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RETRACTED: High glucose induced HIF-1α/TREK1 expression and myometrium relaxation during pregnancy

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Background: The incidence of gestational diabetes mellitus (GDM) is increasing worldwide. GDM patients have a significantly higher rate of cesarean section and postpartum hemorrhage, suggesting changes in uterine contractility. TWIK-1-related potassium channel (TREK1) expressed in the pregnant uterus and its role in uterine contraction. In this study, we examined the expression of HIF-1α and TREK1 proteins in GDM uterine and investigated whether high glucose levels are involved in the regulation of human uterine smooth muscle cells (HUSMCs) contraction through TREK1, and verified the role of HIF-1α in this process.

Methods: Compared the uterine contractility between GDM and normal patients undergoing elective lower segment cesarean section. The HUSMCs were divided into normal glucose group, high glucose group, normal glucose with CoCl2 group, CoCl2 with echinomycin/L-Methionine group, and high glucose with echinomycin/L-Methionine group; Compare the cell contractility of each group. Compared the expression of hypoxia-inducible factor-1α (HIF-1α) and TREK1 protein in each group.

Results: The contractility of human uterine strips induced by both KCl and oxytocin was significantly lower in patients with GDM compared with that in normal individuals, with increased TREK1 and HIF-1α protein expression. The contractility of cultured HUSMCs was significantly decreased under high glucose levels, which was consistent with increased expression of HIF-1α and TREK1 proteins. The contractility of HUSMCs was decreased when hypoxia was induced by CoCl2 and increased when hypoxia was inhibited by echinomycin. The TREK1 inhibitor L-methionine also recovered the decreased contractility of HUSMCs under high glucose levels or hypoxia.

Discussion: The high glucose levels decreased the contractility of the myometrium, and increased expression of HIF-1a and TREK1 proteins play a role in changes in uterus contractility.

KEYWORDS

gestational diabetes mellitus, myometrium contractility, human uterine smooth muscle cells, HIF-1α, TREK1

1 Introduction

Gestational diabetes mellitus (GDM) is defined as diabetes diagnosed in the second or third trimester of pregnancy, which is not overt diabetes prior to gestation (1). GDM is one of the most common pregnancy complications, and the prevalence of GDM is increasing globally (2).

Several studies have shown that the rate of cesarean section and postpartum hemorrhage in patients with GDM is significantly higher than that in women without GDM (3–6), and more than 30% of cesarean sections in patients with GDM are due to failed vaginal birth (7). Failed vaginal delivery trial and postpartum hemorrhage are associated with uterine atony (8, 9), suggesting that GDM affects uterine contractility. An *in vitro* trial of isolated uteri in patients with diabetes confirmed that high glucose levels caused poor uterine contractility (10). In a non-pregnant murine model of non-obese type-1 diabetes mellitus, isolated uteri isometric contraction showed a significant reduction in spontaneous motility and hypo-contractility compared with controls (11). Telma et al. (12) found that the myometrium had a reduced number and irregular arrangement of myogenic fibers and decreased contractility in pregnant female rats with diabetes compared with controls. These results indicate that hyperglycemia may affect myometrial contractility of pregnant and nonpregnant uteri. Although these studies indicate that diabetes reduces uterine contractility, the underlying mechanisms remain unclear.

Uterine smooth muscle cells are stimulated by specific signals to cause membrane depolarization and generate action potentials, which trigger electro-mechanical coupling, leading to cell contraction. The TWIK-1-related potassium channel (TREK1) is a double-pore potassium channel protein expressed in human uterine tissue, which plays an important role in potassium efflux and maintaining the resting potential of smooth muscle cells; TREK1 is regulated by temperature, PH, stretching, arachidonic acid, L-methionine, progesterone, and other factors (13, 14). We previously demonstrated that the incubation of human pregnant myometrial strips with the TREK1 activator arachidonic acid significantly reduced myometrial strip contractility, whereas incubation with the TREK1 inhibitor L-methionine significantly enhanced myometrial strip contractility, confirming the importance of TREK1 in regulating uterine contractility (15–17). Several studies (15, 16) have shown that TREK1 expression is altered in the pregnant uterus and that this change is associated with changes in the contractility of pregnant uterine tissue (18). However, it is unknown whether the expression of TREK1 protein changes in GDM uterine tissues and whether the glucose-related changes in myometrium contractility are also associated with TREK1.

Studies comparing patients with diabetes and individuals without diabetes have reported significantly lower contractility of the myocardium, vascular smooth muscle, and gastrointestinal smooth muscle among patients with diabetes, with an increased incidence of heart failure (19), decreased ventricular systolic function (20), impaired vascular smooth muscle contraction (21, 22), and gastroparesis (23). Tissue or cell injury caused by diabetes was found to be associated with an increased expression of hypoxia-inducible factor (HIF) triggered by glycemia (24–27). HIFs are nuclear factors that reflect the degree of tissue or cell hypoxia and consist of alpha and beta subunits, which include mainly HIF-1 and HIF-2 (28, 29). HIF-1α plays a central role in hypoxia and regulates many targets, promoting erythropoietin, cell proliferation, and angiogenesis for tissue or cells to respond to hypoxia (29–32). HIF-1α is rapidly degraded by hydroxylation under proline hydroxylase, and $CoCl_2$ can prevent this hydroxylation (28, 33). Lim et al. (34) showed that upregulated expression of HIF-1α in vascular smooth muscle leads to reduced contractility of vascular smooth muscle and that vascular smooth muscle strips incubated with the HIF-1α inhibitors echinocandin and U0126 restored vascular smooth muscle contractility. In uterine tissue, Alotaibi reported significantly decreased contractility in hypoxic myometrium (35). Osman et al. (36) found that the uterine contractility was significantly reduced after incubation with cyanide to induce hypoxia, and the contractility was restored when the uterine was removed from the cyanide environment. Studies have shown that cyanide induced the nuclear accumulation of HIF-1α (37). Eun et al. (38) found that HIF-1α mRNA and protein overexpression significantly increased TREK1 mRNA and protein expression in primary astrocytes using $CoCl_2$. Astrocyte elevated gene-1 (AEG-1) is a major mediator of hypoxia-regulated TREK1 expression in astrocytes, and HIF-1α binds directly to the AEG-1 promoter. AEG-1 knockdown dramatically decreased the mRNA and protein levels of TREK1, suggesting that TERK1 is regulated by HIF-1α. However, the expression of HIF-1α protein in uterine tissues of GDM and the association between HIF-1α protein changes and changes in uterine contractility in patients with GDM are unclear.

In this study, we examined the expression changes of HIF-1α and TREK1 proteins in uterine tissues of patients with GDM, investigated whether high glucose levels are involved in the regulation of uterine smooth muscle contraction during pregnancy through TREK1, and verified the role of HIF-1α in this process.

2 Materials and methods

2.1 Ethics statement

The study was reviewed and approved by the First Affiliated Hospital of Anhui Medical University Ethics Committee for the Protection of Human Subjects in Research and Tissue Collection (PJ2020-06-12). Uterine tissues were collected from pregnant women undergoing elective lower segment cesarean section at the First Affiliated Hospital of Anhui Medical University, Hefei, China. All participating women provided written informed consent to participate in the study.

Abbreviations: AEG-1, astrocyte elevated gene-1; AUC, area under the curve; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; GDM, gestational diabetes mellitus; HIF-1α, hypoxia-inducible factor-1 alpha; HUSMCs, human uterine smooth muscle cells; miRNA/miR, microRNA; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; SEM, standard error of the mean; TREK1, TWIK-1-related potassium channel.

2.2 Tissue collection

Diagnosis of GDM was made according to the Chinese Society of Obstetrics and Gynecology and the Chinese Medical Association consensus (39). A 75-g oral glucose tolerance test was performed between the 24th and 28th weeks of gestation. The values meeting the diagnostic criteria for GDM were as follows: fasting plasma glucose ≥5.1 mmol/L (92 mg/dL), 1-h plasma glucose ≥10.0 mmol/L (180 mg/dL), and 2-h plasma glucose ≥8.5 mmol/L (153 mg/dL). As previously described (40), patients aged 18 years or older with a singleton pregnancy, vertex presentation, and GDM diagnosis were included. The following exclusion criteria were applied: gestational age at birth of less than 35 weeks, patients with GDM with uncontrolled blood glucose levels, multiple pregnancies, placenta previa, scarred uterus, and medical/surgical comorbidity as an indication for cesarean section. All gestational ages were verified using the last menstrual period and confirmed using the first-trimester sonographic measurement of crown-rump length.

After safe delivery of the fetus and placenta by elective lower segment cesarean section, tissue specimens from the lower uterine margin were resected and immediately placed in a cryopreservation incubator in a refrigerated Krebs solution for transport to the laboratory. The uterine tissue was trimmed to a 7 mm × 3 mm muscle strip, and contraction of the uterine muscle strip was measured. Since oxytocin is synthesized in the decidual tissue immediately adjacent to the myometrium, it was necessary to separate the decidual tissue from the surface of the myometrial strip before measuring the contractility of the myometrium. The uterine tissue was trimmed to 3 mm × 3 mm × 3 mm segments for western blot analysis.

2.3 Measurement of uterine contraction

The method for the measurement of uterine contraction has been previously described (15). Briefly, the uterine muscle strips are fixed to the constant temperature bath and multi-channel physiological signal acquisition and processing system while continuously ventilated with 95% O_2 and 5% CO_2 at 37.0 ± 0.5°C. Our previous study has confirmed that uterus strip contractility performance is most appropriate at a 2-g stretch (15). After the appearance of a stable and regular contraction curve, the strips were stimulated with 96 mM KCl and different concentrations of oxytocin (from 10^{-11} to 10^{-6} mM), and the contraction response was recorded. Finally, the uterine muscle strips were weighed at the end of the experiment for calibration.

Quantitative analysis of the contractility of the uterine muscle strips was performed by a multichannel physiological signal acquisition system (RM6240E, Chengdu, China) by calculating the area under the curve (AUC) of the contraction curve presented as AUC/g tissue weight. The AUC was measured at time 0 and was subtracted from the AUC measured after 5 min of application of KCl or each oxytocin concentration.

2.4 Cell culture

Isolation of primary human gestational uterine smooth muscle cells was achieved by the enzymatic dispersion method. Myometrium was obtained from women after elective cesarean delivery in late pregnancy. The endometrium and epithelium were slightly scraped off the surface of the myometrium with a sterile blade, and the myometrium was then cut up with tissue scissors. The cut uterine tissue was digested using 15 ml of digestion solution, followed by shaking for 1 h at 37°C on a shaker. Then, the digested solution was filtered through a 100-μm filter to remove the tissue fragments, and the filtrate was transferred to a sterile centrifuge tube and centrifuged at 1000 rpm/min for 5 min before discarding the upper layer. The cell precipitate was resuspended in normal glucose medium, and the cell suspension was placed in a 25 cm^2 cell culture flask (Corning) and incubated at 37°C, 5% CO_2 incubator until fusion.

2.5 Cell contraction assay

According to the protocol (Cell Contraction Assay Kit, Cell Biolabs, San Diego), the collagen solution, 5× phosphate-buffered saline (PBS), and neutral solution were mixed and diluted in proportion and then placed on ice. The number of uterine smooth muscle cells was adjusted to 800,000 cells/100 μl cell suspension. The cell suspension and diluted collagen solution were mixed (1:4) to configure the gel, and the gel was added to a 24-well plate at 500 μl/well and then incubated for 1 h in an incubator at 37°C to allow the gel to solidify. The gel was divided into 7 groups: normal glucose group, high glucose group, normal with $CoCl_2$ group, $CoCl_2$ with echinomycin group, high glucose with echinomycin group, $CoCl_2$ with L-methionine group, and high glucose with L-methionine group. After collagen solidification of the first three groups, they were cultured in the corresponding medium. The gels of the latter four groups were first incubated in a $CoCl_2$ medium or high glucose medium for 4 h, then changed to the corresponding medium containing echinomycin or L-methionine, and incubated for 4 h. KCl and oxytocin were added to every group at 4 h after gel solidification. The gel areas of each group were observed and recorded with a camera at the time of solidification (0 h) and the addition of KCl/Oxytocin at 4 h; the areas were measured using Image J.

2.6 Western blot analysis

Total protein was extracted with RIPA lysis buffer containing benzoyl fluoride and phosphatase inhibitors (Beyotime Biotechnology, China) from uterine muscle strips. The supernatant was collected by centrifugation at 4°C and 12,000 rpm/min for 10 min. The BCA protein assay was used to determine the total protein concentration. The supernatant and loading buffer were mixed (1:4) and heated at 100°C for 10 min. Protein homogenates were electrophoresed on 10% SDS (sodium dodecyl sulfate) polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated in PBS-Tween buffer containing 5% skim milk for 2 h to block non-specific sites and then incubated overnight at 4°C in primary antibody solution containing the

monoclonal mouse antibody to GAPDH (1:5000, Abcam, England) or the monoclonal rabbit antibody to TREK1 (1:200, Sigma-Aldrich, American) or HIF-1α (1:500, Abcam, England). The PVDF membranes were then washed 3 times in PBS-Tween for 15 min each and then incubated with horseradish peroxidase-coupled goat anti-rabbit secondary antibody (1:10,000, Abcam, England) or goat anti-mouse secondary antibody (1:10,000, Abcam, England) for 2 h. The membrane blots were washed with PBS-Tween and visualized by enhanced chemiluminescence (ECL, Biosharp, China). A band of 37 kDa for GAPDH, 110 kDa for HIF-1α, and 47 kDa for TREK1 was detected according to the corresponding protocols of the antibody products and were confirmed by the Marker. GAPDH was used as the internal control. Reaction bands corresponding to GAPDH, TREK1, and HIF-1α were analyzed with Image J.

2.7 Solutions and drugs

The composition of all solutions in this study is summarized as follows. Normal Krebs solution contained the following (in mM): 120 NaCl, 5.9 KCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 11.5 dextrose, 2.5 $CaCl_2$, 1.2 $MgCl_2$ (Biosharp, China). High KCl solution (96 mM) was prepared as normal Krebs but with equimolar substitution of NaCl with KCl. Oxytocin (MedChemExpress, China) was dissolved in deionized water to prepare 10^{-11} to 10^{-6} M concentration for isometric contractions. Digestion solution was prepared: 2 mg/ml type II collagenase, 1 mg/ml BSA, and 0.5 mg/ml deoxyribonuclease I was dissolved in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, American). The normal glucose medium consisted of DMEM with 5.5 mmol/L glucose, 10% fetal bovine serum (FBS) (Sigma-Aldrich, American), and 1% penicillin/streptomycin (Gibco, American) solution. The high glucose medium consisted of DMEM with 25 mmol/L glucose, 10% FBS, and 1% penicillin/streptomycin solution. $CoCl_2$ medium was prepared in normal glucose containing 200 μmol/L $CoCl_2$ (Sigma-Aldrich, American). High glucose with echinomycin medium was prepared in high glucose containing 10 nmol/L echinomycin (MedChemExpress, China). $CoCl_2$ with echinomycin medium was prepared in $CoCl_2$ medium containing 10 nmol/L echinomycin. $CoCl_2$ with L-methionine medium was 10 μmol/L L-methionine (Sigma-Aldrich, American) dissolved in $CoCl_2$ medium. High glucose with L-methionine medium was 10 μmol/L of L-methionine dissolved in high glucose medium. High KCl (96 mM) and oxytocin (10^{-7} M) were dissolved in the respective medium for cell contractions.

2.8 Statistical analysis

All data were analyzed and presented as mean ± standard error of the mean (SEM) using Prism (v.8.01; GraphPad Software, San Diego, CA), with the "n" value representing the number of subjects. For uterine contraction experiments, individual concentration-contraction curves were constructed, and sigmoidal curves were fitted to the data using the least squares method. Data were first analyzed using the analysis of variance with multiple classification criteria between the normal and high glucose groups. When a statistical difference was observed, the data were further analyzed using Bonferroni's *post-hoc* test for multiple comparisons. Unpaired Student's t-test was used for the comparison of two means. Differences were considered significant if $P<0.05$.

3 Results

3.1 Uterine contractility decreased in patients with GDM

High concentrations of 96 mM KCl cause depolarization of cell membranes and stimulate Ca^{2+} influx through voltage-gated Ca^{2+} channels (41). Normal human pregnancy uterine strips respond rapidly to KCl stimulation, with contractility peaking rapidly and then decreasing but remaining at a high level. Uterine strips in patients with GDM also respond rapidly to KCl, although the peak contractility is lower than normal. The sum of contractility produced by KCl stimulation of the uterine muscle strips at 5 min (AUC, 5 min) was also significantly lower ($P<0.05$) in patients with GDM than in normal individuals (Figures 1A, C).

The contractility of uterine muscle strips in normal individuals and patients with GDM responds to oxytocin in a concentration-dependent manner, reaching a maximum at 10^{-7} M. The contraction curve of the muscle strips induced by oxytocin shows a cyclic oscillation, with an increase in contraction frequency and peak contractility with increasing oxytocin concentration. The peak and sum of uterine strip contractility (AUC, 5 min) induced by all concentration subgroups showed that GDM was weaker than normal (Figures 1B, D, E) ($P<0.05$).

3.2 Increased expression of HIF-1α and TREK1 protein in the uterus of pregnant women with GDM

We extracted total proteins from pregnant uterine tissues for western blot analysis and detected a band corresponding to HIF-1α at the 110 kDa and TREK1 at the 47 kDa position. Western blot analysis showed that HIF-1α ($P<0.05$) and TREK1 ($P<0.05$) protein levels were significantly higher in the uterine tissues of patients with GDM than in those of normal individuals (Figures 2A, B).

3.3 High glucose levels decreased cell contraction and increased protein expression of HIF-1α and TREK1 in human uterine smooth muscle cells

To further confirm the modulation of contractility of human uterine smooth muscle cells (HUSMCs) with increased glucose levels, we performed cell-collagen contraction experiments and

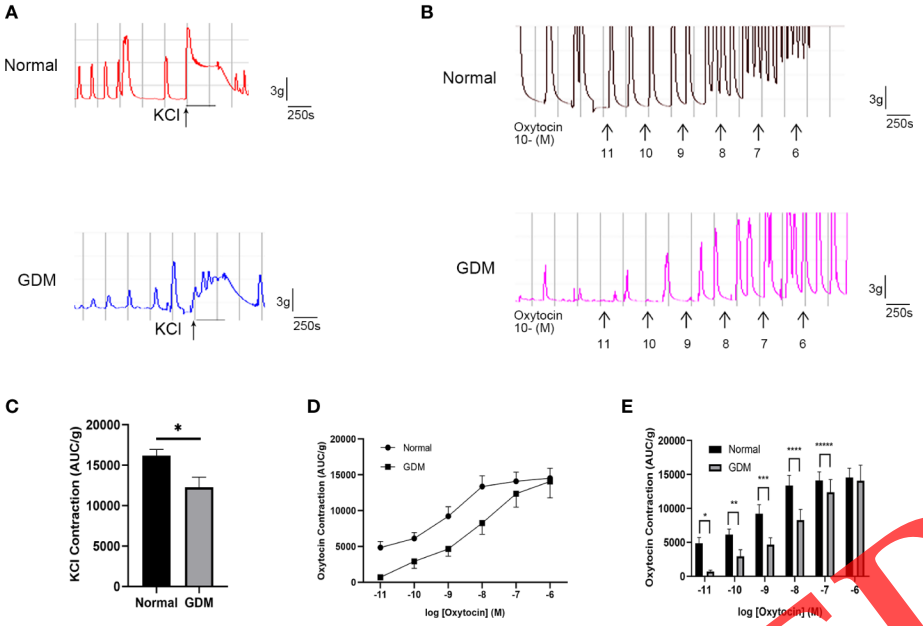

FIGURE 1
Decreased uterine contractility in patients with GDM. Uterine strips from normal (Nor) individuals and patients with GDM were stimulated with 96 mM KCl **(A)** followed by washing 3 times in Krebs solution and then stimulated with increasing concentrations (10^{-11} to 10^{-6} M) of oxytocin **(B)**. The contraction to KCl **(C)** and oxytocin **(D, E)** was measured as AUC/g tissue. Data are presented as means ± SEM, n = 6 to 7. * to ***** mean GDM vs. Nor and P<0.05. AUC, area under the curve; GDM, gestational diabetes mellitus; SEM, standard error of the mean.

divided the gels into the normal glucose group and the high glucose group. The total proteins of HUSMCs in the normal glucose and high glucose groups were extracted and analyzed for HIF-1α and TREK1 by western blot.

We observed the gels of both normal glucose and high glucose groups after stimulation with mM KCl or 10^{-7} M oxytocin for 4 h; the gel area in the normal glucose group was significantly smaller (P<0.05) than that in the high glucose group (Figures 3A, B), indicating that the normal glucose group has greater cell contractility. Western blot analysis of HUSMCs showed that high glucose levels significantly increased (P<0.05) HIF-1α (Figure 3C) and TREK1 (Figure 3D) protein expression.

3.4 Induced cell hypoxia decreased the contraction and increased TREK1 protein expression in HUSMCs

High glucose levels cause increased HIF-1α/TREK1 expression and decreased cell contractility. $CoCl_2$ was used to induce cell

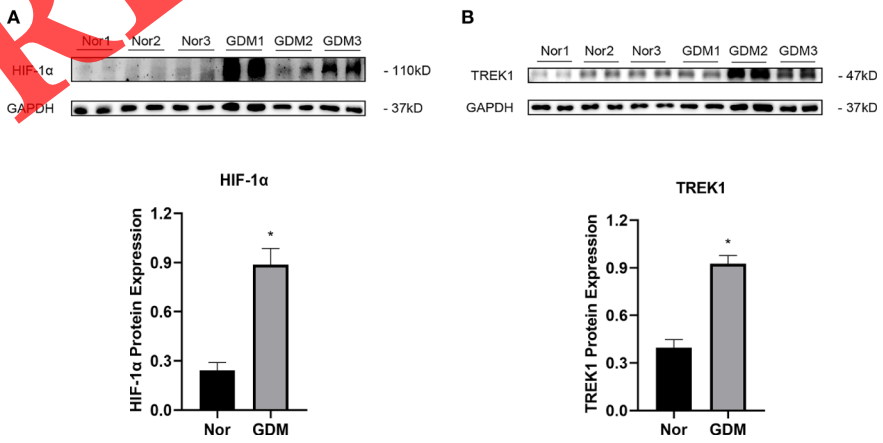

FIGURE 2
Expression of HIF-1α and TREK1 protein increased in the uterine tissues of patients with GDM. Uterine tissues from normal (Nor) individuals and patients with GDM. The total protein of each group was extracted, and HIF-1α **(A)** and TREK1 **(B)** protein expression was measured by western blot analysis. Data are presented as means ± SEM, n = 6 to 7. * means GDM vs, Nor and P<0.05. GDM, gestational diabetes mellitus; HIF-1α, hypoxia-inducible factor-1 alpha; SEM, standard error of the mean; TREK1, TWIK-1-related potassium channel.

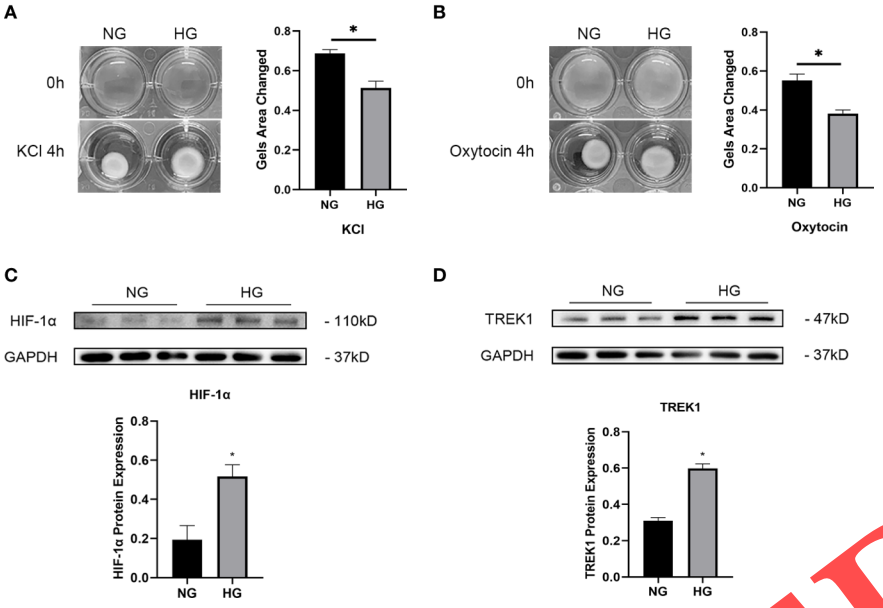

FIGURE 3
High glucose level decreased cell contraction and increased protein expression of HIF-1α and TREK1 in HUSMCs. HUSMCs mixed with collagen were cultured in normal glucose (5.5 mmol/L) (NG) medium and high glucose (25 mmol/L) (HG) medium in a 24-well culture plate. The medium was added after collagen polymerization (0 h). The images of collagen stimulated by KCl **(A)** or oxytocin **(B)** for 4 h. The contraction of HUSMCs was assessed by measuring the mean gel area change ([the area of KCl 4 h − the area of 0 h]/the area of 0 h and [the area of oxytocin 4 h − the area of 0 h]/the area of 0 h). The HUSMCs of each group were cultured in NG medium at the beginning, and the HG group was replaced with HG medium for 24 h when the cell density reached 60%–70%. Then, the total protein of each group was extracted, and HIF-1α **(C)** and TREK1 **(D)** protein expression was measured by western blot analysis. Three or four independent experiments were conducted, and the average of each group was calculated. Data are presented as means ± SEM, n = 4 for each group. * means HG vs. NG and $P<0.05$. HIF-1α, hypoxia-inducible factor-1 alpha; HUSMCs, human uterine smooth muscle cells; SEM, standard error of the mean; TREK1, TWIK-1-related potassium channel.

hypoxia to determine whether the decrease in cell contractility was related to hypoxia. We divided the HUSMCs into the normal glucose group and the normal glucose with $CoCl_2$ group. In collagen contraction, following incubation with KCl or oxytocin for 4 h, the gel area of the normal glucose group was significantly smaller ($P<0.05$) than that of the normal glucose with $CoCl_2$ group (Figures 4A, B). Western blot analysis showed that normal glucose levels with $CoCl_2$ induced cell hypoxia, which was detected as increased HIF-1α protein expression ($P<0.05$) (Figure 4C). This induced hypoxia also significantly increased ($P<0.05$) TREK1 protein expression (Figure 4D).

3.5 Hypoxia inhibition recovered the decreased cell contractility under high glucose conditions

Echinomycin was used to inhibit HIF-1α. High glucose levels induced cell hypoxia and increased HIF-1α. We used echinomycin to inhibit HIF-1α and detected KCl- or oxytocin-induced cell contraction for 4 h. We divided the HUSMCs into the normal glucose group, the high glucose group, and the high glucose with echinomycin group. The gel area was significantly smaller ($P<0.05$) when echinomycin was used in the high glucose group compared with that obtained without echinomycin (Figures 5A, B), indicating a recovery of cell contractility that had decreased with high glucose levels. Echinomycin inhibited cell hypoxia, decreasing HIF-1α and TREK1 expression ($P<0.05$) (Figures 5C, D).

3.6 Hypoxia inhibition recovered the $CoCl_2$-decreased cell contractility in HUSMCs

$CoCl_2$-induced hypoxia decreased cell contractility and increased ($P<0.05$) the HIF-1α and TREK1 expression. Echinomycin was then used to inhibit HIF-1α expression, and gels were incubated with KCl or oxytocin for 4 h. We divided the HUSMCs into the normal glucose group, the normal glucose with $CoCl_2$ group, and the normal glucose + $CoCl_2$ with echinomycin group. We observed that the gel area in the echinomycin group was significantly smaller ($P<0.05$) than without echinomycin (Figures 6A, B), indicating that the contractility of HUSMCs was significantly recovered by echinomycin after hypoxia. The HIF-1α and TREK1 protein expression variations were consistent (Figures 6C, D), which increased ($P<0.05$) under hypoxia and decreased when hypoxia was inhibited.

3.7 TREK1 inhibitor L-methionine increased HUSMC contractility that was decreased by high glucose levels or hypoxia

High glucose levels and hypoxia induced TREK1 protein expression of HUSMCs and decreased cell contractility. To check whether the TREK1 variation contributed to contractility change, we used L-methionine to inhibit the TREK1 function. We divided

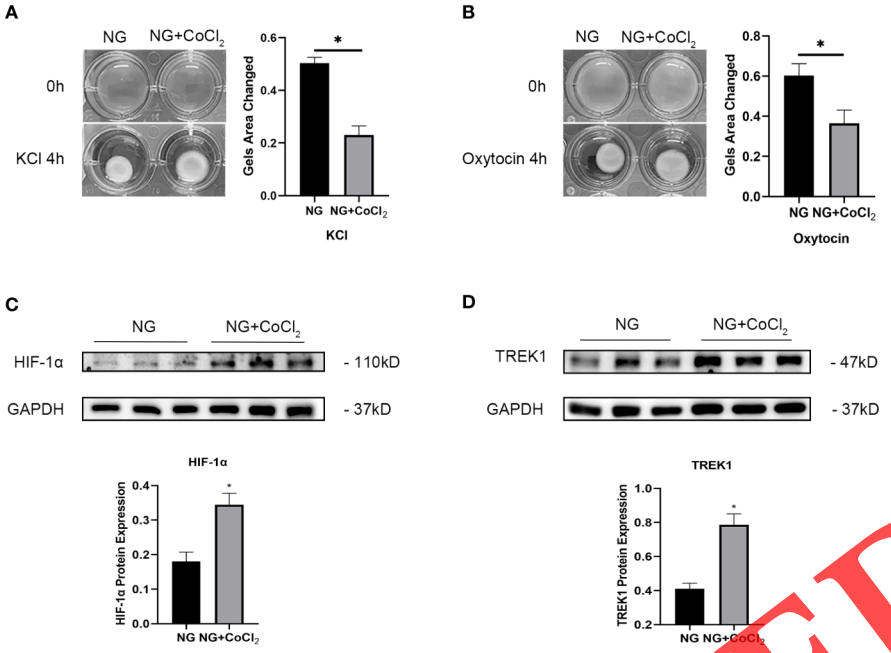

FIGURE 4
Induced cell hypoxia decreased the contraction and increased TREK1 protein expression in HUSMCs. HUSMCs mixed with collagen were cultured in normal glucose (5.5 mmol/L) (NG) medium and normal glucose with $CoCl_2$ medium (NG+$CoCl_2$) which induced hypoxia in a 24-well plate. The medium was added after collagen polymerization (0 h). The images of collagen stimulated by KCl **(A)** or oxytocin **(B)** for 4 h. The contraction of HUSMCs was assessed by measuring the mean gel area change. The HUSMCs of each group were first cultured in NG medium, and the NG + $CoCl_2$ group was replaced with NG + $CoCl_2$ medium for 24 h when the cell density reached 60%–70%. Then, the total protein of each group was extracted, and HIF-1α **(C)** and TREK1 **(D)** protein expression was measured by western blot analysis. Data are presented as means ± SEM, n = 4 for each group. * means NG+$CoCl_2$ vs. NG and P<0.05. HIF-1α, hypoxia-inducible factor-1 alpha; HUSMCs, human uterine smooth muscle cells; SEM, standard error of the mean; TREK1, TWIK-1-related potassium channel.

the gels into the normal glucose group, the high glucose group, and the high glucose with L-methionine to check the function of TREK1 in a high glucose environment. We then divided the gels into the normal glucose group, the normal glucose with $CoCl_2$ group, and the normal glucose group + $CoCl_2$ with L-methionine group to check the function of TREK1 in the $CoCl_2$ environment. After KCl or oxytocin was added to the gel for 4 h, the gel area of the high glucose with L-methionine group (Figures 7A, B) and the $CoCl_2$ with L-methionine group (Figures 7C, D) was significantly smaller (P<0.05) than that of the high glucose group and the normal glucose with $CoCl_2$ group.

4 Discussion

Our study showed that 1) the hyperglycemic environment in patients with GDM causes a significant decrease in uterine contractility in late pregnancy and increases HIF-1α/TREK1 protein expression and that 2) hyperglycemia promotes hypoxia in HUSMCs, causing increased TREK1 expression and decreased HUSMCs contractility.

Patients with GDM have a significantly higher incidence of prolonged labor, cesarean section, and postpartum hemorrhage than women without GDM, and this may be associated with abnormal uterine contractility caused by hyperglycemia (3–6). However, previous studies (11, 42–46) on the regulation of uterine contractility by diabetes have reported inconsistent findings. In diabetic animal models, uterine tissues of non-pregnant diabetic rats or mice induced by KCl or oxytocin produced significantly weaker contractility than normal rats or mice (11, 42, 43). The differences in contractility between diabetic pregnant and normal rats vary considerably during different gestation periods. The contractility of uterine tissue in diabetic rats did not differ from normal rats at 22 days of gestation (44, 45), significantly increased at 15 days of gestation (45), and significantly decreased at 10 days of gestation (46). The differences in uterine contractility changes in diabetic animal models have been observed in isolated human uterine tissues by *in vitro* experiments. Sarioglu et al. (47) found no difference in spontaneous uterine contractility between patients with GDM and normal individuals, but the study did not compare uterotonic-induced uterine contractions. Al-Qahtani et al. (10) found that spontaneous, KCl-induced, and oxytocin-induced uterine contractility were significantly weaker in patients with GDM and concluded that reduced uterine contractility was associated with reduced calcium channel expression, intracellular calcium signaling, and decreased muscle mass. These results are consistent with our present data. In 96 mM KCl- and 10^{-7} M oxytocin-induced uterine contractions, the contractility was significantly weaker in patients with GDM than in normal individuals. We also found that HUSMCs contractility decreased in an environment with high glucose levels, showing consistent outcomes with those observed in GDM uterine tissues.

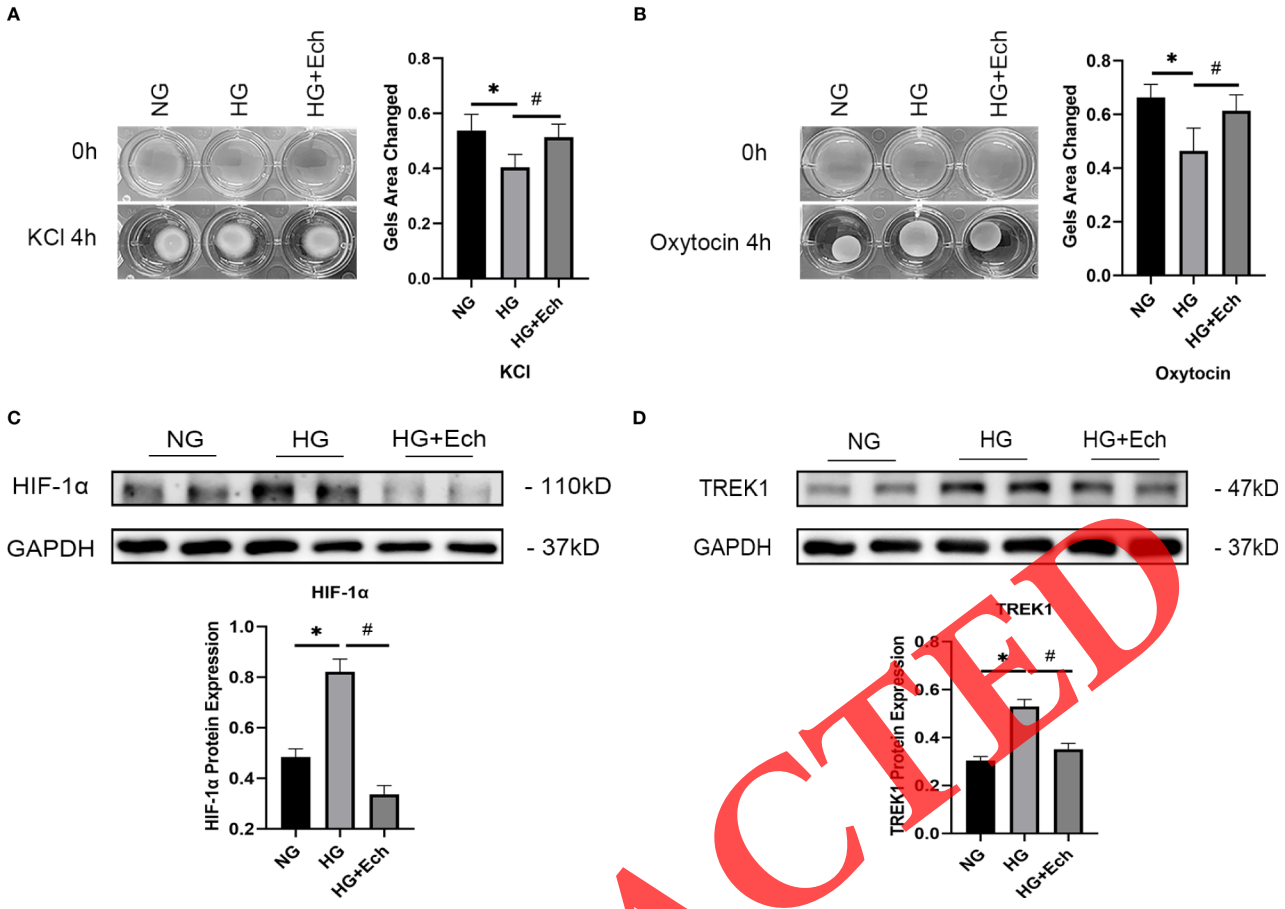

FIGURE 5
Hypoxia inhibition recovered the decreased cell contractility under high glucose levels. We divided HUSMCs into the normal glucose (NG, 5.5 mmol/L) group, the high glucose (HG, 25 mmol/L) group, and the high glucose with echinomycin (HG+Ech) group to inhibit hypoxia in a 24-well culture plate. The medium was added after collagen polymerization (0 h). The images of collagen stimulated by KCl **(A)** or oxytocin **(B)** for 4 h. The contraction of HUSMCs was assessed by measuring the mean gel area change. The HUSMCs of each group were cultured in NG medium at the beginning. Then, the NG medium in the HG and HG + Ech groups was replaced with HG medium for 24 h when the cell density reached 60%–70%, after which echinomycin was added to the HG + Ech group for 4 h. The total protein of each group was then extracted, and HIF-1α **(C)** and TREK1 **(D)** protein expression was measured by western blot analysis. Data are presented as means ± SEM, n = 4 for each group. * means HG vs. NG and $P<0.05$. # means HG+Ech vs. HG and $P<0.05$. HIF-1α, hypoxia-inducible factor-1 alpha; HUSMCs, human uterine smooth muscle cells; SEM, standard error of the mean; TREK1, TWIK-1-related potassium channel.

Previous studies (15, 48) have shown that uterine tissue contractility is regulated by the potassium channel TREK1, and high TREK1 expression in pregnant uterine tissues can cause uterine tissue diastole. However, it is not clear whether decreased uterine contractility in patients with GDM is caused by TREK1 and hyperglycemia. This study found that TREK1 protein expression in uterine smooth muscle was significantly higher in patients with GDM than in normal individuals, while TREK1 protein expression was also significantly higher in HUSMCs in the high glucose group. When TREK1 function was inhibited with L-methionine, HUSMC contractility was restored significantly. These results indicate that high glucose levels promote the expression of TREK1 proteins in uterine smooth muscle, which leads to reduced contractility of uterine smooth muscle.

Hyperglycemia can lead to hypoxia in various tissues or cells, and HIF-1α is considered a marker closely associated with hypoxia (28, 29). There is inconsistent evidence on the effects of hyperglycemia on HIF-1α in different tissues or cells, with some studies showing that hyperglycemia promotes HIF-1α expression and others reporting its role in HIF-1α degradation in some tissues (49–53). We found that HIF-1α protein expression was significantly increased in uterine tissues of patients with GDM compared with those of normal individuals. Moreover, HIF-1α protein expression in HUSMCs was also significantly increased in the high glucose group. When $CoCl_2$ was used to simulate HUSMC hypoxia, cell contractility was also reduced, as in the high glucose group. This finding is consistent with the observation by Alotaibi et al. (35) of a significant decrease in uterine strip contractility during hypoxia. However, whether the TREK1 protein, which regulates cell contractility, also functions during hypoxia-induced reduction of contractility in uterine smooth muscle cells remains unclear.

TREK1 expression was found to be significantly elevated in astrocytes after ischemia and hypoxia (38). However, this finding did not establish whether hyperglycemia-induced HIF-1α

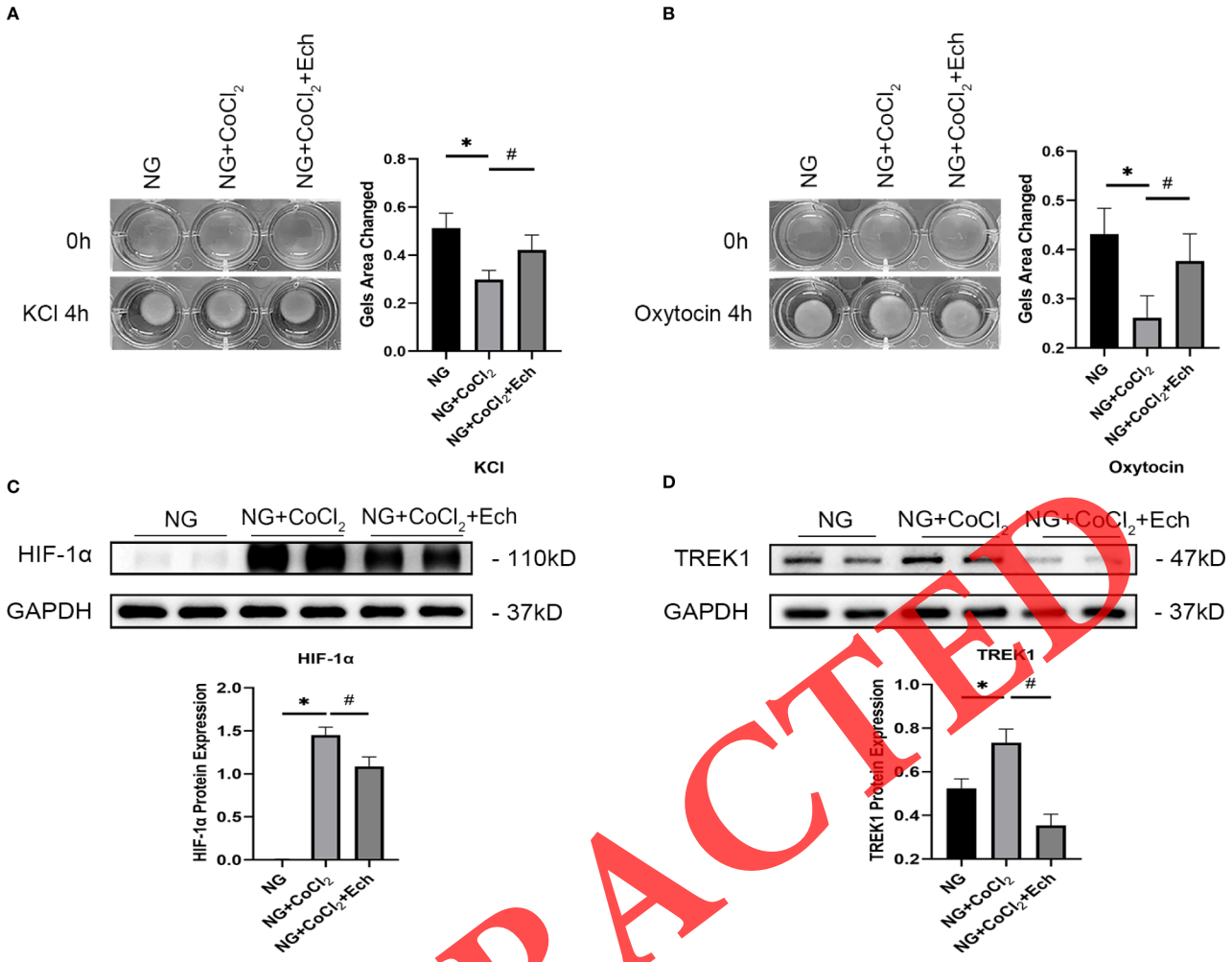

FIGURE 6
Hypoxia inhibition recovered the $CoCl_2$-decreased cell contractility in HUSMCs. We divided HUSMCs into the normal glucose (NG, 5.5 mmol/L) group, the normal glucose with $CoCl_2$ (NG+$CoCl_2$) group, and the normal glucose + $CoCl_2$ with echinomycin (NG+$CoCl_2$+Ech) group in a 24-well culture plate. The medium was added after collagen polymerization (0 h). The images of collagen stimulated by KCl **(A)** or oxytocin **(B)** for 4 h. The contraction of HUSMCs was assessed by measuring the mean gel area change. The HUSMCs of each group were cultured in NG medium at the beginning. Then, the NG medium in NG+$CoCl_2$ and NG+$CoCl_2$+Ech groups was replaced with NG+$CoCl_2$ medium for 24 h when the cell density reached 60%–70%, after which echinomycin was added to the NG+$CoCl_2$+Ech group for 4 h. The total protein of each group was then extracted, and HIF-1α **(C)** and TREK1 **(D)** protein expression was measured by western blot analysis. Data are presented as means ± SEM, n = 4 for each group. * means NG+$CoCl_2$ vs. NG and $P<0.05$; # means NG+$CoCl_2$+Ech vs. NG+$CoCl_2$ and $P<0.05$. HIF-1α, hypoxia-inducible factor-1 alpha; HUSMCs, human uterine smooth muscle cells; SEM, standard error of the mean; TREK1, TWIK-1-related potassium channel.

expression in the uteri of patients with GDM also regulates uterine tissue contractility by modulating TREK1 expression. To determine whether TREK1 is regulated by HIF-1α in uterine tissue, we inhibited hypoxia and investigated TREK1 expression. Our results showed that both HIF-1α and TREK1 protein expression in HUSMCs were significantly decreased by echinomycin. This demonstrates that TREK1 is regulated by HIF-1α in HUSMCs and that HIF-1α modulating TREK1 protein expression may contribute to changes in uterine contractility.

The collagen gel contraction assay was used to verify cell contractility; the data indicated that hypoxia decreased cell contractility and that the decrease was recovered by the TREK1 inhibitor L-methionine. The induced hypoxia was associated with the changes in contractility observed with high glucose levels. The cell contraction data confirmed that high glucose induced myometrium hypoxia and decreased contractility through TREK1. Interestingly, a recent study (54) reported that hypoxia and increased HIF-1α expression promote the contraction of myometrial and smooth muscle cells. These findings contradict our experimental results; however, we found some inconsistencies in the supplement Figure 4 of their paper and the conclusion about the cell contraction. In a study related to HIF and venous contraction, Lim et al. (34) found that induced HIF-1α overexpression was associated with reduced venous contraction. Our study still has some limitations. We did not use TREK1 shRNA/siRNA treatment in HUSMCs to specifically inhibit endogenous TREK1 to examine the changes in cell contractility under high glucose or $CoCl_2$ conditions. We also did not propose a

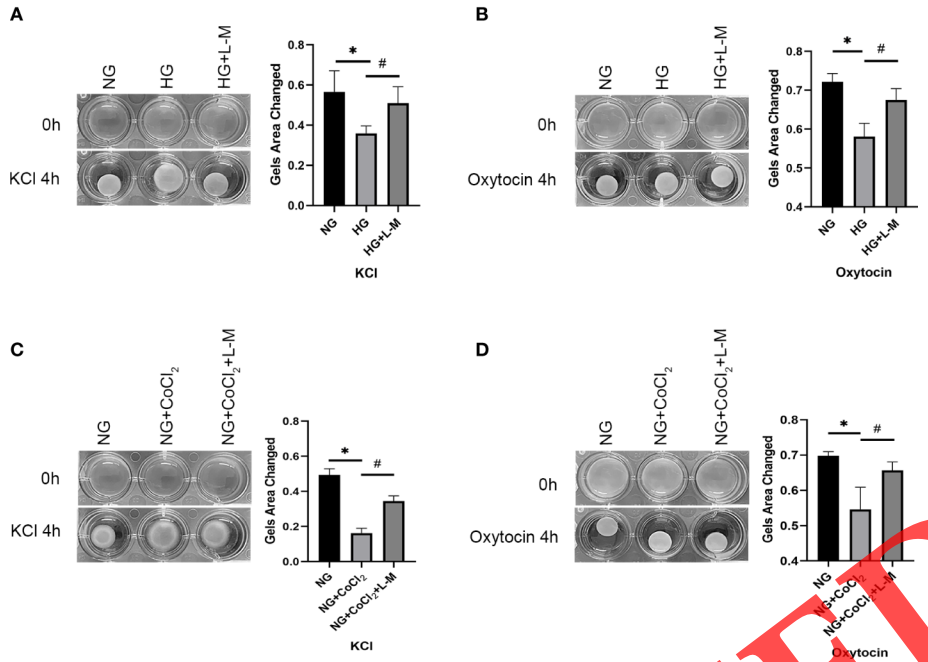

FIGURE 7
TREK1 inhibitor L-methionine increased the HUSMC contractility decreased by high glucose levels or hypoxia. We divided HUSMCs into the normal glucose (NG, 5.5 mmol/L) group, high glucose (HG, 25 mmol/L) group, high glucose with L-methionine (HG+L-M) group, normal glucose with $CoCl_2$ ($NG+CoCl_2$) group, and normal glucose + $CoCl_2$ with L-methionine ($NG+CoCl_2$+L-M) group in a 24-well culture plate. The medium was added after collagen polymerization (0 h). The images of collagen stimulated by KCl **(A)** or oxytocin **(B)** for 4 h. The contraction of HUSMCs was assessed by measuring the mean gel area change. The medium was added after collagen polymerization (0 h). The images of collagen stimulated by KCl **(C)** or oxytocin **(D)** for 4 h. The contraction of HUSMCs was assessed by measuring the mean gel area change. Data are presented as means ± SEM, n = 4 for each group. **(A, B)** * means HG vs. NG and P<0.05. **(A, B)** # means P<0.05 HG+L-M vs. HG and P<0.05. **(C, D)** * means P<0.05 $NG+CoCl_2$ vs. NG and P<0.05. **(C, D)** # means P<0.05 $NG+CoCl_2$+L-M vs. $NG+CoCl_2$ and P<0.05. HUSMCs, human uterine smooth muscle cells; SEM, standard error of the mean.

specific mechanism for HIF-1α regulation of TREK1. Mammalian microRNAs (miRNA/miR) play especially powerful roles in smooth muscle cells (55). Hypoxia induces further downregulation of miR-124 (56), and Bucharest et al. (57) revealed an inverse correlation between miR-124 and TREK1 expression in neurons. Whether miR-124 is involved in the regulation of TREK1 by HIF-1α in the uterus may be a future research direction.

In conclusion, our study confirms that the HIF-1α and TREK1 protein expression is significantly increased in the gestational diabetic uterus and HUSMCs cultured under high glucose and immediate hypoxia conditions. Hypoxia is involved in the regulation of uterine smooth muscle contraction through TREK1, which is an important pathway allowing hyperglycemia to regulate the process of uterine smooth muscle contraction in patients with GDM. Intervention with hypoxia and TREK1 restores the contractility of uterine smooth muscle. Therefore, hypoxia and TREK1 may be potential targets for future modulation of uterine contractility and reduction in the complications of GDM.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by First Affiliated Hospital of Anhui Medical University Ethics Committee for the Protection of Human Subjects in Research and Tissue Collection (PJ2020-06-12). The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZY, DL, and TL wrote the manuscript and researched data. JF, HY, XW, JB, and FC researched data and contributed to the discussion. TL and JF contributed equally to this work. ZY and DL contributed equally to this work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1115619/full#supplementary-material

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