Regulation of Contractile Protein S-Nitrosation in Preterm Myometrium Underlies the Dysfunctional Relaxation to Nitric Oxide

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Abstract

Preterm labor leads to preterm delivery in over 50% of cases. Current treatment for preterm labor is inadequate and relies on findings in other smooth muscles. No treatment can prevent labor to allow a fetus to remain in the womb until term. Regulation of contraction/relaxation of the uterine smooth muscle is poorly understood. Recent work in our laboratory has revealed major disparity between term and preterm human uterine smooth muscle that suggests new approach to drug discovery.

Key Words: ADH5, Cyclic GMP, GSNO, GSNOR, Human uterus, Labor, Nitric Oxide, S-Nitrosation, Preterm Labor, Relaxation, Signaling, Smooth Muscle

The Problem

Human gestation requires approximately 40 weeks for complete fetal development. Birth before 40 weeks is a global problem fraught with peril for the afflicted fetus and devastating for the family (1). Fifteen million babies are born preterm worldwide each year and as many as 20,000 babies die annually in the US as a result of preterm birth. Preterm birth (PTB), defined as delivery before 37 weeks accounts for the majority of fetal morbidity and mortality and while there are regional differences and ethnic disparities in the rates of prematurity, as many as one in ten pregnancies results in preterm birth. PTB is preceded by preterm labor. No available medications can reliably interrupt established spontaneous preterm labor (SPTL) and allow an afflicted pregnancy to continue to term. Medications used to prevent early SPTL (tocolytics) on average delay labor for only 48 hours, a window for antenatal steroid treatment to promote lung cell maturation, but hardly an effective treatment to prevent preterm birth (2). Indeed, because these agents are tested on the outcome of preventing labor for only 48 hours, efficacy of the entire class is suspect (3).

What Causes Early labor

The cause(s) of early labor are unknown in most cases. The risk factors associated with prematurity are many and some, such as low socioeconomic status confound a mechanistic understanding of the problem (4). Microbial infection might initiate SPTL, but antibiotic treatment doesn't prevent preterm birth (5,6). In searching for the underlying biological causes of prematurity, it became clear that this is not a problem faced by animal species in the wild suggesting that PTB might be a uniquely human problem. Moreover, there were not attractive animal models in which to examine SPTL. Hormonal differences such as progesterone withdrawal that precedes parturition in many species, does not occur in women (7) limiting the utility of species such as mice and rats. The key it seemed to us was to do the hard work of examining how the human uterus remained quiescent during gestation. Perhaps by understanding relaxation pathways in uterine

muscle, we might discover therapeutic targets to prevent preterm labor.

An NO Signaling Exception

Following the work of Robert Furchgott's discovery of endothelium-derived relaxing factor (8) and its identity as nitric oxide (9), we became interested in the actions of nitric oxide on uterine muscle. After all, the uterus is a smooth muscle like the muscle of blood vessels where the actions of nitric oxide had been discovered to raise cyclic GMP and signal relaxation (10). While examining the mechanisms of relaxation of uterine smooth muscle (myometrium), we discovered something quite unexpected about the action of nitric oxide. Even though treatment of the human uterine smooth muscle in the organ bath resulted in elevation of cyclic GMP as the Nobel work of Furchgott *et al.* had demonstrated in vascular smooth muscle, it was neither necessary nor sufficient to explain the resulting relaxation of myometrium (11). Our findings were not initially well received. After all, science was not ready to accept an exception to work that had been awarded a Nobel. Despite skepticism, our results were repeatable. We quantified the ability of nitric oxide (NO) to relax the muscle even when soluble guanylyl cyclase was blocked to prevent cyclic GMP accumulation in guinea pig, cynomolgus monkey as well as human (12–14).

We reasoned that myometrium represented an interesting signaling exception that might reveal mechanisms to promote quiescence. The Nobel dogma posits that NO activates myometrial soluble guanylyl cyclase, cyclic GMP is generated and crucial proteins such as the smooth muscle myosin phosphatase are phosphorylated (15). However, unlike vascular smooth muscle, term myometrium relaxes to NO and proteins are phosphorylated, but this is not required for relaxation (16). NO-mediated relaxation of pregnant human myometrium together with its cyclic GMP-independence (17), established that another mechanism mediates relaxation to NO in the myometrium (18,19). It is clear now that we were right since a degree of cyclic GMP-independence of NO action is also known for other smooth muscles (20).

Dysfunctional Relaxation in Preterm Tissues

More recently, we have focused our attention on the actions of NO in preterm myometrium. Acquiring samples of myometrium from women undergoing a Cesarean section allows for a direct assessment of physiological and pharmacological parameters. Because some women enter labor preterm and require delivery of their fetus by Cesarean, we have been able to study the effects of NO in preterm as well as term human myometrium. We were surprised to discover that preterm tissues do not respond well to NO (21). This disparity could be the result of gestational timing if the response to NO matures during gestation. If on the other hand, endogenous NO signals quiescence as we suggest, then the failure of preterm tissues to relax could suggest a fundamental biochemical difference in preterm tissues that might offer a pathway to drug development.

A Role for S-Nitrosation

At the time we were learning about the failure of preterm tissues to relax to NO, we were also probing the mechanism of NO-induced relaxation in term myometrium. If cyclic GMP is not the proximate cause of NO-mediated relaxation of myometrium, then what signaled the relaxation? We proposed that NO-induced S-nitrosation of critical contraction-relaxation associated proteins signaled the relaxation. S-nitrosation describes a thiol (*e.g.,* cysteine) converted to a nitrosothiol (R-NO) by a one-electron oxidation from the •NO radical (22). The term often used, nitrosylation is addition of an NO group to a metal-centered protein such as guanylyl cyclase. The majority of proteins modified by NO are S-nitrosation on a cysteine thiol. Although researchers have used both terms to describe NO addition to a protein thiol, we employ the term S-nitrosation and suggest that this term be preferred.

We discovered crucial pregnancy state differences in NO-donor induced S-nitrosations

in myometrium (23). Based on our findings we developed the hypothesis that NO donor (GSNO)-mediated relaxation of term myometrium results in part from altered S-nitrosation of myosin light chain kinase (MLCK), its substrate myosin light chain 9 (MYL9), and the actin binding protein profilin-1 (PFN1). The notion that these proteins, central to the mechanisms of contraction-relaxation of smooth muscle are differentially S-nitrosated in term versus preterm myometrium is supported by our data (23) and links existing notions about the gestational maturation of quiescence signaling in myometrium during pregnancy with a mechanism, *S-nitrosation*.

Regulation of S-Nitrosation

While it is immediately attractive to propose that S-nitrosation differences of crucial proteins such as MLCK could render preterm tissues resistant to the actions of NO, there was little in the literature and less in myometrium to validate our notions. What was the basis of this difference in S-nitrosations? Is preterm tissue fundamentally different, or are we examining a difference based on gestational timing alone. In order to address this conundrum, we examined the principle mechanism of S-nitrosation regulation in smooth muscle, S-nitrosoglutathione reductase (GSNOR).

Glutathione is the major thiol in mammalian cells (up to 10 mM) (24). GSNO is the likely form in which NO crosses the membrane of most cell types to trans-nitrosate proteins (25). GSNO-metabolizing activity is accomplished by the Class-III alcohol dehydrogenase (ADH5) also called GSNOR (26). The principal substrate for ADH5 is GSNO. The enzyme utilizes NADH to carry out a 2^{e-} reduction of GSNO to generate glutathione sulfinamide (27). Posttranslational modification of mammalian GSNOR has not been described. Because GSNOR($^{-/-}$) mice have increased cellular levels of GSNO and SNO-proteins, it is likely that GSNO and some S-NO-proteins are in equilibrium governed by Cys-to-Cys trans-nitrosation, and GSNOR mediated de-nitrosation (26).

Altered GSNOR expression in myometrium associated with SPTL would be expected to alter S-NO-protein levels as we observed (23) and thus, may alter uterine quiescence. We documented both increased S-nitrosation and decreased S-nitrosation of specific myometrial proteins. Up S-nitrosations fit a simple notion of NO reacting with free thiols. Down protein S-nitrosation was unexpected and could be the result of the action of GSNOR, inhibitors of which are in clinical trial (28) for airway smooth muscle disease (29,30). Up and down nitrosations in SPTL may be the result of selective regulation of de-nitrosation by GSNOR as was shown known in nerve (31) or similar de-nitrosases themselves activated by S-nitrosation to act on specific substrates. Our data suggest that the notion that S-nitrosations are obligatory and governed solely by availability of NO and substrate is likely to be wrong. We suggest that S-nitrosations are regulated.

Putting it Together

Our experiments in myometrium from both term and preterm patients revealed and exciting connection between biochemical differences in S-nitrosation measured by quantitative mass spectroscopy and the outcome of these differences in the effect of NO-donor on myometrial relaxation. Patients who enter labor spontaneously preterm without infection have a blunted relaxation response to NO-mediated relaxation suggesting that the mechanism of NO action may be involved in the pathophysiology of preterm labor (21). Ours is the first study to measure the ability of NO to relax preterm vs. term pregnant human myometrium. We find that GSNOR expression is elevated in patients that deliver preterm and that this is not the result of gestational timing (21). Furthermore, differences in expression can be measured as increased enzyme activity. When S-nitrosation is examined in the actin motility assay to address the effect of S-nitrosation of the movement of actin on a myosin substrate (a surrogate for the mechanism of NO action), results are consistent with an effect of increased S-nitrosation to mediate increased muscle activity. Thus, while

NO-donors cannot be useful for preventing preterm labor, the differences in the effect of NO on the uterine smooth muscle from term versus SPTL patients has revealed a mechanistic basis for early labor and further examination may reveal new targets for tocolytic development.

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