Hemeoxygenase-1 Inhibits Human Myometrial Contractility via Carbon Monoxide and Is Upregulated by Progesterone during Pregnancy

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Abstract

Nitric oxide was proposed as an endogenous inhibitor of myometrial contractility during pregnancy. Carbon monoxide (CO) like nitric oxide increases cGMP and is generated during the degradation of heme to biliverdin IX by hemeoxygenases (HO). Here we report that the expression of both HO-1 (inducible) and HO-2 (constitutive) were > 15-fold higher in pregnant myometrium compared to nonpregnant myometrium ($n = 4$, $P < 0.001$, $P < 0.005$, respectively). Moreover, the activation of the HO-CO pathway by the HO inducer, hemin (10 μM), completely inhibited spontaneous contractility ($n = 3$). Oxytocin-stimulated contractions ($n = 5$) were also significantly reduced ($P < 0.05$) in myometrial strips mounted for isometric recording under 2 g tension in Krebs solution. Reverse transcription-PCR analysis revealed that mRNA encoding HO-1 and HO-2 was undetected in explant cultures of nonlaboring pregnant myometrium under basal conditions, however, exposure to progesterone, but not estradiol-17β, induced the expression of HO-1 and HO-2 mRNAs. Progesterone also significantly induced HO-1 protein synthesis ($n = 4$, $P < 0.001$) while estradiol-17β had no effect ($n = 4$). In term (37–42-wk gestation) nonlaboring myometrial explants, CO production was stimulated by progesterone (10^{-6} M) ($n = 2$) and hemin (10 μM) ($n = 3$) after 2 h of incubation and the effect of hemin was inhibited by 1 h of preincubation with the HO inhibitor tin protoporphyrin IX (20 μM). This study clearly demonstrates the expression of HO in the human myometrium and shows that its induction produces CO that limits uterine contractility in pregnant myometrium indicating a role for the HO-CO-cGMP pathway in the maintenance of the quiescent state of the uterus during pregnancy. (*J. Clin. Invest.* 1998. 101:949–955.) Key words: uterus • labor • carbon monoxide • smooth muscle • progesterone

Introduction

Spontaneous preterm birth (before 37-wk gestation) is the major factor contributing to both perinatal mortality and morbidity (1). Premature babies account for two-thirds of the neonatal mortality, and ~ 60% of the morbidity in newborns is attributable to prematurity (2–4) . Despite extensive efforts to prevent preterm delivery there is as yet no effective therapy. Tocolytics, including β_2 adrenergic agonists and magnesium sulfate, do not prolong pregnancy for more than a matter of days and fail to improve neonatal outcome (5, 6). Newer agents such as oxytocin receptor antagonists, antibiotic therapy, and glyceryl trinitrate patches have not been fully evaluated but preliminary data do not indicate any dramatic improvement. In recent years, attention has been focused on endogenously occurring muscle relaxants which may be natural tocolytics. The presence of such agents is supported by the observation that the uterus is maintained in a quiescent state during pregnancy despite progressive stretching of the myometrial muscle as the fetus grows.

The endogenous nitric oxide (NO)[1]-cGMP pathway was proposed as the pathway for the relaxation of uterine smooth muscle cells during pregnancy and as a possible modulator of labor (7, 8). However, in a recent study it was demonstrated that pregnancy increases myometrial cGMP by a pathway independent of NO in the guinea pig (9). Like NO, carbon monoxide (CO) is an endogenously produced molecule that activates soluble guanylate cyclase leading to an increase in intracellular cGMP. CO has been implicated in vascular smooth muscle relaxation (10) and BP regulation (11) in rats, which suggests that CO may have a possible role in smooth muscle contractile physiology. CO is generated during the degradation of heme to biliverdin IX by hemeoxygenases (HOs). HO is a microsomal enzyme that exists as two homologous isozymes. HO-1 is inducible and is highly expressed in spleen and liver where it is responsible for the catabolism of heme from red blood cells, whereas HO-2 is constitutively expressed and is widely distributed in the body with highest concentration in the brain (12). Although both NO and CO exert their

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1. *Abbreviations used in this paper:* CO, carbon monoxide; COHb, carboxyhemoglobin; HO, hemeoxygenase; LD, lactate dehydrogenase; NO, nitric oxide; NOS, nitric oxide synthase; RT, reverse transcription; SnPP, tin protoporphyrin IX.

actions via a guanylate cyclase-cGMP mechanism to induce smooth muscle relaxation, there is as yet no evidence to propose the role of the HO-CO pathway in the maintenance of uterine quiescence during pregnancy and its implication in the initiation of labor in humans. Therefore, the purpose of this study was to investigate the biological role of HO in the control of uterine contractility and to determine the effect of sex steroid hormones on the expression of HO-1 and HO-2 isozymes in human pregnant myometrium. Our observations provide a new insight into the mechanisms involved in the maintenance of uterine quiescence during pregnancy and may have therapeutic relevance in preventing preterm labor.

Methods

Materials and drug preparation. All chemicals were obtained from Sigma Chemical Co. (Poole, UK) or as otherwise cited. Hemin, tin protoporphyrin IX (SnPP), and hemoglobin were dissolved in 0.1 M NaOH to which normal sterile saline was added, and the pH was adjusted to 7.4. Progesterone and estradiol-17β were dissolved in 100% ethanol to prepare a 1 M solution and a stock solution of 10^{-2} M was prepared by dilution (1:100) in phenol-red–free DME medium and stored at −20°C. From this stock, serial dilutions in phenol-red–free DME medium were prepared to the concentration desired of the hormones.

Tissue collection and preparation. Myometrial samples were obtained from women undergoing either elective (before the onset of labor) or emergency (after the onset of labor) lower segment cesarean section at term (37–42-wk gestation). The reasons for cesarean section included breech presentation, previous cesarean section, and fetal distress or prolonged labor. All biopsies were taken in the midline from the upper lip of the incision in the lower uterine segment. Only women having regional anesthesia (spinal or epidural nerve block) were included in the study. There was no significant difference in age, weeks of pregnancy, parity, birth weight, or infant sex between the two groups. Nonpregnant myometrium was obtained from uteri from normal cycling premenopausal patients undergoing hysterectomy for benign disease. Immediately after collection, the tissue was either snap-frozen in liquid nitrogen and stored at −80°C until required for protein and RNA analysis, placed in Krebs-Henseleit physiological salt solution for contractility studies, or collected in phenol-red–free DME medium for tissue explant experiments. Informed consent was obtained from the patients and the study had the approval of the South Birmingham Ethical Committee (Birmingham, UK).

Western blotting analysis. Total protein was extracted from tissue in high-salt buffer (0.4 M KCl, 20 mM Hepes, 1 mM DTT, 20% glycerol, 0.5 μg/ml bacitracin, 40 μg/ml PMSF, 5 μg/ml pepstatin, and 5 μg/ml leupeptin). Equal amount of total protein (100 μg) for each sample was loaded in 10% SDS-polyacrylamide minigel and separated by electrophoresis. Proteins from the gels were electrotransferred to prewetted nitrocellulose membrane (Amersham, International plc., Bucks, UK) and subjected to immunoblot analysis. The membranes were blocked in Tris-buffered saline containing 1% Tween, 10% dry skimmed milk, 5% BSA, and 2% goat serum for 1 h to prevent nonspecific binding of the antibodies. Membranes were then incubated with either polyclonal rabbit anti–rat HO-1 antibody (1:1000) or polyclonal rabbit anti–rat HO-2 (1:1000) antibody (StressGen Biotechnologies Corp., Victoria, Canada). Bands were detected with the nonradioactive ECL Western blotting system (Amersham International plc.), exposing the membranes to X-Omat AR x-ray film (Eastman Kodak Co., Rochester, NY) to record chemiluminescence, and bands were quantitated using a laser densitometer UVP ImageStore 5000 combine system. Recombinant rat heat shock protein 32, also known as HO-1, and recombinant rat HO-2 (StressGen Biotechnologies Corp.) were used as positive controls. Protein determination was carried out by the Bio-Rad protein assay (Bio-Rad Laboratories Ltd., Herts, UK) using BSA as standard.

Immunohistochemistry. Serial 4-μm sections of formalin-fixed, paraffin-embedded myometrial biopsies were deparaffinized by incubation for 10 min in xylene. Endogenous peroxidase activity was quenched by incubating the sections in 0.3% (vol/vol) of hydrogen peroxide in methanol for 20 min. Nonspecific protein binding sites were blocked with 10% normal goat serum in PBS for 30 min at room temperature in a humidified chamber. Sections were incubated with polyclonal anti–HO- (1:500) or polyclonal anti–HO-2 (1:500) (StressGen Biotechnologies Corp.) in 10% goat serum and 0.3% Triton X-100 for 60 min at room temperature. Primary antibody signal was amplified by incubation with a goat anti–rabbit biotinylated secondary antibody and the localization of HO was detected using a streptavidin-biotin immunoperoxidase kit for rabbit IgG (DAKO Ltd., Bucks, UK). Diaminobenzidine was used as the chromogen. The immunostained sections were counterstained with Mayer's hematoxylin. Specificity of the immunoreactions was determined by comparing the reactivity of the antibodies with that of 10% goat serum in PBS in the absence of the primary antibody as well as with antibodies adsorbed with excess (10 μg/ml) of purified HO-1 and HO-2 recombinant proteins, respectively.

Amplification of myometrial HO-1 and HO-2 cDNA fragments. Total RNA from myometrium was prepared using RNAzol™ and quantified spectrophotometrically at 260 nm. HO-1 and HO-2 cDNA were synthesized from 5 μg of total RNA by reverse transcription (RT) using oligo$(DT)_{12–18}$ and then amplified by PCR with the SuperScript™ preamplification system (Life Technologies Ltd., Paisley, UK) in 35 thermal cycles. Primers were designed according to the published sequence of human monocyte HO-1 (13) and rat testis HO-2 (14). The forward (5′-GAATTCAGCATGCCCCAGGATTTG-3′) and the reverse (5′-TCTAGACTAGCTGGATGTTGAGCAGGA-3′) primers for HO-1 were used to amplify a 615-bp fragment, and the forward (5′-GAATTCGGGACCAAGGAAGCACAT-3′) and the reverse (5′-TCTAGACTATGTAGTACCAGGCCAAGA-3′) primers for HO-2 were used to amplify a 828-bp fragment (10). The PCR products were subcloned and sequenced to confirm their identity. PCR amplification of mouse β-actin mRNA was run in parallel using the same cDNA generated by RT for each sample to confirm cDNA input in each reaction. Total RNA from rat brain was used as positive control.

Tissue bath experiments. Myometrial contractile activity was assessed as described previously (15). Briefly, myometrial strips from term nonlaboring uteri ($\sim 2 \times 2 \times 10$ mm) were dissected from each sample and mounted for isometric recording under 2 g tension in Krebs-Henseleit physiological solution maintained at 37°C and gassed with 95% O_2 and 5% CO_2. Strips were allowed to equilibrate for 2 h before contractile activity was assessed. The mechanical responses were measured by calculation of the integral of the area under the curve for a designated period of time using a combined MacLab™ hardware unit and Chart v3.4β5 software system. After the equilibration period myometrial strips were exposed to KCl (45 mM, added to the bath) for 20 min and the integrated tension was measured for 10 min. The strip was thoroughly washed out and base line activity was reestablished for 30 min. Strips were then exposed to oxytocin (0.5 nM) and the contractile pattern was observed for 30 min and then incubated with hemin (HO inducer) (13) for the time desired. The integrated tension was measured for periods of 30 min. The effect of oxytocin and hemin on contractile activity is demonstrated by expressing this 30-min integral as a percentage of the average KCl-induced 10-min integral for each strip. Control experiments were run in parallel where the strip was incubated with the vehicle.

Measurement of carboxyhemoglobin (COHb) in conditioned media. Production of CO by nonlaboring pregnant myometrium at term was determined by the estimation of the proportion of COHb in the media as described previously (10, 16). Myometrial explants were exposed to hemin (10 μM), SnPP (20 μM), progesterone (10^{-6} M), or the vehicle for up to 24 h and hemoglobin (50 μM) added for the last

hour of incubation. Percentage of COHb was determined spectrophotometrically and expressed as milligrams per milliter of total protein.

Tissue viability: Lactate dehydrogenase (LD) assay. Leakage of LD to the medium in myometrial explants was measured as an index of cytotoxicity as reported by others (17). To determine LD activity, 100-μl aliquots of tissue homogenate or medium from myometrial explants exposed to progesterone (10^{-4} and 10^{-6} M), estradiol-17β (10^{-6} and 10^{-7} M), and vehicle (control) for 24 h were mixed with LD reagent (Sigma Diagnostics, St. Louis, MO) kept at 30°C. The rate of increase in absorbance at 340 nm due to NADH formation was recorded for 60 s to give LD activity expressed as U/liter. The cytotoxicity index was expressed as the ratio of LD medium to LD tissue and a value of $< 5\%$ was regarded to indicate that the tissue was viable.

Analysis of results. Laser densitometric analysis is expressed as arbitrary units. Data are shown as the mean±SEM. Statistical analysis of the data was done by two-tailed unpaired Student's *t* test. A value of $P < 0.05$ was regarded as significant.

Results

Western blot analysis demonstrates the expression of both HO-1 and HO-2 immunoreactive protein in human myometrium (Fig. 1 *A*). Corresponding laser densitometry revealed that HO-1 and HO-2 proteins increased by 17- and 16-fold, respectively, in pregnant compared to nonpregnant myometrium. However, there was no significant difference in expression between nonlaboring and laboring groups (Fig. 1 *B*). Immunohistochemical localization showed that both isozymes were uniformly localized in the myometrial smooth muscle and in the endothelium of the blood vessels (data not shown). The staining pattern was similar in the pregnant (nonlaboring and laboring) and in the nonpregnant myometrium for HO-1 and HO-2. Within each group ($n = 8$), however, there was variability in the intensity of staining among patients. In negative control and preadsorbed sections no detectable immunostaining was observed for HO-1 and HO-2 (data not shown).

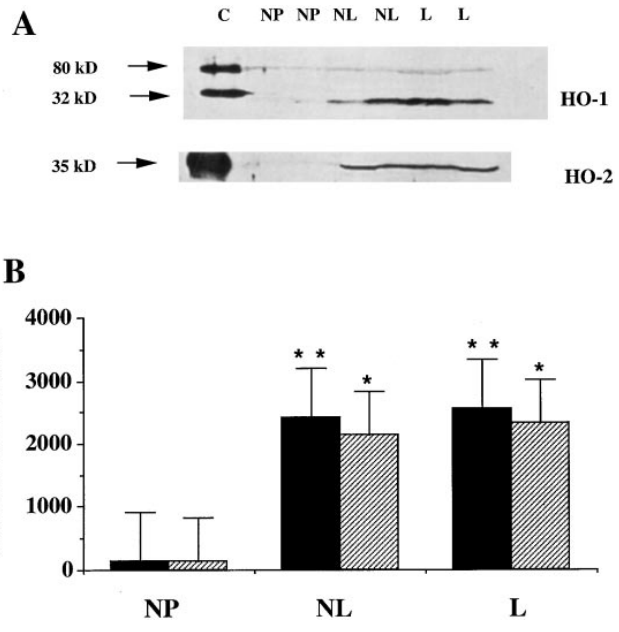

Figure 1. Western blot analysis for HO-1 and HO-2 in human myometrium. (*A*) Expression of HO-1 (a 32-kD heat shock protein) and HO-2 (35 kD) proteins in nonpregnant (*NP*), nonlaboring (*NL*), and laboring (*L*) pregnant term human myometrium. A band of ~ 80 kD was also detected in both the positive control and samples. (*B*) Corresponding laser densitometric analysis of HO-1 (*solid bars*) and HO-2 (*dashed bars*). Recombinant proteins for HO-1 and HO-2 were used as positive controls (*C*). Data are shown as the mean±SEM of four experiments. $^*P < 0.05$, $^{**}P < 0.005$ vs. NP.

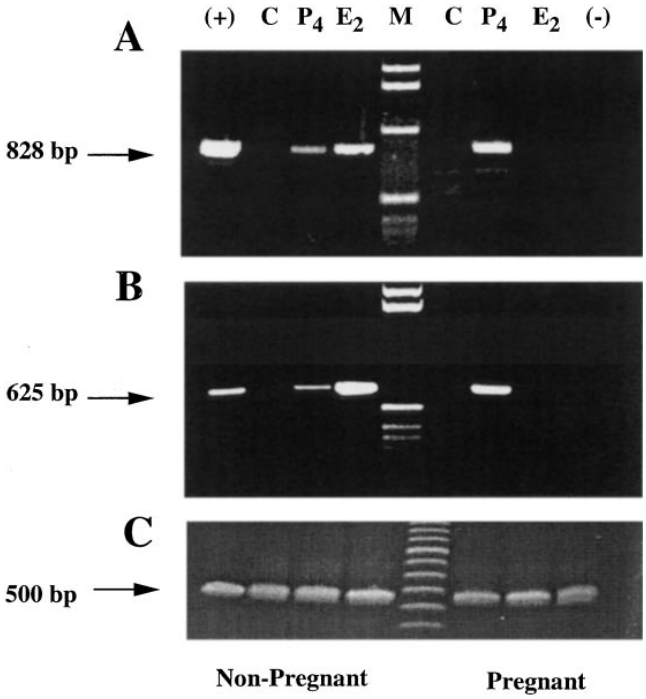

Figure 2. Amplification of HO-1 and HO-2 mRNA by RT-PCR in human myometrial explants. Nonpregnant and term nonlaboring pregnant myometrial strips were cultured in phenol-red–free DME medium and exposed to 10^{-6} M progesterone (P_4) or 10^{-8} M estradiol-17β (E_2) for 24 h. A 828-bp fragment was amplified for HO-2 (*A*) and a 625-bp fragment was amplified for HO-1 (*B*). A 500-bp fragment for β-actin was amplified from cDNA generated by the RT for each sample to verify cDNA input in each PCR reaction (*C*). (+) Rat brain total RNA as positive control, (−) no RNA input as negative control, (*M*) 100-bp ladder, $n = 3$.

The influence of sex steroid hormones on HO protein and gene expression was also assessed in myometrial explants. Tissue from nonpregnant and nonlaboring patients at term were exposed to increasing concentrations of estradiol-17β (10^{-8}–10^{-6} M) and progesterone (10^{-8}–10^{-4} M) for 24 h in phenol-red–free DME medium containing 0.1% BSA at 37°C in a 95% O_2/5% CO_2 incubator. RT-PCR analysis revealed that under basal conditions mRNA encoding HO-1 and HO-2 was not detected, however, exposure to progesterone (10^{-4} M) but not estradiol-17β (10^{-6} M) induced the expression of mRNA encoding both enzymes in the nonlaboring pregnant myometrium explants. In contrast, the mRNA encoding for HO-1 and HO-2 was induced by both sex steroid hormones in the nonpregnant myometrium (Fig. 2, *A* and *B*). Western blot analysis confirmed that HO-1 protein synthesis was induced by progesterone in a dose-dependent manner but not by estradiol-17β in the nonlaboring pregnant myometrium (Fig. 3). Corresponding laser densitometric analysis showed that HO-1

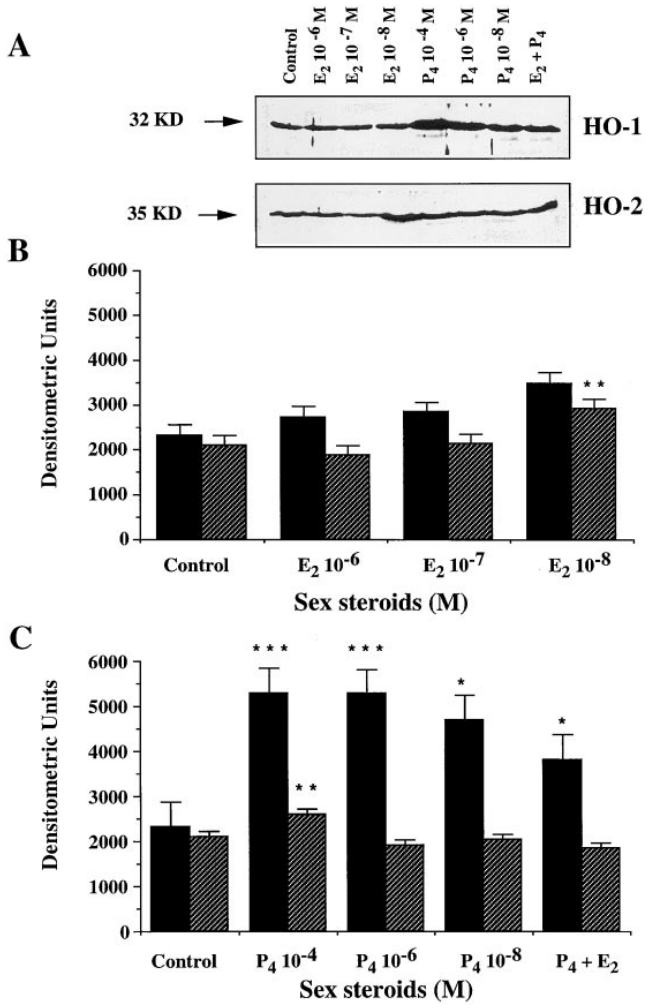

Figure 3. Steroid regulation of HO-1 and HO-2 proteins in human nonlaboring pregnant myometrium. Strips of myometrium were cultured in phenol-red–free DME medium and exposed to increasing concentrations of either progesterone (P_4) (10^{-8}–10^{-4} M) or estradiol-17β (E_2) (10^{-8}–10^{-6} M) alone, or the combination of both (progesterone, 10^{-6} and estradiol-17β, 10^{-7} M), or the vehicle (control) for 24 h. Progesterone induced HO-1 protein expression in a dose-dependent manner with a maximal increase when the tissue was exposed to 10^{-4} and 10^{-6} M progesterone compared to control. (*A*) Western blot analysis for HO-1 (32 kD) and HO-2 (35 kD). (*B*) Corresponding laser densitometric analysis for tissue exposed to estradiol-17β. (*C*) Corresponding laser densitometric analysis for tissue exposed to progesterone (P_4) and combination of estradiol-17β and progesterone (E_2 and P_4). Data are shown as the mean±SEM of four experiments. $^*P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$ vs. control.

protein expression was highly significantly induced ($P < 0.001$) when the tissue was exposed to high doses of progesterone (10^{-4} and 10^{-6} M). However, HO-1 was also significantly induced when the tissue was exposed to a lower dose of progesterone (10^{-8} M) and when progesterone (10^{-6} M) and estradiol-17β (10^{-7} M) were used in combination ($P < 0.05$) (Fig. 3 *C*). The expression of HO-2 (constitutive) was also significantly induced by the lowest dose of estradiol-17β and the highest dose of progesterone ($P < 0.05$) (Fig. 3, *B* and *C*, respectively). Toxicity to progesterone and estradiol-17β was

Table I. Measurement of LD Activity in Tissue Homogenates and Media from Myometrial Explants Exposed to Estradiol-17β (E_2; 10^{-6} and 10^{-7} M), Progesterone (P_4; 10^{-4} and 10^{-6} M), or Vehicle (Control)

	LD activity		Index of cytotoxicity
	Tissue	Media	LDm/LDt × 100
	U/liter	*U/liter*	
Control	93.6	2.8	3%
E_2 10^{-6} M	157.0	5.4	3%
E_2 10^{-7} M	138.6	5.7	4%
P_4 10^{-4} M	145.1	2.8	2%
P_4 10^{-6} M	159.6	4.8	3%

LD activity is expressed in U/liter. An index of cytotoxicity (LDm/LDt) < 5% was regarded as viable tissue.

evaluated by measuring the leakage of LD to the medium in myometrial explants. As shown in Table I, neither progesterone nor estradiol-17β significantly increased LD leakage as measured by LD medium/LD tissue ratio. The values were < 5%.

Myometrial contractile activity was assessed in human myometrial strips as described in Methods. Addition of hemin (10 μM) completely inhibited spontaneous contractions after 2 h of incubation, whereas the contractile activity was maintained constant in the control experiments where the strip was incubated with the vehicle (Fig. 4). Tissue responsiveness was assessed before and after hemin or vehicle incubation with the addition of oxytocin (0.5 nM) and separately with KCl (45 mM), that produced regular phasic and tonic contractions, respectively. In addition to spontaneous contractions being inhibited by hemin, oxytocin-induced contractions were also significantly reduced (Fig. 5). Myometrial strips were exposed to oxytocin (0.5 nM) for 30 min and then incubated with hemin (3 μM) for 2.5 h. Hemin concentration was maintained constant throughout the experiment by the addition of fresh hemin every 30 min (Fig. 5, *arrows*). Fig. 5 demonstrates that oxytocin-induced contractions were reduced by 45% (1.8-fold) after incubation with hemin for 30 min, with a maximal inhibition of 68% (3-fold) after 2 h. The effect of oxytocin and hemin on myometrial contractile activity was calculated as a 30-min integral and expressed as a percentage of the KCl maximal contraction.

To confirm whether pregnant myometrium produces CO in response to HO activation, term nonlaboring myometrial explants were exposed to either hemin (10 μM), progesterone (10^{-6} M), or the vehicle (control) for up to 24 h and CO production was calculated by measuring the percentage of COHb in the conditioned media as described in Methods. Fig. 6 *A* shows a maximal induction of sixfold increase in CO production after 2 h of incubation with hemin which gradually decreased over time to reach basal levels at 12 h (Fig. 6 *A*). A similar trend of CO production was observed when the tissue was incubated with progesterone (10^{-6} M) (Fig. 6 *B*). After 2 h of induction with progesterone there was a substantial increase (5.7-fold) in CO production which decreased progressively over time reaching basal levels at 12 h (Fig. 6 *B*). Hemin-induced CO production was significantly inhibited by the HO inhibitor,

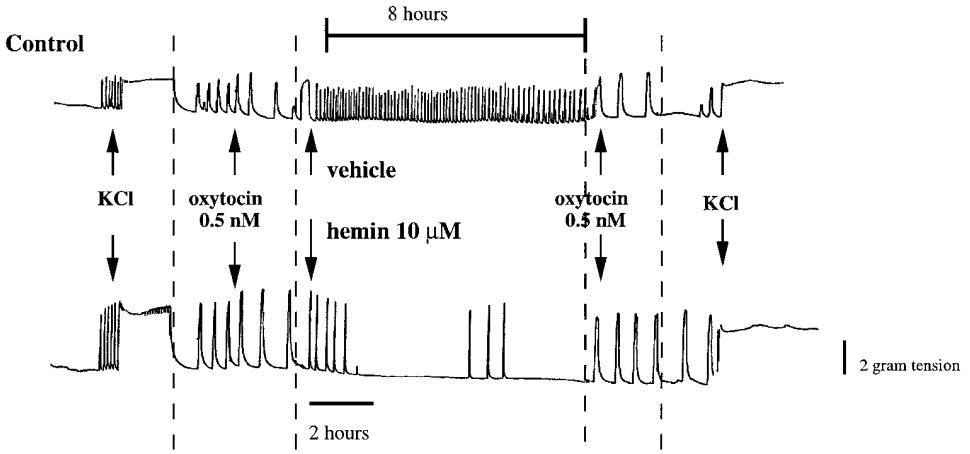

Figure 4. The effect of hemin on spontaneous contractions in human nonlaboring pregnant myometrium at term. Myometrial strips were mounted for isometric recording under 2 g tension and after 2 h of equilibration, contractile activity was assessed. Addition of hemin (10 μM) completely inhibited spontaneous contractions after 2 h of incubation, whereas the contractile activity was maintained constant in the control experiments (vehicle) (*arrows*). Oxytocin (0.5 nM) and KCl (45 mM) were used to assess the responsiveness of the tissue before and after hemin incubation. Washes are indicated by dashed lines. Speed of the recording chart was slowed during hemin and vehicle incubation for publishing purposes. The tracing represents one of three similar experiments.

SnPP, in the myometrial strips preincubated for 1 h with SnPP (20 μM) (Fig. 7).

Discussion

HOs have been identified in a variety of tissues including spleen (12), liver (18), brain (19), and vascular smooth muscle (10). This study for the first time demonstrates the expression of HO-1 and HO-2 in human uteri. It shows that the expression of HO is markedly higher in the pregnant as compared to the nonpregnant myometrium, and progesterone, but not estradiol-17β, induces both gene and protein expression of HO. More importantly, incubation with the HO inducer, hemin, inhibits both spontaneous and oxytocin-induced contractions in nonlaboring pregnant myometrial strips. Furthermore, the induction of HO by progesterone and hemin induces CO production in nonlaboring pregnant myometrial strips that was inhibited by preincubation with the HO inhibitor SnPP, indicating that HO-induced myometrial relaxation was due to CO release.

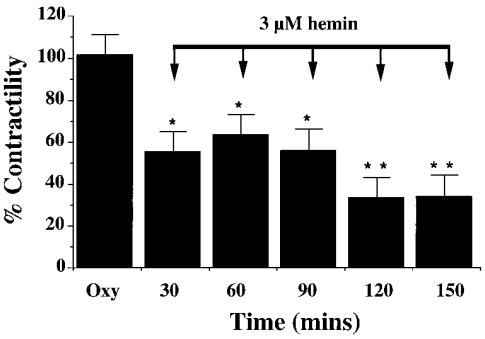

Figure 5. Effect of hemin on oxytocin-induced contraction in nonlaboring pregnant myometrium at term. Myometrial strips were mounted for isometric recording under 2 g tension and after 2 h of equilibration, contractile activity was assessed. The effect of oxytocin (*Oxy*) (0.5 nM) and hemin (3 μM) on contractile activity is demonstrated by expressing a 30-min integral as a percentage of the average KCl-induced 10-min integral for each strip (see Methods). Control experiments were run in parallel where the strip was incubated with the vehicle. Hemin concentration was maintained constant throughout the experiment by adding fresh hemin at 30-min intervals after washing out the strip (*arrows*). Data are shown as the mean±SEM of five experiments. $*P < 0.05$, $**P < 0.005$ vs. oxytocin.

An NO-cGMP pathway was proposed as the contributor to the relaxation of uterine smooth muscle during pregnancy (20). Studies in rodents have suggested that NO synthesis in the uterus may contribute to the maintenance of myometrial quiescence during the antepartum period and that a reduction in NO synthesis by the uteroplacental unit may be an important event in the initiation of labor (21, 22). Functional NO is generated by the conversion of L-arginine to L-citrulline by NO synthase (NOS) in the presence of molecular oxygen and cofactors. Work from our laboratory showed a marked increase in the levels of immunoreactive NOS in pregnant compared to nonpregnant myometrium (23) and is in agreement with the data indicating the involvement of NO in the maintenance of myometrial quiescence during pregnancy (24). The role of NO in the initiation of labor in women is less clear. Western blot analysis showing no significant difference in the levels of immunoreactive NOS in the laboring and nonlaboring human myometrium (23) is in agreement with the pharmacological data of Jones and Poston (25) which also do not support the role of NO in the initiation of labor in women as suggested by others (24). Jones' and Poston's conclusion that NO was not playing a role in the control of tension in myometrium was based, not just on the results of the addition of L-arginine, but on the observation that the addition of an NOS inhibitor (L-NAME) did not increase tension in spontaneously contracting myometrial strips obtained either from term (nonlabor) or preterm (nonlabor) deliveries (25). In contrast to the earlier rat studies (8), no effect was observed. In this study the levels of HO immunoreactivity showed a similar pattern of expression to that of NOS immunoreactivity (23). There was no significant difference in the level of HO expression between nonlaboring and laboring myometrium. Both NOS and HO expression were assessed in myometrial tissues at term when their expression may be already reduced, thus, masking their

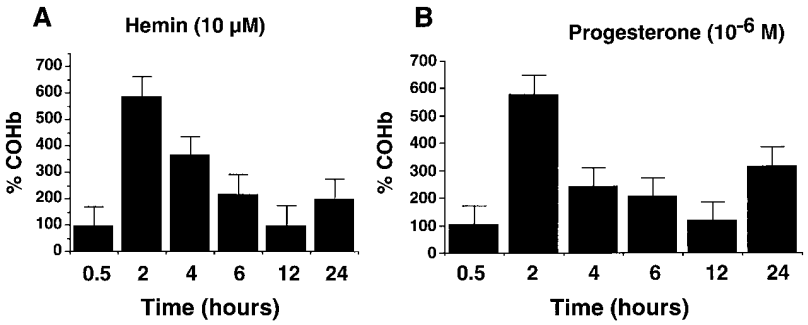

Figure 6. The effect of hemin and progesterone on CO release in the conditioned media of nonlaboring pregnant myometrium at term. (*A*) Tissue was exposed to hemin (10 μM) and CO levels determined as a measure of the percentage of COHb in the media. (*B*) Tissue exposed to 10^{-6} M progesterone. Data in *A* are shown as the mean±SEM of three experiments. Data in *B* are shown as the mean±SEM of one representative experiment of two separate experiments. Results shown as %COHb represent the percentage of control for each time point.

role (if any) in the initiation of labor. The use of glyceryl trinitrate patches was proposed to be effective in pregnant human volunteers in arresting preterm labor (26), however, the same group recently reported no statistically significant improvement in preventing premature births in the glyceryl trinitrate group (65 patients) compared with the ritodrine-treated group (61 patients) in a clinical trial of 137 patients (27). The mean prolongation of pregnancy was also similar (27). The findings in this study raise the possibility that NO donors alone may not be as effective as compared to those combined with HO activators.

In this study we show conclusively the HO-CO system as an endogenous tocolytic agent in the human myometrium during pregnancy. A low dose of hemin completely inhibited spontaneous contractions and reduced significantly oxytocin-induced contractions in nonlaboring myometrial strips at term as determined by the isometric contractions in organ bath experiments. Hemin as an inducer of HO takes 2–4 h to induce the enzyme to produce CO as is confirmed by the time course of myometrial CO release. This explains the delay of 2–4 h that is required to inhibit spontaneous contractions. HO activity was reported to be modulated by inhibitors and donors of NO in rat brain and spleen (28), and more recently it was shown that an NO donor (SIN-1) increased rat liver HO-1 activity (29). Therefore, it is possible that the NOS-NO pathway may exert its effect by the HO-CO system.

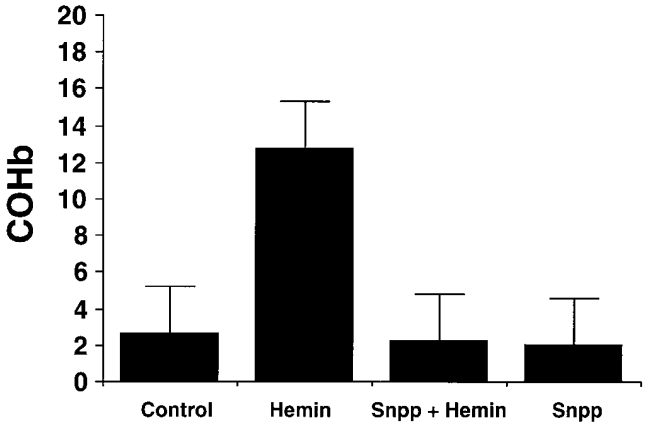

Figure 7. The effect of HO inducer and HO inhibitor on CO production in nonlaboring pregnant myometrial strips at term. Tissue was exposed to the HO inhibitor SnPP (20 μM) for 1 h and then incubated with the HO inducer hemin (10 μM) for 4 h. CO release is expressed as a percentage of COHb in the conditioned media relative to total hemoglobin. Data are shown as the mean±SEM of one representative experiment of three separate experiments.

In nonprimate mammals, the onset of labor is characterized by a shift from progesterone to estrogen dominance (30, 31). Although there is no change in the circulating estradiol and progesterone plasma concentrations to announce parturition in women, a local paracrine system within uterine tissue and fetal membranes producing an increase in the estradiol/progesterone ratio around the time of the onset of labor was suggested by Mitchell and Wong (32). More recently, Karalis et al. proposed a model for functional progesterone withdrawal at the end of human pregnancy, which may be involved in the initiation of labor (33). The shift from progesterone to estrogen dominance leads to the activation of multiple pathways which contribute to onset of labor, including stimulation of oxytocin, oxytocin receptors, and prostaglandin synthesis (for review see reference 31). Myometrial cGMP increases with advancing gestational age, and the greatest increase occurs during the period of greatest fetal growth (9). In this study HO-1 gene and protein expression was consistently upregulated by progesterone in the nonlaboring myometrial explants at term. It is interesting to note that RT-PCR was unable to detect mRNA in the control biopsies while the protein levels of both HO-1 and HO-2 were readily detected by Western blot analysis. It is possible that HO mRNA levels are not detected because of poor RNA stability or very rapid translation into protein. A more likely explanation may be that the control biopsies were deprived of sex steroid influence as they were kept in phenol-red–free medium for a total of 48 h. This may result in down-regulation in the mRNA turnover. That is to say that the transcription from the genomic DNA to mRNA may be inhibited or below the detection threshold of the assay. More interestingly, progesterone and hemin induced HO activity in myometrial explants to produce CO which acts as a messenger to activate soluble guanylate cyclase, increasing intracellular cGMP to relax myometrial smooth muscle cells. It was reported that estradiol can activate guanylate cyclase in the macaque myometrium (34). In this study estradiol-17β also induced HO-2 protein expression in the nonlaboring myometrial strips. It also induced HO-1 and HO-2 mRNA in the nonpregnant myometrium.

It is clear that higher expression of HO protein in the term pregnant uterus together with the findings that progesterone upregulates gene and protein expression and induces CO production in nonlaboring myometrial strips at term suggests that the HO-CO-cGMP system must contribute to the relative

uterine quiescence during pregnancy. Its role in the initiation of labor requires investigation, however, current study indicates that induction of HO-1 in the pregnant uterus or overexpression of the HO-1 gene in women predisposed to preterm labor may provide new therapy for premature birth.

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