GLUCOSE IS AN ESSENTIAL NUTRIENT for milk synthesis in the mammary gland. It is an important energy source and the major precursor of lactose, the primary carbohydrate in milk. In nonruminant animals, glucose is also a key substrate of milk proteins and lipids (37). The glucose used in milk synthesis is taken up from the bloodstream by mammary secretory epithelial cells (MECs), a process that is mediated by facilitative glucose transporters (GLUTs) (37, 39). GLUT1 and GLUT8 are the primary GLUT isoforms expressed in the mammary gland (38, 40). During the onset of lactation from late pregnancy to early lactation, the glucose demand in the mammary gland increases dramatically to support milk synthesis (6, 7, 9). Correspondingly, the mRNA levels of GLUT1 and GLUT8 increase a few hundred-fold and 10-fold, respectively, from 40 days prepartum to 7 days postpartum (39), to accommodate increased glucose demand in the bovine mammary gland.

We originally hypothesized that the increased GLUT expression during the onset of lactation was stimulated by lactogenic hormones: prolactin, glucocorticoids, and insulin. However, our recent study showed that the lactogenic hormone complex did not upregulate GLUT expression in MECs or tissue explants, although they dramatically stimulated the expression levels of milk protein genes and lipogenic genes (25). Therefore, other factors must play roles in the upregulation of GLUT expression in MECs during the onset of lactation.

Oxygen is essential for cell metabolism and efficient energy production in mammals (25). The partial pressure of oxygen (PO2) across the body varies considerably, ranging from ~150 mmHg in the upper respiratory tract (21% oxygen by volume) to as low as 5 mmHg (~1%) in the retina (8). The normal PO2 levels in individual tissues are directly related to their oxygen consumption. When the activity in a tissue is increased and the oxygen demand is greater than the oxygen supply, the oxygen tension in the tissue is reduced, resulting in a local hypoxia. In most tissues, a venous PO2 below 40 mmHg (<6%) triggers hypoxia responses, including increased glucose transport, angiogenesis, and anaerobic glycolysis (8, 26), but the threshold may vary between organs (27). Many of these responses are coordinated by the hypoxia-inducible factors hypoxia inducible factor-1α (HIF-1α) and HIF-1β, which form a heterodimer and bind specifically to hypoxia response elements (HREs) (8, 23). HREs contain the core sequence 5'-RCGTG-3' and are present in the promoter or enhancer regions of target genes, such as the angiogenic factor vascular endothelial growth factor, erythropoietin, GLUT1, and multiple glycolytic enzymes (24). While HIF-1β is not oxygen-responsive, HIF-1α is tightly regulated by oxygen levels, primarily through the stabilization of HIF-1α protein (8).

The mammary gland has a high metabolic rate both during lactation and during its rapid development in the pregnancy stages. The level of mammary O2 uptake has been shown to be increased during late pregnancy and reaches its highest levels during lactation stages (9, 20). The increased O2 uptake during the pregnancy through lactation stages may well result in a chronic local hypoxia. Thus, we hypothesize that the upregulation of GLUT1 and GLUT8 in the mammary gland during the onset of lactation is stimulated by hypoxia. To test this hy-
Detection of hypoxic conditions in the mouse mammary gland. All animal use was approved by the University of Vermont Institutional Animal Care and Use Committee, and all procedures followed the federal guidelines. Hypoxia in the mouse mammary gland was detected using the Hypoxyprobe Plus Kit (Hypoxyprobe, Burlington, MA). Sixteen C57BL/6 female mice at ~3 mo of age (Jackson Laboratory, Bar Harbor, ME) were housed in air- and temperature-controlled cage shelves on a 12:12 h light-dark cycle and were fed rodent diet (Scott Distributing, Hudson, NH) and water ad libitum. Pimonidazole HCl was administered by intraperitoneal injection at a dosage of 60 mg/kg body wt to these mice at virgin (4 mice), midpregnancy (days 10–13, 4 mice), late pregnancy (days 18–20, 4 mice), and early lactation (day 1, 4 mice) stages. The mice were euthanized 90 min after the injection. The thoracic and inguinal mammary glands were collected, fixed in 4% paraformaldehyde, and cut into 10-μm sections using a Cryostat HM 505 N (Microm, Walldorf, Germany) and mounted on gelatin-coated slides. The slides were then immunostained using the antibodies provided by the Hypoxyprobe Plus Kit following the manufacturer’s instructions. Images were taken of the stained mammary gland tissues using a BX50 microscope (Olympus America, Center Valley, PA). Finally, hypoxic areas in the images were quantitated using ImageJ (National Institutes of Health, Bethesda, MD) software. The results are presented as the average percentage of total hypoxic area in the images from three photomicrographs per animal.

Culture of Mac-T cells and primary BMECs. Mac-T [a BMEC line (13)] cells were grown in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 50 μg/ml gentamicin and 5 μg/ml insulin. Primary BMECs were prepared from three individual lactating Holstein cows, as described previously (31) and cultured in the same medium as Mac-T cells. After cells reached 50% confluence (in ~2 days), the old medium was replaced with fresh medium, and the cells were treated with either normal normoxic [control: 95% air (21% O₂) + 5% CO₂] or hypoxia (2% O₂ + 93% N₂ + 5% CO₂).

Cell viability assay. Mac-T cells and primary cells were treated with hypoxia or normoxia for 6, 12, 24, or 48 h. After treatment, the medium was transferred to a 15-ml tube. The cells were detached from the culture dishes by trypsin digestion and combined with the collected medium. Cell viability was measured using a TC10 Automated Cell Counter (Bio-Rad, Hercules, CA).

Glucose uptake assay. Mac-T cells and primary cells were treated with hypoxia or normoxia for 24 h. The cells were detached by trypsin digestion followed by centrifugation at 100 g for 5 min. The cells were then resuspended in 1 ml of PBS (Invitrogen) containing 10 mM of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG, Invitrogen) and incubated at room temperature for 10 min. 2-NBDG is a fluorescently labeled 2-deoxy-D-glucose analog that has been used to monitor glucose uptake in live cells (33–36). After incubation, glucose transport was stopped by adding ice-cold PBS containing 0.1 mM phloretin (Sigma, St. Louis, MO), an inhibitor of facilitative glucose transport. The cells were pelleted by centrifugation at 100 g for 5 min and then resuspended in ice-cold PBS containing 0.1 mM phloretin. The centrifugation and resuspension steps were repeated once, and the cells were kept on ice. The fluorescence in individual cells was measured using a flow cytometer (LSR II flow cytometer; BD Bioscience, San Jose, CA).

Quantitative real-time RT-PCR. RNA was isolated from Mac-T cells and primary cells using the RNaseasy mini kit (Qiagen, Valencia, CA), and RNA concentrations were determined using a NanoDrop spectrophotometer (ThermoScientific, Wilmington, DE). One microgram of total RNA was reverse transcribed to cDNA using the Moloney murine leukemia virus RT (United States Biochemical, Cleveland, OH) following the manufacturer’s protocol. GLUT1 and GLUT8 mRNA expression levels were quantified using the TaqMan Gene Expression Assays with β-actin as an internal control (GLUT1 [Bt03215313], GLUT8 [Bt03217728], and β-actin [Bt03279175]; Applied Biosystems, Carlsbad, CA). HIF-1α mRNA expression was measured by quantitative RT-PCR with the use of SYBR Green (Bio-Rad), in which the primers for HIF-1α were forward 5'-GATCTGGCACCACACCTTCT-3’ and reverse 5’-CCA-GAGGATCATACAGGGACAG-3’ and the primers for HIF-1α were forward 5’-ACTCATCCATGTGACCAGA-3’ and reverse 5’-AGTTTCTCCCCGGGTAGTTA-3’. The real-time PCR reaction, conditions, and calculations were the same, as described previously (25).

RNAi. After reaching 50% confluence, Mac-T cells were transfected with a control siRNA (no. AM4661; Invitrogen) or a custom-designed HIF-1α siRNA (Invitrogen) overnight with the use of Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instruction. The sequence of the HIF-1α siRNA was 5’-GGAGAUGAU- GACUCCACGUUATT-3’ for the sense strand and 5’-UAACUG-GAAGUCUAUCATCAT-3’ for the antisense strand. After 18 h, the transfection medium was replaced with growth medium, and the cells were incubated for an additional 12 h. The medium was then replaced with fresh growth medium, and the cells were treated with normoxia or hypoxia for 12 h. RNA and protein were extracted for qRT-PCR or western blot analysis as described below.

Western blot analysis. After normoxia or hypoxia treatment, cells were washed with ice-cold PBS. The culture dishes (100 mm) were placed on ice, and 1 ml of cytoplasmic extraction buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.6% Nonidet P-40, 0.5 mM PMSF, 5 μg/ml aprotinin and 5 μg/ml leupeptin] was added to each dish. The cells were scraped and transferred to a Dounce homogenizer and placed on ice. The cells were homogenized with 20 strokes on ice followed by centrifugation at 600 g for 5 min at 4°C to separate the nuclear and cytoplasmic fractions. The supernatants were transferred to a 1.5-ml tube, and the debris was removed by centrifugation at 15,000 g for 10 min at 4°C. The resulting supernatant was stored in a −80°C freezer as the cytoplasmic extract. The nuclear pellets from the 600-g centrifugation step were resuspended with nuclear extraction buffer [25% glycerol, 20 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 400 mM NaCl, 0.5 mM PMSF, 5 μg/ml aprotinin and 5 μg/ml leupeptin] and incubated at 4°C for 20 min with rotation. The debris was removed by centrifugation at 15,000 g for 10 min at 4°C. The supernatants containing the nuclear fraction were then transferred to 1.5-ml tubes and stored in a −80°C freezer.

The cytoplasmic and nuclear fractions were resolved on a 12% SDS-polyacrylamide gel and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was blocked with Tris-buffered saline-Tween [TBS-T, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween-20] containing 5% (wt/vol) nonfat dried milk (Bio-Rad) overnight at 4°C, and then incubated with an anti-HIF-1α antibody (Novus Biologicals, Littleton, CO) diluted 1:500 in TBS-T containing 5% (wt/vol) nonfat dried milk for 1 h at room temperature. The membrane was washed 3 times with TBS-T for 5 min at room temperature and then incubated with a goat anti-rabbit IgG conjugated with horseradish peroxidase 1:5,000 diluted in TBS-T containing 5% (wt/vol) nonfat dried milk for 1 h at room temperature, followed by 3 washes with TBS-T for 5 min at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was added onto the membrane and incubated for 5 min, and then the protein bands were visualized using a Gel Doc XR+ System (Bio-Rad).
ELISA. Cytoplasmic extracts were obtained as described above. The GLUT1 protein concentration was measured using an ELISA kit (no. BV2136, Biotang, Lexington, MA) following the manufacturer’s instruction.

Chromatin immunoprecipitation. Mac-T cells were cultured in 150-mm cell culture dishes. After the cells reached 70% confluence, the medium was replaced with 15 ml of fresh medium, and the cells were treated with normoxia or hypoxia for 6 h. Formaldehyde was added to the medium to a final concentration of 1%, followed by 15 min of cross-linking at room temperature. Glycine was then added to the medium to a final concentration of 0.125 M. After a 5-min incubation at room temperature, the dish was placed on ice, the old medium was removed, and the cells were washed 2 times with 20 ml of ice-cold PBS containing a protease inhibitor cocktail (Sigma). After the final wash, 2 ml of ice-cold PBS with protease inhibitors was added to each dish, and the cells were scraped and transferred into a 15-ml tube. Another 2 ml of PBS was added to rinse the dish and then transferred to the same tube. The cells were pelleted by centrifugation at 800 g for 10 min at 4°C, and the supernatant was removed. The cells were resuspended in 1 ml of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.6% IGEPAL CA-630, and protease inhibitors cocktail] and incubated on ice for 15 min, followed by centrifugation at 800 g for 10 min at 4°C. The supernatant was removed, and the cell pellet was resuspended in 500 μg of nuclei lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris·HCl (pH 8.0), and protease inhibitors cocktail]. The DNA was sheared by sonication for 10 s, followed by a 1-min incubation on ice. The sonication was repeated 10 times, and then the debris was removed by centrifugation at 13,000 g for 10 min at 4°C. The supernatant was frozen in liquid nitrogen and stored at −80°C. A total of 50 μl of the sheared chromatin was mixed with 450 μl of ChIP dilution buffer [0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris·HCl (pH 8.0), 167 mM NaCl and protease inhibitors cocktail], and 10 μl of this diluted chromatin was saved at 4°C as an input control. The remaining diluted chromatin was mixed with 10 μl of an anti-HIF-1α antibody (40 μg; Novus Biologicals) or mouse normal IgG (a negative control)

Fig. 1. The mammary gland is under hypoxic conditions at the mid-pregnancy, late-pregnancy, and early lactation stages. A: immuno-histochemical staining (brown color) of hypoxia in the mammary glands of mice at the virgin (V), midpregnancy (MP), late-pregnancy (LP), and early lactation (L) stages. One representative image is shown for each stage. Scale bar = 100 μm. B: quantitative analysis of the percentage areas of hypoxia stained in A. Each mean represents data from three images per animal and four animals per group. Different uppercase letters (A, B) above each bar represent P < 0.01.
and incubated at 4°C overnight with rotation. Then, the chromatin was incubated with 167 µl of Dynabeads Protein A (Invitrogen) for 2 h at 4°C with rotation. The Protein A beads were pelleted with a magnet, and the supernatant was removed. The Protein A beads were then washed with 1 ml each of the ice-cold buffers in the order listed: a low-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl (pH 8.0), and 150 mM NaCl], a high-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl (pH 8.0), and 500 mM NaCl], a LiCl buffer [250 mM LiCl, 1% IGEPAL CA-630, 1% deoxycholic acid, 1 mM EDTA, 20 mM Tris·HCl (pH 8.0)] and TE buffer [10 mM Tris·HCl (pH 8.0), and 1 mM EDTA, and 10 mM Tris·HCl (pH 8.0)] and TE buffer [10 mM Tris·HCl (pH 8.0) and 1 mM EDTA].

Statistical analyses were performed using JMP (SAS Institute, Cary, NC). The means from the control and the hypoxia treatment groups were compared using Student’s t-test. When comparison of three or more treatments was carried out, ANOVA with the Tukey-Kramer honestly significant difference test was used.

RESULTS

The mammary gland is exposed to hypoxic conditions from midpregnancy to early lactation stages. To examine possible hypoxic conditions in the mammary gland during different developmental stages, a hypoxia marker, pimonidazole HCl, was injected into mice at the virgin stage through the early lactation stage. Pimonidazole HCl is a chemical that forms adducts with thiols in proteins, peptides, and amino acids in hypoxic cells (http://www.hypoxyprobe.com/), and these pimonidazole adducts can be detected with specific antibodies. Immunohistochemical analysis showed strong hypoxia staining in the mammary glands of mice at midpregnancy, late pregnancy, and early lactating stages, whereas the staining was barely detectable in the mammary glands of the virgin mice (Fig. 1). Fig. 1A shows representative images of hypoxia staining in the mammary tissues of mice at different developmental stages. Figure 1B shows the percentages of hypoxia-positive area in mammary tissues at each stage. The mean percentages of a hypoxia-positive area in the mammary tissues were significantly higher in the midpregnancy to early lactating stages than in the virgin mice (P < 0.01).

Hypoxia stimulates glucose uptake in MECs. To determine whether hypoxia increases glucose uptake in MECs, Mac-T cells and primary BMECs were treated with 2% oxygen (hypoxia treatment) for 24 h and then incubated with 2-NBDG solution for 10 min. Glucose uptake by individual cells was measured using flow cytometry. As shown in Fig. 2, the glucose uptake was increased 2.1- and 2.5-fold in Mac-T cells and primary BMECs, respectively, following hypoxia treatment (P < 0.05).

Hypoxia does not decrease the viability of MECs. Mac-T cells and primary BMECs were treated with normoxia (control)
or 2% oxygen for 6, 12, 24, or 48 h; in the control group, there were 91, 92, 90, and 87% live Mac-T cells at these time points, respectively, and in the hypoxia treatment group, there were 92, 89, 92, and 86% of cells were alive, and following hypoxia treatment, 90, 90, 88, and 84% of cells were alive after each time point, respectively (Fig. 3). There was no significant difference between the control and the hypoxia treatment groups at any of these time points (Fig. 3).

**Hypoxia increases GLUT1 mRNA and protein expression in MECs.** Mac-T cells and primary BMECs were treated with 2% oxygen for 3, 12, and 24 h, and then the expression level of GLUT1 mRNA was measured by quantitative RT-PCR. Compared with control (normoxia) treatment, hypoxia treatment increased the GLUT1 mRNA expression level 2.3-, 2.6-, and 2.7-fold (P < 0.01) in Mac-T cells (Fig. 4A, left), and 5-, 11-, and 4-fold (P < 0.01) in primary cells (Fig. 4A, right) at 3, 12, and 24 h of treatment, respectively. To determine whether the mRNA response led to a corresponding increase in the GLUT1 protein level, Mac-T cells and primary cells were treated with 2% oxygen for 12, 24, and 48 h, and then the GLUT1 protein levels were measured by ELISA. As shown in Fig. 4B, hypoxia increased the GLUT1 protein level 11% (P < 0.05), 23% (P < 0.01), and 26% (P < 0.01) in Mac-T cells, and 17%, 34%, and 32% (P < 0.01) in primary cells at 12, 24, and 48 h of treatment, respectively. These results suggest that hypoxia increases both GLUT1 mRNA and protein expression in BMECs.

To determine the level of hypoxia needed to elicit the GLUT1 response, Mac-T cells and primary cells were also treated with a range of oxygen concentrations from 2% to 10% for 12 h. Compared with normoxia, treatment with 2% and 5% oxygen increased the GLUT1 mRNA level 2.8- (P < 0.01) and 2-fold (P < 0.05) in Mac-T cells (Fig. 4C, left) and 11- and 3.3-fold (P < 0.01) in primary cells (Fig. 4C, right), respectively, whereas treatment with 10% oxygen had no effect on the GLUT1 mRNA expression level in either cell type (Fig. 4C). The GLUT1 induction in both Mac-T cells and primary cells was also readily reversed by returning the cells to normoxia for 12 h, as shown in Fig. 4D.

Fig. 4. The time and dose effects of hypoxia treatment on the GLUT1 expression levels in Mac-T cells and primary bovine mammary epithelial cells. A: Mac-T cells and primary mammary epithelial cells were exposed to either normoxia (Ctrl, 21% O₂) or 2% O₂ (Hypoxia) for 3, 12, and 24 h and the GLUT1 mRNA expression level was measured using real-time RT-PCR and normalized to the β-actin mRNA level. B: GLUT1 protein levels were measured by ELISA in Mac-T cells and primary mammary epithelial cells treated with either normoxia (Ctrl) or 2% O₂ (Hypoxia) for 12, 24, and 48 h. C: Mac-T cells and primary mammary epithelial cells were treated with 2, 5, or 10% O₂ for 12 h. The bars represent the GLUT1 mRNA expression level in hypoxia-treated cells relative to normoxia-treated cells. D: Mac-T cells and primary mammary epithelial cells were treated with either normoxia (Ctrl) or 2% O₂ (hypoxia) for 12 h and then cultured in normoxia for another 12 h (recovery). The GLUT1 mRNA expression level was measured using real-time RT-PCR. Each mean was from three independent experiments. *P < 0.05; **P < 0.01.
Hypoxia decreases GLUT8 expression in MECs. Mac-T cells and primary BMECs were treated with 2% oxygen for 3, 12, and 24 h, and the expression level of GLUT8 mRNA was measured by qRT-PCR. Compared with the normoxia control, hypoxia treatment decreased the GLUT8 mRNA expression levels by 40% ($P < 0.01$) and 30% ($P < 0.05$) after 12 and 24 h of treatment in Mac-T cells, respectively, and by 50% after both 12 and 24 h in primary cells ($P < 0.01$) (Fig. 5A).

To determine the level of hypoxia needed to elicit changes in GLUT8 expression, Mac-T cells and primary cells were treated with a range of oxygen concentrations (2–10%) for 12 h. Compared with normoxia, treatment with 2% oxygen decreased the GLUT8 mRNA level by 42% in Mac-T cells and 50% in primary cells ($P < 0.01$); treatment with 5% oxygen decreased the GLUT8 mRNA level by 30% ($P < 0.05$) in Mac-T cells and 42% ($P < 0.01$) in primary cells; treatment with 10% oxygen had no effect on the GLUT8 mRNA expression level (Fig. 5B). The decrease in GLUT8 expression in both Mac-T cells and primary cells was also readily reversed by returning the cells to normoxia for 12 h, as shown in Fig. 5C. Thus, unlike GLUT1, GLUT8 expression is downregulated by hypoxia in BMECs.

HIF-1α mediates the upregulation of GLUT1 but not the downregulation of GLUT8 by hypoxia in MECs. Mac-T cells were treated with hypoxia for 3, 12, and 24 h, and then the mRNA levels of HIF-1α were measured by qRT-PCR. As shown in Fig. 6A, the HIF-1α mRNA level was not changed by hypoxia treatment at any time point. The HIF-1α protein level was measured by Western blot after Mac-T cells were treated with hypoxia for 24 h. The results showed that HIF-1α protein was only detected in the nuclei of hypoxia-treated cells (Fig. 6B).

To investigate whether the effects of hypoxia on GLUT1 and GLUT8 expression are mediated by HIF-1α, Mac-T cells were transfected with a HIF-1α siRNA and then cultured under hypoxia conditions for 12 h. As shown in Fig. 6C, the hypoxia-induced HIF-1α protein in the cell nuclei (compare lane 2 to 1) was completely knocked down by the HIF-1α siRNA (compare lane 3 to 2), verifying the effectiveness of the HIF-1α siRNA. Subsequently, the GLUT1 and GLUT8 mRNA levels were measured by qRT-PCR in Mac-T cells treated with both hypoxia and HIF-1α siRNA. As shown in Fig. 6D, the increased GLUT1 mRNA expression in hypoxia-treated cells was completely abolished by the knockdown of HIF-1α. In contrast, the downregulation of GLUT8 mRNA expression by hypoxia in BMECs was not affected by the HIF-1α knockdown.
hypoxia was not changed following HIF-1α siRNA transfection (Fig. 6D).

**Hypoxia-induced HIF-1α binding to an enhancer element of the bovine GLUT1 gene.** An HRE is found at 3.7 kb upstream of the transcription start site of the bovine GLUT1 genes. This sequence shares homology with the sequences in the mouse and rat GLUT1 genes located at 2,788 and 2,785 bp upstream of their transcription start sites, respectively, which have been identified as HIF-1α-bound enhancer elements (5, 10) (Fig. 7A). To examine whether hypoxia induces HIF-1α binding to
this element in BMECs, we performed a ChIP assay in Mac-T cells treated with hypoxia (Fig. 7B). The assay confirmed the binding of HIF-1α to this element in the bovine GLUT1 gene and demonstrated that the binding activity increased 1.8-fold in cells treated with hypoxia compared with normoxia (P < 0.05).

**DISCUSSION**

Pimonidazole HCl has been widely used as a hypoxia marker in preclinical and clinical studies of both normal and malignant tissues (2, 32). In this study, we used this marker to demonstrate for the first time that the mammary gland becomes significantly hypoxic from the midpregnancy stage through the early lactation stage in mice. This observation is consistent with the increase of O2 uptake in the mammary gland during late pregnancy and lactation observed in previous studies in goats (9, 20). The increase of O2 uptake is associated with the increased metabolic rates to support rapid mammary growth, functional differentiation, and lactogenesis (3, 20). Because of the semiquantitative nature of the pimonidazole method, any possible difference in the degree of hypoxia in the mammary gland from midpregnancy to early lactation might not be
including hexokinases (HKs), as in tumor cells (8). An in-
upregulating the expression of several key glycolytic genes,
ular), hypoxia may also promote glycolysis in MECs by
exhibited reduced milk volume and a change in milk compo-
dition. This response would help to accommodate the increased
substrate for lactose synthesis, as well as for protein and lipid
syntheses in nonruminant animals (1). Thus, our results indi-
icate that hypoxia may be a key factor responsible for both the
dramatically enhanced GLUT1 expression and the glucose
uptake observed in the mammary gland from late pregnancy to
early lactation stages (19, 28, 39). This observation is also
detectable by this method. Although our experiment was car-
rried out in mice, the similar results are likely extended to the
bovine mammary gland because dramatically increased O2
uptake was observed in ruminant goat mammary gland during
late pregnancy and early lactation (20, 26). Unfortunately, this
experiment could not be carried out in bovine mammary gland
because of the associated high cost.

We also showed that hypoxia can dramatically increase both
GLUT1 expression and glucose uptake in MECs. These observ-
ations may have significant physiological implications in
mammary development and lactation. The increased O2 con-
sumption during pregnancy through lactation stages results in
chronic local hypoxia, which may, in turn, play an important
role in stimulating glucose uptake in mammary endothelial
cells (17) and MECs, mediated by increased GLUT1 expres-
sion. This response would help to accommodate the increased
 glucose requirements as both an energy source and an essential
substrate for lactose synthesis, as well as for protein and lipid
syntheses in nonruminant animals (1). Thus, our results indi-
cate that hypoxia may be a key factor responsible for both the
dramatically enhanced GLUT1 expression and the glucose
uptake observed in the mammary gland from late pregnancy to
early lactation stages (19, 28, 39). This observation is also
supported by a transgenic study by Seagroves et al. (22), which
found HIF-1α to be a critical regulator of mammary develop-
ment and lactogenesis. In the study by Seagroves et al., mice
with a loss of HIF-1α function specifically in the mammary
gland had lower GLUT1 mRNA expression levels starting from
day 15 of gestation and had impaired mammary secretory
cell differentiation and activation. As a result, these mice
exhibited reduced milk volume and a change in milk compo-
sition.

In the mammary gland (in nonruminant animals, in partic-
ular), hypoxia may also promote glycolysis in MECs by
upregulating the expression of several key glycolytic genes,
including hexokinases (HKs), as in tumor cells (8). An in-
creased glycolysis in MECs may provide more substrates (such
as glucose-6-phosphate, NADPH, α-glycerol, and amino acids)
for mammary syntheses of lactose and other macromolecules.
Because hypoxia is well known for its stimulation of vascular
angiogenesis (8), it is possible that hypoxia may also stimulate
mammary angiogenesis during mammary development and
lactation. However, the vascular system in mammary HIF-1α-
deficient mice showed no differences compared with in wild-
type animals (22). Thus, this evidence supports a model in
which hypoxia regulates mammary development and lactation
through mechanisms other than stimulating mammary angiog-
nogenesis, such as regulating mammary glucose uptake and cell
metabolism.

Hypoxia and HIF-1α have also been shown to induce phos-
phorylation of the transcription factor STAT5 and to enhance
its DNA binding activity in MECs and breast cancer cells (14,
15). STAT5 is well known to play critical roles in mediating
prolactin-induced milk protein synthesis (11, 16, 18, 30). When
prolactin binds to its receptor in MECs, the prolactin receptor
phosphorylates JAK2, which, in turn, phosphorylates STAT5.
The phosphorylated STAT5 then dimerizes and is translocated
to the nucleus, where it stimulates milk protein gene transcrip-
tion (11). STAT5 is also shown to be essential for mammary
gland development and differentiation (12, 29). STAT5-null
mice exhibit impaired lobuloalveolar development and failed
mammary epithelial cell differentiation and milk protein gene
expression (4). Thus, hypoxia may also be involved in mam-
mary development and lactation by regulating STAT5.

Hypoxia can result in the inhibition of oxidative phosphor-
ylation (8). Studies have shown that inhibition of oxidative
phosphorylation can also upregulate GLUT1 expression
through enhancer elements upstream of the transcription start
site that are independent of HIF binding (5, 10). In the mouse
GLUT1 gene, an enhancer region, located ~2.7 kb upstream of
the transcription start site, contains both an HRE and a serum-
responsive element (SRE). The SRE is located ~100 nucleo-

![Fig. 7. Hypoxia treatment enhances HIF-1α binding to the upstream sequence of the bovine GLUT1 gene in mammary epithelial cells. A: Sequence element 3.7 kb upstream of the bovine GLUT1 gene transcription start site is homologous to the identified hypoxia response elements (HREs) of the mouse and rat GLUT1 genes, which are located at 2,788 and 2,785 bp upstream of their transcription start sites, respectively. B: Mac-T cells were treated with either normoxia (Ctrl) or 2% O2 (hypoxia) for 6 h and then underwent chromatin immunoprecipitation using an anti-HIF-1α antibody. The amount of the bovine GLUT1 HRE sequence pulled down by the anti-HIF-1α antibody was analyzed using real-time PCR. Each mean was from three independent experiments. *P < 0.05.](http://ajpregu.physiology.org/doi/fig/10.1152/ajpregu.00093.2014)
tides upstream of the HRE and conveys the responses to mitochondrial inhibitors (10). In the rat GLUT1 gene, an HRE, which responds to hypoxia, is located at 2,785 bp upstream of the transcription start site, whereas a 666 bp sequence at 6 kb upstream of the transcription start site responds to inhibition of oxidative phosphorylation (5). Notably, the induction of GLUT1 expression by low oxygen availability is rapid, while the half-life of GLUT1 mRNA is not affected. In contrast, upregulation of GLUT1 mRNA by inhibition of oxidative phosphorylation has a delayed response, and the GLUT1 mRNA half-life is increased significantly (5). Thus, it is possible that hypoxia may also be able to upregulate GLUT1 expression through the inhibition of oxidative phosphorylation in BMECs. However, our current study showed that HIF-1α is involved in the entire hypoxia-induced GLUT1 expression increase because it can be completely abolished by the knockdown of HIF-1α in the cells. It is also questionable whether hypoxia inhibits oxidative phosphorylation in MECs because the mammary gland has high metabolic rates in late pregnancy and early lactation (20). Nevertheless, identifying whether inhibition of oxidative phosphorylation plays a role in hypoxia regulation of GLUT1 expression in MECs will require further investigation.

Our results showed that hypoxia treatment upregulates GLUT1 but downregulates GLUT8 expression in MECs. These data are consistent with the study by Scheepers et al. (21), in which hypoxia treatment reduced the expression of GLUT8 in 3T3-L1 adipocytes (21). The downregulation of GLUT8 expression was not affected by the siRNA knockdown of HIF-1α protein in contrast to the upregulation of GLUT1 expression. These results indicate that hypoxia may not be responsible for inducing GLUT8 expression during the onset of lactation in the bovine mammary gland (39) and that the expression levels of GLUT1 and GLUT8 are regulated by different mechanisms in the MECs. The specific mechanism by which hypoxia regulates GLUT8 expression remains to be investigated. Although GLUT8 expression was decreased, hypoxia treatment increased the glucose uptake by the cells by more than twofold, suggesting that GLUT1 plays a predominant role in glucose uptake in MECs, as these two transporters are the major GLUTs expressed in the mammary gland and Mac-T cells (38, 39). The role of GLUT8 in mammary glucose utilization remains largely unknown.

Although HIF-1α is clearly required in normal mammary development and lactation, we cannot rule out the possibility that hypoxia per se may not be the only or major stimulus of HIF-1α activity in the mammary gland. In addition to being regulated by hypoxia, HIF-1α stability has been shown to be regulated by other stimuli, such as nitric oxide, reactive oxygen species, nutrient stress, and glycolytic intermediates (26). These stimuli may also play a role in regulating HIF-1α function in the mammary gland.

Perspectives and Significance

In conclusion, the mouse mammary gland is hypoxic from midpregnancy to early lactation stages, and hypoxia increases GLUT1 expression levels through HIF-1 signaling, which results in increased glucose uptake in BMECs. We propose that hypoxia is a key factor in promoting the expression and function of GLUT1 in the mammary gland during the onset of lactation. A further understanding of this regulatory mechanism will help us to increase glucose utilization by MECs for milk synthesis in dairy production and to inhibit glucose utilization by cancer cells in breast cancer treatment and prevention.

GRANTS

This project was supported by the National Research Initiative Competitive Grant no. 2007-35206-18037 from the U.S. Department of Agriculture National Institute of Food and Agriculture (to F.Q. Zhao).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: Y.S., K.M.L., and F.-Q.Z. conception and design of research; Y.S. and T.L.W. performed experiments; Y.S. analyzed data; Y.S., K.M.L., and F.-Q.Z. interpreted results of experiments; Y.S. prepared figures; Y.S. and F.-Q.Z. drafted manuscript; Y.S., T.L.W., K.M.L., and F.-Q.Z. edited and revised manuscript; Y.S., T.L.W., K.M.L., and F.-Q.Z. approved final version of manuscript.

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13. Huynh HT, Robitaille G, Turner JD. Cloning and expression of Stat5 and an additional homologue (Stat5b) in the bovine mammary gland has high metabolic rates in late pregnancy and early lactation (20). Nevertheless, identifying whether inhibition of oxidative phosphorylation plays a role in hypoxia regulation of GLUT1 expression in MECs will require further investigation.

Our results showed that hypoxia treatment upregulates GLUT1 but downregulates GLUT8 expression in MECs. These data are consistent with the study by Scheepers et al. (21), in which hypoxia treatment reduced the expression of GLUT8 in 3T3-L1 adipocytes (21). The downregulation of GLUT8 expression was not affected by the siRNA knockdown of HIF-1α protein in contrast to the upregulation of GLUT1 expression. These results indicate that hypoxia may not be responsible for inducing GLUT8 expression during the onset of lactation in the bovine mammary gland (39) and that the expression levels of GLUT1 and GLUT8 are regulated by different mechanisms in the MECs. The specific mechanism by which hypoxia regulates GLUT8 expression remains to be investigated. Although GLUT8 expression was decreased, hypoxia treatment increased the glucose uptake by the cells by more than twofold, suggesting that GLUT1 plays a predominant role in glucose uptake in MECs, as these two transporters are the major GLUTs expressed in the mammary gland and Mac-T cells (38, 39). The role of GLUT8 in mammary glucose utilization remains largely unknown.

Although HIF-1α is clearly required in normal mammary development and lactation, we cannot rule out the possibility that hypoxia per se may not be the only or major stimulus of HIF-1α activity in the mammary gland. In addition to being regulated by hypoxia, HIF-1α stability has been shown to be regulated by other stimuli, such as nitric oxide, reactive oxygen species, nutrient stress, and glycolytic intermediates (26). These stimuli may also play a role in regulating HIF-1α function in the mammary gland.
REGULATION OF GLUT EXPRESSION BY HYPOXIA IN MAMMARY EPITHELIAL CELLS


