiment, we also isolated ovarian granulosa cells to detect the effect enchinomycin on ET-2 gene expression in response to hCG. As expected, hCG-induced increase of ET-2 gene expression was blocked by this HIF-1α inhibitor, further demonstrating HIF-1α may participate in the regulation ET-2 gene expression and ovarian ovulation.

In addition to these pharmacological interventions, we also used a molecular decoy approach to determine the direct role of HIF-1α in the transcriptional regulation of ET-2 gene. The present results demonstrated that no obvious changes of HIF-1α protein among each groups, but HIF-1 activity in isolated ovarian granulosa cells transfected with a dsODN containing an HIF-1 binding site, 5'-*CGTG*-3' decreased significantly and ET mRNA level increased to a much lesser extent under hCG treatment in transfected cells than those in control cells, because this anti-gene therapy strategy can decoy and thereby block the binding of transcription factors to

their binding sites in promoter or enhancer regions by introducing a synthesized dsODN containing a binding *cis*-element (Morishita *et al*., 1998). These results provide the direct evidence that using cis-element oligodeoxynucleotide transfection to specifically decoy HIF-1α and block HIF-1 binding activity, increased mRNA level of ET-2 in response to hCG was attenuated, which further supports our hypothesis that HIF-1 mediates the transcriptional activation of the ET-2 gene during gonadotropin-induced superovulation.

In summary, the present study is the first time to provide the direct evidences indicating that ET-2 expression is transcriptionally regulated *via* an HIF-1α pathway under hCG treatment. This hCG-induced transcriptional activation may be one of the important mechanisms mediating increased expression of ET-2 during ovulation in the mammalian ovary. Furthermore, HIF-1α antagonism affords an opportunity for the development of novel treatments for fertility control and for some types of ovarian dysfunction, particularly those conditions characterized by pathological angiogenesis and excessive vascular permeability, such as polycystic ovarian syndrome (PCOS), ovarian hyperstimulation syndrome (OHSS) and ovarian neoplasia (Quintero *et al*., 2004; Galanis *et al*., 2008; Alper *et al*., 2009; Miyazawa *et al*., 2009, 2010; Seeber *et al*., 2011).

Methods

Animals

Immature female Sprague-Dawley rats (21-day old) were purchased from Wushi Experimental Animal Supply Co. Ltd. (Fuzhou, P. R. China). The animals were maintained under a 14 h light, 10 h dark schedule giving continuous supply of chow and water. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee, Fujian Normal University.

Experiment design

To induce superovulation, rats were treated ip with 10 IU PMSG (pregnant mare serum gonadotropin, Sigma-Aldrich, St. Louis, MO) and 5 IU hCG (human chorionic gonadotropin, Sigma-Aldrich) after 48 h to induce the ovulatory process. In this protocol, ovulation typically occurs at approximately 12 h after hCG treatment.

Echinomycin dose and time dependent experiment: During gonadotropin-primed superovulation, rats were given either vehicle or a different dose echinomycin (0.01, 0.1, 1 and 10 mg/kg body weigh, BioViotic, Dransfelg, Germany) at 6 h after hCG, and a fixed dose echinomycin (1 mg/kg body weigh) at a different time (3, 6, 9 and 10 h) after hCG. The released eggs were counted at 20 h after hCG treatment.

Effects of echinomycin on HIF-1α expression experiment: Immature rats were given either vehicle or 1 mg/kg body weigh echinomycin at 6 after hCG treatment. Ovaries were collected from each group at 11 h after hCG and freezed in liquid nitrogen for mRNA and protein detection.

Cell culture experiment: Animals were sacrificed after 48 h PMSG-treatment for collecting ovaries and granulosa cells *in vitro* culture experiments with echinomycin and HIF-1α decoy treatment.

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted using TRIzol solution (Life Technologies, Rockville, MD) and then reverse-transcribed (cDNA Synthesis Kit; Bio-Rad). The reverse-transcribed products were amplified using a TaqMan Gene Expression Assays kit (Applied Biosystems). A kit for detecting the levels of 18S ribosomal RNA was used as an endogenous control. The relative gene expressions were calculated in accordance with the ΔΔCt method. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta Ct}$.

Western blot analysis of HIF-1α protein levels

Protein concentrations were determined by a Bio-Rad assay with bovine serum albumin standards. 20 μg protein samples were subjected to 8% SDS-PAGE gel electrophoresis and then electrophoretically transferred onto a PVDF membrane. The membrane was washed and probed with 1:500 specific anti-HIF-1α antibody (1:500, Abcom, Cambridge, MA) overnight at 4°C. After washing, the membranes were incubated with HRP-labeled goat anti-mouse IgG (1:5000, Novus Biologicals, Littleton, CO) for 60 min at room temperature, and then developed the film to obtain the images. To detect immunoblotting signal, 2 ml of enhanced chemiluminescence detection solution was added, and the membrane was wrapped and exposed to Kodak OMAT film.

Isolation and culture of granulosa cells

Granulosa cells were isolated from gonadotropin-primed rats ovaries by follicular puncture. Briefly, granulosa cells were collected in a culture medium, DMEM/F-12 medium (GIBCO, No. 11330) containing 5% Fetal Bovine serum and 20 μg/ml gentamicin. Cells were washed three times in the culture medium then plated on serum-coated six-well plates at a density of approximately 1×10^6 cells per well and cultured in a cell culture incubator with a humidified atmosphere of 5% CO_2 in air at 37.5°C. To examine the effects of echinomycin on HIF-1α expression *in vitro*, granulosa cells were collected from ovaries of gonadotropin-primed rats sacrificed at 48 h after PMSG and cultured with/without 5 IU hCG and other reagents for 6 h. All cultures were performed in duplicate and replicated 3 times on different days.

Decoy of HIF-1α

It has been demonstrated that HIF-1α activates gene ex-

pression by binding to a promoter or an enhancer site, HRE. This *cis* element contains a -*CGTG*-consensus sequence. A standard fluorescein-attached HRE containing oligodeoxynucleotides (ODN) was synthesized with sequences of 5'-GCC CTA CGT GCT GTC TCA-3' (sense) and 5'-TGA GAC AGC ACG TAG GGC-3' (antisense) and scrambled ODN with sequences of 5'-GCC CTT ACA ACT GTC TCA-3' (sense) and 5'-GAG ACA GTT GTA AGG GC-3' (antisense). To make double-strand ODN (dsODN), both sense and antisense ODNs (100 μM in TE, pH 8.0) were heated at 95°C for 5 min and then cooled slowly down to room temperature. These dsODNs were wrapped by using cationic liposomes (Avanti Polar Lipids, Alabaster, AL) and transfected into GCs as described by the manufacturer. dsDNA (10 μg) was first mixed with 50 μl of liposome and then added to 5 ml of serum-free incubation medium.

Preparation of cell nuclear extracts and analyses of HIF banding activity

Cell nuclear protein was prepared using a nuclear extract kit (Panomics, Fremont, CA) and HIF-1 binding activities in the nuclear extracts were detected using an ELISA-based HIF binding kit (Panomics, Fremont, CA). The ELISA-based HIF binding assay kit provides a fast, sensitive, and specific measurement for the HIF-1 binding activities.

Statistics

Data are presented as means ± SE. The significance of differences in mean values within and between multiple groups was evaluated using a one-way ANOVA, followed by a Tukey's multiple range test. Student's *t* test was used to evaluate statistical significance of differences between two groups. $P < 0.05$ was considered statistically significant.

Acknowledgements

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