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Review
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Review

Gasotransmitters in pregnancy: from conception to uterine involution†

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Abstract

Gasotransmitters are endogenous small gaseous messengers exemplified by nitric o**x**ide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S or sulfide). Gasotransmitters are implicated in myriad physiologic functions including many aspects of reproduction. Our objective was to comprehensively review basic mechanisms and functions of gasotransmitters during pregnancy from conception to uterine involution and highlight future research opportunities. We searched PubMed and Web of Science databases using combinations of keywords nitric oxide, carbon monoxide, sulfide, placenta, uterus, labor, and pregnancy. We included English language publications on human and animal studies from any date through August 2018 and retained basic and translational articles with relevant original findings. All gasotransmitters activate cGMP signaling. NO and sulfide also covalently modify target protein cysteines. Protein kinases and ion channels transduce gasotransmitter signals, and co-expressed gasotransmitters can be synergistic or antagonistic depending on cell type. Gasotransmitters influence tubal transit, placentation, cervical remodeling, and myometrial contractility. NO, CO, and sulfide dilate resistance vessels, suppress inflammation, and relax myometrium to promote uterine quiescence and normal placentation. Cervical remodeling and rupture of fetal membranes coincide with enhanced oxidation and altered gasotransmitter metabolism. Mechanisms mediating cellular and organismal changes in pregnancy due to gasotransmitters are largely unknown. Altered gasotransmitter signaling has been reported for preeclampsia, intrauterine growth restriction, premature rupture of membranes, and preterm labor. However, in most cases specific molecular changes are not yet characterized. Nonclassical signaling pathways and the crosstalk among gasotransmitters are emerging investigation topics.

Summary Sentence

Gasotransmitters modulate mammalian pregnancy via conserved ion channels, receptors, and second messenger-mediated signal transduction pathways.

Key words: gasotransmitter, nitric oxide (NO), nitric oxide synthase (NOS), carbon monoxide (CO), heme oxygenase (HO), hydrogen sulfide (H_2S), cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE)

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, 3-mercaptosulfurtransferase (3-MST), pregnancy, decidua, maternal–fetal interface, extravillous trophoblast (EVT), placenta, paraventricular nucleus (PVN), myometrium, calcium-gated potassium channel (BK_{Ca}), ATP-gated potassium channel (K_{ATP}), parturition, uterus.

Nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S; hereafter referred to as "sulfide") are volatile autocrine and paracrine gaseous signaling compounds known as "gasotransmitters" [1–3]. Gasotransmitters participate in myriad aspects of pregnancy, and signaling can vary by gestational age. Genetic or pharmacologic impairment of gasotransmitter biosynthesis adversely impacts placentation [4, 5], maternal- and feto-placental hemodynamic adaptations of pregnancy [6, 7], the timing of parturition [8–10], and long-term neonatal outcomes [11].

The purpose of this review is threefold:

- *First, to survey gasotransmitter molecular and cellular biochemistry.*
- *Second, to evaluate reports of NO, CO, and sulfide in pregnancy from embryo fallopian tube transit though postpartum uterine involution.*
- *Third, to discuss opportunities for future gasotransmitter research in pregnancy.*

Defogger: an overview of gasotransmitter biochemistry and signaling

The consensus among biochemists a century ago was that NO, CO, and sulfide were environmental toxins with no physiologic functions, although they were useful as reagents and indicators. For example, the odor of sulfide indicated bacterial contamination in the early days of food quality control [12]; the reaction of NO and methemoglobin established early pH testing [13], and Archibald Hill exploited CO's hemoglobin affinity to establish enzymatic cooperativity [14]. The first discussion of endogenous biosynthesis of NO, CO, and sulfide emerged in the 1940s when the Fromageot and Smythe labs discovered a cysteine-dependent sulfide synthase [15, 16]. Sjöstrand [17] later observed heme degradation-dependent CO production in human erythrocytes, which inspired Tenhunen and Schmid's characterization of heme oxygenase (HO) in 1969 [18]. Research establishing the second messenger paradigm in the 1950s and 1960s, exemplified by 3′,5′-cyclic adenosine monophosphate (cAMP) [19, 20], then facilitated the seminal discoveries of gasotransmitter signal transduction.

In the 1970s, Furchgott and Zawadzki first postulated the existence of endothelium-derived relaxing factor (EDRF) mediating acetylcholine vasodilation [21]. By 1977, Murad's lab reported that NO stimulates soluble guanylate cyclase (sGC) to produce 3′,5′-cyclic guanosine monophosphate (cGMP) [22]. Ignarro and Chaudhuri confirmed that EDRF is NO [23], and Moncada's group published the biosynthetic route from L-arginine to nitric oxide via NO synthase (NOS) in 1988 [24]. These pivotal discoveries defined the so-called classical NO pathway by which NO activates sGC to make cGMP, causing vasodilation. Subsequently, the Ulrich and McGrath groups reported CO-dependent vasodilation via classical sGC-cGMP signaling [25, 26], and Snyder's lab showed that brain cGMP correlates with HO-2 transcription [27]. Sulfide research resumed in the 1990s with Abe and Kimura's observation of sulfide synthesis in rat brain [28], and Wang's group demonstrated that sulfide is also a vasodilator [29, 30]. These discoveries established the signaling family of gasotransmitters and their general transduction pathways.

Nitric oxide

NO is a nonpolar, selectively reactive gas with an unpaired electron. It reacts with ferrous (Fe^{2+}) hemoglobin, molecular oxygen (O_2), and superoxide (O_2^-), respectively, to promote the three established NO signal transduction mechanisms: classical sGC-cGMP signaling, S-nitrosation, and the peroxynitrite ($ONOO^-$) cytotoxic pathway (Figure 1A) [31].

In the *classical pathway*, NO's unpaired electron attacks the Fe^{2+} heme of sGC, inducing increased cGMP synthesis. cGMP activates protein kinase G (PKG), which phosphorylates targets including myosin light chain phosphatase (MLCP; activating phosphorylation) and voltage-gated calcium (Ca^{2+}) channels (Ca_v; inactivating phosphorylation). Ca_v closure reduces cytosolic Ca^{2+} entry, thereby curtailing Ca^{2+} binding to calmodulin (CaM) and reducing CaM-dependent myosin light chain kinase (MLCK) activity to promote smooth muscle relaxation [32].

S-nitrosation is the addition of a nitroso group (—NO) to a cysteine thiol (—SH) resulting in an S-nitrosothiol (SNO). Models suggest that cysteine S-nitrosation is indirect [33]. NO and O_2 undergo radical–radical coupling to produce nitroso-oxide intermediates that rearrange to nitrous anhydride (N_2O_3). Subsequently, glutathione's (GSH) thiol group nucleophilically attacks N_2O_3 to produce nitrite (NO_2^-) and S-nitrosoglutathione (GSNO), which is the primary agent of S-nitrosation [34]. GSH-independent S-nitrosation has been detected in bacteria [35], but a role in mammals is uncertain. S-nitrosation protects thiols from oxidation and can thereby alter cysteine-dependent enzyme activity, although nitrosation is vulnerable to reducing agents [33, 36, 37]. Mass spectrometry has identified thousands of nitrosated proteins [38, 39], but the biophysical basis for *selective* cysteine modification is unknown [40]. Sulfide and SNO react to form nitrosopersulfide ($ONSS^-$), which enhances NO-dependent cGMP production by an unknown mechanism [41, 42].

In the *cytotoxic pathway*, NO and O_2^- radicals form $ONOO^-$, a strong oxidant that damages DNA and promotes inflammation [43]. All nucleated cells can produce $ONOO^-$ in response to environmental oxidants, infection, or inflammatory mediators [44], but macrophages specifically use cytotoxic $ONOO^-$ to attack microorganisms [45]. NO and O_2^- must accumulate to produce $ONOO^-$ [46]. Therefore, both downregulation of the O_2^--degrading enzyme superoxide dismutase (SOD) and upregulation of NO synthesis favor the cytotoxic pathway [47].

Living systems produce NO via arginine oxidation and nitrate (NO_3^-) reduction [48]. Arginine oxidation to NO and citrulline requires NOS enzymes, which are homodimers containing N-terminal oxidase and C-terminal reductase domains. A salvage pathway also exists through which NO_3^- is reduced to NO_2^- and then NO [48]. The purine catabolic enzyme xanthine oxidoreductase (XOR) can reduce NO_3^- to NO [49]; however, the importance of XOR for endogenous NO synthesis is not known.

There are three NOS isoforms: neuronal (nNOS or NOS1), inducible (iNOS or NOS2), and endothelial (eNOS or NOS3) (Figure 1B–D). All NOSs require the same cofactors (O_2, Fe^{2+}-heme, flavins, NADPH, HSP90, tetrahydrobiopterin, and Ca^{2+}-bound CaM [50]), but each NOS has distinct tissue expression, regulation, and function. Interaction with the membrane protein PSD95 and N-terminal

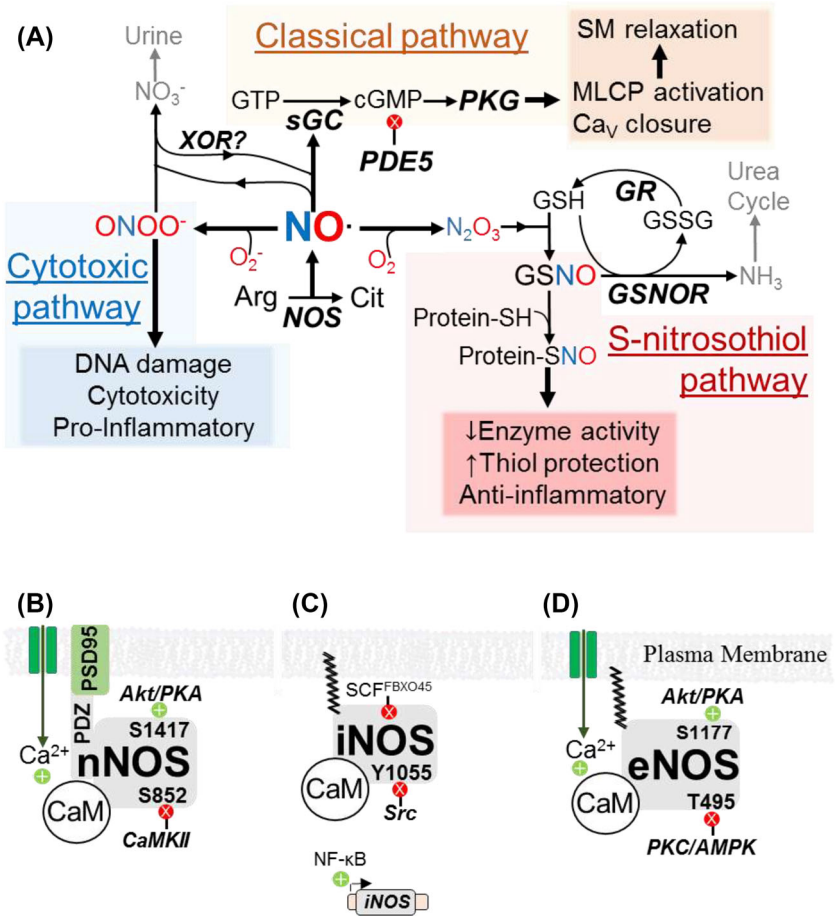

Figure 1. Nitric oxide metabolism and regulation. (A) Intermediates, enzymes (bold, italics), and biochemical effects (shaded boxes) of classical, S-nitrosothiol, and cytotoxic NO signaling. Gray text and arrows indicate excretion pathways. Arg: arginine. Cit: citrulline. GR: GSH reductase. SM: smooth muscle. (B–D) Transcriptional and post-translational regulation of neuronal (B), inducible (C), and endothelial (D) nitric oxide synthase (NOS) isoforms. Kinases, phosphoregulatory sites, and inhibitory ubiquitin ligases are shown. Green +'s and red X's indicate positive and negative regulation, respectively.

acylation enable membrane localization of nNOS and iNOS/eNOS, respectively [51]. Neuronal NOS and eNOS are constitutively expressed (nNOS in neurons and skeletal muscle; eNOS in endothelium, myocardium, and syncytiotrophoblast [STB]). In contrast, iNOS expression requires inflammatory signaling [52–56]. The cellular redox state is important for all three NOSs, as oxidative stress can "uncouple" the flow of electrons to produce O_2^- and promote the cytotoxic pathway [57]. Although nNOS, iNOS, and eNOS each require CaM, the EC_{50} for CaM activation of nNOS/eNOS is 100–300 nM Ca^{2+}, whereas iNOS is essentially Ca^{2+}-independent [58, 59]. Because resting intracellular [Ca^{2+}] is 10–100 nM [60], full nNOS/eNOS activation requires external Ca^{2+} entry via membrane channels (Ca_V) or internal release from endoplasmic reticulum stores. Phosphorylation and ubiquitination also regulate NOS activity. Phosphorylation at Akt/PKA consensus sites (S1417 of nNOS and S1177 of eNOS) reduces nNOS/eNOS Ca^{2+} dependence [61, 62]. Conversely, phosphorylation by CaMKII (S852 of nNOS) and PKC/AMPK (T495 of eNOS) diminishes NOS activity even at maximal Ca^{2+} concentrations [63–65]. The tyrosine kinase Src decreases iNOS activity via Y1055 phosphorylation, and the ubiquitin ligases ECS-SPSB and SCF^{FBXO45} facilitate iNOS and eNOS/nNOS degradation [66–69]. Compared with synthesis and signaling, NO diffusion and catabolism have received less attention. NO binds reversibly to circulating hemoglobin [70], so NO carried by red blood cells may exert endocrine actions in addition to autocrine and paracrine effects. Inhibition of the cGMP-degrading enzyme phosphodiesterase-5 (PDE5) by drugs like sildenafil (Viagra®) enhances penile erection [32], underscoring the importance of the classical pathway in vascular biology. NO spontaneously oxidizes to NO_3^-, which is eliminated in urine. GSNO reductase (GSNOR) couples GSH oxidation to SNO reduction [71], and S-nitrosation decreases GSNOR activity [72].

Carbon monoxide

Carbon monoxide (CO) is a relatively nonpolar, chemically stable gas composed of carbon triple bonded to oxygen. The two known CO signaling mechanisms are the classical (cGMP) pathway and a cGMP-independent pathway (Figure 2A). *Classical CO signaling* is mechanistically identical to classical NO signaling: CO activates sGC to increase cGMP stimulation of PKG. CO and NO bind sGC with similar affinity, and both elicit smooth muscle relaxation. However, NO-sGC is 25–50 times more active than CO-sGC [73]. Hence, in some circumstances CO competes with NO and can attenuate

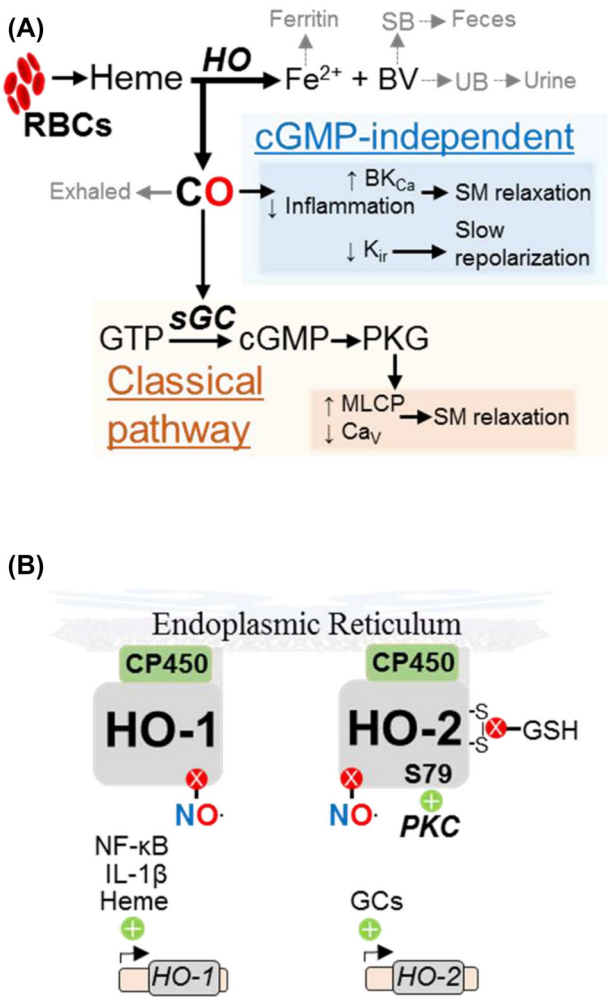

Figure 2. Carbon monoxide metabolism and regulation. (A) Intermediates, enzymes (bold, italics), and biochemical effects (shaded boxes) of classical and cGMP-independent CO signaling. RBCs: red blood cells, (B) Transcriptional and post-translational regulation of HO-1 and HO-2.

NO-mediated cGMP production [74]. In the *cGMP-independent CO pathway*, CO directly modulates protein function. CO binds heme groups of Ca^{2+}-gated large conductance K^+ channels (BK_{Ca}), which increases BK_{Ca} channel opening [75, 76]. CO also inhibits K^+ inward rectifier channels (K_{ir}) by an unidentified cGMP-independent mechanism [77]. Organometallic compounds (e.g. heme) probably mediate nonclassical CO signaling; CO is not very reactive in the absence of transition metals except at extreme temperature and pressure [78]. CO cannot covalently modify proteins. Thus, cGMP-independent NO and CO pathways do not directly interact or compete, and it is not known if the cGMP-independent NO and CO signals are physiologically additive or antagonistic.

Two homodimeric heme oxygenases, HO-1 and HO-2 (Figure 2B), synthesize CO. HO-1 and HO-2 localize to the endoplasmic reticulum and require O_2 and NADPH to oxidize Fe^{2+}-heme to CO, biliverdin IX-α (BV), and free Fe^{2+}. HO-1 and HO-2 associate with cytochrome P450 reductase (CP450), which reduces ferric (Fe^{3+}) heme from red blood cells to the HO substrate Fe^{2+} heme [79]. The HO reaction producing CO is the first step of porphyrin degradation [18]. NO inhibits both HO enzymes [80, 81], suggesting complex interactions between NO and CO.

Due to differences in protein structure and tissue expression, HO-1 and HO-2 are not redundant. Like iNOS, HO-1 is expressed during inflammation and oxidative stress. HSF1, NF-kB, HIF-1α, and heme upregulate HO-1 transcription and translation [82], and HO-1 in turn upregulates anti-inflammatory cytokines such as IL-10 [83]. HO-2 is constitutively expressed by neurons, glia, vascular endothelium, and endometrium [84]. Glucocorticoids stimulate HO-2 transcription, and post-translational modification activates (e.g. PKC serine-79 phosphorylation) or inhibits (e.g. GSH reduction of intramolecular disulfides) HO-2 activity [85, 86].

Excretion of HO products involves multiple organ systems. Endogenous CO, chemically stable and too dilute to influence O_2 transport, is exhaled during respiration [87]. Intracellular ferritin sequesters Fe^{2+} for use in iron-containing proteins. Porphyrin degradation enzymes process BV to urobilin and stercobilin, which are eliminated in the urine and feces, respectively [88].

Sulfide

In living systems, sulfide is a mixture of polar hydrogen sulfide gas (H_2S), hydrogen sulfide anion (HS^-), and nonpolar polysulfide (H_2S_n). The [H_2S]:[HS-] ratio approaches unity in vivo, and H_2S spontaneously oxidizes to H_2S_n. Because these are all bioactive and difficult to distinguish [89], they are collectively referred to as "sulfide." Sulfide signals through *cysteine persulfidation* (Cys-SH + sulfide → Cys-SSH, also called sulfhydration) and by transactivation of *classical cGMP signaling* via 8-HS-cGMP (Figure 3A). Persulfidation and S-nitrosation sometimes compete at target cysteines that alter enzyme activity [90, 91]. NFκB persulfidation reduces TNFα-stimulated apoptosis [90], while ATP-gated K^+ (K_{ATP}) and BK_{Ca} channel persulfidation hyperpolarizes cell membranes [92]. 8-HS-cGMP forms by persulfidation of 8-nitro-cGMP, a cGMP derivative that promotes autophagy and oncogenesis [93]. Compared with cGMP, 8-HS-cGMP resists degradation by PDE5. As such, 8-HS-cGMP augments cGMP signaling [3]. Recent reports suggest PDE5 inhibition contributes to sulfide-dependent smooth muscle relaxation [94, 95].

Three enzymes synthesize sulfide by cysteine oxidation: cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) which are primarily cytosolic, and 3-mercaptosulfurtransferase (3-MST) which is mitochondrial (Figure 3B–D) [89]. CBS and CSE can produce sulfide from numerous sulfur-containing amino acids, but cysteine and homocysteine (Hcy) are preferred substrates [96]. CBS is predominant in brain and kidney, whereas CSE is more abundant in liver and blood vessels [97]. CBS and CSE are also widely expressed as key enzymes in the reverse transsulfuration (RTS) pathway by which methionine (Met) is recycled to cysteine. 3-MST generates sulfide from 3-mercaptopyruvate (3-MP), a product of cysteine deamination. Expressed in all cell types, 3-MST is most abundant in liver, kidney, and brain [98].

Sulfide biosynthetic enzymes are subject to transcriptional and post-translational regulation. Oxidative stress stimulates ATF4- and Nrf2-dependent CSE transcription [99, 100], and estrogen (E_2) promotes CSE activity in human osteoblasts and mouse liver and vasculature [101, 102]. Multiple allosteric mechanisms regulate CBS activity. CBS binds S-adenosyl methionine (SAM) with high affinity, increasing CBS activity and exposing an NO/CO sensitive inhibitory

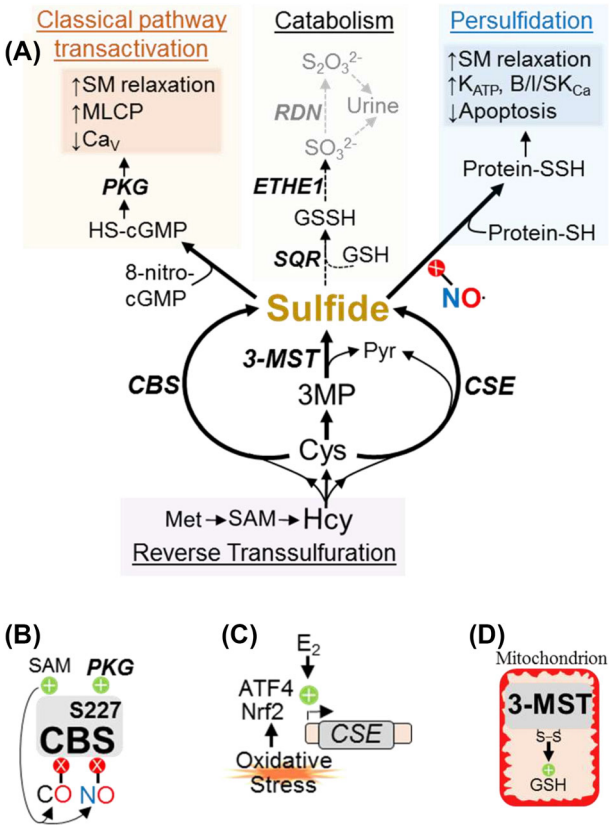

Figure 3. Sulfide metabolism and regulation. (A) Intermediates, enzymes (bold, italics), and biochemical effects (shaded boxes) of classical and persulfide-based sulfide signaling. B/I/SK_{Ca}: Ca^{2+}-gated large, intermediate, and small conductance K^+ channels. GSSH: GSH persulfide. Protein-SSH: Proteins with persulfidated cysteine residues. ROS: reactive oxygen species. (B–D) Transcriptional and post-translational regulation of CBS (B), CSE (C), and 3-MST (D).

heme moiety [103, 104]. On the other hand, PKG phosphorylation of CBS S227 increases CBS activity [105]. These apparently opposing mechanisms suggest that CBS activity is maintained within an ideal range; high NO inhibits CBS to prevent overactivation of the classical pathway, while CBS phosphorylation enables sulfide synthesis to resume when NO decreases. Rat liver CBS translocates to mitochondria during hypoxia [106], which could bolster ATP production and prevent apoptosis by supplying sulfide as an alternate electron donor. CSE also translocates to mitochondria during hypoxia [107], and 3-MST may be an oxidant sensor because 3-MST cysteine oxidation increases mitochondrial GSH levels [108].

Sulfide catabolism requires a trifecta of mitochondrial enzymes. Sulfide-quinone reductase (SQR), sulfide dioxygenase (ETHE1), and rhodanese (RDN) utilize a soluble thiol (likely GSH) to oxidize sulfide to sulfite (SO_3^{2-}) and thiosulfate ($S_2O_3^{2-}$), which are excreted in urine [109–111]. Since SQR, ETHE1, and RDN1 localize to mitochondria, alternate pathways exist in cells with few mitochondria. For example, erythrocytes couple methemoglobin reduction to sulfide oxidation [112].

Key points

- NO, CO, and sulfide are gaseous paracrine and autocrine signaling molecules.

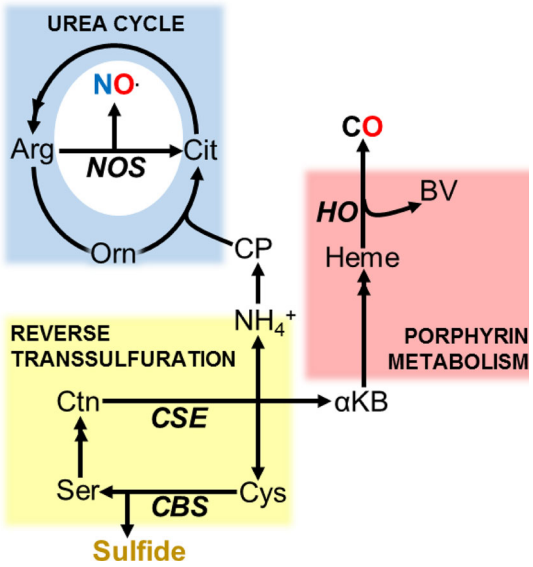

Figure 4. Integration of gasotransmitter metabolism. Sulfide synthesis via reverse transsulfuration generates NH_4^+ and αKB, which can produce NO and CO via the urea cycle and heme metabolism, respectively. αKB: α-ketobutyrate. CP: carbamoyl phosphate. Ctn: Cystathionine. Orn: ornithine. Ser: Serine. Double arrowheads indicate pathways in which multiple intermediates have been omitted for clarity

- NO is produced by arginine oxidation or NO_3^- reduction. The NO metabolites GSNO and $ONOO^-$ mediate S-nitrosation and cytotoxicity, respectively.
- CO is made via heme oxidation.
- Sulfide is generated by cysteine oxidation. Sulfide augments the classical cGMP pathway and increases target protein activity by thiol persulfidation.
- All three gasotransmitters activate the classical sGC-cGMP-PKG pathway. All three can modify ion channels by covalent modification or heme binding.

Gasotransmitter crosstalk

As discussed above, reactions among gas molecules can alter signaling (e.g. O_2^- and NO produce $ONOO^-$). Gasotransmitters can also regulate each other's production or activity (e.g. CO reduces NO stimulated sGC activity) or metabolism (e.g. sulfide prolongs NO signals by inhibiting PDE5 activity). Other interactions likely exist among gasotransmitter metabolic pathways (Figure 4).

Having reviewed gasotransmitter biochemistry, in the next section we turn to specific roles of NO, CO, and sulfide in pregnancy.

Fueling pregnancy: gasotransmitters in conception, placentation, and vascular adaptation

Fallopian tube transit, decidualization, and implantation

Gasotransmitters and their biosynthetic enzymes change as the nascent conceptus travels to the uterus (Figure 5A). Women exhale twice as much CO in the luteal phase compared to the follicular phase [113]. The reason for this change is unknown but may reflect regulation of tubal transit or early systemic adaptations

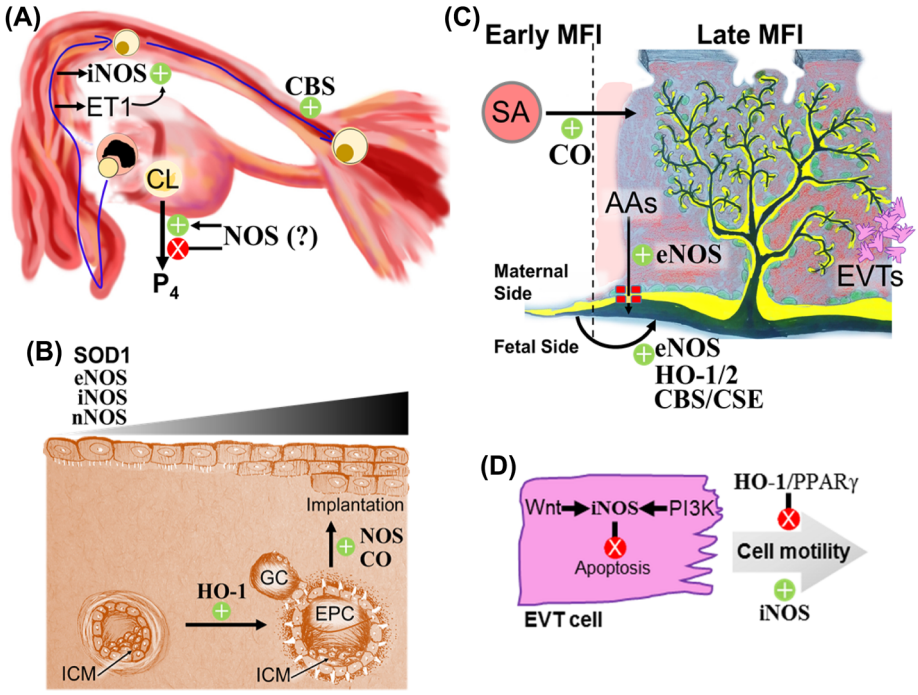

Figure 5. Gasotransmitters regulate fallopian tube transit, decidualization, implantation, and placentation. (A) *Fallopian tube transit.* ET1 from the fallopian endothelium upregulates iNOS in the fallopian ampulla. Deficiencies in CBS hinder transit of fertilized embryos. Depending on its concentration, NO stimulates or inhibits pregnancy-sustaining progesterone (P_4) by the corpus luteum (CL). (B) *Decidualization and implantation.* HO-1 drives proliferation of ectoplacental cone (EPC) cells. Decidual eNOS, nNOS, iNOS, and SOD1 increase in abundance as the endometrium decidualizes, and NOS activity facilitates implantation. ICM: inner cell mass, GC: Giant cells. (C) Developmental changes at the maternal-fetal interface (MFI). During pregnancy, CO facilitates remodeling of maternal spiral arteries (red circle) into low-resistance, high-flow canals (red patch) surrounding the placental villi (yellow, green, and black branched structure). Endothelial NOS potentiates placental amino acid transport from maternal circulation (red) to fetal circulation (black). On the fetal side, eNOS, both HO isoforms, CBS, and CSE facilitate dilation and angiogenesis of fetal vasculature (black). AAs: amino acids. SA: spiral arteries. Green circles and yellow layers represent cytotrophoblasts and the syncytiotrophoblast, respectively. (D) *EVT motility.* Wnt and PI3K pathways both activate iNOS, which inhibits EVT apoptosis and promotes cell motility. HO-1 and PPARγ inhibit EVT motility. Green +'s and red X's denote up- and downregulation, respectively.

of pregnancy. In cattle, endothelins induce iNOS accumulation in the ampulla [114]. Human fallopian tubes express CBS, which produces sulfide to relax fallopian tube smooth muscle [115]. In mice, CBS inhibition causes morula retention in the tube. This suggests NO and sulfide can counteract procontractile endothelins to facilitate tubal peristalsis and embryo transit.

Decidualization is endometrial maturation under the influence of progesterone (P_4) secreted by the corpus luteum (Figure 5B). It includes mesenchymal-to-epithelial transdifferentiation of endometrial fibroblasts into secretory stromal cells, invasion of leukocytes that assist tissue reorganization, and capillary angiogenesis to potentiate implantation [116]. Human decidua expresses both eNOS and iNOS in the vascular endothelium and glandular epithelium [117]. In nonhuman primates, decidualization enhances endometrial nNOS, iNOS, and eNOS expression [118], while in mice only iNOS and eNOS accumulate. In rats, the nonselective NOS inhibitor L-nitroarginine methyl ester (L-NAME) blocks decidualization [119, 120]. NO (probably from eNOS) also promotes secretory endometrial differentiation and endometrial vessel dilation to increase perfusion during early pregnancy. Additionally, first trimester decidua from women with spontaneous miscarriage shows higher prostaglandin $F_{2\alpha}$ and lower Cu-Zn SOD (SOD1) than normal pregnancies [121]. This suggests SOD1 could suppress cytotoxic $ONOO^-$ production [46], preventing feed-forward synergy between rising NO and prostaglandin $F_{2\alpha}$.

The rigid glycocalyx surrounding the preimplantation embryo (the zona pellucida) is disassembled just before decidual implantation, a process called "hatching." In mice, both NO donors and NOS inhibitors prevent hatching [122], suggesting hatching requires an optimal NO concentration. Implantation occurs when trophoblast cells of a hatched blastocyst attach to the decidualized endometrium [123] (Figure 5B). Trophoblast comprises multinucleated STB and mononucleated cytotrophoblasts (CTBs), some of which differentiate into extravillous trophoblasts (EVTs). In the preimplantation murine embryo, giant cells are analogous to human EVTs, and the ectoplacental cone is analogous to STBs and CTBs. If implantation is successful, EVT invasion extends chorionic villous STBs and CTBs deep into the decidua as the blastocyst inner cell mass undergoes gastrulation. Although giant cells and the ectoplacental cone express iNOS, and NOS inhibitors abolish implantation [119, 124], iNOS knockout mice have no pregnancy defects [125]. In contrast, conceptus-derived CO is crucial for implantation; HO-1 knockout embryos make fewer ectoplacental cone cells and implant less efficiently, a phenotype rescued by exogenous CO inhalation. In HO-1 heterozygous dams, 40% of HO-1 null fetuses die by E14, compared with only 20% of HO-1 heterozygous fetuses and 10% of wild-type fetuses [11]. CO upregulates and is upregulated by vascular endothelial growth factor (VEGF), a family of angiogenic signaling proteins [126]. However, CO likely promotes trophoblast proliferation independent from VEGF and angiogenesis since inhibition of HO

enzymatic activity prevents differentiation of immature trophoblast stem cells into giant cells [11].

After ovarian follicle oocyte release, the corpus luteum forms from the remnant steroidogenic theca and granulosa cells. Luteal P_4 sustains all mammalian pregnancies prior to placentation and is required for the entire pregnancy in rodents. In humans, by 10–12 weeks' gestation the placenta replaces the corpus luteum as the main source of P_4. Low concentrations of NO donors enhance luteal P_4 secretion in horses, sheep, and rats, whereas high concentrations of NO donors decrease P_4 [127–129]. High NO bolus doses (e.g. 0.1–1 mM) or NOS activators diminish P_4 and increase apoptosis of corpora lutea obtained from eumenorrhoeic women [130, 131]. Similar to blastocyst hatching, intermediate NO levels may optimally prevent luteolysis.

Key points

- Fallopian tube iNOS and CBS levels increase during embryo transit to the uterine cavity. Sulfide facilitates tubal peristalsis.
- NO promotes decidualization, and eNOS increases in endometrial vascular endothelium and glandular epithelium from conception to placentation.
- CO enhances trophoblast proliferation and aids embryo implantation and early survival.
- Elevated NO prevents P_4 synthesis and can be luteolytic in early pregnancy.

Feto-placental angiogenesis and hemodynamics

Primates, rodents, and lagomorphs exhibit hemochorial placentation, in which maternal and fetal blood are separated by placental CTB/STB and chorionic villus capillary endothelial cells (Figure 5C). Gas, nutrients, and wastes are transferred between maternal blood from spiral arteries (which become low-resistance conduits to the intervillous space after remodeling) and fetal blood in placental capillaries. Inadequate placental vascularization limits nutrient extraction (particularly O_2) required for normal fetal growth. Abnormal placentation is associated with intrauterine growth restriction (IUGR), gestational diabetes, preeclampsia, and preterm birth [132].

Decidual invasion by EVTs is critical for normal pregnancy (Figure 5D), and in vitro iNOS stimulates EVT motility and invasiveness while inhibiting EVT apoptosis. Interdependent PI3K-Akt-mTORC1 and Wnt-β-catenin pathways activate EVT iNOS [133–135], and conversely iNOS and sGC inhibition inactivate Akt, destabilize β-catenin, and increase EVT apoptosis [136, 137]. The invasive EVT leading edge accumulates iNOS and matrix metalloprotease-9 (MMP9). Although MMP9 inhibition curtails EVT motility in cell culture models [138, 139], this has not been tested in vivo. Murine pregnancy does not require iNOS [125], but there is a correlation between stimuli that reduce trophoblast invasion and reduced placental iNOS and MMP9 [140]. On the other hand, HO-1 expression (and likely CO production) is inversely related to trophoblast invasiveness. By an unknown mechanism, HO-1 and PPARγ reduce EVT motility. HO-1 and PPARγ are decreased in first trimester EVTs, while overexpression or knockdown of HO-1 respectively enhances or suppresses PPARγ [141, 142], and HO-2 neutralizing antibodies inhibit CTB migration [143]. In contrast, iNOS-dependent EVT invasiveness is sensitive to CO activation of PPARγ [144].

Development of placental chorionic villi (composed of fetal vessels surrounded by CTB and STB) requires trophoblast remodeling (Figure 5C). Feto-placental angiogenesis and vasodilation occur throughout pregnancy to maintain fetal nutrient delivery; lower HO-2 in placental vascular endothelium correlates with preeclampsia and IUGR [145, 146]. Placental microvasculature is less developed in HO-1 knockout mice [147]. In rats, placenta-permeable HO inhibitors decrease placental growth factor (PLGF, a VEGF homolog [148]) and increase placental oxidative stress [149]. These effects are probably due to CO deficiency, as exogenous CO prevents HO inhibitor-induced giant cell apoptosis, and low dose inhaled CO in pregnancy rescues the HO-1 null placental structural defects [11]. Placental sulfide may also stimulate placental angiogenesis via miRNAs. Relative to term healthy placentas, CBS and CSE protein and VEGF transcript levels are lower and miRNAs 20a and 200c increase in term preeclamptic placentas. Sulfide donors rescue VEGF levels and decrease miR20a/200c and sFlt1. CBS or CSE siRNA knockdown in human placental explants also blocks sulfide donor effects [150, 151]. Further, CBS knockout dams resorb most CBS heterozygous embryos, underscoring the importance of maternal sulfide production [5]. Based on these data, CO and sulfide likely promote placental angiogenesis via PLGF and VEGF, respectively. Both VEGF [152] and villous trophoblast sulfide [153] stimulate Akt-dependent activation of eNOS, and eNOS-deficient mice exhibit placental hypoxia [4]. This suggests NO also mediates placental angiogenesis.

Along with placental angiogenesis, normal pregnancy requires regulated placental hemodynamics. NO donors dilate term human placental arteries in a sGC and BK_{Ca}-dependent manner [154]. VEGF increases eNOS expression in human umbilical vein endothelial cells and increases NO production from excised human umbilical vein [155, 156]. In IUGR placentas, NO effects are attenuated, and the proportion of phosphorylated eNOS S1177 (more active) decreases [157]. IUGR placental arteries also show eNOS promoter hypermethylation [158] and impaired Ca^{2+}-dependent eNOS activation [159], suggesting that eNOS mediates placental NO production. Similarly, CO dilates human placental vessels via cGMP-PKG [160]. Although hypoxia does not induce HO-1 or HO-2 in term placental explants [161], HO inhibitors do reduce vasodilation and decrease placental perfusion [162–164]. Sulfide also regulates placental vascular tone. In human preeclamptic placentas, miRNA 20 suppresses CSE accumulation [165], and stem villus artery CSE loss is associated with human IUGR [166]. Placental explants subjected to hypoxia-reoxygenation show reduced actin and myosin, but sulfide donors prevent those changes [166]; thus, placental vascular sulfide may protect against IUGR. K_{ATP} channel and NOS may synergize to mediate placental sulfide signaling since glibenclamide (a K_{ATP} channel blocker) and L-NAME both block sulfide donor-dependent placental artery vasodilation [165]. Sulfide can augment classical signaling via PDE5-resistant 8-nitro-cGMP [3, 94, 95] and $ONSS^-$ stimulation of cGMP synthesis [41, 42]. These data show that gasotransmitters have both acute (vasodilation) and chronic (angiogenesis and cytoskeletal remodeling) effects on placental vasculature.

Key points

- NO inhibits EVT apoptosis and stimulates EVT motility via Akt and Wnt pathways.
- HO-1 and PPARγ reduce EVT motility.
- HO enzymes and sulfide promote placental angiogenesis via PLGF and VEGF expression.
- Endothelial NOS produces NO to dilate placental capillaries, and CSE produces sulfide to augment NO production and signaling

and further relaxes placental vascular smooth muscle by activating K_{ATP} channels.

- IUGR correlates with lower HO-1, HO-2, CBS, and CSE expression and decreased eNOS S1177 phosphorylation in placental vessels.

Maternal hemodynamics

Maternal vascular resistance decreases in pregnancy as cardiac output and blood volume increase, thus maintaining blood pressure. Central nervous system NO may regulate maternal hemodynamics. The hypothalamic paraventricular nucleus regulates autonomic sympathetic output [167, 168], which increases peripheral vascular resistance. In rats, paraventricular nNOS declines during pregnancy [169], and paraventricular expression of a dominant negative nNOS mutant increases vascular resistance [170]. Sympathetic neurotransmission also rises in pregnant women [171, 172], whereas parasympathetic output decreases [173–175]. This suggests decreased paraventricular nNOS in pregnancy disinhibits the sympathetic nervous system [169, 176] despite the overall decrease in vascular resistance during pregnancy. This is probably because of hormone-dependent increased NO production in the vasculature. Serum relaxin, a smooth muscle relaxant, and estrogen (E_2) increase several hundred-fold during pregnancy [177–179]. Relaxin induces vasodilation via PI3K-Akt phosphorylation of eNOS S1177 [180]. E_2 reduces paraventricular nNOS protein expression in rats [181] and increases eNOS transcription via E_2 receptors (ERs) [181, 182]. E_2 promotes phosphorylation of eNOS at S1177 by activating plasma membrane ERα-$G_{\alpha i}$ to stimulate a Src-PI3K-Akt pathway [183–185]. While ERα promotes activating eNOS S1177 phosphorylation, ERβ curtails inhibitory phosphorylation of eNOS T495 in pregnant bovine uterine artery endothelial cells [186]. Pregnant women express more uterine artery eNOS than age-matched nonpregnant women [187, 188], and E_2 promotes uterine artery eNOS accumulation in ovariectomized sheep [189]. Moreover, eNOS knockout mice are hypertensive [190]. These studies suggest decreased paraventricular nNOS helps maintain vascular resistance to balance the increased peripheral vasodilation necessary for pregnancy (Figure 6A).

CO and sulfide also regulate maternal vascular tone, and low respiratory and serum CO and sulfide are associated with hypertension in pregnancy. Exhaled CO is inversely correlated with gestational hypertension [191], while longer HO-1 promoters attenuate HO-1 expression and are predictive of preeclampsia [146]. In mice, HO inhibition at mid-pregnancy does not affect placental histology or fetal growth, but does increase maternal vascular resistance [192]. Therefore, while early placentation requires embryonic HO, maternal HO curtails vasoconstriction later in pregnancy. Uterine artery CBS increases during pregnancy [188], and decreased maternal serum sulfide and placental CBS and CSE protein levels correlate with preeclampsia [165, 193, 194]. CSE knockout mice exhibit reduced VEGF-dependent angiogenesis [195], and sulfide donors block sFlt1-induced hypertension in rats [196]. sFlt1 is a soluble VEGF receptor that reduces free serum VEGF, antagonizes VEGF signaling, and is linked to preeclampsia [196]. Sulfide may also regulate maternal vascular tone by directly persulfidating smooth muscle K_{ATP} channels [197, 198]. During pregnancy, K_{ATP} and CBS levels increase in human and ovine uterine artery smooth muscle cells [199, 200]. Endogenous sulfide stimulates K_{ATP} opening in rat mesenteric artery smooth muscle cells [201], and sulfide donors inhibit ATP-dependent

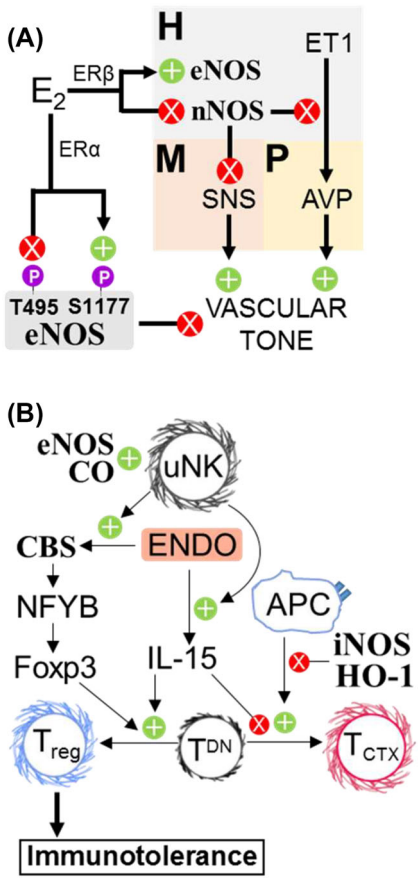

Figure 6. Gasotransmitters regulate maternal vascular tone and immunotolerance. (A) Prior to pregnancy, nNOS in the paraventricular nucleus of the hypothalamus (H) limits sympathetic activity in the rostral ventrolateral medulla (M) and inhibits endothelin 1 (ET1)-mediated release of vasopressin from the pituitary (P), both of which reduce peripheral vascular resistance. During pregnancy, rising E_2 inhibits PVN nNOS and stimulates PVN eNOS expression via ERβ. ERα facilitates E_2-mediated eNOS activation in the periphery by promoting S1177 and curtailing T495 phosphorylation. AVP: Vasopressin. (B) CO promotes recruitment of uNKs to the decidua, which enhance fetal alloimmune tolerance via endometrial IL-15 and CBS. Inducible NOS and HO-1 activity attenuate T_{CTX} maturation, while persulfidation of NFYB enhances naïve T^{DN} differentiation into T_{regs}. Endo: endometrium. T^{DN}: double-negative ($CD4^-$ $CD8^-$) naïve T cells.

Ca^{2+} entry into porcine vascular smooth muscle cells [202]. Hence, K_{ATP} persulfidation may increase uterine blood flow by antagonizing vasoconstriction.

Key points

- E_2 promotes maternal vascular eNOS transcription, protein accumulation, and NO production.
- E_2 downregulates hypothalamic nNOS protein expression in pregnancy. This increases sympathetic tone and thereby maintains blood pressure.
- HO activity decreases maternal vascular resistance during pregnancy.
- CSE- and CBS-produced sulfide enhance VEGF signaling in endothelial cells and K_{ATP} channel opening in vascular smooth muscle to lower vascular resistance.

Maternal angiogenesis and vascular remodeling

The maternal side of the maternal-placental interface undergoes vasodilation and angiogenesis akin to the fetal side to support adequate placental exchange. Uterine blood flow increases dramatically during pregnancy to about 20% of cardiac output by the third trimester. This is accommodated by increased myometrial radial artery angiogenesis, as well as remodeling of endometrial spiral arteries to low-resistance, high-flow canals that maximize maternal blood flow to the intervillous space (Figure 5C). Inadequate placental perfusion may result in placental ischemia and oxidative stress and promote the release of placental factors that cause preeclampsia [203]. Both NO and CO regulate uterine blood flow.

Although eNOS expression is similar in uterine artery endothelial cells from pregnant and virgin sheep, VEGF only stimulates uterine artery eNOS during pregnancy. VEGFR2 increases PI3K-dependent Ca^{2+} entry, which stimulates eNOS independently of ERK1/2, Akt, or eNOS S1177 phosphorylation [204, 205]. Pregnant eNOS knockout mice exhibit IUGR that is rescued by administration of an endothelin receptor A antagonist [190]. Similar to fallopian tube peristalsis, maternal uterine artery expansion could involve dynamic interplay between endothelin-dependent contraction and eNOS-dependent relaxation. Additionally, the placental basal plate expresses both HO isoforms, which influence placental perfusion [145, 162–164, 206]. HO-1 knockout mice exhibit reduced spiral artery remodeling, and human IUGR correlates with longer maternal HO-1 promoters (associated with decreased HO expression) but not with HO activity [11, 145, 146, 164]. CO inhalation (50 ppm) in early pregnancy nevertheless increases placental bed angiogenesis in HO-1 knockout mice [6, 11], so CO may drive uteroplacental vascular remodeling [149].

Spiral artery remodeling is associated with the appearance of decidual uterine natural killer cells (uNKs) in early pregnancy. The precise function of uNKs is still uncertain, but they mediate allorecognition and tolerance during placentation and also assist spiral artery remodeling [207]. They promote endometrial accumulation of VEGF-C that induces lymphangiogenesis [208, 209]. All three gasotransmitters regulate uNKs. Titers of uNKs are lower in mice with genetic deletion of eNOS, HO-1, or CBS. Decreased decidual uNKs in eNOS knockouts [4] is consistent with eNOS-dependent human NK resistance to apoptosis [210] and indicates NO autocrine or paracrine facilitation of uNK proliferation. Remarkably, early pregnancy CO inhalation (50 ppm) rescued uNK levels in pregnant HO-1 heterozygous mice and normalized the growth of HO-1 knockout conceptuses [6, 211]. CO, therefore, appears necessary for early pregnancy local immune cell changes but dispensable in late pregnancy. Uterine NKs upregulate decidual transcription of CBS and CSE [208], and early pregnant CBS knockout mice produce fewer uNKs [5]. Despite these connections, mechanisms are speculative since uNK origins are not clearly understood. While CO and NO cause uNK proliferation, CBS regulation of uNKs may be indirect.

Key points

- VEGF induces eNOS to facilitate maternal uterine artery vasodilation.
- HO promotes spiral artery remodeling.
- CO increases uNKs in pregnancy that in turn upregulate endometrial CBS and CSE expression.

Immune adaptation

The developing conceptus is an allograft sharing only half of the maternal genes. Since maternal leukocytes can cross the placenta and enter fetal circulation [212] and may identify the placenta and fetus as foreign, maintaining maternal immune tolerance is critical for successful gestation. Pregnancy reshapes the maternal adaptive immune system (Figure 6B). Early in pregnancy, the ratio of T helper cell type-1 (T_h1) cytokines (i.e. IFNγ) to T_h2 cytokines (i.e. IL-4 and IL-10) decreases. Since T_h2 cells transduce inflammatory signals less efficiently than T_h1s [213], this shift likely contributes to maternal tolerance. In animal models, NOS and HO facilitate the T_h1/T_h2 shift. Lymphocyte NO synthesis increases during bovine pregnancy, and in pregnant goat serum NO_3^- correlates with T_h2 cytokines [214, 215]. Maturation of rat naïve T cells into cytotoxic effectors (T_{CTX}) requires downregulation of antigen-presenting cells' iNOS and HO-1 [216]. CO and sulfide also regulate T regulatory cell (T_{reg}) suppression of T_{CTX} maturation. HO inhibitors raise dendritic cell titers and induce embryo resorption in mice [217], but exogenous CO prevents antigen-presenting cell activation and T_{CTX} maturation by a cGMP-independent pathway [218, 219]. Similarly, CBS- or CSE-produced sulfide *persulfidates* NFYB, driving Tet1/2 methylcytosine dioxygenase expression and de-repressing Foxp3 expression. Foxp3 (a forkhead transcription factor) commits $CD4^-$ $CD8^-$ progenitors to become T_{regs} [220]. Because CBS loss impairs murine fertility and uNK titers [5], CBS may regulate the uterine T_{reg} niche.

Key points

- Inducible NOS and HO-1 suppress naïve T-cell differentiation into T_{CTX}.
- HO-1 increases naïve T-cell differentiation to $T_{regs.}$
- Sulfide increases T_{reg} cells via NPYB persulfidation and increased Foxp3.

Rising to delivery: gasotransmitters in later pregnancy and parturition

Myometrial quiescence

The myometrium is the uterine visceral smooth muscle between the endometrium and the serosa. Endocrine signals (e.g. corticotrophin releasing hormone, P_4 withdrawal) drive myometrial contractility through expression of contraction-associated proteins and altered ion channel activity. The myometrium is quiescent for about 90% of pregnancy until maternal-fetal-placental signals stimulate expression of G_q protein-coupled oxytocin receptor (OTR), prostaglandin $F_{2\alpha}$ receptor, cyclooxygenase enzymes, and the gap junction protein Connexin 43 [221]. The OTR and prostaglandin $F_{2\alpha}$ receptors activate phospholipase C to produce inositol triphosphate and diacylglycerol, which promote myocyte depolarization, voltage-sensitive L-type Ca_v channel activation, and regular intracellular Ca^{2+} spikes. Increased Ca^{2+} activates CaM-dependent myosin light chain phosphorylation and myosin-actin cross-bridge cycling to increase contractile force (Figure 7A). In contrast, relaxation occurs when the contractile phenotype has not been activated or when intracellular $[Ca^{2+}]$ is insufficient to activate MLCK. In addition to actin, myosin, and regulatory kinases and phosphatases, cytoskeletal accessory proteins can influence myocyte contractility. Connexin 43 increases electrochemical connections among myocytes, allowing depolarization to spread and amplify during labor [222]. About half of preterm labor leading to preterm delivery involves precocious

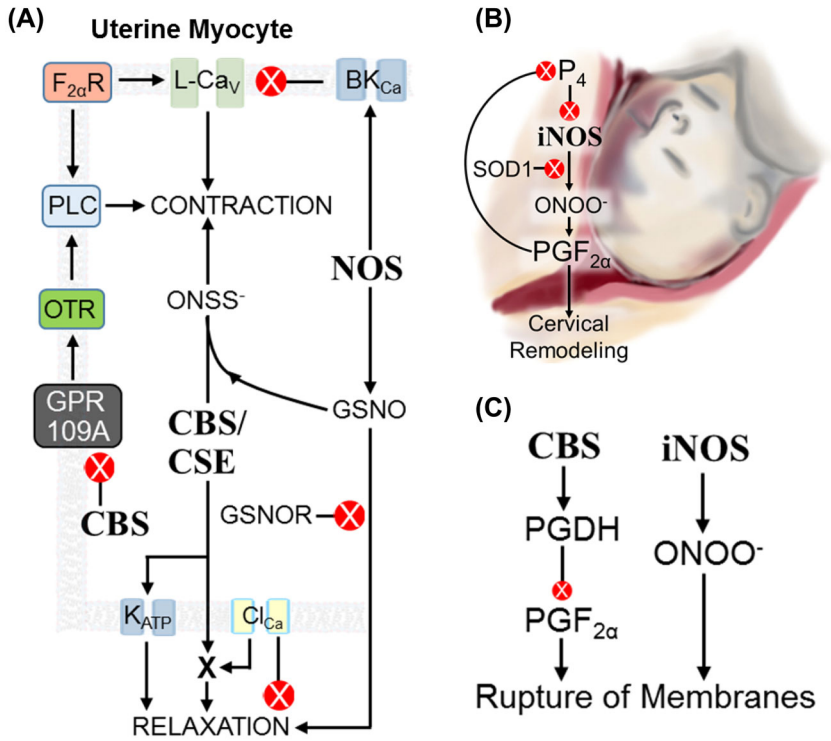

Figure 7. Gasotransmitters in parturition. (A) Regulation of myometrial contractility (depicted cell is a uterine myocyte). NOS activity in myocytes or adjacent endothelium inhibits contraction by stimulating BK_{Ca} and raising GSNO levels. GSNO is tocolytic. Myocyte CBS curtails GPR109A activity, which reduces OTR signaling. CBS and/or CSE also activate K_{ATP} and potentiate an unidentified protein downstream of Cl_{Ca} that induces tocolysis. Sulfide and GSNO produce $ONSS^-$, which promotes uterine contractility. $F_{2\alpha}R$: receptor for prostaglandin $F_{2\alpha}$ PLC: phospholipase C. (B) Cervical remodeling. In early pregnancy, P_4 inhibits iNOS synthesis. More iNOS and less SOD1 near parturition allows $ONOO^-$ to accumulate, which stimulates prostaglandin $F_{2\alpha}$ synthesis. Prostaglandin $F_{2\alpha}$ in turn promotes PR-A accumulation and thus blocks P_4 perception. (C) Rupture of fetal membranes. Inducible NOS activity drives formation of $ONOO^-$, which accelerates membrane rupture. By an unknown mechanism, CBS activity promotes PGDH-dependent prostaglandin $F_{2\alpha}$ degradation, which slows fetal membrane rupture.

activation of uterine contractions. To prolong pregnancy, investigators have studied mechanisms of tocolysis (the cessation of myometrial contraction). Exogenous NO and sulfide potently relax oxytocin-stimulated and spontaneous myometrial contractions [223, 224].

Endothelial NOS may be the primary uterine NO source in pregnancy. In humans, E_2 increases myometrial eNOS expression [225], while in mice both P_4 and E_2 increase uterine eNOS [226]. Human pregnant myometrium expresses vascular eNOS [225, 227] and myometrial eNOS [228, 229]. Since NO can freely diffuse between cells, localization may not be critical. Whereas rat uterine iNOS and eNOS increase until labor at E22, nNOS decreases by E18 [230, 231]. Like vascular and gastrointestinal smooth muscle, the myometrium contains autonomic (predominantly sympathetic) nerve endings. However, female puberty coincides with E_2-dependent reduction of myometrial sympathetic innervation [232, 233], and uterine neuronal signals are nearly undetectable during pregnancy [234–236]. Collectively, there is little evidence for neuronal control of the myometrium, and nNOS is probably not a major quiescence mediator.

Pregnant myometrium expresses both HO isoforms as well as CBS and CSE. Myometrial explants from term laboring (TL) and term nonlaboring (TNL) pregnancy express more HO-1 and HO-2 and produce more CO than explants from nonpregnant women. Exogenous heme relaxes term human and E22 rat myometrial strips [237], but exogenous CO gas has no effect [238]. Hence, other HO products (Fe^{2+} or BV) are likely the tocolytic agent(s). TNL explants also express more CBS and CSE and lower levels of contraction-associated proteins (e.g. OTR) compared with TL tissue, and there is one report of endogenous cysteine-dependent relaxation of TL myometrium via CBS [239, 240]. Compared with wild-type animals, $CBS^{+/-}$ dams express OTR earlier and deliver earlier. Recent publications also report differential S-nitrosation of myometrial cytoskeletal proteins during pregnancy that may influence contractility [241–243].

There is some debate regarding molecular mechanisms of gasotransmitter-induced tocolysis. Classical NO signaling is insufficient to explain NO tocolysis because pharmacological inhibition of cGMP pathways does not inhibit uterine NO effects [223, 244]. Nonclassical NO and sulfide myometrial targets fall into three groups: cytoskeleton-associated proteins, ion channels, and membrane receptors. MLCK, vinculin, and galectin-1 SNOs are less abundant in preterm laboring human myometrium than in TNL tissue [244], while pregnant guinea pig myometrium exhibits increased desmin, vimentin, and transgelin SNOs relative to nonpregnant myometrium [38]. Myometrial GSNOR expression is higher in women with preterm labor than TNL [245], suggesting myometrial SNOs maintain uterine quiescence. Active site cysteine SNOs decrease the activity of numerous enzymes, including GAPDH [246], SIRT1 [247], and PDK1 [248], but effects of noncatalytic cysteine

SNOs on actin-myosin accessory proteins are complex. S-nitrosation decreases skeletal muscle myosin cross-bridge cycling velocity and increases myosin stall force [249], and a cofilin-1 SNO depolymerizes actin fibers [250]. Myometrial protein SNOs correlate with pregnancy status, but their relationship to contractility is uncertain. $ONSS^-$ promotes *contraction* of rat myometrium despite *relaxing* blood vessels [42]. This may be due to unique cell-specific signaling mechanisms in vascular and myometrial smooth muscle. Altered protein expression in the two muscle types has been described; for example, α-actin and vimentin contents are higher in vascular muscle compared to visceral muscle [251]. Whatever the underlying mechanism, there are clear difficulties with directly extrapolating vascular findings to uterine myometrial function.

Uterine smooth muscle BK_{Ca} and K_{ATP} channels are important mediators of uterine quiescence [252, 253]. Contractile stimuli induce membrane depolarization [254] that promotes opening of voltage-sensitive Na_v and Ca_v channels. As Ca^{2+}-CaM dependent contraction proceeds, Ca^{2+} activates BK_{Ca} to increase K^+ outflow, hyperpolarize the membrane, and close Ca_v channels. Ca^{2+} pumps reduce cytosolic [Ca^{2+}], and the smooth muscle cell relaxes [255, 256]. At rest, BK_{Ca}, Na_v, Ca_v, and Ca^{2+}-gated Cl^- channels (Cl_{Ca}) are closed, K_{ir} channels are intermittently open, and the resting membrane potential matches the K^+ reversal potential. NO donors and arginine increase BK_{Ca} channel opening, and PKG/NOS inhibitors block BK_{Ca} current in myometrial cells from pregnant women [257]. PKG agonists and cGMP analogs stimulate BK_{Ca} activity in pregnant myometrial myocytes, but not in nonpregnant myocytes [258]. K_{ATP} is active when the ATP:ADP ratio decreases, and increased K^+ permeability maintains uterine myocyte hyperpolarization and quiescence [259, 260]. Cysteine and sulfide donors activate myometrial K_{ATP} via persulfidation, but tocolysis is sensitive to the K_{ATP} inhibitor glibenclamide [9, 239, 261]. The mechanism may be more complex, as antagonists of myometrial Cl_{Ca} channels (which conduct outward Cl^- current and are therefore depolarizing/procontractile) [262, 263] paradoxically *inhibit* sulfide relaxation of rat myometrium [9]. Sulfide may, therefore, act downstream of Cl_{Ca} channels.

G protein-coupled receptors and receptor kinases are expressed throughout the myometrium (e.g. OTR, VEGF receptor) and can be regulated by gasotransmitters. Intraperitoneal sulfide reduces murine preterm birth induced by the Toll-like receptor agonist LPS [10, 264, 265], but the mechanism is not known. The G protein-coupled niacin receptor GPR109A promotes inflammatory pathways and accumulates in the placenta and uterus of pregnant CBS heterozygous dams [266, 267]. Intriguingly, GPR109A deletion rescues OTR overexpression and premature delivery in pregnant CBS heterozygous mice [266], suggesting sulfide antagonizes GPR109A-activated contractility. Complete understanding of uterine receptor modulation by gasotransmitters will require additional investigation.

Key points

- Uterine eNOS, CBS, and CSE levels increase during pregnancy and decrease during labor.
- The HO substrate heme is a tocolytic, but direct CO application is not.
- NO activates both classical pathway and non-cGMP dependent uterine relaxation.
- NO activates myometrial smooth muscle BK_{Ca} activity, and sulfide increases myometrial K_{ATP} activity.
- S-nitrosation of cellular contractile proteins correlates with preterm labor.

Cervical remodeling

Cervical remodeling is the softening, shortening, and dilation of the uterine cervix before delivery. It begins in mid pregnancy with glycosaminoglycan degradation, decreased collagen fiber production, and neutrophil/macrophage invasion [268–270]. Inflammatory mediators such as IL-8, IL-1α, and prostaglandin $F_{2\alpha}$ augment cervical effacement (i.e. shortening) [271, 272] and relax the myocervical circular smooth muscle [273, 274]. NO promotes cervical remodeling.

Pregnant human cervix expresses all three NOS isoforms, and cervical fluid NO_3^- is lower in TNL women [275]. Cervical epithelia contain nNOS. Inducible NOS and eNOS accumulate in vascular endothelial cells, and cervical leukocytes also express iNOS. Cervical iNOS increases 2-fold between the first trimester and term [276], and human cervical fibroblasts treated with IL-1α upregulate iNOS transcription 16-fold. L-NAME blocks IL-1α stimulated secretion of matrix metalloprotease-1 [277], suggesting that iNOS can facilitate cervical ripening. P_4 suppresses iNOS transcription in RAW macrophages [278] and prevents NO-stimulated prostaglandin E_2 production in human cervical explants [279, 280]. In rodents, peripartum luteolysis reduces P_4, which then permits iNOS accumulation [231]. P_4 receptor (PR) antagonists accelerate cervical remodeling in humans and animals and increase iNOS transcription fourfold in pregnant rats [281]. P_4 and cervical iNOS are inversely related near the end of pregnancy and intimately associated with inflammation signals prior to parturition. In humans, P_4 levels do not decline, but PR switching before labor from active PR-B to truncated PR-A causes *functional withdrawal* of P_4 signaling [282, 283].

Both classical and cytotoxic ($ONOO^-$) NO signaling influence cervical remodeling. NO donors increase cervical cGMP [284] and promote human myocervical relaxation ex vivo [285–288]. Low concentrations of NO donors (10 μM) suppress and high concentrations (3 mM) promote uterine prostaglandin synthesis in mice [289]. Cervical cytokines and reactive oxygen species increase as labor approaches [271, 272, 290], while cervical SOD1 decreases. Thus, increased cervical iNOS producing NO along with increased O_2^- and oxidative stress causes $ONOO^-$ formation [43, 57, 276, 291], suggesting the cytotoxic pathway mediates cervical change [292] (Figure 7B). However, there is no preterm labor phenotype in iNOS knockout mice [125]. This may reflect compensatory upregulation of nNOS/eNOS.

Key points

- P_4 signaling, via PR switching, and SOD1 decrease in the cervix at parturition, promoting $ONOO^-$ production.
- NO may stimulate prostaglandin synthesis via either classical or cytotoxic NO pathways.
- There is no evidence for sulfide or CO regulating cervical remodeling.

Rupture of membranes

The fetal membranes comprise the fused inner amnion and outer chorion. They provide a structural barrier to infection and the maternal immune system and contain the amniotic fluid reservoir and fetus [293]. Membrane cells continue to multiply in late gestation, and membrane stretch accommodates rapid fetal growth.

As telomeres shorten in the cells comprising fetal membranes, apoptosis ensues, and the membranes rupture [294–296]. This releases inflammatory cytokines to enhance uterine contractions [221]. Loss of amniotic fluid is a strong signal for delivery [297], and NO and sulfide may influence the timing of membrane rupture (Figure 7C).

As in the cervix, iNOS in fetal membranes correlates with labor. In humans, TL membranes after vaginal delivery express more iNOS than intact TNL membranes from cesarean delivery [298]. Oxytocin stimulates iNOS expression and $ONOO^-$ synthesis in human membrane explants [299]. Inducible NOS expression stimulates p38 MAPK-dependent chorion cell apoptosis [300], while NOS inhibitors delay membrane apoptosis [301]. $ONOO^-$ protein nitration and p38 MAPK signaling increase in mouse fetal membranes exposed to cigarette smoke extract [302], implicating the cytotoxic pathway as a mechanism for preterm premature rupture of membranes in smokers. In contrast, sulfide reportedly maintains chorion/amnion integrity. Human and rat fetal membranes express CBS and CSE. CBS is more abundant in rats [303]. CBS and CSE expression is lower in TL membranes compared with TNL cesarean controls [304]. Since sulfide attenuates oxidative stress and inhibits prostaglandin synthesis, CBS expression might reduce inflammation and delay membrane rupture [305].

Key points

- Fetal membrane iNOS increases in late gestation and may facilitate rupture of membranes via the cytotoxic pathway.
- Membrane sulfide may curtail inflammatory signaling, thereby delaying membrane rupture.

Uterine involution

After birth, the uterus must continue contracting to limit postpartum bleeding [306]. Over several weeks, uterine involution proceeds with the myometrium and vasculature returning to the pregravid state. In pigs, uterine iNOS increases from postnatal day 7 to 35 [307], which may be a return to baseline and/or may represent increased inflammation and phagocytosis. Inducible NOS-expressing uterine M1 macrophages, however, accumulate sharply at parturition and then decrease after delivery [308]. Thus, the source, direction, and function of iNOS/NO in uterine involution are unclear. In mice, eNOS-derived NO may prevent postpartum uterine blood vessel narrowing. Parous wild-type dams have uterine arteries with wider lumens than virgin mice, but eNOS knockout uterine arteries remain similar to nulligravid wild-type mice [7]. Whether this is due to defects in eNOS-dependent vascular remodeling during pregnancy or altered postpartum involution involving NO is uncertain. If NO does facilitate uterine involution, it is probably not via the classical pathway in myocytes since involution is a contractile process. Inflammation in the postpartum uterus is prominent, and cytotoxic NO signaling may occur if sufficient O_2^- is present to yield $ONOO^-$. A mechanism by which sulfide promotes autophagy and apoptosis has been identified [309], but this mechanism has not been studied in the postgravid uterus.

Key points

- Few studies have examined the mechanisms of uterine involution or the effect of gasotransmitters in the postpartum uterus.
- Uterine iNOS increases postpartum and may be associated with general tissue inflammation.

The winds of tomorrow: gasotransmitters in perinatal research

We have summarized the evidence for gasotransmitter regulation of pregnancy from conception to postpartum involution. Where possible, we have described specific mechanisms. However, important gaps remain in the present literature. Here we describe some of the challenges, questions, and future opportunities for gasotransmitter pregnancy research.

- *Novel gasotransmitters and signaling pathways*. New small gas transmitters have been proposed in recent years, but almost none have been studied in pregnancy. For example, brain, kidney, and liver produce ammonia (NH_3), the abundance of which influences renal pH even in healthy people [310]. Methane (CH_4), a stable but rare volatile, may protect against hypoxia-reperfusion injury [311]. Carbonyl sulfide (COS) is rapidly converted to sulfide and bicarbonate by carbonic anhydrase [312], and carbon disulfide (CS_2) prevents NFκB-mediated inflammation [313]. The relevance of these gasses and the novel products of gasotransmitter combinations require exploration. The product of NO and sulfide, $SSNO^-$, is both a uterotonin and a vasodilator. This finding complicates the conventional view of NO/sulfide synergy [165, 314]. Methylated sulfides such as trimethylsulfonium [315] relax smooth muscle but also activate specific antioxidant enzymes [316], suggesting persulfidation may not be the only mechanism of sulfide signal transduction. As our technical capabilities expand, time and attention will become limiting resources to measure and identify novel gasotransmitters.
- *Nongaseous products of gasotransmitter enzymes*. The gasotransmitter synthetic reactions create other nonvolatile products with possible signaling roles. Citrulline (produced with NO by NOS enzymes) is a hydroxyl radical scavenger [317]. Homocysteinemia and cystathioninuria occur with CBS and CSE deficiency, respectively, because of altered RTS that changes intracellular cysteine levels, affects glutathione and taurine metabolism, and perturbs the intracellular redox state [318, 319]. Biliverdin produced by HO is an antioxidant that modulates oxidative damage [320], while free Fe^{2+} can generate reactive free radicals. These nongaseous co-products may mediate some effects of gasotransmitter enzyme activity or deficiency, which deserves consideration in future experiments.
- *Current enzyme tools and pharmacology*. For many commercially available gasotransmitter enzyme agonists and antagonists, isoform selectivity is low. For example, the NOS inhibitors 7-nitroindazole (7-NI), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), and diphenyleneiodonium (DPI) are reportedly selective for nNOS, iNOS, and eNOS. Unfortunately, the IC_{50s} show considerable overlap, which risks incorrect conclusions for tissues expressing multiple NOS isoforms [321]. Similar issues exist for HO and CSE/CBS inhibitors. Genetic knockout models can be informative, but confounding effects require attention (e.g. dramatically increased cystathionine in CSE knockout mice may be a cause of the phenotype). Experimental design using multiple drugs or approaches will produce more robust findings.
- *Gasotransmitter measurement and reporters*. Specific, real-time detection of gasotransmitters in living cells and tissues can yield artifact since many gasotransmitter-reactive dyes also interact with other redox mediators. In vitro enzymatic assays are reliable, but they do not assess bioactivity in the natural intracellular milieu. New genetically encoded fluorescent biosensors for NO [322], CO [323], and sulfide [324] are promising, but their selectivity

in living cells requires verification. This issue is well illustrated by a sulfide biosensor that is 25 times more selective for sulfide than GSH [324], even though intracellular GSH is more than 1000 times more abundant than sulfide [325]. Chemiluminescence [326–328], amperometry [329–331], and stable metabolite quantification [332, 333] accurately quantify gasotransmitters [334], but implementation is complicated and limited in scale. Multiple approaches and reagents are probably needed in present gasotransmitter research.

- *Limited perinatal research*. After the initial excitement surrounding NO in the 1990s, few labs have ongoing basic investigation of gasotransmitters in pregnancy. Indeed, many seminal discoveries regarding gasotransmitters in pregnancy still require independent verification. Although many lines of promising research show therapeutic potential for obstetrical syndromes, few reproductive sciences labs are active in this area. Devoting increased effort and resources to basic mechanisms, including expanding the number of laboratories and investigators, could better inform clinical trials and thereby advance therapeutic and diagnostic tools for pregnancy.

Key points

- In different cells and tissues, gasotransmitters can antagonize or synergize with one another.
- Novel gasotransmitters have been identified, but their function in reproductive biology has not been tested.
- The important effects of by-products of gasotransmitter enzymatic activity deserve separate consideration from gasotransmitters themselves.
- Experimental design should account for limitations of current enzyme inhibitors and activators and gasotransmitter tracers.
- The most accurate and sensitive tools to measure NO, CO, and sulfide are ozone chemiluminescence, palladium-catalyzed fluorescence, and amperometry, respectively.
- Study of gasotransmitters in reproductive biology, especially with independent confirmation of findings, will accelerate opportunities for accurate development of pregnancy therapies and diagnostics.

Conclusion

NO, CO, and sulfide influence multiple aspects of pregnancy physiology. In the decades since the earliest discoveries showing that NO potently relaxes the uterus, we have developed a deeper and more complex knowledge of gasotransmitter production, regulation, and interactions (Figure 8). NO regulates P_4 secretion to maintain early pregnancy and augments endometrial decidualization. NO and sulfide affect fallopian tube peristalsis. NO and CO balance trophoblast invasion and proliferation during implantation, and CO promotes spiral artery remodeling. All three gasotransmitters facilitate placental angiogenesis and augment maternal uterine blood flow, together maximizing utero-placental transfer. All three also modulate maternal immune function in pregnancy, activating uNK and T_{reg} populations while suppressing alloimmunity. As the myometrium acquires resistance to NO and sulfide quiescence at labor, NO weakens the fetal membranes preparing for rupture and promotes cervical remodeling. The precise role of gasotransmitters in specific perinatal pathologies is not well established, and recently recognized reactions *among* gasotransmitters and established second messengers are largely unexplored. There are many opportunities for further study, and we anticipate the development of new pregnancy therapies from increased understanding of perinatal gasotransmitter signaling.

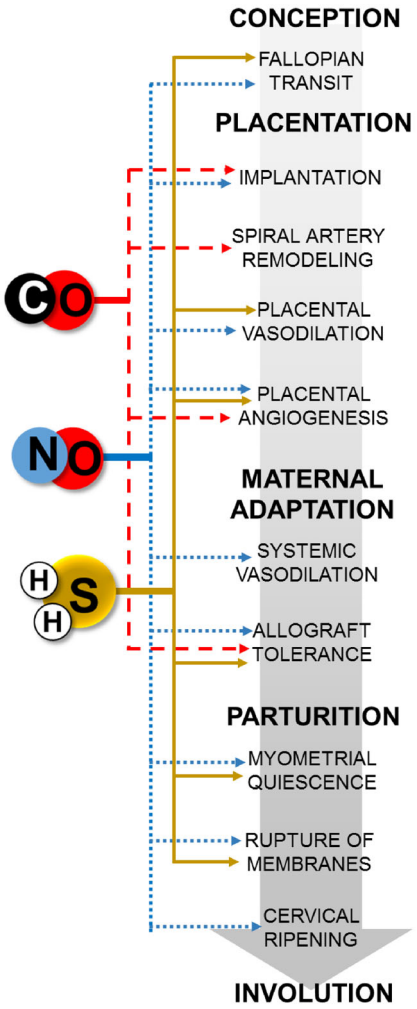

Figure 8. Summary schematic of gasotransmitter-mediated processes in pregnancy. Blue dotted, red dashed, and yellow solid lines denote NO, CO, and sulfide, respectively.

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