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Original article

Megalin expression in human term and preterm placental villous tissues: Effect of gestational age and sample processing and storage time

Amal A. Akour [a], Phillip Gerk [b], Mary Jayne Kennedy [a,*]

[a] Department of Pharmacotherapy and Outcomes Science, School of Pharmacy, Virginia Commonwealth University, Richmond, VA, United States
[b] Department of Pharmaceutics, School of Pharmacy, Virginia Commonwealth University, Richmond, VA, United States

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ABSTRACT

Introduction: The aims of this study were to characterize megalin expression in human term and preterm placental villous tissues and to assess the impact of gestational age and sample storage on receptor expression. **Methods:** Placental tissue samples were collected from pregnant women undergoing term and preterm Cesarean deliveries. Placental villous tissues were used to quantify megalin protein and mRNA expression by western blotting and quantitative polymerase chain reaction (q-PCR), respectively. Stability of megalin expression was also evaluated under various processing and storage conditions. **Results:** Megalin mRNA was detected in term and preterm placental villous tissues. Expression in early preterm samples was 6-fold higher than in late preterm and term samples. Refrigeration of processed term samples at 4 °C for up to 18 h had a slight impact on megalin mRNA expression with stored samples exhibiting mRNA levels approximately 1.5-fold lower than those frozen immediately after processing. A greater decrease in mRNA expression (up to 33-fold) was observed when processed samples were snap-frozen immediately and thawed at 4 °C. Processing of samples prior to refrigeration also appeared to improve mRNA stability with significantly higher expression levels noted in processed vs. unprocessed samples at all points for up to 48 h. **Discussion:** These data suggest that expression of megalin mRNA in term placental villous tissue is relatively stable for up to 18 h when samples are processed immediately and refrigerated at 4 °C prior to freezing. Processing prior to storage also appears to improve mRNA stability. This paper demonstrates the practical feasibility of analyzing stored tissue samples, thus, it will help with placental mRNA analysis. Additionally, megalin expression appears to vary inversely with gestational age with the greatest expression noted in the most premature samples. Age-dependent differences in placental megalin may therefore influence fetal exposure.

1. Introduction

Intra-amniotic infections (IAIs) are common complications of labor and delivery occurring in up to 10% of all pregnancies and 25% of preterm births (Armer & Duff, 1991; Gibbs, Dinsmoor, Newton, & Ramamurthy, 1988; Soper, Mayhall, & Dalton, 1989). If inadequately treated, these infections can lead to significant morbidity in both the mother and the fetus. Serious, life-threatening complications may also occur in fetuses and infants born to mothers with IAI. It is estimated that up to 4% of deaths in term infants and more than 10% of those in preterm infants are directly related to IA (Newton, 1993). Approximately 20–40% of early-onset newborn sepsis and pneumonia cases are also associated with IAI and occur, most likely, as a consequence of infection acquired in utero (Newton, 1993). While there is unequivocal evidence that maternal antibiotic administration during delivery significantly improves outcomes in both the fetus and the newborn, (Gibbs et al., 1988) the impact of intrapartum antibiotics on morbidity and mortality is highly dependent on attainment of therapeutic drug concentrations in the fetus. It is therefore essential that any maternally-administered antibiotics readily cross the placenta so that adequate fetal levels are achieved.

Aminoglycoside (AG) antibiotics (gentamicin, tobramycin, amikacin) are frequently used during pregnancy to treat maternal infections and are recommended by the American College of Obstetrics and Gynecology (ACOG) as one of the first line antibiotic treatments for documented or suspected IAI (ACOG educational bulletin, 1998). These agents are highly active against gram-negative bacteria and, when used in combination with a β-lactam, provide excellent coverage against the most frequently isolated pathogens in early-onset newborn sepsis (*E. Coli* and *Group B*

Abbreviations: AGs, aminoglycosides; ANOVA, analysis of variance; DPBS, Dulbecco's phosphate buffered saline; HDL-C, high lipoprotein cholesterol; HepG2, human hepatocellular carcinoma cells; HK-2, human kidney cells type 2; IAI, intra-amniotic infection; IRB, Institutional Review Board; LRP, low-density lipoprotein receptor-related protein; ML, mouse liver; MW, molecular weight; NTC, non-template control samples; PBS, phosphate buffered saline; PTMs, posttranslational modifications; PVT, placental villous tissue; q-PCR, quantitative polymerase chain reaction; RK, rat kidney; RT, reverse transcriptase enzyme; VCU, Virginia Commonwealth University.

* Corresponding author at: Department of Pharmacotherapy and Outcomes Science, School of Pharmacy, Virginia Commonwealth University, 410 N. 12th Street, PO Box 980533, Richmond, VA 23298-0533, United States. Tel.: +1 804 628 3316.
E-mail address: mjkennedy@vcu.edu (M.J. Kennedy).

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Streptococcus). AGs also readily cross the placenta achieving maximum fetal serum concentrations that are 21-48% of maternal peak levels within 1 to 2 h of maternal administration (Good & Johnson, 1971; Kauffman, Morris, & Azarnoff, 1975; Weinstein, Gibbs, & Gallagher, 1976; Yoshioka, Monma, & Matsuda, 1972). This level of exposure is above the minimum inhibitory concentration (MIC) for the most common pathogens in IAI and is sufficient to provide rapid, bactericidal activity in the fetus.

The highest and most persistent fetal AG levels, however, are found in renal tissue where concentrations of up to 7.2 μg/g (3.5 times the recommended trough serum level) have been observed for up to 34 h after maternal administration (Bernard et al., 1977; Bernard et al., 1977). AG levels are also detectable in fetal kidney tissue within 2 h of maternal administration and continue to steadily increase despite decreasing serum levels (Bernard, Abate, et al., 1977; Bernard, Garcia-Cazares, et al., 1977). There is a direct correlation between the rate and extent of AG accumulation in renal tissue and individual susceptibility to nephrotoxicity, a common and well-characterized side effect of AG treatment (Schentag, Cerra, & Plaut, 1982). The fetus is therefore particularly vulnerable to the nephrotoxic effects of AGs given the rapid and significant renal tissue accumulation that occurs following drug exposure. Persistence of AGs in renal tissue after birth also increases the susceptibility of the newborn to injury during the early postnatal period when AGs are administered as the standard of care to prevent and/or treat infections acquired in utero. Consequently, the need to achieve therapeutic AG levels in the fetal serum must be carefully balanced with the inherent risk of developmental nephrotoxicity.

It has been recently demonstrated that megalin, an endocytic receptor expressed on the apical surfaces of absorptive epithelia, is responsible for the uptake of AGs into renal proximal tubular epithelial cells, the physiologic site of AG induced renal injury (Nagai, Tanaka, Nakanishi, Murakami, & Takano, 2001). Pharmacologic blockade of the megalin receptor has been shown to limit renal accumulation of AGs and prevent nephrotoxicity in animal models (Watanabe et al., 2004). This strategy may be useful in preventing drug accumulation in the fetal kidney during intrapartum AG administration but only if placental drug transport remains unaltered by megalin receptor blockade. It is therefore important to understand the molecular mechanisms involved in placental AG transport so that targeted strategies to limit renal accumulation without compromising placental transport can be developed.

Expression of the megalin receptor has been previously demonstrated in human term placenta (Larsson et al., 2003) and it is reasonable to speculate, given its role in renal AG uptake, that it is similarly involved in the placental uptake of AGs. It is not known, however, whether megalin is expressed in human preterm placenta and if its expression varies with gestational age. The objective of this study, therefore was to characterize megalin expression in human term and preterm placental villous tissues and to assess the impact of gestational age on receptor expression.

2. Methods

2.1. Study subjects

Pregnant adult females (18 to 45 years) admitted to Virginia Commonwealth University (VCU) Medical Center for labor and delivery were eligible for enrollment. Women delivering at term (≥36 weeks) and preterm (<36 weeks) were included. Subjects were excluded if any of the following criteria were met: (Soper et al., 1989) maternal history of diabetes, pre-eclampsia, hypertension or HIV infection; (Gibbs et al., 1988) maternal history of tobacco, drug or alcohol abuse; and/or (Armer & Duff, 1991) documented or suspected placental disorders. The research protocol and informed consent were reviewed and approved by the VCU Institutional Review Board prior to study initiation. Term placentas were collected from women undergoing scheduled Cesarean section at VCU Medical Center and written informed consent was obtained prior to sample and data collection. Given the unplanned nature of preterm deliveries, a waiver of consent was granted by the VCU IRB for the collection of preterm placentas. The following information was collected from the subject's medical record: maternal race, placental weight and gestational age.

2.2. Collection, processing and storage of placental tissue

Upon collection, placental tissues were inspected for the presence of any gross abnormalities. Any tissues with visible infarcts, calcifications, hematomas or other abnormalities were excluded from analysis. Pieces of placental tissue were snap-frozen in liquid nitrogen and stored at −80 °C. Placental villous tissue fragments were also prepared using methods previously described by our laboratory (Vaidya, Walsh, & Gerk, 2009). Briefly, the umbilical cord was cut gently to release any blood and subsequently excised. Triangular wedges of tissue (approximately 100 g) were cut and the basal and chorionic plates removed. Tissues were then rinsed with ice-cold sterile saline and blotted with sterile gauze. Tissue wedges were then gently cut into small pieces, washed in antibiotic-supplemented Dulbecco's phosphate buffered saline (DPBS) filtered through gauze and subsequently minced into smaller pieces. After several cycles of mincing and washing, 300–400 mg of the resulting villous tissue was frozen until analysis (maximum of 4 months).

Standard protocol at VCU Medical Center requires that all preterm placentas be evaluated by the Department of Pathology prior to becoming available for research purposes. Because of the inherent variation in processing/storage of preterm samples, stability is therefore a concern. Consequently, we collected, processed and stored term samples under varying conditions to determine the impact of variation in these parameters on megalin stability. To evaluate the effect of storage conditions, term placental tissues were collected and divided into two groups. In the first group, samples were processed immediately and then stored at 4 °C for 1, 2, 4, 6 and 18 h (n = 3 per time point). Samples were then frozen at −80 °C until analysis (Fig. 1-A). In the second group, tissues were snap-frozen immediately after processing and then thawed in the refrigerator for 1, 2, 4, 6 and 18 h prior to analysis (n = 3 per time point).

We also evaluated the effect of sample processing pre- and post-refrigeration on megalin stability. Term placental tissue samples were collected and pieces of tissue from each sample were processed using 2 different methods. In the first group, samples were processed immediately after delivery, stored in the refrigerator for 1, 2, 4, 6, 10, 24, and 48 h (n = 3 per time point) and then frozen at −80 °C. In the second group, samples were left unprocessed at 4 °C for 1, 2, 4, 6, 10, 24, and 48 h (n = 3 per time point) and after each time point were subsequently processed and frozen at −80 °C (Fig. 1-B). mRNA expression in these samples were then compared to a snap-frozen sample of the same placental tissue.

2.3. Protein separation and in-gel western blotting

Frozen placental villous tissue samples were homogenized on ice in 1:10 tissue protein extraction buffer (t-PER®; Thermo Scientific Pierce Inc, Rockford, IL) containing 1:100 Halt® Protease inhibitor (Thermo Scientific, Rockford, IL). Homogenization was done for approximately 1 min at a speed setting of 6.5 using a Polytron PT 10–35 homogenizer with a PTA 10 TS generator (Kinematica, Lucerne, Switzerland). Protein concentrations were determined in tissue supernatants using the BCA protein assay kit (Thermo Scientific, Rockford, IL) with bovine serum albumin (BSA) as a standard. Approximately 100–200 μg of membrane protein were loaded onto a 4−12% polyacrylamide BioRad® tris−glycine denaturing gel (Biorad, Hercules, CA) and electrophoresed at 100 V for 45 min to 1 h. Separated proteins were subsequently fixed with 50% isopropyl alcohol and 12% acetic acid for 15 min at room temperature and after fixation, the gel was washed with ultra-pure water. Binding of the primary (rabbit anti-human megalin, 1:200; mouse