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Serum amyloid A upsurge precedes standard biomarkers of hepatotoxicity in ritodrine-injected mice

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ABSTRACT

The tocolytic agent ritodrine acts on the β2-adrenoceptor and is an effective treatment option for preterm labor. However, several adverse effects of ritodrine therapy, including liver damage, have been noted. To elucidate the underlying mechanisms of ritodrine-induced adverse effects, development of sensitive biomarkers of these adverse events is necessary. Here, we report the development and analysis of an animal model of ritodrine-induced liver damage. Female mice received daily ritodrine injections for 2 weeks; liver samples were then collected and subjected to DNA microarray analysis. Ritodrine significantly altered the expression of genes related to steroid and lipid metabolism, as well as the metabolism of ritodrine itself. Importantly, expression of the acute-phase reactant serum amyloid A (SAA) significantly increased after ritodrine injection, with values indicating the largest fold-change. This large increase in blood SAA levels serves as a more sensitive biomarker than conventional liver enzymes, such as aspartate aminotransferase and alanine aminotransferase. The increase in SAA expression is specific to ritodrine-induced liver damage, because SAA expression was not induced by other hepatotoxic drugs such as acetaminophen, valproic acid, or metformin. Our in vitro studies showed that cyclic adenosine 3′,5′-monophosphate (cAMP) accumulation was not a primary cause of the ritodrine-induced SAA increase. Instead, SAA expression was enhanced by indirect phosphorylation of the signal transducer and activator of transcription-3 (STAT3) mediated by interleukin-6. Therefore, our study provides a method for sensitive and early detection of hepatic injury, and may thus help preclude serious liver damage due to ritodrine use in preterm labor.

1. Introduction

The tocolytic agent ritodrine activates heptahelical β-adrenoceptors, which are coupled predominantly to stimulatory G_s proteins. The β-adrenoceptor family is currently categorized into 3 subtypes: β1, β2, and β3 (Kobilka, 2011). Several previous studies revealed that ritodrine preferentially binds the β2 receptor over either β1 or β3 receptors (Baker, 2010; Inoue et al., 2009), and that the β2-adrenoceptor is the predominant subtype expressed in the human myometrium (Liu et al., 1998). On binding to the receptor, β2-adrenergic agonists activate adenylate cyclase to catalyze the conversion of adenosine triphosphate to intracellular cyclic adenosine 3′,5′-monophosphate (cAMP). Subsequently, intracellular cAMP elevation induces the activation of specific kinases, which in turn phosphorylate various substrates, finally resulting in the attenuation of the interaction between myosin and actin and relaxation of the myometrium (Ferro, 2006; Jeyabalan and Caritis, 2002; Simhan and Caritis, 2007).

β2-sympathomimetics, such as ritodrine, are widely used to treat preterm labor, which is one of the most serious and frequent problems in pregnancy (Caughey and Parer, 2001; Higby et al., 1993; Simhan and Caritis, 2007; Yaju and Nakayama, 2006). However, occasional occurrences of adverse events during ritodrine therapy necessitate discontinuation of subsequent treatments, often compromising fetal survival (Caughey and Parer, 2001; Higby et al., 1993; Jeyabalan and Caritis, 2002; Simhan and Caritis, 2007). Among the various cardiovascular and non-cardiovascular adverse effects of ritodrine, hepatotoxicity is a major adverse effect. It is accompanied by elevations in serum levels of liver enzymes, such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (De Arcos et al., 1996; Hakuno et al., 1994; Lotgering et al., 1986; Shen et al., 1996; Verriello et al., 2009). Several clinical reports have indicated that several days are required to detect elevation of serum AST and ALT levels in patients with preterm labor

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after ritodrine treatment (De Arcos et al., 1996; Hakuno et al., 1994; Lotgering et al., 1986; Shen et al., 1996; Verriello et al., 2009). This means that liver tissue has already been damaged at this point, because serum AST and ALT are produced during tissue breakdown. Therefore, more sensitive markers of liver damage are needed in order to provide better care for mothers at risk for preterm delivery. Despite extensive prior research, the mechanisms underlying the adverse effects of tocolytic agents are largely unknown, and the development of predictive biomarkers of such adverse events during therapy is challenging.

Here, we report the findings of a DNA microarray analysis of ritodrine-treated mouse liver. Notably, gene expression of the acute phase reactant, serum amyloid A (SAA), was markedly increased by ritodrine. We demonstrate that SAA is a reliable biomarker and that it is more sensitive to the harmful effects of ritodrine than the preexisting markers, AST and ALT. SAA level remains upregulated for a longer duration than those of either AST or ALT in ritodrine-treated mice. Therefore, the findings of our study may help prevent the adverse effects of ritodrine injection in women who experience preterm labor.

2. Material and methods

2.1. Animals

Eight-week-old, pathogen-free female C57BL/6J mice (weighing 18–20 g; SLC Japan, Hamamatsu, Japan) were used. The experiments involving mice were approved by the Animal Care and Use Committee of Jichi Medical University. The animals were housed in a temperature- and light-controlled room (25 ± 1 °C; 14-h light and 10-h dark cycle; lights were switched on at 7 a.m.), with food and water provided ad libitum.

2.2. Drugs and cell line

Ritodrine hydrochloride, acetaminophen (4-acetamidophenol), valproic acid sodium salt, and lipopolysaccharide (LPS) from *Escherichia coli* serotype O55:B5 (500,000 EU/mg) were obtained from Sigma–Aldrich (St. Louis, MO). Salbutamol sulfate, metformin (1,1-dimethylbiguanide hydrochloride), and human recombinant interleukin-6 (IL-6) were obtained from Wako Pure Chemical Industries (Osaka, Japan). The HUH-6 clone-5 hepatoblastoma cell line was obtained from Health Science Research Resources Bank (HSRRB) (Osaka, Japan).

2.3. Microarray analysis

Ritodrine (200 mg/kg) was dissolved in saline and was administered to mice by intraperitoneal (i.p.) injection between 10 a.m. and 11 a.m. for 14 days. Mice in the vehicle control group received saline injections for 14 days. At the end of the experiment (10 a.m. to 11 a.m. on day 15), the animals were anesthetized, and their blood and liver samples were collected. Measurements of AST, ALT, alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were performed by SRL Inc. (Tokyo, Japan) by using serum samples. Total RNA from the liver was obtained using TRIzol (Invitrogen, Carlsbad, CA), and quality was evaluated with an Agilent Bioanalyzer 2000 Nano kit (Agilent Technologies, Santa Clara, CA). For the oligonucleotide microarray analysis, liver total RNA (100 ng) from ritodrine-injected or control mice was subjected to a reverse transcription and signal staining protocol provided by Affymetrix (Cleveland, OH). For each sample, the biotin-labeled cRNA product (20 μg) was fragmented and hybridized onto a GeneChip Mouse Genome 430 2.0 Array (Affymetrix). After the chips were scanned, the raw intensity of each probe was analyzed using Gene Spring software GX10. Genes were annotated and gene ontology (GO) terms were analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) according to previously published methods (Huang da et al., 2009).

2.4. Quantitative polymerase chain reaction

Gene expression was examined by real-time quantitative polymerase chain reaction (PCR) as described previously (Koshimizu et al., 2010). In brief, drugs were dissolved in saline, and then mice were treated once with ritodrine (100 or 200 mg kg^{-1} day^{-1}), salbutamol (200 mg kg^{-1} day^{-1}), or vehicle, or were treated daily with each for 14 days, and their livers were collected. Total RNA was extracted from the livers by using TRIzol and purified using RNeasy mini columns (QIAGEN, Hilden, Germany) according to the manufacturers' protocol. Total RNA from HUH-6 clone-5 cells was extracted with RNeasy mini columns, following incubation with a medium containing bovine serum albumin (BSA) (0.3%), with or without research compounds for the indicated durations at 37 °C in a 5% CO_2 incubator. The integrity of the RNA was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE). DNase-treated total RNA (1 μg) was reverse-transcribed with a mixture of oligo-dT primers and random hexamers by using PrimeScript RT-PCR kit (Takara Bio, Shiga, Japan). The SYBR green fluorescent signal was monitored during PCR amplification with a Thermal Cycler Dice Real-Time System (Takara Bio), and the results were normalized against expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH expression was similar in drug-treated samples and controls. To exclude the possibility of contamination with genomic DNA, DNase-treated RNA samples without reverse transcriptase were included as negative controls. PCR primers were designed using the BatchPrimer3 software package (You et al., 2008). The specific primers used in this study were as follows: mouse SAA (indistinguishable between *Saa*1 and *Saa*2 because of their nearly identical sequences, Uhlar and Whitehead, 1999), forward (5′-GGGGGAACTATGATGCTG-3′) and reverse (5′-GTGGGATACACACATGAG-3′); human SAA, forward (5′-TGGTTTTCTGCTCCTTGGTC-3′) and reverse (5′-GCTTTGTATCCCTGCCCTGA-3′); mouse metallothionein 2 (*Mt2*), forward (5′-CTTCCTGCAAGAAAAGCTG-3′) and reverse (5′-ATTAATCAAGTCAACGGCT-3′); mouse lipocalin-2 (*Lcn2*) forward (5′-AGGCAGCTTTACGATG-3′) and reverse (5′-GGTTGTAGTCCGTGGT-3′); human β2-adrenoceptor (*Adrb2*), forward (5′-GAGCACAAAGCCCTCAAGAC-3′) and reverse (5′-TGGAAGGCAATCCTGAAATC-3′); human interleukin-6 receptor (*Il6r*), forward (5′-CTCCTGCCAGTTAGCAGTCC-3′) and reverse (5′-GGACTCCTGGATTCTGTCCA-3′); and mouse GAPDH (*Gapdh*), forward (5′-CACCACAGTCCATGCCATCAC-3′) and reverse (5′-TCCACCACCCTGTTGCTGTA-3′).

2.5. Immunoblot analysis

For the immunoblot analysis of serum SAA protein, sera were collected from the mice successively injected with ritodrine (200 mg/kg/day i.p.) for 14 days or saline-injected mice. Sera (1 μL) were loaded onto an SDS-polyacrylamide gel in separate lanes, and blotting was performed as described previously (Yamada et al., 1999). After blocking in nonfat milk, the membranes were incubated for 60 min at room temperature with primary antibodies diluted with nonfat milk (SAA, 1:100) (Yamada et al., 1999), washed, and incubated for 30 min at room temperature with horseradish peroxidase (HRP)-conjugated anti-rat IgG (Biosource, CA; 1:5000).

In addition, for the detection of phosphorylated signal transducer and activator of transcription-3 (STAT3), ritodrine- (200 mg/kg), LPS- (1 mg/kg), or saline-treated mice were killed 0, 1, 3, 6, 12, or 24 h after i.p. injection. The livers were dissected and homogenized in lysis buffer (66 mM Tris–HCl, pH 7.5, 0.9% NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL each of leupeptin, aprotinin, and pepstatin). Protein (10 μg) was loaded onto a 10% SDS-polyacrylamide gel in separate lanes, and blotting was performed as described previously (Tsuchiya et al., 2008). After blocking in nonfat milk, the membranes were incubated for 60 min at 37 °C with primary antibodies diluted with nonfat milk (STAT3; 1:1000 [Cell Signaling Technology, Danvers, MA], and phosphorylated STAT3 [Cell Signaling Technology]; 1:1000), washed, and incubated for 60 min at room temperature with HRP-conjugated anti-rabbit IgG (GE Healthcare, UK; 1:5000). As a normalizing control, an anti-actin antibody (Chemicon, 1:2000) was used. The membranes were stained with an ECL kit (GE Healthcare) and visualized with an LAS system (Fujifilm, Japan). The densities of the bands were quantified using Image Gauge software (Fujifilm).

2.6. Enzyme immunoassay

Mouse SAA in serum was quantified by a sandwich enzyme immunoassay (EIA) by using a modified version of a previously described method (Yamada et al., 1999). A new chimeric rat/mouse anti-mouse SAA monoclonal antibody was produced according to the previous protocol (Yamada et al., 1999). A clone, BI-2, was used as the secondary antibody instead of a polyclonal antibody. A plastic 96-well microtiter plate was coated with the previously produced monoclonal antibody, clone M10, diluted in phosphate-buffered saline (PBS) overnight at 37 °C, and then blocked with 1% BSA in PBS for 2 h at 37 °C. The mouse serum, diluted more than 1:100 in BSA-PBS containing 0.05% Tween-20, was added to the plates and reacted for 2 h at 37 °C. The plate was washed and then incubated with a peroxidase-conjugated secondary antibody, clone BI-2, diluted in 1% BSA-PBS containing 0.05% Tween-20 for 1 h at 37 °C. After a final wash, color was developed in ortho-phenyldiamine and read at 490 nm. The assay was standardized primarily by comparison against a high-density lipoprotein from inflammation-induced mice, the SAA content in which was determined by SDS-PAGE. When mouse serum was assayed at a dilution of 1:100, the lower detection limit was 0.04 mg/L, sensitive enough for evaluation of SAA concentration under physiological conditions.

2.7. Adenylate cyclase assay

The measurements of cAMP concentration in the HUH-6 clone-5 cells were made using a modified version of a previous method (Koshimizu et al., 2010). The cells were collected in PBS, centrifuged at 1000 × *g* for 2 min, and resuspended in buffer A (DMEM containing 10 mM HEPES [pH 7.4] and 500 μM isobutyl methylxanthine) at 10^5 cells/mL. After incubation in buffer A at 37 °C for 30 min, 10^5 cells/well were stimulated for 5 min at 37 °C with ritodrine or forskolin. The reaction was terminated by heating at 100 °C for 5 min, and cellular supernatants were stored at −20 °C until