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Induction of Pyruvate Dehydrogenase Kinase 1 by Hypoxia Alters Cellular Metabolism and Inhibits Apoptosis in Endometriotic Stromal Cells

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Abstract

Endometriosis is a common gynecological disease, which is defined as the growth of endometrial tissues outside the uterine cavity. It often causes dysmenorrhea, dyspareunia, chronic pelvic pain, and infertility in reproductive-age women. However, the pathogenesis of endometriosis remains largely unclear. Since our previous study revealed that ectopic endometriotic stromal cells experience greater hypoxic stress than their eutopic counterparts, we aim to investigate whether the metabolic properties are changed in the ectopic endometriotic stromal cell when compared to its eutopic counterpart. Here, we found the expression of pyruvate dehydrogenase kinase 1 (PDK1), a critical enzyme in regulating glucose metabolism, was increased in ectopic stromal cells. Molecular characterization reveals that overexpression of PDK1 is induced by hypoxia through transcriptional regulation. Upregulation of PDK1 in ectopic endometriotic stromal cells was accompanied by increases in lactate production and oxygen consumption rate when compared to eutopic endometrial stromal cells. Furthermore, our data showed that inhibition of PDK1 activity by treatment with dichloroacetate inhibits the lactate production and oxygen consumption rate of ectopic stromal cells. In addition, hypoxia-induced PDK1 expression prevented cells from H_2O_2- and low nutrient-induced cell death. These data indicate that ectopic endometriotic cells may adapt to hypoxic microenvironment via upregulating PDK1 and reprogramming metabolism, which provides a survival advantage in the hostile peritoneal microenvironment.

Keywords: PDK1; apoptosis; cellular metabolism; glycolysis; oxygen consumption.

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Nucleotide (RefSeq)
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Abstract

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Keywords

PDK1, oxygen consumption, glycolysis, cellular metabolism, apoptosis

Introduction

Endometriosis is a common gynecological disease in reproductive-age women. It is characterized by the growth of endometrial tissues in a location outside the uterine cavity. It occurs in 10% of women,[1,2] but the exact prevalence is difficult to estimate because of the wide variety of symptoms. The classic symptoms of endometriosis are dysmenorrhea, dyspareunia, chronic pelvic pain, and infertility. Although endometriosis was discovered in 1860,[3] the pathogenesis of this disease has not been entirely established. The most accepted hypothesis is based on the retrograde menstruation theory, which posits that the peeled endometrial fragments proceed retrograde into the peritoneal cavity through the fallopian tubes during uterine contraction.[4] However, there is evidence that retrograde menstruation occurs in 90% of women during the perimenstrual period of the cycle.[5] The discrepancy between the ~10% prevalence of endometriosis and the 90% prevalence of retrograde menstruation indicates that more complex factors are involved in the development of endometriosis.

According to the retrograde menstruation theory, retrograde endometrial fragments suffer from hypoxic stress more than their eutopic counterparts before the growth of vascular vessels.[6,7] How ectopic endometriotic cells change metabolism to overcome and adapt to unfavorable microenvironments is

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poorly understood. Hypoxia inducible factor 1 α (HIF-1α) is a major transcription factor generated in response to hypoxia and drives hypoxic gene expression and cellular metabolic reprogramming. It has been reported that cancer cells, due to repeated hypoxia-normoxia exposure, prefer to use glycolysis even under normal oxygen conditions, in what is known as the Warburg effect.[8] Recently, Young et al reported the observation of a Warburg-like effect in endometriotic lesions and the adjacent peritoneum, likely due to stimulation by transforming growth factor-β.[9] However, whether hypoxia-induced metabolic reprogramming is adopted by endometriotic cells remains unproven.

We have previously reported that the expression of HIF-1α is elevated in ectopic stromal cells, and HIF-1α transcriptionally activates leptin expression to enhance ectopic stromal cell proliferation.[6] Also, HIF-1α inhibits dual-specificity phosphatase-2 and induces microRNA-20a expression, subsequently stimulating prostaglandin E_2 production in ectopic stromal cell and further promoting angiogenesis.[10,11] These findings indicate that endometriotic cells grow in the hypoxic microenvironment and that several critical genes essential for cell survival are upregulated. Thus, it would be interesting to determine whether the genes involved in energy metabolism are altered in ectopic endometriotic cells.

Pyruvate dehydrogenase kinase (PDK) is a mitochondrial enzyme that regulates the entry of pyruvate into the tricarboxylic acid (TCA) cycle. Four isoforms of PDK, encoded by different genes, have been identified in humans. They inhibit the activity of pyruvate dehydrogenase (PDH) complex by phosphorylating the serine residue of PDH. Pyruvate dehydrogenase controls the rate-limiting step of pyruvate to the acetyl-CoA conversion; thus, the phosphorylation of PDH by PDK promotes glucose anaerobic metabolism. The expression levels of PDKs vary in a tissue-specific manner, suggesting that they may have different functions.[12,13] Pyruvate dehydrogenase kinase 1 is expressed mainly in the heart and pancreatic islets. Pyruvate dehydrogenase kinase 2 is ubiquitously expressed with a particularly high expression in the heart, liver, and kidney, whereas PDK3 has a relatively limited tissue distribution (testis, kidney, and brain). Pyruvate dehydrogenase kinase 4 is predominantly expressed at high levels in the heart, skeletal muscle, liver, and kidney. Though PDK-mediated hypoxic adaptation through enhancing glycolytic metabolism has been reported in cancer cells,[14-16] the regulation and function of PDK and its impact on the progression of endometriosis remain unclear.

Materials and Methods

Clinical Specimens

Tissues were obtained from the Department of Obstetrics and Gynecology of National Cheng Kung University Hospital. Endometriosis was confirmed by a pathologist and was classified according to the revised American Society for Reproductive Medicine scoring system. Patients had not received hormonal treatment within 6 months prior to gynecological surgery. Endometrial biopsies obtained from the patients diagnosed with other benign gynecological disease were recruited as normal controls. Fifty-two women with endometriosis and 6 nonendometriosis women (defined as normal in this study) were recruited (Table 1), and stromal cells were purified from biopsies obtained from these patients. Because of the limitations of culture passaging and growth of primary stromal cells, we used numerous primary stromal cells from different patients with endometriosis to conduct each experiment. This study was approved by the Clinical Research Ethics Committee at the National Cheng Kung University Medical Center, and informed consent was obtained from each patient.

Cell Culture

The procedure for isolation of endometrial stromal cells of patients with or without endometriosis was as previously described.[17] Cells were grown in Dulbecco modified Eagle medium/nutrient mixture F-12 supplemented with 10% fetal bovine serum, 1X GlutaMAX (Life Technologies, Inc, Carlsbad, California) and 1X Antibiotic-Antimycotic Solution (Caisson Laboratories, Inc, North Logan, Utah) in a humidified atmosphere with 5% CO_2 at 37°C. In the hypoxia treatment, eutopic stromal cells were cultured in a hypoxia chamber (1% O_2, 94% N_2) for various time periods. The cell lysates were collected at different time points for further analysis.

RNA Isolation and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated using TRIsure reagent (Bioline, London, United Kingdom) according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed to become complementary DNA and then quantified using real-time PCR to quantify the gene expression levels. Each reaction contained 2 μL of real-time products, 0.3 μmol/L specific primers, and 10 μL of FastStart Universal SYBR Green Master (Roche, Basel, Switzerland). The 18S ribosomal RNA (rRNA) was used for normalization. The sequences of the primers used were: PDK1: forward 5'-CGGATCAGAAACCGACACA-3'; reverse 5'-ACTGAACATTCTGGCTGGTGA-3'; PDK2: forward 5'-AAGGACACCTACGGCGATG-3'; reverse 5'-ATGGAGA TGCGGCTGAGG-3'; PDK3: forward 5'-TTAATAAGTCCG CATGGCGC-3'; reverse 5'-TGAAGCATCCCTGGGTTCAC-3'; PDK4: forward 5'-CAATGGCACAAGGAATCATAG-3'; reverse 5'-GGTTCATCAGCATCCGAGTAG-3'; 18S rRNA: forward 5'- GTGTGCCTACCCTACG-3'; reverse 5'-TGACCCGCACTTACTG-3'.

Western Blotting

An equal amount of protein (30 μg) was loaded to sodium dodecyl sulfate polyacrylamide gel and then transferred to a polyvinyl difluoride membrane. After transfer, nonspecific binding on the membrane was blocked with 5% skim milk for

Table 1. The Diagnosis, Menstrual Cycle, and Age of the Patients Recruited in This Study.

Sample	Diagnosis	Phase	Age
Endometriosis-free			
1	Normal (myoma)	L	39
2	Normal (left mucinous cystadenoma)	F	28
3	Normal (chronic pelvic pain)	L	34
4	Normal (leiomyoma)	F	45
5	Normal (fallopian tube obstruction)	F	33
6	Normal (leiomyoma)	F	45
Endometriosis			
7	Severe endometriosis (IV)	L	26
8	Severe endometriosis (IV)	F	29
9	Severe endometriosis (IV)	F	39
10	Severe endometriosis (IV)	F	33
11	Severe endometriosis (IV)	L	37
12	Minimal endometriosis (I)	L	34
13	Moderate endometriosis (III)	F	38
14	Moderate endometriosis (III)	L	33
15	Severe endometriosis (IV)	L	37
16	Severe endometriosis (IV)	F	31
17	Moderate endometriosis (III)	F	36
18	Moderate endometriosis (III)	L	25
19	Minimal endometriosis (I)	L	35
20	Severe endometriosis (IV)	F	37
21	Moderate endometriosis (III)	F	26
22	Mild endometriosis (II)	L	39
23	Moderate endometriosis (III)	F	33
24	Moderate endometriosis (III)	F	43
25	Severe endometriosis (IV)	L	26
26	Severe endometriosis (IV)	F	44
27	Severe endometriosis (IV)	F	33
28	Moderate endometriosis (III)	L	25
29	Moderate endometriosis (III)	L	44
30	Moderate endometriosis (III)	L	29
31	Moderate endometriosis (III)	F	29
32	Severe endometriosis (IV)	L	24
33	Moderate endometriosis(III)	F	35
34	Severe endometriosis (IV)	L	39
35	Moderate endometriosis (III)	L	31
36	Moderate endometriosis (III)	L	39
37	Moderate endometriosis (III)	F	26
38	Severe endometriosis (IV)	F	34
39	Moderate endometriosis (III)	F	22
40	Moderate endometriosis (III)	F	38
41	Moderate endometriosis (III)	L	33
42	Moderate endometriosis (III)	L	27
43	Severe endometriosis (IV)	L	41
44	Moderate endometriosis (III)	F	35
45	Moderate endometriosis (III)	L	29
46	Moderate endometriosis (III)	L	26
47	Mild endometriosis (II)	F	38
48	Severe endometriosis (IV)	F	32
49	Minimal endometriosis (I)	L	36
50	Severe endometriosis (IV)	F	36
51	Moderate endometriosis (III)	L	23
52	Moderate endometriosis (III)	F	30
53	Moderate endometriosis (III)	F	31
54	Severe endometriosis (IV)	F	41
55	Severe endometriosis (IV)	L	37
56	Severe endometriosis (IV)	L	37
57	Moderate endometriosis (III)	L	23
58	Moderate endometriosis (III)	L	23

Abbreviations: F, follicular phase; L, luteal phase.

1 hour. Then, the membrane was probed with different primary antibodies at 4°C overnight. The primary antibodies included PDK1 1:5000 (Enzo Life Sciences, Farmingdale, New York), PDK3 1:5000 (Novus Biologicals, Littleton, Colorado), phosphorylated Serine 293 of PDH E1-alpha subunit 1:500 (Abcam, Cambridge, United Kingdom), PDH 1:2000 (Cell Signaling Technology, Inc, Danvers, Massachusetts), and β-actin 1:10000 (Sigma, Saint Louis, Missouri). The membrane was then washed with phosphate buffered saline with Tween 20 (PBST) before probing with horseradish peroxidase-conjugated appropriate secondary antibodies for 1 hour at room temperature. The membrane was then washed with PBST, and about 1 mL of enhanced chemiluminescence reagent was added. The signal was detected by X-ray film and quantified by AlphaView software (ProteinSimple, San Jose, California).

Promoter Activity Assay

Eutopic endometrial stromal cells were plated into 24-well plates with 1×10^5/well before transfection. Plasmids containing *pdk1* promoters and internal control construct pCMV-β-gal were cotransfected into eutopic stromal cells by lipofectamine 2000 (Thermo Fisher Scientific Inc, Waltham, Massachusetts) the next day. After transfection for 6 hours, the transfected cells were cultured under either normoxia or hypoxia for another 24 hours. After incubation, cells were washed by 1× phosphate buffered saline and lysed with 100 μL of 1× Passive Lysis Buffer (Promega, Madison, Wisconsin). The luciferase activity was evaluated with the Dual Luciferase Reporter Assay System (Promega, Madison, Wisconsin) according to the manufacturer's protocol.

Hypoxia Inducible factor 1α Knockdown

Eutopic stromal cells were transfected with either control small-interfering RNA (siRNA) or HIF-1α siRNA using Lipofetamine 2000 as described previously.[6] After 24 hours incubation, the cells were cultured under either normoxia or hypoxia (1% O_2) for another 24 hours. Cells were then harvested for further evaluation.

High-Resolution Respirometry

The cells were trypsinized and suspended in fresh culture medium. Approximately 1×10^6 cells were suspended in 2 mL of culture medium and then added into a chamber to measure cellular oxygen consumption with the OROBOROS Oxygraph-2k (OROBOROS INSTRUMENTS Corp, Innsbruck, Austria). The signal was recorded when the cells were added into the chamber. First, the cellular oxygen consumption rate was measured under a regular condition called ROUTINE state. Two microliters of oligomycin (final concentration: 0.4 μg/mL) were added into the chamber with a syringe to inhibit the adenosine triphosphate (ATP) synthase. The oligomycin-induced oxygen consumption rate was called the "LEAK" state. After that, 2 μL of rotenone (final concentration: 0.8 μg/mL) was added to shut down the electron transport chain in the mitochondria. The

oxygen consumption rate measured after adding the rotenone was called the residual oxygen consumption (ROX) state. All of the signals were detected for at least 10 minutes. The average of oxygen consumption rate in 10 minutes was expressed as per million cells, per second. The ATP-linked respiration was calculated using the ROUTINE state minus the LEAK state, and the uncoupled respiration was calculated with the LEAK state minus the ROX state.

Glucose Uptake

Cells were seeded into 6-well plates and cultured for 48 hours. Fluorescent D-glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG) (BioVision Incorporated, Milpitas, California) in glucose-free medium was then added, and the sample was incubated for 6 hours at 37°C. The 2-NBDG uptake was stopped by washing twice with PBS. The cells were resuspended and subjected to flow cytometry analysis. The population of the cell with positive green fluorescent protein signal was presented as the 2-NBDG-uptake ability.

Lactate Production

Stromal cells were cultured for 48 hours and then trypsinized and counted. The lactate concentration in the collected medium was measured with a lactate measurement device, Lactate ProTM 2 (Arkray, Alere AB, Lidingö, Sweden). The lactate production was presented as the lactate amount normalized to the cell number.

ATP Determination

Approximately 2×10^5 stromal cells were plated in a 6-cm dish and cultured for 48 hours. After 48 hours, the cells were trypsinized, and the cell pellets were stored at −80°C until ATP detection. Before the ATP determination, the cell pellets were dissolved with 1× lysis buffer (10 mmol/L Tris, 0.1 mol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, and 0.06% Triton), and the total protein amount was measured using the Lowry assay. The intracellular ATP was detected with a commercial kit (Molecular Probes, Inc, Eugene, Oregon) according to the manufacturer's protocol. The luminescence intensities were determined on the Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc, Winooski, Vermont). Following the luminescence intensity, based on a standard curve, the amount of ATP was calculated and was normalized to the individual protein amount.

Mitochondrial Membrane Potential

Cells were labeled by tetramethylrhodamine (Thermo Fisher Scientific Inc.), a cell permeable, positively charged, red–orange dye that readily accumulates in active mitochondria due to their relatively negative charge and then incubated in the dark for 20 minutes at 37°C. After a single washing with PBS, the cells were resuspended in flow buffer and analyzed for tetramethylrhodamine intensity using a flow cytometer. The mean intensity of signal indicated the relative inner mitochondrial potential.

Apoptosis Assay

The ectopic stromal cells were treated with 1 mmol/L dichloroacetate (DCA; Santa Cruz Biotechnology, Dallas, Texas) for 48 hours. The eutopic stromal cells were cultured under hypoxia with or without DCA treatment for 24 hours. The treated cells were then challenged with either 100 μmol/L H_2O_2 for another 6 hours or a low-nutrient condition (medium contains 1 mM glucose, 1% fetal bovine serum, and 1X GlutaMAX) for another 24 hours. After H_2O_2 or low nutrient challenge, the cells were collected and stained with annexin-V (BioLegend, San Diego, California) as apoptotic marker and analyzed with a flow cytometer.

Statistical Analysis

Data are shown as mean ± standard error of the mean. All statistical analyses were performed using GraphPad Prism version 4.02 (GraphPad Software, La Jolla, California). The unpaired Student t test was used to compare differences between eutopic and ectopic groups. Differences between DCA treatment groups were analyzed using the paired t test. A significant difference was considered at $P < .05$.

Results

Cellular Metabolism Is Altered in Ectopic Stromal Cells

To explore whether the metabolic features were different in eutopic and ectopic stromal cells, we measured the cellular oxygen consumption using high-resolution respirometry. The results showed that the oxygen consumption rate was higher in the ectopic stromal cells than in the eutopic stromal cells (Figure 1A). In addition, we found that the oxygen consumption rate for the eutopic stromal cells of patients with endometriosis or disease-free controls (normal) were similar (Supplementary Figure S1). Consequently, we only compared the metabolic differences between the eutopic and the ectopic stromal cells from patients with endometriosis. For further assessment, we added oligomycin and rotenone to detect the oxidation that is coupled and uncoupled to ATP synthesis, respectively. Both the ATP-coupled respiration and the uncoupled respiration were increased in the ectopic stromal cells (Figure 1B and C). In addition, the typical characteristics of the Warburg-effect such as glucose uptake and lactate production were evaluated in both the eutopic and the ectopic stromal cells. The results showed that the glucose uptake ability of the eutopic and ectopic stromal cells was low and that there was no difference (Figure 1D). However, the lactate production of the ectopic stromal cells was elevated (Figure 1E). Interestingly, the resting oxygen- and ATP-linked consumption rates were both increased in the ectopic endometriotic stromal cells (Figure 1A, B), but there was no difference in the total cellular ATP content between eutopic and ectopic stromal cells

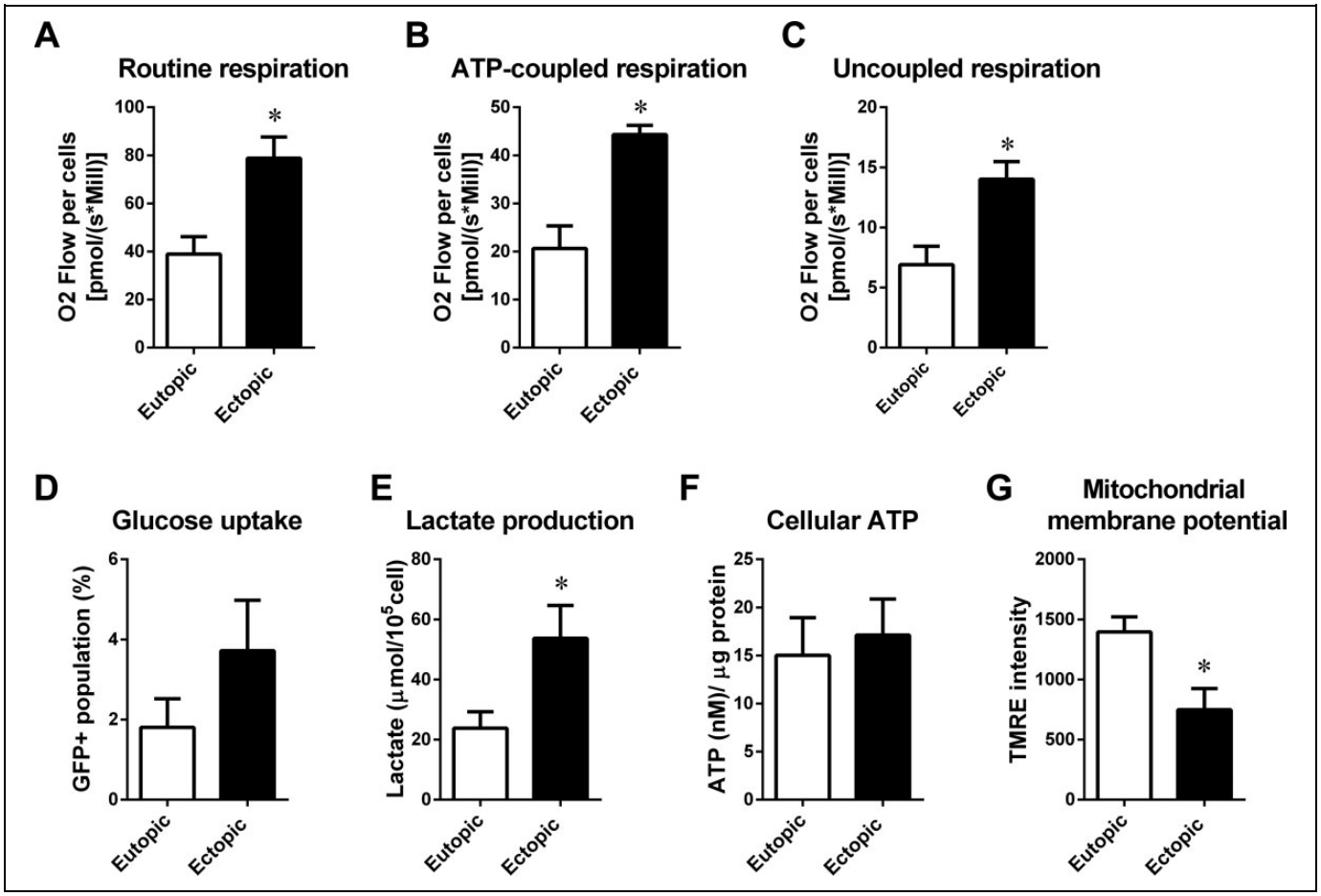

Figure 1. The metabolic features of the eutopic endometrial stromal cells and ectopic endometriotic stromal cells are different. A, Routine respiration (n = 6 biological repeats for eutopic stromal cells, n = 7 biological repeats for ectopic stromal cells) was detected using ORO-BOROS Oxygraph-2 k. The changed rate in the oxygen concentration was normalized to cell number; thus the unit was represented in pmol/(s*million cells). B, After adding the oligomycin, the oxygen consumption rate (OCR) was measured. The ATP-coupled respiration was calculated as routine OCR minus oligomycin-mediated OCR. The oxygen consumption rate was normalized to cell number. C, The oxygen consumption rate was measured after adding the rotenone. The uncoupled respiration was calculated as oligomycin-mediated OCR minus OCR in the presence of rotenone. The oxygen consumption rate was normalized to cell number. D, Cells (n = 6 biological repeats) were incubated with 2-NBDG (fluorescent glucose analog) for 6 hours, and the GFP signal was recorded with flow cytometry. The results were shown as the population of cells which uptake 2-NBDG. E, Lactate production in the culture medium was detected using lactate measurement device Lactate Pro 2 and was normalized to cell number (n = 7 biological repeats). F, ATP generation was measured with an ATP determination kit. The cellular ATP production was normalized to the amounts of individual protein (n = 7 biological repeats). G, Cells were incubated with tetramethylrhodamine ethyl ester (TMRE) for 20 minutes and then subjected to fluorescent intensity quantification with a flow cytometer (n = 7 biological repeats). Asterisks indicated significant between-groups differences at $P < .05$. 2-NBDG indicates 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose; ATP, adenosine triphosphate.

(Figure 1F). To investigate whether the similar ATP content in the eutopic and ectopic stromal cell was due to an inefficiency in the mitochondrial electron transporting system in the ectopic stromal cells, we measured the mitochondrial membrane potential of the eutopic and ectopic endometrial stromal cells. As seen in Figure 1G, the membrane potential was lower in the ectopic endometriotic stromal cells. Concomitantly, the uncoupled respiration of the ectopic stromal cells was higher than that of the eutopic stromal cells (Figure 1C). Taken together, these data indicated that the metabolic pathway was altered in the ectopic stromal cells.

Pyruvate Dehydrogenase Kinase 1 Is Upregulated in Ectopic Endometriotic Stromal Cells

To investigate the underlying mechanism causing the increase in the lactate production and oxygen consumption rate, we evaluated the PDK expression level in the eutopic endometrial stromal cells taken from the endometriosis-free patients as well as the eutopic and ectopic endometrial stromal cells from the patients with endometriosis. The results indicated that both PDK1 and PDK3 messenger RNA (mRNA) levels were significantly higher in the endometriotic stromal cells (Figure 2A), while the level of PDK2 mRNA did not differ in these 3 groups.

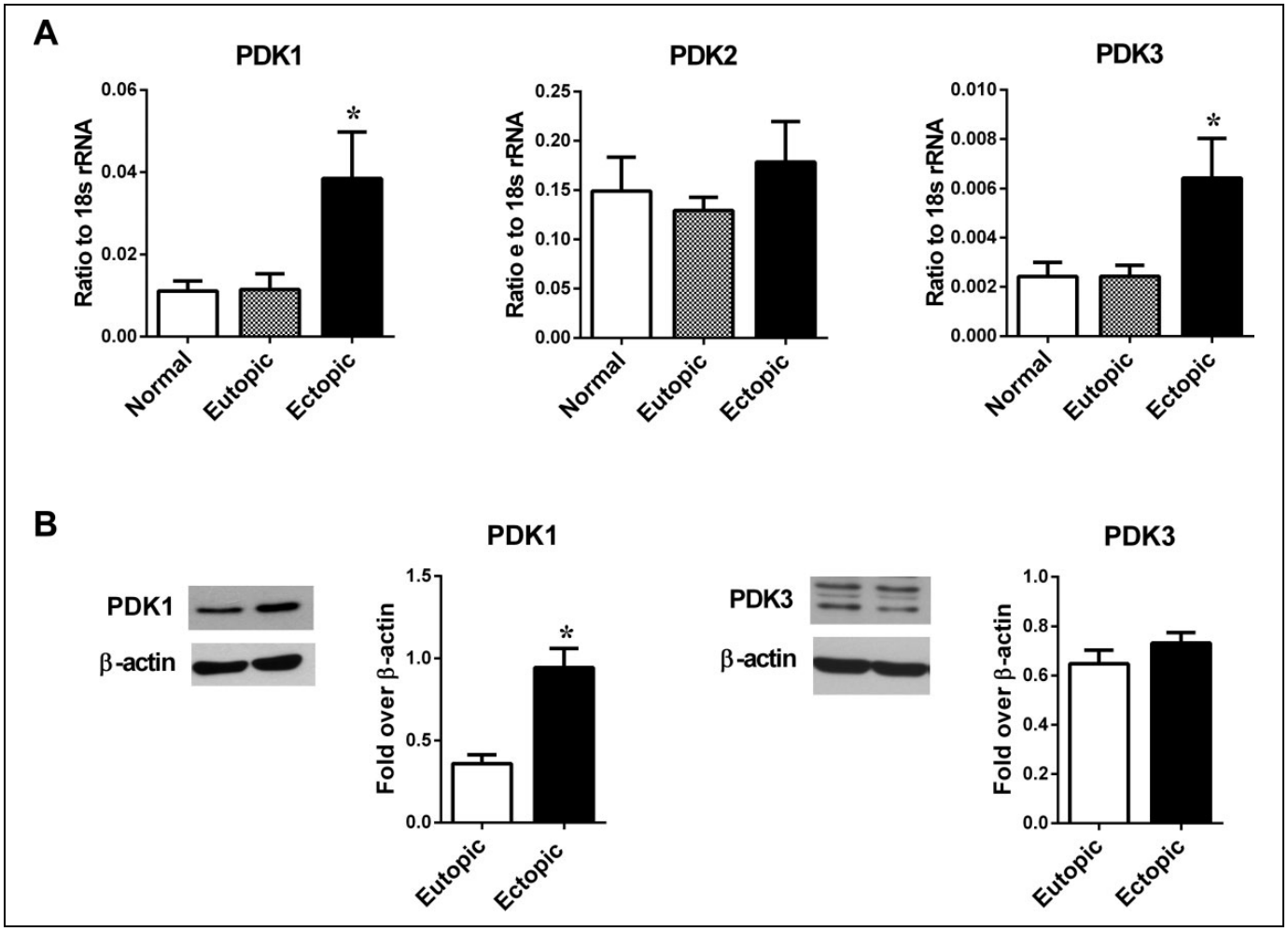

Figure 2. Pyruvate dehydrogenase kinase 1 (PDK1), a key enzyme in regulating glucose metabolism, is elevated in endometriotic ectopic stromal cells. A, The mRNA levels of 4 PDK isoforms were detected in disease-free endometrial stromal cells (normal) and eutopic and ectopic stromal cells from patients with endometriosis. The PDK4 mRNA level was under the detection limit; thus, it is not shown (n = 6 biological repeats for normal controls, n = 17 biological repeats for eutopic stromal cells, n = 18 biological repeats for ectopic stromal cells). B, A representative Western blot shows the protein levels of PDK1, PDK3, and β-actin in paired eutopic and ectopic stromal cells (n = 5 biological repeats in each group). The levels of PDK1 and PDK3 were normalized to the internal control, β-actin. Asterisks indicate $P < .05$; mRNA, messenger RNA.

The PDK4 mRNA level was too low to be detected in any of the samples. Consequently, we compared the PDK1 and PDK3 protein levels in the paired eutopic and ectopic endometrial stromal cells. Western blot results showed that only PDK1 was significantly increased in the ectopic endometriotic stromal cells (Figure 2B).

Hypoxia-Induced PDK1 Upregulation Is Mediated by HIF-1α at the Transcriptional Level

We next investigated the mechanism of PDK1 upregulation in ectopic stromal cells. Because our previous data showed that HIF-1α was elevated in ectopic endometriotic stromal cells,[6] we therefore cultured eutopic stromal cells in either hypoxia (1% O_2) or normoxia (21% O_2) for various time periods. Pyruvate dehydrogenase kinase 1 mRNA started to increase at 8 hours after the hypoxia treatment and remained elevated for up to 48 hours (Figure 3A). Concomitantly, the PDK1 protein level was increased at 16, 24, and 48 hours after the hypoxia treatment (Figure 3B). To further determine the mechanism of PDK1 elevation under hypoxia, the promoter activity assay of the eutopic endometrial stromal cells was performed. By using the in-house bioinformatics platform (The Binding Element Searching Tool), 2 predicted hypoxia response elements (HRE) in the PDK1 promoter region were identified (Figure 3C). The hypoxia treatment dramatically increased the promoter activity in the wild-type human PDK1 reporter, which was abolished by mutating the HRE2 sequence (Figure 3D). Furthermore, the mRNA level of PDK1 was induced under the hypoxia treatment, and the induction was abolished by knockdown of HIF-1α (Figure 3E). These results demonstrated that hypoxia-induced PDK1 expression via HIF-1α-mediated transcriptional regulation in the endometrial stromal cells.

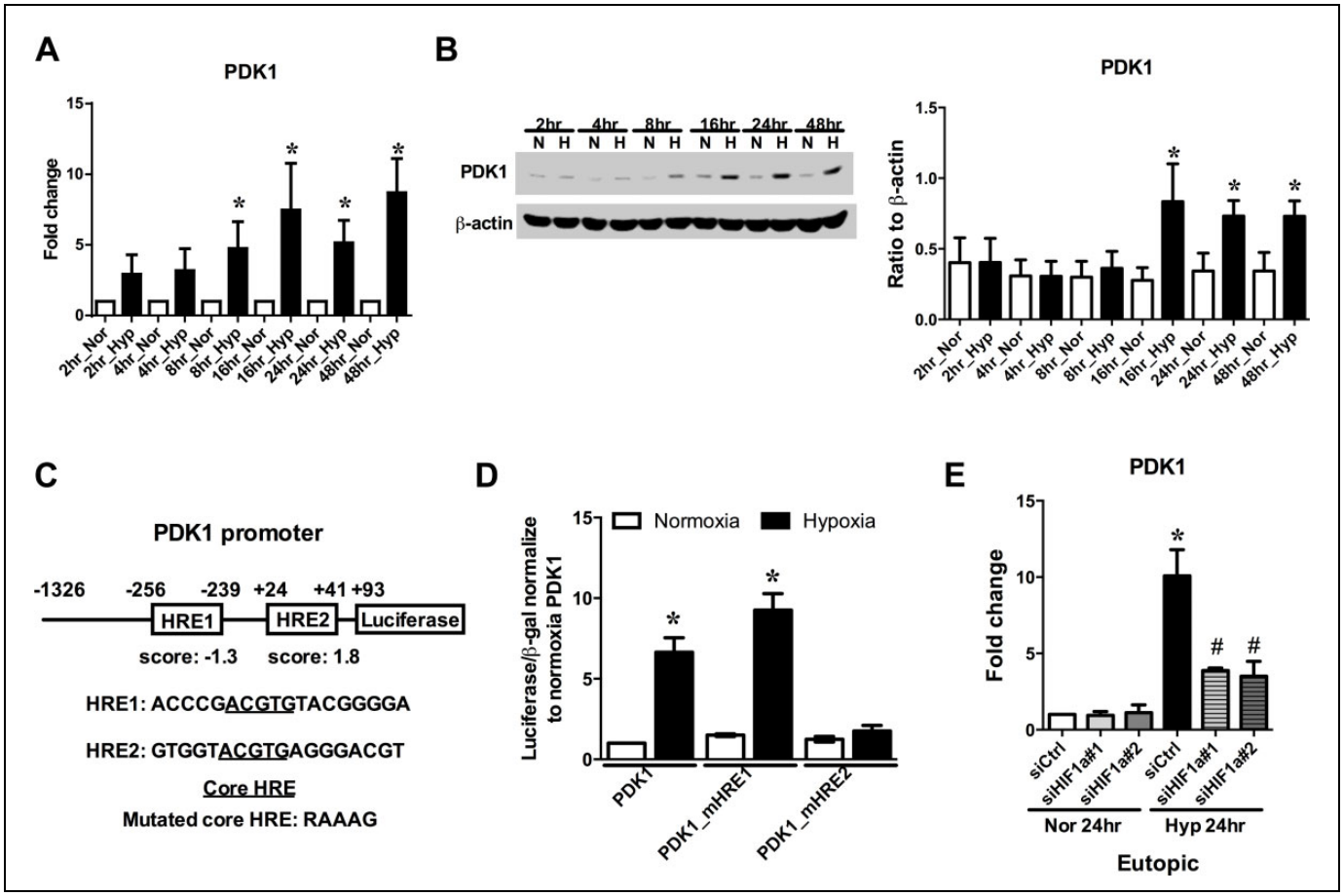

Figure 3. Hypoxia mediates PDK1 induction via transcriptional regulation. A, The mRNA level of PDK1 was detected at different time points for hypoxia-treated eutopic endometrial stromal cells (n = 4 biological repeats). The level of PDK1 mRNA was normalized to 18S rRNA. B, A representative Western blot (left panel) and the quantitative results (right panel) showed the protein levels of PDK1 and β-actin in the hypoxia-treated eutopic stromal cells (n = 5 biological repeats). C, Schematic drawing showing the promoter construct of PDK1 with predicted HRE and the score for each HRE. The HRE and mutated core HRE (mHRE) sequences are shown at the bottom of schematic drawing. D, Eutopic stromal cells were transiently transfected with the PDK1 promoter reporter constructs and internal control plasmids containing β-galactosidase (β-gal) and then cultured under normoxia (21% O_2) or hypoxia (1% O_2) for 24 hours (n = 5 biological repeats). The promoter activity was shown as luciferase activity normalized to the β-gal. E, Eutopic stromal cells were transfected with siRNA against HIF-1α or control siRNA and incubated under either normoxia or hypoxia for 24 hours (n = 4 biological repeats). The level of PDK1 mRNA was normalized to 18S rRNA. *$P < .05$; #$P < .05$ compared to hypoxia control siRNA group. Nor indicates normoxia; Hyp, hypoxia; HRE, hypoxia response element; mHRE, mutated HRE; mRNA, messenger RNA; rRNA, ribosomal RNA; siRNA, small interfering RNA; PDK1, pyruvate dehydrogenase kinase 1.

Inhibition of PDK Activity Attenuates the Lactate Production and Oxygen Consumption Rates in Ectopic Endometriotic Stromal cells

To investigate whether the elevated lactate production and oxygen consumption rate in the ectopic endometriotic stromal cells were mediated by upregulation of PDK1, the cells were treated with DCA, a PDK inhibitor. The treatment of the ectopic endometriotic stromal cells with DCA dose-dependently inhibited PDK activity as indicated by the reduced level of phosphorylated PDHE1α (Figure 4A). Lactate production, total respiration, and ATP-linked respiration were significantly reduced in the DCA-treated cells without affecting the uncoupled respiration (Figure 4B-E). Moreover, glucose uptake, the upstream event of glycolysis, was not affected by DCA treatment (Figure 4F). These results indicated that PDK activity was involved in metabolic feature change in the ectopic endometriotic stromal cells.

Pyruvate Dehydrogenase Kinase 1 Inhibits Apoptosis in Endometriotic Stromal Cells

To test whether increased PDK1 expression provides any beneficial effect for ectopic stromal cells, we challenged the ectopic stromal cell with either 100 μmol/L of hydrogen peroxide or a low-nutrient condition, in either the presence or absence of DCA. The apoptotic population was increased in the DCA-

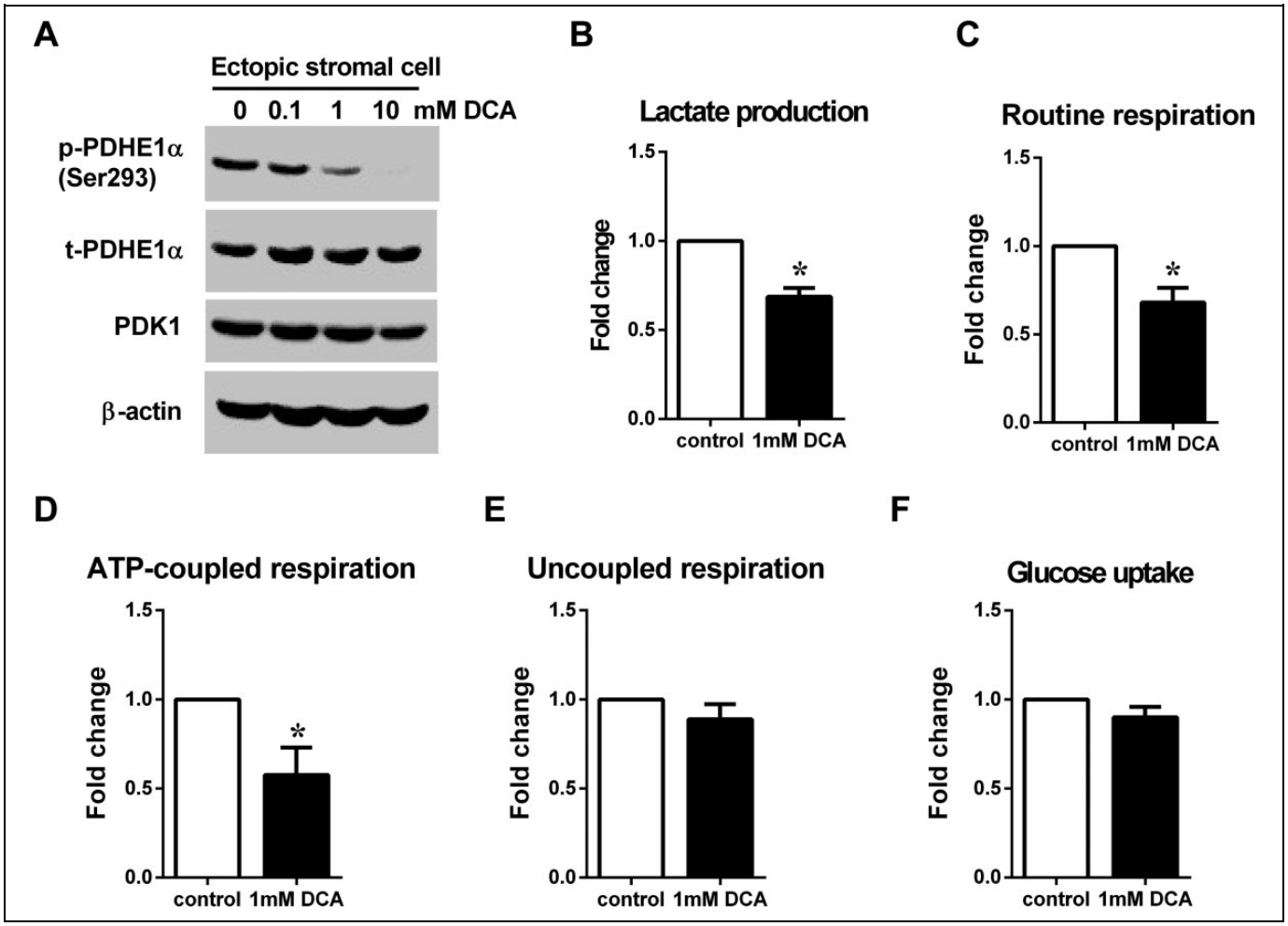

Figure 4. Upregulated PDK1 plays a role in metabolic-related cellular processes in ectopic endometriotic stromal cell. A, A representative Western blot shows the levels of total and phosphorylated PDHE1α in stromal cells treated with different doses of DCA. PDHE1α is known as a substrate of PDK. Lactate production (B), Routine respiration (C), ATP-coupled respiration (D), uncoupled respiration (E), and glucose uptake (F) were measured in ectopic stromal cells treated with 1mM DCA for 48 hours. Data are mean and SEM from 6 independent experiments using different batches of cells. *$P < .05$. PDHE1α indicates pyruvate dehydrogenase component α subunit; DCA, dichloroacetate; ATP, adenosine triphosphate; PDK, pyruvate dehydrogenase kinase.

treated ectopic stromal cell compared to the control group (Figure 5A). Moreover, we treated the eutopic stromal cells with hypoxia to elevate the PDK1 level with or without DCA and then challenged them with 100 μmol/L of hydrogen peroxide or the low nutrient condition. As was seen in the ectopic cells, the apoptotic population was increased in the DCA-treated cells (Figure 5B). These results suggested that upregulation of PDK1 by hypoxia can protect stromal cells from oxidative stress-induced apoptosis.

Discussion

According to the retrograde theory, ectopic endometriotic fragments suffer from hypoxic stress before the growth of blood vessels. Our previous study showing that HIF-1α is elevated in ectopic tissue[6] provides experimental evidence to support the premise that ectopic endometriotic lesions are indeed situated in a low-oxygen microenvironment. It is therefore important to investigate how ectopic endometriotic cells survive in a hypoxic environment, and whether the biochemical properties of ectopic cells remain the same or are altered to adapt to the low-oxygen conditions. Herein, we demonstrated that ectopic endometriotic stromal cells have greater oxygen consumption abilities that lead to the production of a similar amount of ATP to that produced by their eutopic counterparts when cultured under normoxic conditions. In addition, lactate production is increased in ectopic endometriotic stromal cells. Our results showed that this metabolic switch is likely to be mediated by hypoxia-induced PDK1 upregulation, since treatment with the PDK inhibitor, DCA, abolished these effects. More importantly, upregulation of PDK1 provides a survival advantage in oxidative stress and low-nutrient microenvironments. To our knowledge, this is the first report to thoroughly characterize the metabolic profile and the physiological impacts of eutopic and ectopic endometriotic stromal cells.

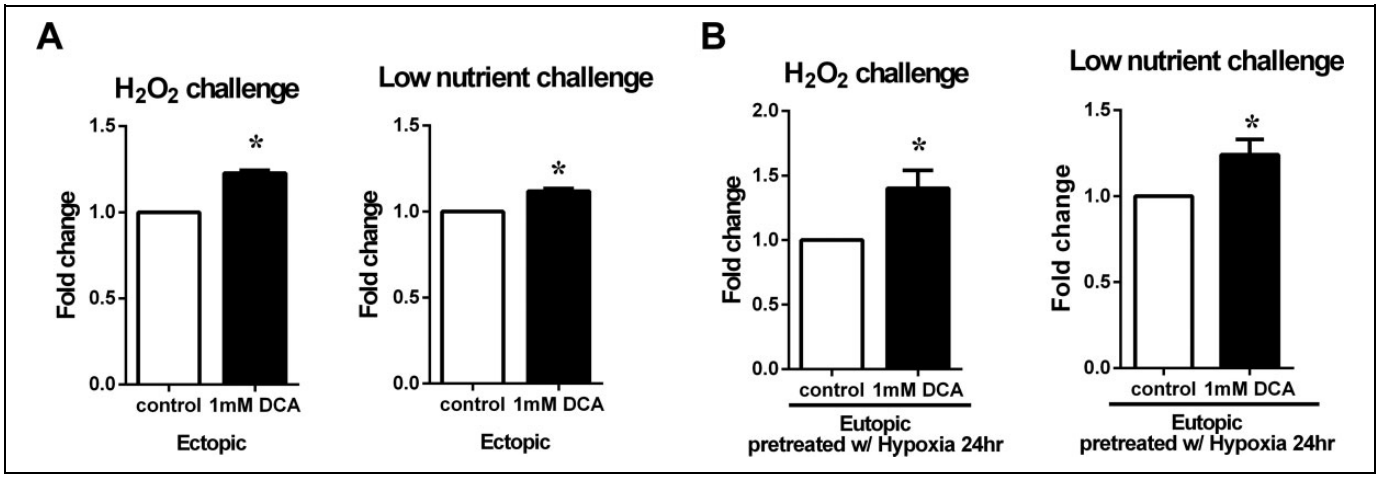

Figure 5. Blocking the PDK activity attenuated antiapoptotic ability of stromal cells. A, Ectopic endometriotic stromal cells were pretreated with 1mM DCA for 48 hours (n = 5 biological repeats). After pretreatment, the H_2O_2 was added into the medium and incubated for another 6 hours, or the medium was changed to low-nutrient medium containing DCA for another 24 hours. After challenge, the cells were collected and stained with annexin-V as an apoptotic marker and analyzed using flow cytometry. B, Eutopic stromal cells were pretreated with hypoxia for 24 hours (n = 5 biological repeats). The pretreated cells were challenge with either 100 μM of H_2O_2 for 6 hours or low-nutrient medium for 24 hours. After the challenge, the cells were collected and stained with annexin-V as an apoptotic marker and analyzed using flow cytometry. *$P < .05$. ANXA5 indicates Annexin A5 (or annexin V); DCA, dichloroacetate; PDK, pyruvate dehydrogenase kinase.

Cellular metabolism and mitochondrial energy production are pivotal for cell survival under various types of stresses. Pyruvate dehydrogenase kinase controls the critical step in cellular glucose metabolism by regulating the diversion point of 2 distinct pathways; the lactate production pathway and the mitochondria-dependent oxidative phosphorylation pathway. Elevation in PDK phosphorylates PDH and thus inhibits its ability to convert pyruvate to acetyl-CoA. The accumulated pyruvate is then converted by lactate dehydrogenase to produce lactate. In contrast, when PDK levels are low, unphosphorylated PDH will convert pyruvate into acetyl-CoA, which then enters the TCA cycle to be fully oxidized to generate more ATPs. Usually, when oxygen concentration is low, glucose will be metabolized by the glycolytic pathway, which yields less ATP per molecule of glucose.[16,18] In this study, we found that ATP-coupled respiration and uncoupled respiration were increased in endometriotic ectopic stromal cells. This observation is interesting for 2 reasons. First, the ATP-coupled oxygen consumption rate was higher in ectopic stromal cells, but the total ATP content was similar to that for the eutopic endometrial cells. We reasoned this was likely due to a leakage of the electron transfer system in the mitochondrial membrane, leading to low ATP production efficiency. Indeed, the low mitochondrial membrane potential in the ectopic stromal cells provides evidence to support our hypothesis (Figure 1F). Second, we observed that the overall glucose uptake ability of the endometriotic stromal cells was low. Given that the ectopic endometriotic stromal cells consumed more oxygen than the eutopic stromal cells, the glucose uptake was not significantly different. It is possible that endometriotic stromal cells may prefer utilizing other nutrients such as fatty acids as energy source, which bypass the conversion of pyruvate to acetyl-CoA.[19] β-Oxidation of fatty acid can generate acetyl-CoA, which then can enter the TCA cycle. This also explains why ectopic endometriotic stromal cells consume more oxygen, because the TCA cycle is still running at its basal rate. However, the detailed mechanism of the energy source switch in endometriotic stromal cell and the effect of PDK on fatty acid utilization are not known and warrant further investigation.

A HIF-1α-mediated increase in glycolysis is considered as a prerequisite for cellular adaption to hypoxia[16,18] and is one of the most common phenomena in cancer cells.[14,20-22] A recent study by Young et al reported that HIF-1α, PDK1, and lactate dehydrogenase A are increased in endometriotic lesions and peritoneal mesothelial cells, and this is concomitant with an increase in lactate production in the peritoneal fluid of women with endometriosis.[9] However, the relationship between HIF-1α and PDK1 or HIF-1α and lactate production was not addressed. Our current results report that lactate production is elevated in endometriotic stromal cells. In addition, the additional lactate production observed in ectopic endometriotic stromal cells is likely to be due to HIF-1α-induced PDK1 upregulation. Indeed, in the current study, when PDK1 activity was blocked by DCA, the production of lactate was reduced. However, the glucose uptake ability was low in both the eutopic and the ectopic stromal cells. This phenomenon raised a question about the source of pyruvate. Glutaminolysis can be a pathway to lactate accumulation.[23] Glutamine is converted to glutamate and then transaminated into α-ketoglutarate, which promotes the synthesis of citrate and malate. Both citrate and malate can be exported to mitochondria and further converted to pyruvate. This mitochondrial functional switch from oxidative phosphorylation and ATP production to synthesis of anabolic intermediates has been reported in cancer cells.[24] In addition,

glutaminolysis is enhanced under hypoxia.[25,26] This suggests that endometriotic stromal cells can switch their metabolic features and usage of nutrients.

Our data showed that both lactate production and the oxidative phosphorylation pathway are elevated in endometriotic stromal cells. Although this phenomenon was first reported in endometriosis, it is not unprecedented, since a similar result was also seen during tissue repair.[27,28] It has been proposed that initially, a partial glycolytic phenotype allows cell growth, and then hypoxia strengthens the glycolytic phenotype. Consequently, the nutrient shortage forces the restoration of oxidative phosphorylation via promoting glutaminolysis.[29,30] In addition, enhanced oxidative phosphorylation, glutaminolysis, and β-oxidation have been observed in the metastatic phenotype of melanoma cells.[31] In endometriotic stromal cells, hypoxic stress may trigger a similar effect as that reported in tissue repair or other pathological conditions. As a result, both lactate production and oxygen consumption rate are upregulated.

Hypoxia-induced PDK1 expression alters glucose metabolism and prevents excessive production of reactive oxygen species in cancer cells, which contributes to the pathogenesis and malignancy of cancer.[16,18] Thus, administration of DCA to inhibit PDK activity has been reported to synergize the treatment effect of several anticancer drugs.[32-34] In addition, upregulated PDK causes the accumulation of lactate, which has been reported to exert the antiapoptosis ability in germ cells.[35] Our result showed that inhibition of PDK activity by DCA reduces lactate production and oxygen consumption, which ultimately leads to an increase in H_2O_2- and low nutrient-induced apoptosis in both ectopic stromal cells and hypoxic eutopic stromal cells. These data suggest that ectopic endometriotic stromal cells can survive in the hypoxic microenvironment of the peritoneal cavity due to a hypoxia-induced, PDK1-mediated metabolic switch. In conclusion, our current findings provide new insights demonstrating that metabolic reprogramming may help cells cope with the hostile microenvironment in the endometriosis disease model.

Authors' Note

H.-C. Lee and S.-C. Lin designed and performed experiments. M.-H. Wu and S.-J. Tsai conceived and coordinated the project. M.-H. Wu performed the clinical diseases evaluations. H.-C. Lee and S.-J. Tsai wrote the manuscript.

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Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

References

1. Giudice LC. Clinical practice. Endometriosis. *N Engl J Med*. 2010;362(25):2389-2398.
2. Eskenazi B, Warner ML. Epidemiology of endometriosis. *Obstet Gynecol Clin North Am*. 1997;24(2):235-258.
3. Acien P, Velasco I. Endometriosis: a disease that remains enigmatic. *ISRN Obstet Gynecol*. 2013;2013:242149.
4. Giudice LC, Kao LC. Endometriosis. *Lancet*. 2004;364(9447): 1789-1799.
5. Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde menstruation in healthy women and in patients with endometriosis. *Obstet Gynecol*. 1984;64(2):151-154.
6. Wu MH, Chen KF, Lin SC, Lgu CW, Tsai SJ. Aberrant expression of leptin in human endometriotic stromal cells is induced by elevated levels of hypoxia inducible factor-1alpha. *Am J Pathol*. 2007;170(2):590-598.
7. Hsiao KY, Lin SC, Wu MH, Tsai SJ. Pathological functions of hypoxia in endometriosis. *Front Biosci (Elite Ed)*. 2015;7: 309-321.
8. Warburg O. On the origin of cancer cells. *Science*. 1956; 123(3191):309-314.
9. Young VJ, Brown JK, Maybin J, Saunders PT, Duncan WC, Horne AW. Transforming growth factor-beta induced Warburg-like metabolic reprogramming may underpin the development of peritoneal endometriosis. *J Clin Endocrinol Metab*. 2014;99(9): 3450-3459.
10. Wu MH, Lin SC, Hsiao KY, Tsai SJ. Hypoxia-inhibited dual-specificity phosphatase-2 expression in endometriotic cells regulates cyclooxygenase-2 expression. *J Pathol*. 2011;225(3): 390-400.
11. Lin SC, Wang CC, Wu MH, Yang SH, Li YH, Tsai SJ. Hypoxia-induced microRNA-20a expression increases ERK phosphorylation and angiogenic gene expression in endometriotic stromal cells. *J Clin Endocrinol Metab*. 2012;97(8):E1515-E1523.
12. Bowker-Kinley MM, Davis WI, Wu P, Harris RA, Popov KM. Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. *Biochem J*. 1998;329 (pt 1):191-196.
13. Gudi R, Bowker-Kinley MM, Kedishvili NY, Zhao Y, Popov KM. Diversity of the pyruvate dehydrogenase kinase gene family in humans. *J Biol Chem*. 1995;270(48):28989-28994.
14. Lu CW, Lin SC, Chen KF, Lai YY, Tsai SJ. Induction of pyruvate dehydrogenase kinase-3 by hypoxia-inducible factor-1 promotes

metabolic switch and drug resistance. *J Biol Chem*. 2008;283(42): 28106-28114.
15. Lu CW, Lin SC, Chien CW, et al. Overexpression of pyruvate dehydrogenase kinase 3 increases drug resistance and early recurrence in colon cancer. *Am J Pathol*. 2011;179(3):1405-1414.
16. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab*. 2006;3(3):177-185.
17. Tsai SJ, Wu MH, Lin CC, Sun HS, Chen HM. Regulation of steroidogenic acute regulatory protein expression and progesterone production in endometriotic stromal cells. *J Clin Endocrinol Metab*. 2001;86(12):5765-5773.
18. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab*. 2006;3(3):187-197.
19. Krivoruchko A, Zhang Y, Siewers V, Chen Y, Nielsen J. Microbial acetyl-CoA metabolism and metabolic engineering. *Metab Eng*. 2015;28:28-42.
20. Denko NC.Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nat Rev Cancer*. 2008;8(9):705-713.
21. Weljie AM, Jirik FR. Hypoxia-induced metabolic shifts in cancer cells: moving beyond the Warburg effect. *Int J Biochem Cell Biol*. 2011;43(7):981-989.
22. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell*. 2010;40(2):294-309.
23. Doherty JR, Cleveland JL. Targeting lactate metabolism for cancer therapeutics. *J Clin Invest*. 2013;123(9):3685-3692.
24. DeBerardinis RJ, Mancuso A, Daikhin E, et al. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci U S A*. 2007;104(49):19345-19350.
25. Metallo CM, Gameiro PA, Bell EL, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature*. 2011;481(7381):380-384.
26. Wise DR, Ward PS, Shay JE, et al. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. *Proc Natl Acad Sci U S A*. 2011;108(49):19611-19616.
27. Shyh-Chang N, Zhu H, Yvanka de Soysa T, et al. Lin28 enhances tissue repair by reprogramming cellular metabolism. *Cell*. 2013; 155(4):778-792.
28. de Moura MB, Uppala R, Zhang Y, Van Houten B, Goetzman ES. Overexpression of mitochondrial sirtuins alters glycolysis and mitochondrial function in HEK293 cells. *Plos One*. 2014;9(8): e106028.
29. Smolkova K, Plecita-Hlavata L, Bellance N, Benard G, Rossignol R, Jezek P. Waves of gene regulation suppress and then restore oxidative phosphorylation in cancer cells. *Int J Biochem Cell Biol*. 2011;43(7):950-968.
30. Wagner BA, Venkataraman S, Buettner GR. The rate of oxygen utilization by cells. *Free Radic Biol Med*. 2011;51(3):700-712.
31. Rodrigues MF, Obre E, de Melo FH, et al. Enhanced OXPHOS, glutaminolysis and beta-oxidation constitute the metastatic phenotype of melanoma cells. *Biochem J*. 2016; 473(6):703-715.
32. Bonnet S, Archer SL, Allalunis-Turner J, et al. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer Cell*. 2007;11(1):37-51.
33. Choi YW, Lim IK. Sensitization of metformin-cytotoxicity by dichloroacetate via reprogramming glucose metabolism in cancer cells. *Cancer Lett*. 2014;346(2):300-308.
34. Stander XX, Stander BA, Joubert AM. Synergistic anticancer potential of dichloroacetate and estradiol analogue exerting their effect via ROS-JNK-Bcl-2-mediated signalling pathways. *Cell Physiol Biochem*. 2015;35(4):1499-1526.
35. Erkkila K, Aito H, Aalto K, Pentikainen V, Dunkel L. Lactate inhibits germ cell apoptosis in the human testis. *Mol Hum Reprod*. 2002;8(2):109-117.