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Elevation of Both Cyclooxygenase-2 and Prostaglandin E_2 Receptor EP3 Expressions in Rat Placenta after Uterine Artery Ischemia–Reperfusion☆

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Intrauterine growth restriction (IUGR) has a multifactorial pathogenesis and is an important cause of perinatal mortality. The relationship between fetal weight and placental blood flow in an animal model of IUGR has been investigated, showing that fetal growth is regulated by placental blood flow. The aim of the present study was to determine whether ischemia–reperfusion (I/R) injury stimulates the prostaglandin E_2 (PGE2) system or the vascular endothelial growth factor (VEGF) system in the placenta of a rat IUGR model. COX-2 is reported to be involved in ischemic damage in many organs. There are 4 types of PGE2 receptor (EP1, EP2, EP3 and EP4). It is well known that EP1 and EP3 is associated with vasoconstriction.

In the present study, vessels were occluded in the right uterine horn on day 17 of pregnancy in rats, and the clamps were removed after 30 min of ischemia. At 24 h, 48 h, and 5 days after I/R injury, the live fetuses and placentas were obtained by cesarean section. This study revealed that I/R injury caused IUGR 5 days after the treatment. COX-2 expression and EP3 receptor expression were significantly elevated at 24 h after I/R injury, but VEGF mRNA expression was not altered in the placenta from the ischemic horn compared with the non-ischemic horn. These results suggested that induction of the COX-2-EP3 system in the placenta may be one of the causes of IUGR induced by uterine ischemia, because the EP3 receptor and PGE2 are well known to mediate vasoconstriction in many organs.

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INTRODUCTION

Intrauterine growth restriction (IUGR) has a multifactorial pathogenesis and is an important cause of perinatal mortality. Ischemia–reperfusion (I/R) injury is possibly a pivotal mechanism of IUGR as well as pre-eclampsia, because I/R injury is now a well-recognized consequence of malperfusion in many organ systems. Moreover, the constancy of placental perfusion may be a more important factor than the absolute rate of blood flow, because both the fetus and the placenta extract considerable quantities of oxygen during mid to late gestation so the placental tissues will soon develop local hypoxia during periods of vasoconstriction. It has been reported that prostaglandin E_2 (PGE2) (acting via the EP1 or EP3 receptor) are associated with vasoconstriction [1]. It has been reported that TXA2 plays a salient role in I/R-induced IUGR in a rat model [2]. Although PGE2 is a vasoactive factor, it is unknown whether PGE2 also has a role in the pathophysiology of this model.

Vascular endothelial growth factor (VEGF) expression is well known to be upregulated by hypoxia/ischemia. It is presently unclear whether pre-eclampsia causes the expression of VEGF to be elevated or reduced because there are opposing reports regarding the direction of VEGF expression, i.e., upregulation or down-regulation [3,4].

The aim of the present study was to determine whether I/R injury stimulated the PGE2 system or VEGF system in the placental tissues of a rat IUGR model.

MATERIALS AND METHODS

Animal model and induction of transient ischemia

Sprague–Dawley rats ($n = 40$) weighing 250–350 g were purchased from Hokudo Co. (Sapporo, Japan) on day 13 of pregnancy. They were housed in an environmentally controlled

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vivarium and had free access to a standard pellet diet and water. Animal care complied with the "Guidelines of for Experimental Animal Care" issued by the Office of the Prime Minister of Japan. IUGR was induced by the previously described method [5]. On day 17 of pregnancy, the rats were anesthetized with ether. The skin was sterilized with povidone—iodine solution before a low abdominal midline incision was made. The uterine horns were inspected, and the number of live fetuses in each horn was recorded. Two small artery clamps were used to occlude the uterine vessels near the lower and upper ends of the right horn, and the clamps were removed after 30 min of ischemia. On day 18 of gestation (24 h after I/R injury), day 19 (48 h after I/R injury), and day 22 (5 days after I/R injury), the live fetuses and placentas were obtained by cesarean section with the dam under ether anesthesia and were weighed. In each experiment, the placentas from the right horn served as the ischemic group and those from the left horn formed the control group (non-ischemic group).

Northern blotting of COX-2

Total placental RNA was extracted by the guanidine/phenol method using Ultraspec RNA from Biotex Laboratories, Inc. (Houston, Texas, USA), electrophoresed on 1% agarose/formamide gel (100 V; 2 h), and transferred onto a nylon membrane (Nytran-Plus: Schleicher & Schuell, Knee, NH, USA) overnight in 20× SCC (3 M sodium chloride, 0.3 M trisodium citrate). Then the membranes were fixed by a UV linker, prehybridized for 4 h, and hybridized overnight at 42 °C with a radiolabeled cDNA probe.

Rat cox-2 cDNA probes were radiolabeled with ^{32}P-dCTP from Amersham (Buckinghamshire, England) using Prime-it II random prime labeling kits from Stratagene (La Jolla, CA, USA). Filters were washed in 2× SSC containing 0.1% SDS twice for 15 min. at RT, and twice for 15 min with 0.2× SSC containing 0.1% SDS at 65 °C. Next, the filters were exposed to Fuji RX X-ray film at −70 °C for 1—2 days. For quantitative analysis, the radioactivity of specific mRNA bands was measured with a BAS 2000 Bio-imaging Analyzer (FUIX, Tokyo, Japan) and was adjusted for the radioactivity of L38 (ribosomal protein) which is a housekeeping protein [6]. All two signals were examined on the same filters.

cDNA probes for rat cox-2 [7] and for human VEGF [8] were used for northern blot analysis. These probes were kind gifts from H. Ristimaki, and M. Shibuya, respectively.

EP3 mRNA detection by real-time PCR

Total RNA extracted from samples was reverse-transcribed before real-time PCR amplification, as described previously [9]. Real-time PCR was performed using the DyNAmo HS SYBR Green qPCR kit (Finnzymes, Espoo, Finland) and fluorescent signals were detected by the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of the primers for EP3 were as follows (forward, 5′-CCAGCTTATGGGGATCATGT-3′; reverse, 5′- AACGGCGATTAGGAAGGAAT-3′).

The thermal cycling conditions included an initial denaturation step at 95 °C for 15 min, followed by 40 cycles at 94 °C for 10 s, 56 °C for 30 s, and 72 °C for 30 s. Experiments were performed in triplicate, and the values were used to calculate the ratio of EP3 to rat beta-actin, with a value of 1 as the control.

Immunohistochemical staining for COX-2 and EP3

Placental tissues were immediately fixed in 4% paraformaldehyde (pH 7.4) for 12 h at 4 °C and were subsequently embedded in paraffin. Then the tissues were cut into 5-μm sections and mounted onto gelatin-precoated slides.

Immunohistochemistry was performed by the avidin—biotin—peroxidase complex technique using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). The slides were deparaffinized, and endogenous peroxidase activity was blocked by incubation with 0.6% H_2O_2 in methanol for 30 min at room temperature. Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan) was then done for 30 min at room temperature to avoid nonspecific staining. After rinsing several times with PBS, primary antibodies were applied to the sections.

Polycloned anti-rat cox-2 antibody and anti-rat EP3 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). These antibodies were applied for 60 min at RT. After incubation with biotinylated goat anti-rabbit IgG and avidin—biotin—peroxidase complex, antigen—antibody complexes were visualized by using diaminobenzidine as a chromogen. The sections were lightly counterstained with Meyer's hematoxylin. As controls, normal rabbit serum was used or the primary antibody was omitted (data not shown).

Western blotting of COX-2 and EP3

Placental extracts (20 μg of protein) were separated by 12.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes using an electroblotting apparatus. Nonspecific binding was blocked by immersing the membrane overnight at RT in PBS containing 0.05% Tween 20 (T-PBS) and 5% skim milk in an orbital shaker, following by washing five times with T-PBS. Then the membrane was incubated with the primary antibody of anti-rat COX-2 or anti-rat EP3 for 90 min at RT in a humidified chamber and washed five times with T-PBS. The membrane was then incubated with an HRP-conjugated antibody at RT in a humidified chamber and washed another five times with T-PBS. The membrane was finally incubated with a chemiluminescence reagent, and exposed to X-ray film. Bands of Western blot were analyzed using NIH Image (Version 1.61).

Data analysis

Results represent the mean ± SD of data from replicate experiments. Statistical differences were evaluated by one-way analysis of variance, followed by Scheffe's *F*-test post hoc