Expression of Cytosolic NADP$^+$-Dependent Isocitrate Dehydrogenase in Bovine Mammary Epithelium: Modulation by Regulators of Differentiation and Metabolic Effectors

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The cytosolic NADP$^+$-dependent isocitrate dehydrogenase (IDH1) catalyzes the conversion of isocitrate to α-ketoglutarate in the cytosol, and generates NADPH as a primary source of reducing equivalents for *de novo* fatty acid synthesis in bovine mammary gland. The enzymatic activity of IDH1 increases dramatically in early lactation in bovine mammary tissue. We hypothesized that the expression of IDH1 in bovine is modulated by regulators of mammary epithelial differentiation. To test this hypothesis, we first examined the changes in IDH1 expression in late pregnancy (20 days) and at various stages (14, 90, 120, and 240 days) of lactation in bovine mammary tissue. IDH1 mRNA levels increased by 2.3-fold after parturition compared to late pregnancy and remained elevated thereafter. Next, we examined the effects of extracellular matrix and lactogenic hormones on the expression of IDH1 in cultured BME-UV bovine mammary epithelial cells. We found that expression of IDH1 mRNA increased in parallel with β-casein expression induced by extracellular matrix. Fetal calf serum and insulin repressed, whereas prolactin stimulated the expression of IDH1 mRNA in a dose-dependent fashion. The inhibitory effects of insulin on IDH1 mRNA levels were antagonized by cotreatment with prolactin. In contrast, treatment with prolactin in the presence of extracellular matrix further increased IDH1 mRNA and protein accumulation. Prolactin-induced IDH1 expression was inhibited by the mitogen-activated protein kinase (MAPK) inhibitors PD98059 and U0126, and Janus tyrosine kinase 2 (Jak2) inhibitor AG490, suggesting that both MAPK and Jak2 contribute to regulation of IDH1 expression by prolactin. Finally, we report that treatment of BME-UV cells with α-ketoglutarate and palmitic acid reduced IDH1 transcript levels. Taken together, our data suggest that the expression of IDH1 in bovine mammary epithelium is modulated by regulators of differentiation including extracellular matrix and lactogenic hormones as well as metabolic effectors.** Exp Biol Med 231:599–610, 2006

Key words: isocitrate dehydrogenase; lactogenic hormones; differentiation; bovine mammary epithelium

Introduction

Isocitrate can serve as a substrate for a variety of synthetic and energy-yielding pathways. The oxidative decarboxylation of isocitrate is catalyzed by the enzyme complex isocitrate dehydrogenase (IDH) (1). Three distinct forms of the enzyme have been found in living organisms including the NAD$^+$-dependent IDH (EC 1.1.1.41), which is located exclusively in the mitochondria, and the NADP$^+$-dependent IDH (EC 1.1.1.42) which is found in both mitochondria (IDH2) and cytosol (IDH1) (2). These isozymes differ in their specificity for cofactors and are proposed to exert different functions (3). The NAD$^+$-dependent IDH is a key enzyme of the Krebs cycle and catalyzes the decarboxylation of isocitrate to α-ketoglutarate (α-KG) in the mitochondria (4–6). Gene disruption studies in yeast documented that NADP$^+$-dependent IDH did not compensate for lack of functions controlled by the NAD$^+$-dependent IDH (7), highlighting the distinct roles exerted by these IDH isozymes.

The IDH1 isoform catalyzes the conversion of isocitrate to α-KG in the cytosol (1). This reaction requires NADP as a cofactor and generates NADPH, which is a primary source of reducing equivalents utilized for fatty acid synthesis in bovine mammary gland. In fact, the activity of enzymes of the pentose phosphate pathway, which also generates

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NADPH (8, 9), has been shown to be lower in bovine mammary tissue compared to rat and sow (10). In addition, the partition of glucose for fatty acid synthesis in the ruminant mammary gland is limited due to low activity of the ATP-citrate lyase (11, 12). Consequently, only a small fraction of NADPH necessary for fatty acid synthesis can be produced by malate dehydrogenase in bovine mammary tissue (10, 13).

Little is known about the mechanisms responsible for regulation of IDH1 expression in bovine mammary epithelial cells. Previous studies documented that enzymatic activity of IDH1 increased dramatically in early lactation in bovine mammary tissue. Similarly, the activity of this enzyme has been shown to increase 3- to 8-fold during transition from late pregnancy to midlactation in rabbit and guinea pigs (14). These findings suggested that IDH1 may be under the control of hormonal and nutritional changes that accompany the transition from late pregnancy to lactation. To test this hypothesis, we examined the levels of IDH1 mRNA at late pregnancy and various stages of lactation in bovine mammary tissue. We also investigated the roles of extracellular matrix, lactogenic hormones and metabolic effectors on the expression of IDH1 in cultured bovine mammary epithelial cells.

Materials and Methods

Reagents and Chemicals. BME-UV cells, a bovine mammary epithelial cell line (15), were kindly provided by Dr. Politis (University of Vermont, Burlington, VT). Bovine prolactin (PRL) was obtained from the National Hormone & Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK; isolated from bovine pituitary gland, 90%–95% monomeric, lot #AF7170E). Bovine insulin (INS), hydrocortisone (HC), α-KG, palmitic acid (PA) and cell culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO). TriReagent was purchased from Molecular Research Center Inc. (Cincinnati, OH). Random hexamer primers, Moloney murine leukemia virus reverse transcriptase, and RNase inhibitor were purchased from Life Technologies Inc. (Gaithersburg, MD). Reverse transcription buffer was obtained from Ambion Inc. (Austin, TX). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA). PD98059 and AG490 were purchased from Calbiochem Co. (San Diego, CA). U0126 and the antibody against phospho-ERK and ERK were purchased from Cell Signaling Technology Inc. (Beverly, MA). The antibody against the mammalian IDH1 was kindly provided by Dr. McAlister-Henn (University of Texas Health Science Center, San Antonio, TX). Growth factor–depleted Matrigel and Dispase were obtained from Collaborative Research Inc. (Bedford, MA). Bovine mammary tissues were obtained at slaughter from 20 Holstein cows of the Beltsville Agricultural Research Center dairy herd (Agricultural Research Center, Beltsville, MD). Use of animals for these investigations was approved by the Beltsville Agricultural Research Center Animal Care and Use Committee. Portions of mammary tissues were frozen in liquid nitrogen and stored at –80°C until RNA extraction.

Cell Culture and Growth Studies. BME-UV cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS) at 37°C and 5% CO_2 atmosphere. For cell growth studies, cells were seeded at a density of 1×10^5/well in 12-well culture plates. After 24 hrs, media were removed and cells washed with Dulbecco's phosphate buffered saline (DPBS). At the end of each treatment period, cells were harvested by trypsinization and viable cells counted by trypan blue exclusion. Matrigel was thawed at 4°C and aliquoted into 6-well culture plates with 1 ml/well. After polymerization of the matrix at 37°C, one-half million BME-UV cells were seeded on top of Matrigel-coated plates or on plastic dishes, and incubated with or without lactogenic hormones for 72 hrs. To dissolve the Matrigel matrix, plates were incubated with Dispase and cells recovered by low-speed centrifugation. Cell pellets were resuspended in TriReagent for extraction of total RNA.

RNA Preparation. Bovine mammary tissues (0.5–1 g) were first minced in PBS and homogenized prior to RNA isolation. For cell culture experiments, BME-UV cells were plated on 60-mm plastic tissue culture dishes (1×10^6 cells/dish) or 6-well (5×10^5 cells/well) plates. Total cellular RNA was extracted from bovine tissue or BME-UV cells using TriReagent followed by DNAse digestion. The integrity of the RNA was confirmed by electrophoretic analysis of the 28s and 18s ribosomal subunits. RNA quality and concentration were determined using the Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA).

Semiquantitative RT-PCR. Reverse transcription was performed using total RNA incubated with random hexamers primers, Moloney murine leukemia virus reverse transcriptase, RNase inhibitor, and reverse transcription at 42°C for 1 hr. Semiquantitative PCR amplification was used to examine the expression of IDH1 and β-casein. Oligonucleotides used to amplify IDH1 fragment (384 bp) were (forward) 5′-GTCTGTGGTAGAGATGCAAGG-3′ and (reverse) 5′-CCATAAGCATGACGACCTATG-3′. PCR reactions were performed using Vent DNA polymerase. Oligonucleotides used to amplify β-casein (203 bp) were (forward) 5′-CCAGGATAAAATCCACCCCT-3′ and (reverse) 5′-AGGGAAGGGCATTTCTTTGT-3′. The authenticity of the IDH1 (Accession No. AF136009) and β-casein (Accession No. XO6359) PCR products were confirmed by direct sequencing and BLAST analysis against deposited sequences in the Genbank database. Ribosomal 18s RNA or S15 were amplified to control for PCR conditions and equal loading. Relative expression levels of IDH1 and β-casein mRNA were estimated by Kodak 1D Image (Eastman Kodak Co., Rochester, NY) analysis and corrected for expression of the control RNA.

Real-time PCR. Total RNA was isolated as described above and the integrity of the RNA was confirmed by electrophoretic analysis. Real-time PCR was performed as previously described (16). Briefly, cDNA was prepared by reverse transcription of sample RNA using the Bio-Rad iScript cDNA Kit (Bio-Rad Laboratories, Hercules, CA) in a reaction volume of 40 µl. DNA standards were prepared from PCR amplicons purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). Product concentrations were determined using the Agilent Bioanalyzer and DNA 500 kits (Agilent Technologies) and diluted to contain 1×10^2 to 1×10^8 molecules per µl. The quantity of cDNA in unknown samples was calculated from the appropriate external standard curve run simultaneously with samples. Real-time PCR was performed using the Bio-Rad MyiQ Real-Time Single Color PCR Detection System in Bio-Rad 96-well plates in a 25 µl volume. Optimal reaction conditions were obtained with the Bio-Rad iQ SYBR Green Supermix Kit at an annealing temperature of 57°C. The PCR program was as follows: 95°C initial denaturation for 3 mins, followed by 45 cycles of 94°C for 15 secs, 57°C for 30 secs, 72°C for 30 secs, then 95°C for 1 min, 55°C for 1 min and holding at 4°C. One microliter of sample cDNA or standard was added to 24 µl of reaction mix in the wells. Oligonucleotides used to amplify IDH1 (149 bp) were (forward) 5′-CAAGGCGGGTCTGTGGTAG-3′ and (reverse) 5′-TGGTCGTTGGTGGCATCG-3′.

Ribonuclease Protection Assay. Oligonucleotides used to amplify the bovine IDH1 were ICDHF4 which contained a 5′ linker for *Hin*dIII (5′-GTCAAGCTTTTTGCAAATGCTTTGGAAGAAG-3′) and ICDHR9 which contained a 5′ linker for *Bam*HI (5′-ATAGGATCCTTAAAGTTTGGCCTGAGCTAG-3′). The PCR fragment was of the expected length (183 bp) and its identity was confirmed by direct sequencing. The *Hin*dIII-*Bam*HI fragment was cloned in the antisense orientation into plasmid pTRIplescript obtained from Ambion Inc. (Austin, TX). Levels of IDH1 mRNA were measured in total RNA (10 µg) by ribonuclease protection assay using the Hybspeed RPA kit obtained from Ambion. The cyclophilin mRNA was used as an internal standard. The cyclophilin ribonucleotide probe transcribed from pTRI-cyclophilin (Ambion) protected a fragment of 103 bp.

Western Blotting. BME-UV cells were plated in 6-well plates at a density of 5×10^5/well. Three wells were assigned to each experimental treatment. At the end of incubation periods, cells were washed with ice cold DPBS three times and 75 µl/well fresh 1 × SDS-PAGE lysis buffer (prepared from 2 × SDS-PAGE lysis buffer containing 125 m*M* Tris pH 6.8, 4 m*M* EDTA, 30% sucrose, 20% glycerol and 6% SDS; 10% β-mercaptoethanol was added just prior to use) was added directly to washed cells. Then, cell suspensions were scraped into microfuge tubes and boiled for 10 mins followed by centrifugation at room temperature for 10 mins to remove any precipitate. Aliquots of cell lysates were mixed with loading buffer and 1µl of β-mercaptoethanol for every 10 µl of cell lysate and boiled for 1 min before loading on gels. Protein content of cell lysates was normalized with β-actin by incubating immunoblots with β-actin antibody-1 (Oncogene Research Products, Cambridge, MA). Protein samples were separated on 14% SDS-PAGE, and electrotransferred to nitrocellulose membrane. Separate blots were run for each immunoblotting. The membrane was blocked for 60 mins at room temperature with 10% milk in TBS-Tween, and incubated with rabbit polyclonal antibody against IDH1, phospho-ERK or total ERK for 2 hrs at room temperature. After washing for 10 mins, the membrane was incubated with goat anti-rabbit horseradish peroxidase conjugated antibody (Bio-Rad Laboratories, CA) for 45 mins followed by 60 mins washing. Then, the membrane was incubated with ECL chemiluminescent substrate for 2 mins, and exposed to film (Amersham Biosciences, Amersham, UK).

Statistical Analysis. Fold-changes in expression of IDH1 mRNA and cell proliferation are presented as means ± standard errors. Comparisons of means following a significant ($P < .05$) analysis of variance (ANOVA) were performed by Fisher's protected least significant difference test.

Results

Expression of IDH1 mRNA in Bovine Mammary Tissue at Different Stages of Lactation. Previous studies have documented that IDH1 enzymatic activity in bovine mammary tissue increased after parturition (14), but little information is available regarding the underlying mechanisms of regulation. To investigate whether or not gene expression of this enzyme is regulated around parturition, we examined the mRNA levels of IDH1 in bovine mammary tissue in late pregnancy and at various stages of lactation. Total RNA samples were extracted from late pregnancy (–20 days) and lactating (14, 90, 120, and 240 days) bovine mammary tissues, and RT-PCR was performed. At each time point, samples were obtained from four different animals ($n = 20$). Preliminary experiments were carried out to determine the optimal conditions for PCR amplification of IDH1. The results of Figure 1 indicated that amplification of IDH1 during a 29-PCR cycle reaction was within linear range. In subsequent experiments, 29-PCR cycles were used for semiquantitative RT-PCR analysis of IDH1 mRNA levels. As depicted in Figure 2, compared to samples collected at –20 days, the mRNA levels of IDH1 at 14 days of lactation increased ($P < 0.05$) 2.3-fold and remained elevated thereafter. These results documented that transition from the late stages of pregnancy to early lactation was accompanied by a significant increase in expression of IDH1.

Effects of Extracellular Matrix and Lactogenic Hormones on Expression of IDH1 in Bovine Mammary Epithelial Cells. The results of the animal studies suggested that IDH1 expression may be upregulated

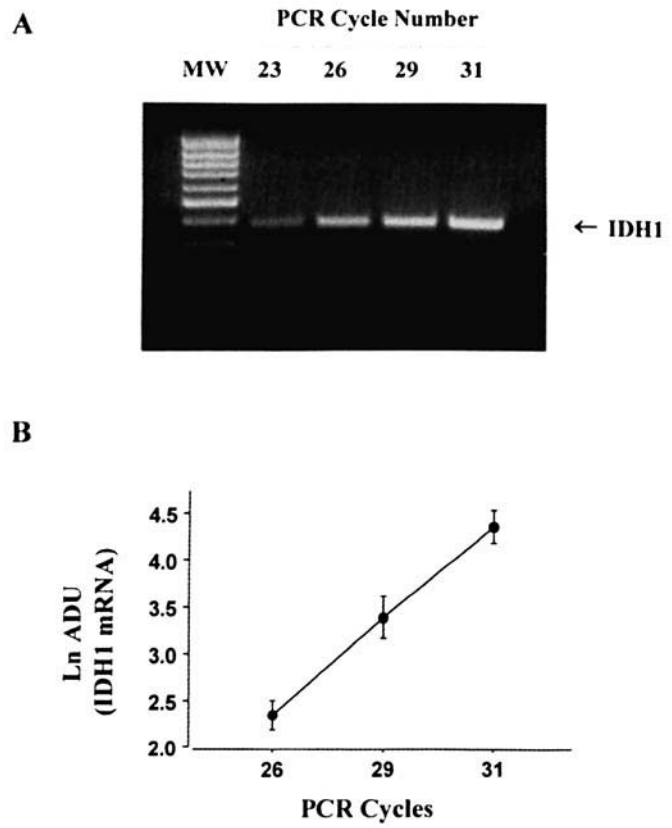

Figure 1. Semiquantitative RT-PCR analysis of cytosolic $NADP^+$-dependent IDH (IDH1). Total RNA was obtained from BME-UV bovine mammary epithelial cells as described in Materials and Methods. (A) Effects of number of cycles on amplification of IDH1 PCR products. (B) Data represent logarithmic arbitrary densitometry units (ADU) of IDH1 PCR products ($n = 3$) ($R^2 = 0.899$, $P < 0.0001$).

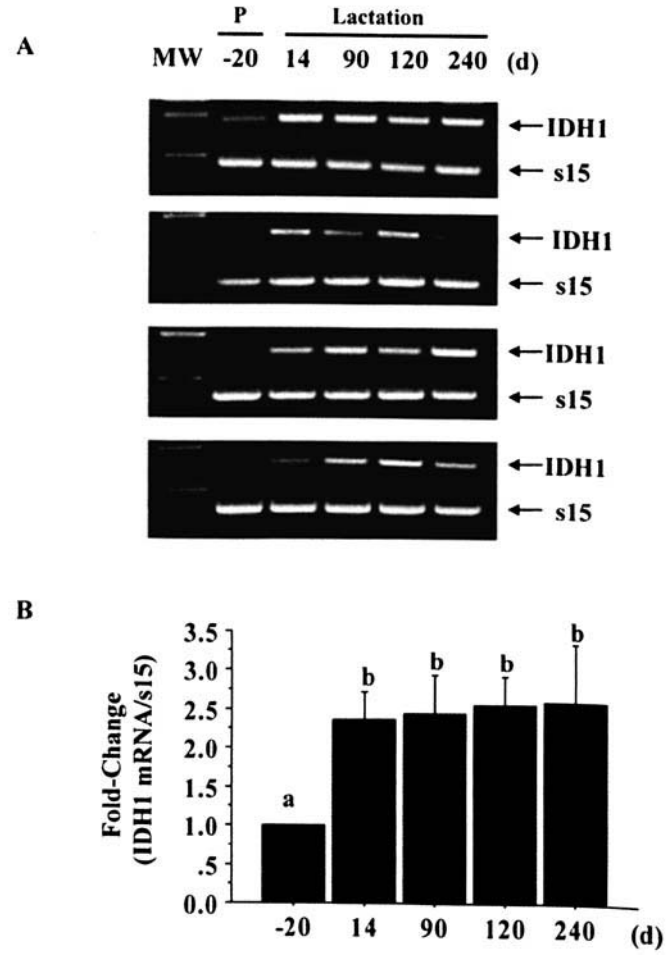

Figure 2. IDH1 mRNA levels in late pregnancy and lactating bovine mammary tissue. Bovine mammary tissues were obtained from late pregnancy ($P = -20$ days) and lactating (14, 90, 120 and 240 days) cows. Samples from four animals were examined for each time point. For each sample, two independent RT-PCRs were performed. (A) The bands represent IDH1 and S15 RT-PCR products from total RNA extracted from tissues at the indicated time points. (B) Bars represent the means ± standard errors of IDH1/S15 arbitrary units ($n = 4$). Means without a common letter differ ($P < 0.05$).

during lactogenesis. We hypothesized that the expression of IDH1 is modulated by regulators of differentiation in bovine mammary epithelium. To test this hypothesis the bovine mammary epithelial cell line BME-UV (15) was used in subsequent experiments. Using a ribonuclease protection procedure, we found that mRNA levels of IDH1 were reduced by 2-, 4- and 3.5-fold when BME-UV cells were cultured in control medium (DMEM) containing 10% FCS for 24, 48, and 72 hrs, respectively, as compared to cells cultured in serum-free medium (Fig. 3A). Parallel experiments using semiquantitative RT-PCR confirmed that IDH1 mRNA levels were reduced 2.5-fold as early as 24 hrs in the presence of 20% FCS (Fig. 3B). These cumulative data suggested that FCS stimulated biological responses or contained factors that exerted a negative effect on IDH1 expression. Based on these results, serum-free media were used in subsequent experiments to examine the effects of regulators of differentiation on IDH1 expression.

Differentiation to a lobuloalveolar structure, which is one of the hallmarks of differentiation of mammary epithelial cells, requires the interactions of cells with extracellular matrix components. However, the presence of lactogenic hormones prolactin, hydrocortisone and insulin is also required to achieve maximal differentiation. Therefore, we examined the effects of extracellular matrix and lactogenic hormones, alone or in combination, on the expression of IDH1 in bovine mammary epithelial cells. BME-UV cells were seeded on Matrigel-coated plates at a density of 5×10^5/well in 6-well plates in the presence or absence of INS (1 μg/ml), PRL (1 μg/ml), and HC (0.1 μ*M*), and incubated for 72 hrs as previously described (17). BME-UV cells were also plated on 6-well plastic dishes and cultured in DMEM, with or without lactogenic hormones, for 72 hrs. Cells plated on plastic exhibited the typical polygonal shape, whereas in the presence of extracellular matrix BME-UV cells formed spherical clusters (data not shown) similar to those reported by our group in earlier studies (17). At the end of the incubation period (72 hrs), cells were harvested and mRNA for IDH1 was measured by ribonuclease protection assay. The results of Figure 4A illustrated that in the presence of extracellular matrix the expression of IDH1 mRNA was increased 3-fold compared

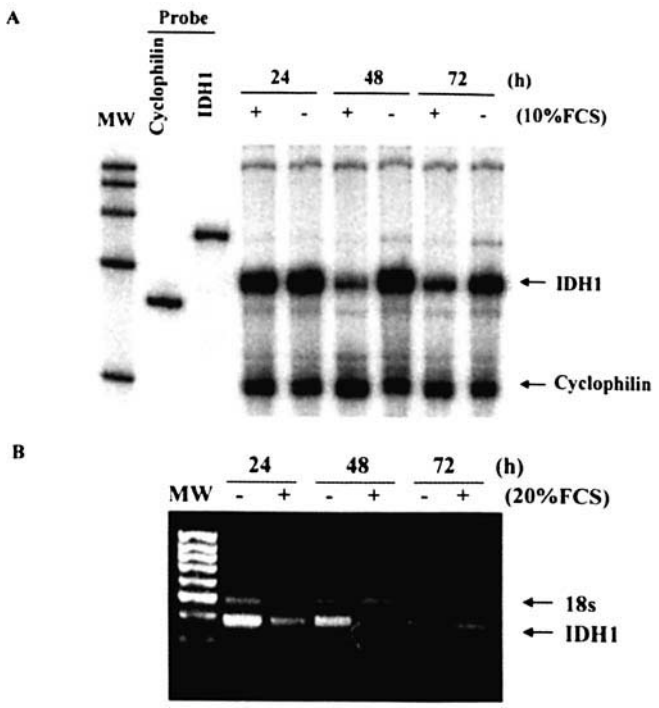

Figure 3. Regulation of IDH1 mRNA expression by fetal calf serum in BME-UV bovine mammary epithelial cells. Cells were cultured on plastic in the absence or presence of the indicated concentrations (10% and 20%) of fetal calf serum (FCS) for 24, 48 or 72 hrs. Cells were harvested and total RNA extracted at the end of the incubation periods. mRNA levels for IDH1 gene were evaluated by (A) RNase Protection Assay or (B) RT-PCR. Arrows indicate mRNAs for IDH1 or internal standard cyclophilin or 18s ribosomal RNA. Data are representative of two independent experiments.

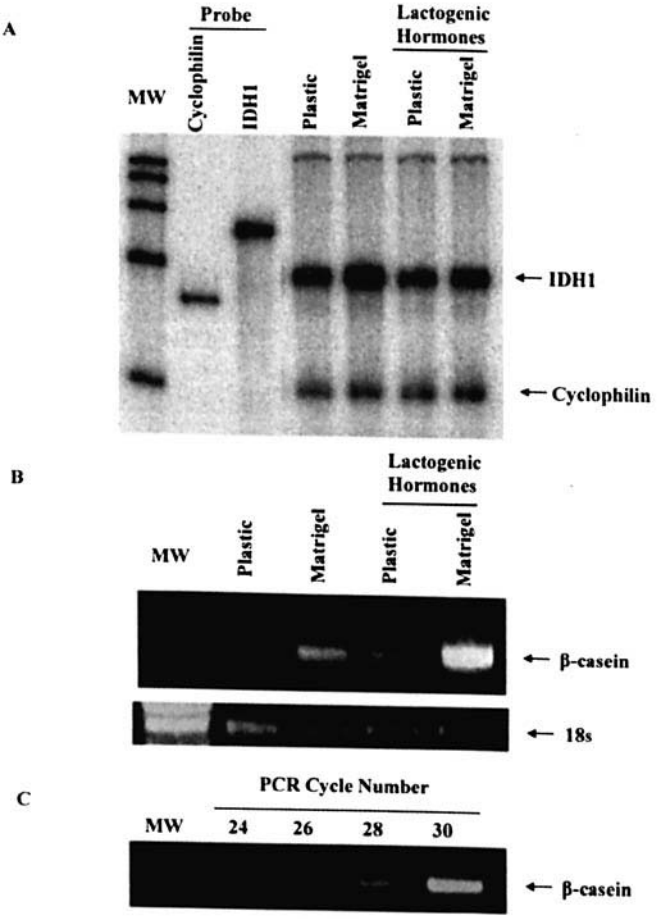

Figure 4. Effects of extracellular matrix and lactogenic hormones on IDH1 and β-casein expression in BME-UV cells. Cells were plated on plastic or Matrigel in presence or absence of the lactogenic hormones insulin (INS, 1 μg/ml), prolactin (PRL, 1 μg/ml) and hydrocortisone (HC, 0.1 μ*M*). At the end of the incubation period (72 hrs), cells were harvested for extraction of total RNA as described in Materials and Methods. In (A) expression of IDH1 was measured by ribonuclease protection assay. Bands represent RNA molecular weight (MW) markers, cyclophilin and IDH1 antisense probes, and protected fragments for IDH1 and cyclophilin. In (B), bands represent RT-PCR products for β-casein (203 bp) and control 18s in total RNA samples from BME-UV cells cultured on plastic or Matrigel. Data in (A) and (B) were confirmed in two separate experiments. (C) Effects of number of cycles on amplification of β-casein PCR products. Conditions for linear amplification of β-casein were confirmed in three separate experiments.

to cells cultured on plastic. These data suggested that transition to a differentiated state was sufficient to activate IDH1 expression. In contrast, mRNA levels of IDH1 decreased in BME-UV cells cultured in the presence of lactogenic hormones both on plastic and Matrigel-coated plates. As a positive control, we measured the expression of the β-casein gene, whose expression has been shown to increase in differentiated cells (18). Preliminary PCR reactions were carried out to determine the optimal number of cycles (28 cycles) for linear PCR amplification of β-casein (Fig. 4C). Levels of β-casein mRNA were induced 3-fold in BME-UV cells plated on Matrigel compared to plastic, and were increased further on Matrigel in the presence of lactogenic hormones (Fig. 4B). These cumulative data indicated that extracelluar matrix alone was sufficient to stimulate IDH1 mRNA levels, but the combination of lactogenic hormones exerted a negative effect on IDH1 expression.

The positive role of the lactogenic hormones INS, PRL, and HC in lactogenesis have well been documented (19). Therefore, we expected that, similar to β-casein, the combination of lactogenic hormones and Matrigel would increase mRNA levels of IDH1. However, the results of Figure 4 indicated that incubation of BME-UV cells with lactogenic hormones decreased IDH1 mRNA levels both on plastic and Matrigel. Therefore, we performed a series of experiments to investigate the specific effects of each hormone on the expression of IDH1 in BME-UV cells cultured on plastic. First, we examined the effects of INS and found that it reduced IDH1 transcripts in a dose-dependent fashion (Fig. 5A). In contrast, PRL stimulated IDH1 mRNA levels in a dose-dependent manner (Fig. 5B), whereas we did not detect changes in IDH1 transcript levels in BME-UV cells treated with HC (Fig. 5C). Coincubation of BME-UV cells with increasing concentrations of PRL (0.5, 1.0 and 1.5 μg/ml) reversed the inhibitory effects of INS on IDH1 mRNA expression (Fig. 5D). Results of real-

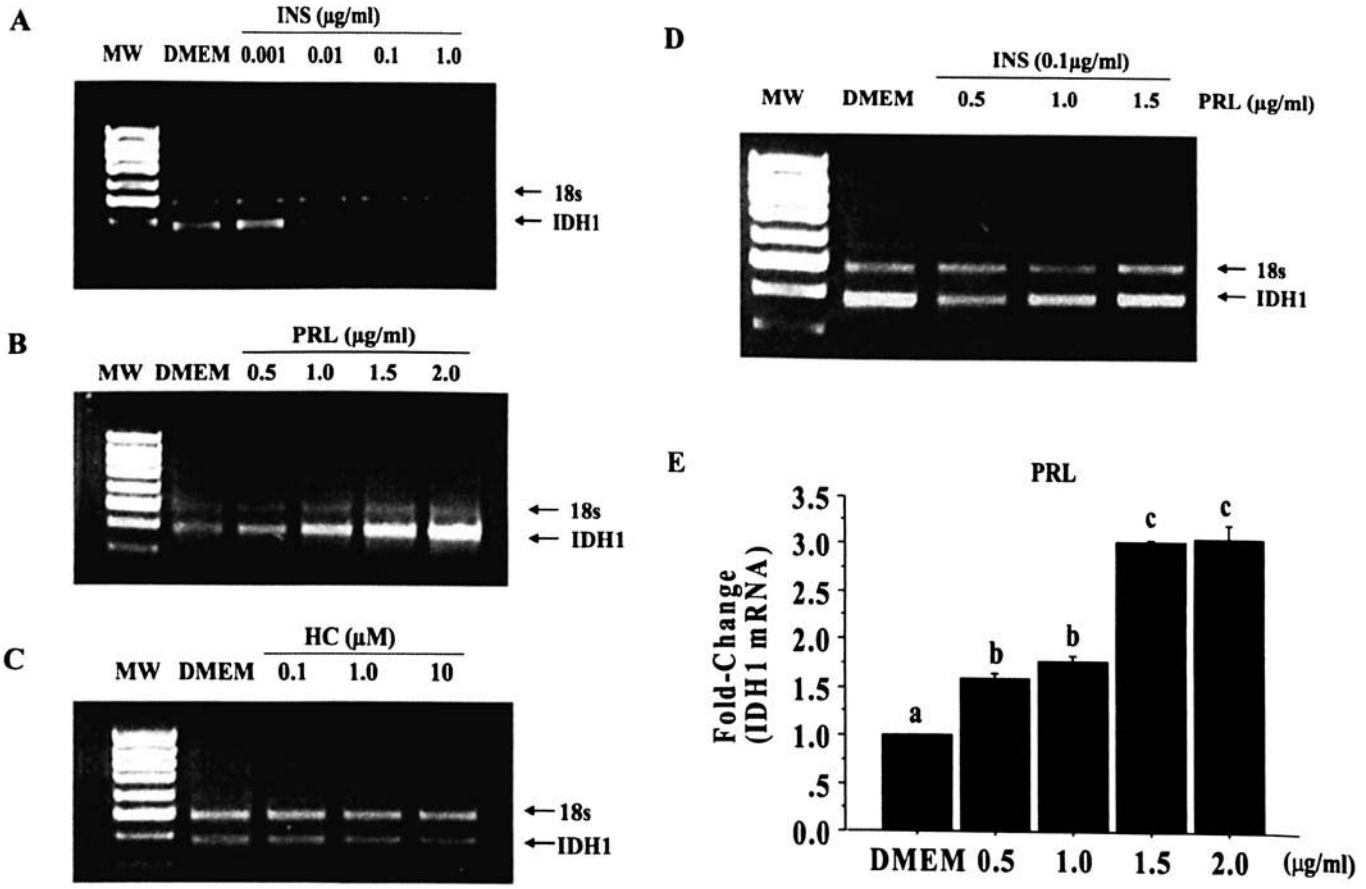

Figure 5. Regulation of IDH1 mRNA expression in BME-UV bovine mammary epithelial cells by lactogenic hormones. Cells were incubated on plastic with various amounts of (A) insulin (INS), (B) prolactin (PRL), (C) hydrocortisone (HC), and (D) PRL plus 100 ng/ml INS for 72 hrs. At the end of the incubation period, cells were harvested and mRNA analyzed using semiquantitative RT-PCR as described in Materials and Methods. Arrows indicate IDH1 or control 18s ribosomal RNA. (E) Real-time PCR was performed to confirm the results of (B). Data represent means ± standard errors from two replicate experiments performed in triplicate. Means without a common letter differ ($P < 0.05$).

time PCR confirmed that IDH1 mRNA expression was upregulated by PRL (Fig. 5E).

To further explore the mechanisms through which these hormones exerted their effects on expression of IDH1, we conducted cell growth studies. The results depicted in Figure 6 document that the treatment with INS stimulated cell growth (Fig. 6A) in a dose-dependent manner, whereas PRL and HC had no effects on cell proliferation (Fig. 6B and C). However, the coincubation with increasing concentrations of PRL abolished the stimulatory effects of INS on cell growth. (Fig. 6D). These cumulative results indicated that the stimulation of IDH1 expression by PRL occurred without affecting cell proliferation, whereas INS and FCS reduced IDH1 expression while stimulating cell growth.

The results of Figures 4 and 5 suggested that both extracellular matrix and PRL played a positive role in regulation of IDH1 expression. To further investigate the effects of PRL on IDH1 expression, BME-UV cells were cultured on plastic or in the presence of extracellular matrix. As depicted in Figure 7, IDH1 mRNA and protein levels were increased in BME-UV cells cultured on plastic in response to treatment with PRL or in cells cultured on extracellular matrix. When BME-UV cells were cultured with PRL in the presence of Matrigel, the expression of IDH1 was further increased.

Involvement of Mitogen Activated Protein Kinase (MAPK) in PRL-Induced IDH1 Expression. Based on the information that the mitogen activated protein kinase (MAPK) pathway modulates the stimulatory effects of PRL on expression of milk proteins (20), we examined the role of MAPK in the PRL-dependent activation of IDH1 expression. In BME-UV cells cultured in the presence of PD98059 (20 μM), a MAPK pathway inhibitor, the stimulatory effects of PRL on IDH1 mRNA expression were abolished, whereas no changes in IDH1 mRNA levels were observed when cells were treated with PD98059 (Fig. 8). The inhibitory effects of PD98059 on PRL-induced expression of IDH1 mRNA were confirmed by both semiquantitative RT-PCR (Fig. 8A) and real-time PCR (Fig. 8B). To confirm the effects of PRL and involvement of MAPK on IDH1 expression, Western blotting of IDH1 and phosphorylated ERK were performed in cells treated with PRL in the presence or absence of the ERK inhibitors PD98059 and U0126. Figure 8C and D indicated that IDH1

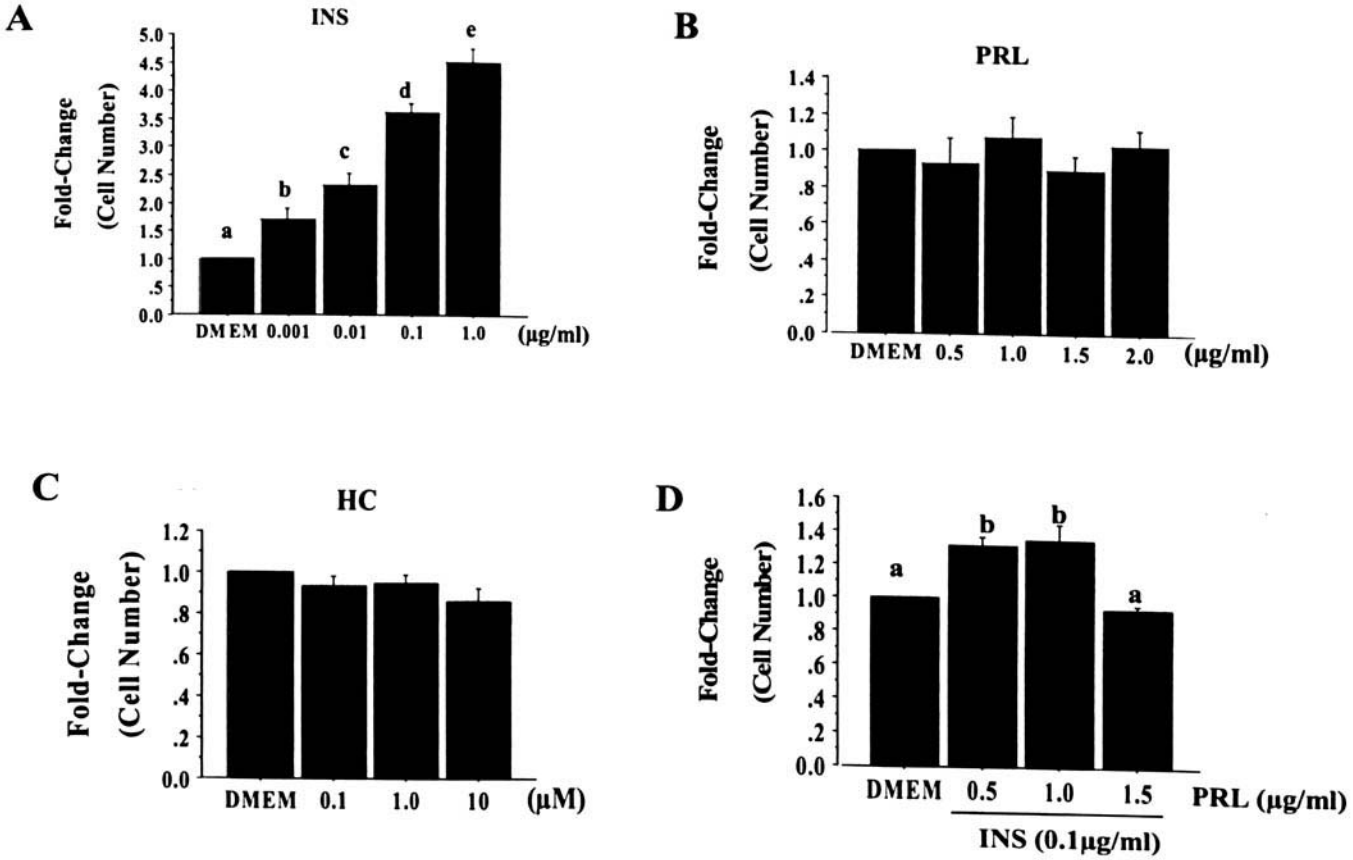

Figure 6. Effects of lactogenic hormones on proliferation of BME-UV bovine mammary epithelial cells. Cells were cultured on plastic in the presence of various concentrations of (A) INS, (B) PRL, (C) HC, and (D) PRL plus 100 ng/ml INS for 72 hrs. Cells were harvested by trypsinization and counted by trypan blue exclusion. Data represent means ± standard errors from two replicate experiments performed in triplicate. Means without a common letter differ ($P < 0.05$).

and phosphorylated ERK protein levels were increased in cells treated with PRL, whereas the cotreatment with PD98059 or U0126 counteracted the activation by PRL. The treatment with PD98059 or U0126 alone had no effects on the protein levels of IDH1. Immunoblot analysis with antibodies against ERK1/2 showed that the total level of ERK1/2 was not changed. Treatment with AG490, a selective Jak2 inhibitor, reduced the phosphorylation of ERK1/2 and IDH1 expression induced by PRL (Fig. 8E), supporting the upstream role of Jak2. These results highlighted the contribution of MAPK to activation of IDH1 expression by PRL and coupling between accumulation of IDH1 mRNA and protein.

Effects of Metabolic Effectors on Expression of IDH1 in BME-UV Cells. Because IDH1 catalyzes the dehydrogenation of isocitrate to α-KG, we examined the time-dependent effects of this metabolic product on IDH1 expression. Treatment of BME-UV cells with α-KG (0.5 m*M*) reduced ($P < 0.05$) IDH1 transcripts levels by 50% at 12 hrs and 30%–35% at 24, 48, and 72 hrs respectively (Fig. 9A). We also examined the effects of palmitic acid (PA) and found that PA did not induce significant changes in IDH1 expression at 12 hrs compared to untreated cells. However, exposure of BME-UV cells to PA for 24 and 72 hrs decreased the mRNA levels of IDH1 by 34% and 65% ($P < 0.05$) respectively (Fig. 9B), whereas there were no differences between control DMEM and PA-treated cells at 48 hrs. Overall, these data suggested that α-KG and PA may act as metabolic repressors of IDH1 expression and that these effects may be time-dependent.

Discussion

IDH1 catalyzes the dehydrogenation of isocitrate to α-KG and generates NADPH in the cytoplasm. Because the activity of the enzymes involved in pentose phosphate pathway and ATP-citrate lyase pathway are relatively low, whereas the enzymatic activity of IDH1 in bovine mammary gland increased dramatically during lactogenesis (10, 14), IDH1 has been considered a primary source of reducing equivalents required for *de novo* fatty acid synthesis in the lactating bovine mammary gland. However, no information is currently available concerning the underlying mechanisms that regulate IDH1 expression. In mammalian species, the onset of lactation is achieved by a process of functional differentiation of mammary epithelial cells controlled by several regulators including extracellular matrix, circulating hormones and metabolic profile (21, 22). We hypothesized that the expression of IDH1 in bovine mammary epithelium

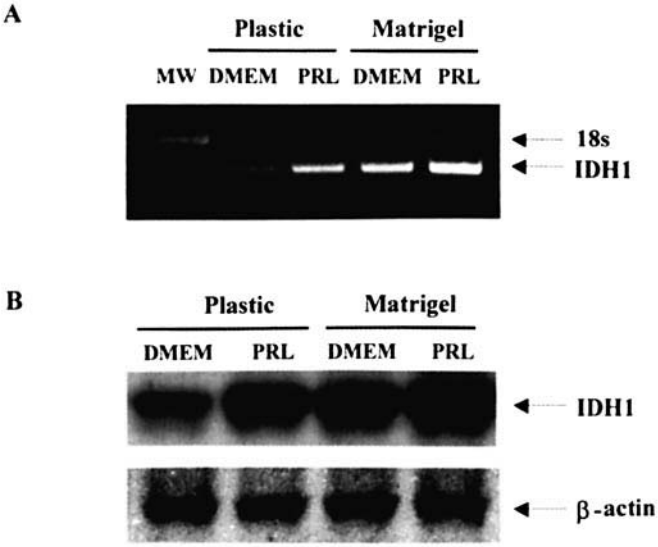

Figure 7. Effects of extracellular matrix and prolactin on IDH1 expression in BME-UV bovine mammary epithelial cells. Cells were cultured on Matrigel-coated or plastic dishes with or without prolactin (PRL) for 72 hrs. Changes in expression of IDH1 were analyzed by (A) RT-PCR and (B) Western blotting. Controls were 18s ribosomal RNA and β-actin.

is modulated by regulators of mammary epithelial differentiation. One objective of the present work was to examine the temporal pattern of IDH1 mRNA expression in bovine mammary tissues during different stages of lactation. We found that IDH1 mRNA levels increased 2.3-fold at 14 days after parturition compared to those measured in mammary tissues collected at 20 days before parturition. These data suggest that the increase in IDH1 enzymatic activity observed in previous studies in lactating bovine mammary glands (14) is likely paralleled by increased expression of IDH1. This may be one mechanism, operating at the onset of lactation, which leads to production of the reducing equivalents required for *de novo* fatty acid synthesis in ruminant mammary tissue (10).

We then used a bovine mammary epithelial cell line (BME-UV) to investigate the roles of regulators of mammary epithelial differentiation on IDH1 expression. For these *in vitro* experiments, we first examined whether

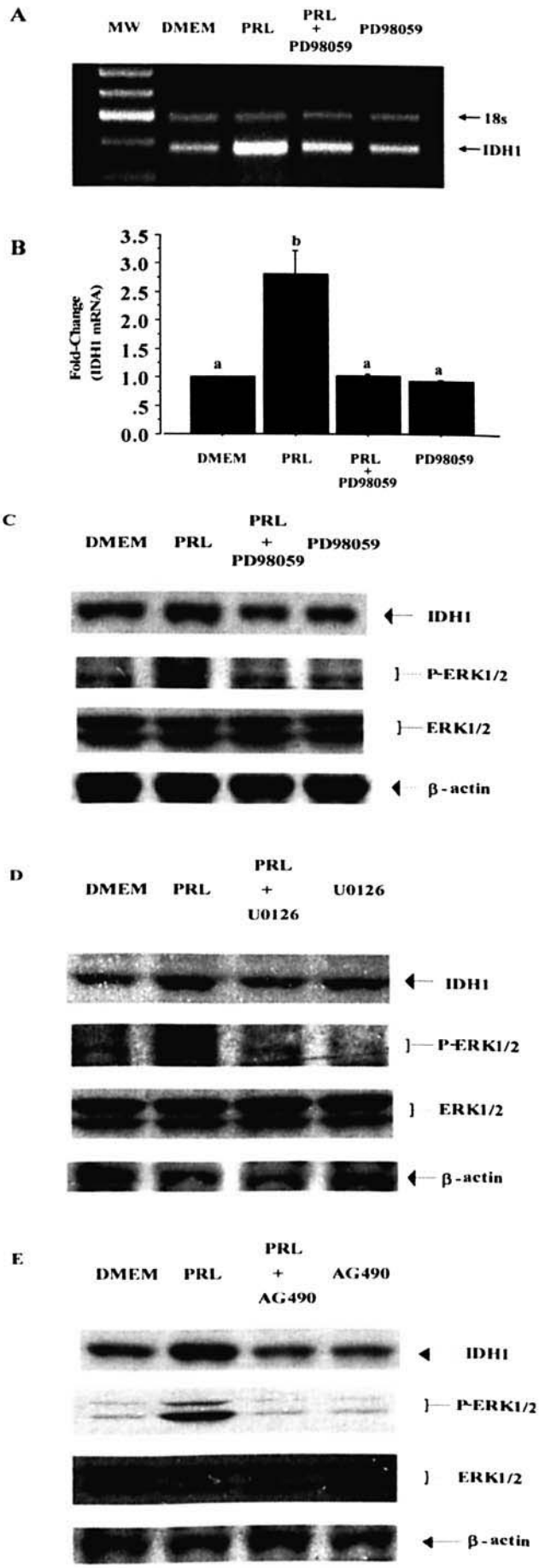

Figure 8. Role of mitogen activated protein kinase (MAPK) pathway in prolactin-dependent regulation of IDH1 expression. IDH1 mRNA levels were measured by (A) semiquantitative RT-PCR, and (B) real-time PCR following treatment of BME-UV cells cultured on plastic with 2 μg/ml prolactin (PRL) for 72 hrs in the presence or absence of the MAPK inhibitor PD98059 (20 μ*M*). In (A) controls were 18s ribosomal RNA. In (B) data represent means ± standard errors from two replicate experiments performed in triplicate. Means without a common letter differ ($P < 0.05$). In (C–E) bands represent Western blotting analysis for IDH1, phosphorylated ERK1/2 (pERK1/2), total ERK1/2 (ERK1/2), and control β-actin in cells treated with PRL in the presence or absence of PD98059 (C), U0126 (D), or the Janus tyrosine kinase 2 (Jak2) inhibitor AG490 (E).

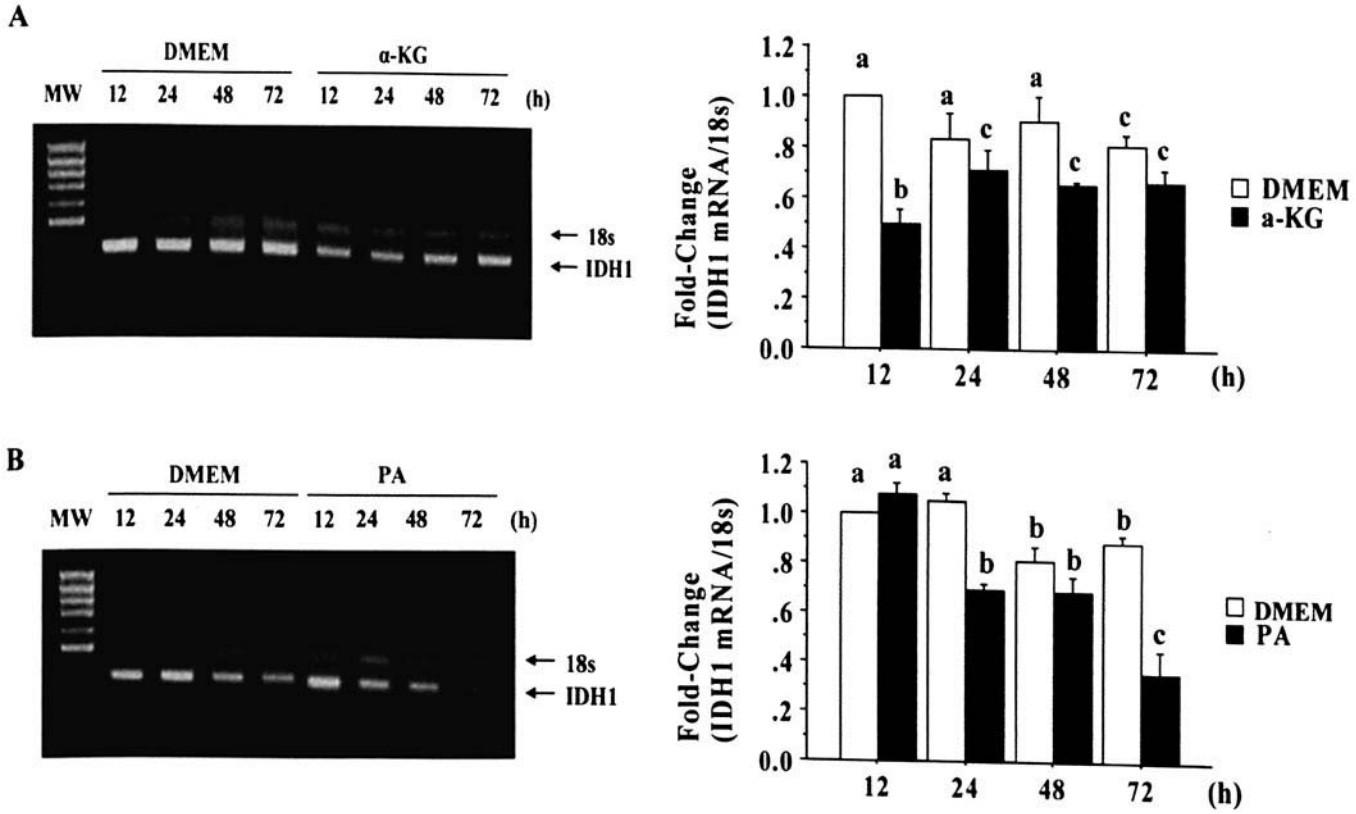

Figure 9. α-ketoglutarate (KG) and palmitic acid (PA) repress IDH1 mRNA levels. BME-UV cells were incubated on plastic dishes with (A) α-KG (0.5 m*M*) or (B) PA (100 μ*M*) for 12, 24, 48, and 72 hrs. At the end of each incubation period, cells were harvested and RT-PCR was performed as described in Materials and Methods. Arrows indicate IDH1 or control 18s ribosomal RNA. Data represent means ± standard errors from two replicate experiments performed in triplicate. Means without a common letter differ ($P < 0.05$).

IDH1 expression was modulated by FCS in cultured BME-UV cells. We found that FCS exerted an inhibitory effect on IDH1 mRNA levels. This finding was consistent with previous reports (23) documenting that IDH1 transcript levels were decreased in human hepatoma HepG2 cells cultured in presence of FCS. These authors speculated that lipids, among other factors contained in FCS, were likely responsible for the repressive effects on IDH1 expression. Therefore, in the current study we used serum-free media to examine the regulation of IDH1 expression.

It has been well established that extracellular matrix is an important regulator of mammary morphogesesis (24–27). Therefore, we examined the effects of extracellular matrix on IDH1 expression in BME-UV cells. We found that in the presence of extracellular matrix, cells formed spherical clusters (data not shown) and the expression of IDH1 was increased in parallel with β-casein expression. These data indicated that plating cells on extracellular matrix was sufficient to induce IDH1 expression. This increase in IDH1 was associated with increased expression of β-casein expression. The combination of extracellular matrix and lactogenic hormones (INS, PRL, and HC) increased further the expression of β-casein. These results were consistent with the notion that both extracellular matrix (28) and hormonal signals (29) activate transcription of β-casein. In contrast, the treatment with the combination of INS, PRL, and HC decreased IDH1 mRNA levels in BME-UV cells plated on extracellular matrix or plastic. Therefore, we investigated the effects of each lactogenic hormone on regulation of IDH1 expression in BME-UV cells cultured on plastic. We found that PRL stimulated IDH1 mRNA levels. The stimulatory effects of prolactin were in keeping with its role in the establishment of lactation (30–33). Previous studies (34–36) documented that PRL increased both the rate of lipogenesis and activity of acetyl-CoA carboxylase, a rate-limiting step in fatty acid synthesis. One possible interpretation of our results is that PRL may activate expression of IDH1 to meet the increased requirements for NADPH necessary for *de novo* fatty acid synthesis during early lactation. To further investigate the role of PRL in regulation of IDH1 expression, we examined mRNA and protein levels in cells cultured on Matrigel-coated or plastic plates. We found that both extracellular matrix and PRL alone induced IDH1 expression. The combination of extracellular matrix and PRL further stimulated IDH1 mRNA and protein accumulation. These results suggest that extracellular matrix or PRL is sufficient to activate IDH1 expression and a positive interaction between these two stimuli may trigger signaling pathways that further enhance the expression of this gene. The effects of signal

transduction pathways activated by extracellular matrix on IDH1 expression need to be further investigated.

Although INS is one of the three hormones required to induce a lactogenic-like response in cultured mammary tissue, its role in regulation of milk lipid synthesis *in vivo* remains controversial (37). The glucogenic-insulin theory proposes that INS stimulates the partition of nutrients to adipose tissue, causing milk fat depression (38). However, results from recent experiments using glucose clamp techniques revealed that infusion with INS had no effect on milk fat yield, but increased milk protein secretion in ruminants (39, 40). We observed that the mRNA levels of IDH1 decreased when bovine mammary epithelial cells were cultured in the presence of INS. One possible interpretation of these data is that by repressing IDH1 expression, INS may exert an inhibitory effect on cytosolic dehydrogenation of isocitrate and NADPH production. Therefore, INS may act as a metabolic switch that reduces the pool of NADPH available for fatty acid synthesis in the cytoplasm, while sparing reducing equivalents that can be used for NADH synthesis in the mitochondria. In turn this may lead to increased carbon flux through the Krebs cycle and production of ATP necessary for growth and protein synthesis. We found that PRL and INS exerted opposing effects on cell proliferation and expression of IDH1. Since both INS and FCS lowered mRNA transcripts for IDH1 in BME-UV cells while stimulating cell proliferation, we concluded that expression of IDH1 may not be compatible with promotion of cell growth.

Glucocorticoids can synergize with PRL to induce milk secretion in many species (41, 42). In our study, HC did not influence IDH1 mRNA levels, suggesting that glucorticoids may not play a key role in regulation of fatty acid synthesis although cortisol plus INS maintained the rate of lipogenesis in cultured mammary explants (35).

The role of MAPK in PRL-mediated effects on gene expression in the mammary epithelium has been the target of several investigations (20, 43–46). However, the signal transduction pathways examined in these studies were those involved in regulation of genes encoding for milk proteins. In the present study, PRL was found to activate ERK1/2 in BME-UV cells. Co-treatment with the MEK inhibitors PD98059 or U0126, which block ERK activation (47), abrogated the ability of PRL to activate IDH1 expression. These data provided evidence that activation of MAPK by PRL contributed to upregulation of IDH1 expression. We further demonstrated that inhibition of Jak2 with AG490 blocked PRL-induced IDH1 expression. Moreover, the treatment with AG490 decreased PRL-induced phosphorylation of ERK, thus confirming an upstream role of Jak2 in activation of the ERK pathway (43, 46).

The concept of metabolite regulation suggests that genes encoding for enzymes of metabolic importance may be regulated, at least in part, by the concentration of specific end products. We found that α-KG reduced IDH1 mRNA levels within 12 hrs, whereas PA, one of the end products of fatty acid synthesis reduced IDH1 mRNA levels at later time points. A possible implication of these data is that the expression of IDH1 may be regulated by α-KG and PA through negative feedback mechanisms. Previous studies in yeast reported that α-KG reduced the enzymatic activity of IDH1 by 60%, confirming a negative role for this metabolic product in oxidative decarboxylation of isocitrate (48). Moreover, our data support previous suggestions that lipids may exert repressive effects on IDH1 expression (23).

In summary, we explored the possible mechanisms involved in regulation of IDH1 expression in bovine mammary epithelium. In the present work, we documented that IDH1 expression increased after parturition in bovine mammary gland and in bovine mammary epithelial cells cultured in the presence of extracellular matrix. Our cell culture data suggest that PRL induces the expression of IDH1, possibly through activation of a MAPK-dependent pathway. Also, we reported that metabolic effectors such as α-KG and PA repressed IDH1 expression. Since IDH1 generates the primary source of reducing equivalents required for *de novo* fatty acid synthesis in bovine mammary gland and approximately 40%–60% of milk fat found in cow milk is synthesized *de novo*, these results further our understanding of the mechanisms involved in the regulation of fatty acid synthesis in bovine mammary gland. We are currently investigating the coordinated effects of differentiation and peptide hormones that have been shown to modulate lactogenesis on regulation of IDH1 expression. Ongoing investigations include evaluation of the roles of growth hormone and insulin-like growth factor-1 (49–52), and the molecular mechanisms through which the IDH1 gene is transcriptionally regulated.

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