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Antenatal montelukast treatment reduces uterine activity associated with inflammation in a pregnant rat model☆

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ABSTRACT

Objective: The potency of acute montelukast treatment, a leukotriene receptor antagonist, has been demonstrated as tocolytic on *in vitro* myometrial contractility. This study assessed the ability of a 48 h montelukast treatment to modify *in vitro* contractions under inflammatory conditions in a pregnant rat model.

Study design: Pregnant Sprague-Dawley rats were injected intraperitoneally (gestational days 20–22) with lipopolysaccharides (LPS) 200 μg/kg (4 treatments at 12 h intervals) alone or combined with montelukast 10 mg/kg/day or a saline solution for a 48 h period. Uterine rings ($n = 72$) were obtained by median laparotomy at day 22. Spontaneous contractile activities were compared using pharmacological compounds (oxytocin, nifedipine) along with assessment of contractile parameters. Myometrial subcellular fractions were also analyzed by Western blot to quantify oxytocin, cysteinyl leukotriene receptors and inflammation markers.

Results: In *in vitro* experiments, the area under the curve, the amplitude and the duration of phasic contractions were significantly reduced following 48 h of LPS + montelukast treatment comparatively to the LPS group. Moreover, in this same group, oxytocin (10^{-9}–10^{-7} M) largely decreased uterine sensitivity ($p = 0.04$). Following LPS and montelukast treatment, the tocolytic effectiveness of nifedipine (10^{-9}–10^{-7} M) was increased ($p < 0.01$). Western blot analysis confirmed the presence of type 1 CysLT receptors in all treated groups. Hence, montelukast treatment restored TNF-α and COX-2 basal levels.

Conclusion: Our results strongly suggest that montelukast treatment could facilitate a relative uterine quiescence by decreasing its sensitivity to uterotonic agent or by increasing tocolytic efficiency under proinflammatory conditions.

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Introduction

Despite sustained research efforts over the past decade, preterm birth still remains a major public health problem. Spontaneous preterm labor occurs in 45% of preterm birth cases leading to neonatal mortality, morbidity and disability [1,2]. From a clinical standpoint, a new therapeutic approach is thus needed to prevent the threat of preterm labor. The exact mechanisms leading to preterm labor are poorly understood. However, infection and underlying inflammatory conditions are present in 40–60% of cases [3,4]. Among these pregnant women, the level and expression of inflammatory cytokines are increased in both amniotic fluid and fetal membranes. Indeed, TNF-α, IL-1β, IL-6, IL-8 and matrix metalloproteinases (MMPs) are significantly increased [3,5]. To reproduce these inflammatory processes, a pregnant rat inflammatory model with administration of lipopolysaccharides (LPS) has been widely used [6–8]. Indeed, binding of LPS with its membrane receptor activates the secretion of multiple cytokines such as TNF-α, IL-1β and IL-6. Furthermore, IL-1β is known to increase the expression of COX-2 [9]. Other inflammatory markers such as NFκB have been described during active labor, irrespective of the presence of infection [10]. Recently, MacIntyre et al. demonstrated an increase in COX-2 expression during preterm labor [11]. Increasing COX-2 expression allows arachidonic acid conversion into prostaglandins which are responsible for the initiation of active labor and maintenance of strong effective contractions [12–14]. However, there is a paradox in that blocking

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the COX pathway is ineffective in situations of preterm labor [15] whereas inflammatory mediators, prostaglandins, are in fact key components of prematurity; hence, the need to explore alternative pathways.

Alternatively to prostaglandin metabolites, arachidonic acid can be metabolized by 5-lipoxygenase (5-LOX) into leukotrienes which are proinflammatory compounds known for their positive modulation of contractile activity [16]. Plasma levels of cysteinyl leukotrienes (LTC4, LTD4 and LTE4) have moreover been shown to be increased at the end of pregnancy and during parturition [16,17].

In a recent study, we demonstrated that montelukast, a specific antagonist of type 1 cysteinyl leukotriene receptors, displayed tocolytic properties in an *in vitro* human model of uterine tissues [18]. The net inhibitory effect of montelukast reached upward of 60%, with a further additive effect in the presence of low concentrations of nifedipine. In addition to being a cysLTR1 antagonist, montelukast also presents secondary anti-inflammatory effects. Indeed, montelukast has been reported to inhibit 5-LOX in inflammatory cells [19,20] and interfere with COX-2 activity, resulting in decreased prostaglandin levels [21]. In 2010, we demonstrated that inhibition of 5-LOX and COX largely reduced contractions *in vitro* [22]. Moreover, specific antagonists of cysLTR1, namely pranlukast and montelukast, have also been shown to decrease LPS-induced cytokines such as TNF-α and IL-6 [23,24]. Montelukast is furthermore widely used during pregnancy since it is prescribed for the treatment of asthma, with absence of any toxicity in pregnant women [25–27]. To date, our previous work had demonstrated that, in physiological conditions, montelukast relaxes *in vitro* myometrial contractility. To better characterize the effect of this drug, the next step was to test the efficacy of montekukast under actual inflammatory conditions.

The present study aimed to quantify the effects of antenatal montelukast treatment in an animal model under proinflammatory conditions on *in vitro* contractile activity and on inflammation marker levels.

Materials and methods

Animal groups

Timed pregnant rats were obtained from Charles River Laboratories (Saint-Constant, Canada). Rats were randomized into three groups ($n = 9$ per group): control, lipopolysaccharides (LPS) and LPS + montelukast. Four successive LPS administrations of 200 μg/kg in 0.9% saline solution were performed intraperitoneally (IP), from gestational days 20 to 22 (G20–G22), at 12 h-intervals. In the LPS + montelukast group, the first and third LPS injections were followed by an IP injection of montelukast (10 mg/kg; $N = 9$ rats). Following the 48 h treatment period, median laparotomies were performed 8 h after the last injection, at G22. Biopsies from 6 rats per group were used for isometric tension measurement, while uterin tissues from the 3 remaining rats were snap-frozen for preparation of sub-cellular fractions and Western blot analysis. The study was approved by our institutional Ethics Committee for research on animal subjects (project # 337-13). All animals were maintained under standard laboratory conditions (12 h light/12 h dark) and an environmental temperature of 21–23 °C. Food and water were available *ad libitum*.

Tissue collection and isometric tension measurements

Myometrial rings ($n = 72$) were obtained by median laparatomy at G22 from Sprague-Dawley rats as previously described [28]. Each uterine horn was dissected in 6 uterine rings. Once collected, all tissues were placed in Krebs' physiological solution at pH 7.4. The myometrial rings were mounted in isolated baths between 2 inox stirrups for isometric tension recording. For each sample, 1 stirrup was fixed to the bottom of the chamber, and the other was connected to an isometric force transducer coupled to Polyview software (Grass-Astro-Med Inc., West Warwick, RI). The organ baths (Radnoti Glass Tech., Monrovia, CA) contained 7 ml of Krebs solution thermostated at 37 °C and bubbled continuously with carbogen (95% O_2–5% CO_2; pH 7.40) as previously described [28]. Once baseline values for amplitude and frequency of contractions were recorded, cumulative concentrations of drugs or chemical agents, in pH-balanced saline, were added to the tissue baths.

SDS-PAGE and Western blot analysis

SDS-PAGE and Western blot analyses were performed exactly as previously reported [17]. Blots were incubated 2 h with rabbit antiserum raised against CysLTR1 (Assay Biotech, CA, USA), OXTR (Abcam, MA, USA), 5-LOX, COX-2, TNF-α, and PPARγ, (Cayman Chemical, MI, USA). β-Actin was used for density ratio analysis.

Drugs and chemical reagents

Lipopolysaccharides from *Escherichia coli* serotype 0127: B8 (Sigma–Aldrich, St. Louis, MO) were dissolved in sterile 0.9% NaCl solution at a final concentration of 50 μg/ml and administered IP to pregnant rats at a dose of 200 μg/kg, as previously described [7]. For the control group without LPS treatment, rats received IP injections of sterile saline (0.9% NaCl) only. The final bath concentration of EtOH was 0.3%, and the final drug concentration of nifedipine and oxytocin ranged from 10^{-9} to 10^{-7} M for which simultaneous controls were run as outlined above.

Data analysis and statistics

Contractile activities were quantified by calculating the area under the curve over 10-min periods using Sigma Plot 12.0 (SPSS-Science, Chicago, IL). For each drug (oxytocin or nifedipine) modifications of spontaneous contractile activities were compared to its initial basal period and then relative responses were normalized. The normality of data was assessed and confirmed using the Shapiro–Wilk test. A non-parametric test was chosen since the sample size was small ($n < 30$). If the difference was significant, a Mann–Whitney *U*-test with Bonferroni correction was used, therefor a p-value <0.016 was considered statistically significant. Krustal Wallis test was used for comparative analysis.

Results

Fig. 1 depicts the analysis of contractile parameters. First, the effect of LPS treatment increased contractile activity, as witnessed by a significant increase in the amplitude (Fig. 1A), the time to peak (Fig. 1B) and the duration to 90% relaxation (Fig. 1C) relative to the control group ($p < 0.01$). In contrast, under antenatal treatment with montelukast, a decrease of all contractile parameters was observed when compared to the LPS-treated group. Both time to peak and duration recovered their basal values while a significant decrease was observed in the amplitude and frequency compared to the control group. Of note, a decrease in the frequency of phasic contractions was also observed under proinflammatory conditions (LPS stimulation, Fig. 1D).

In order to determine the impact of antenatal montelukast treatment on the expression of both cysteinyl leukotriene receptor type 1 (CysLTR1) and inflammation markers (TNF-α, COX-2, 5-LOX and PPARγ), Western blot analysis was performed on uterine tissues obtained from control, LPS and LPS + montelukast groups. Western blot analysis revealed that CysLTR1 was present in all microsomal fractions of the three groups (Fig. 2A), justifying the