Published in final edited form as:

Reprod Sci. 2010 May ; 17(5): 465–470. doi:10.1177/1933719110361960.

Neutrophil Expression of Cyclooxygenase-2 in Preeclampsia

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Abstract

The objective of this study was to determine: 1) if neutrophils of preeclamptic women express cyclooxygenase-2 (COX-2), and 2) if COX-2 mediates neutrophil interleukin-8 (IL-8) production. Neutrophils were isolated from heparinized blood collected from women with preeclampsia and normal pregnancy, and from normal nonpregnant women. COX-2 protein was measured in the neutrophils of the three groups by specific ELISA. To study the role of COX-2 in IL-8 production, neutrophils of normal pregnant women were activated with arachidonic acid and incubated overnight in Medium 199 with and without NS398, a specific COX-2 inhibitor. We found that neutrophils from preeclamptic women expressed significantly more COX-2 than neutrophils from normal pregnant or normal nonpregnant women. Arachidonic acid activated neutrophils to produce IL-8 which was completely inhibited by NS398. These data demonstrate that COX-2 is expressed in neutrophils of preeclamptic women and that COX-2 mediates IL-8 production by activated neutrophils.

Keywords

Preeclampsia; pregnancy; neutrophils; COX-2; interleukin-8

INTRODUCTION

Maternal circulating neutrophils are activated in pregnancy and further activated in preeclampsia^{1–5}. One possibility for neutrophil activation is that they are activated as they circulate through the intervillous space and are exposed to oxidized lipids secreted by the placenta^{6–9}. Another possibility is that they are activated by elevated levels of arachidonic acid present in the plasma of preeclamptic women^{10, 11}. These conditions could induce expression of cyclooxygenase-2 (COX-2) in neutrophils.

The cyclooxygenase enzyme is present in three isoforms: COX-1, COX-2 and COX-3^{12, 13}. COX-3 is a splice variant of COX-1. COX-1 and COX-2 are encoded by different genes, but both catalyze the conversion of arachidonic acid to prostaglandin H₂. COX-1 is present constitutively in cells and is responsible for the biosynthesis of prostaglandins involved in homeostatic regulation, whereas COX-2 is an induced form and produces prostaglandins and thromboxane involved in inflammation. Therefore, increased expression of COX-2 in neutrophils could be important because neutrophils infiltrate systemic vascular tissue in women with preeclampsia and this is associated with vascular inflammation^{14, 15}. Release of COX-2 inflammatory products by neutrophils that have infiltrated the vasculature might be responsible for vascular dysfunction and vasoconstriction.

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Interleukin-8 (IL-8) is a potent chemotactic agent and an important product of activated neutrophils. IL-8 levels are elevated in the plasma of preeclamptic women¹⁶. Release of IL-8 by neutrophils that have infiltrated into the vasculature would provide a chemotactic gradient to attract more neutrophils. This could establish a positive feedforward mechanism to worsen vascular inflammation. IL-8 is known to be regulated by transcription factors, such as nuclear factor- κ B (NF- κ B), but we recently found that cyclooxygenase metabolites play an important role in IL-8 production under conditions that induce COX-2 in human vascular smooth muscle cells¹⁷. Therefore, it is possible that COX-2 also plays an important role in the production of IL-8 by activated neutrophils.

In the present study, we measured the expression of COX-2 in neutrophils obtained from preeclamptic women and compared it to expression in neutrophils obtained from normal pregnant women and normal nonpregnant women. We also evaluated the role of COX-2 in neutrophil production of IL-8. We hypothesized that neutrophils obtained from preeclamptic women would have significantly higher expression of COX-2 as compared to neutrophils obtained from normal pregnant women or normal nonpregnant women, and that COX-2 would play a key regulatory role in the production of IL-8 by activated neutrophils obtained from pregnant women.

MATERIALS AND METHODS

Study Participants

Blood samples were obtained from 5 normal nonpregnant women, 14 normal pregnant women and 14 preeclamptic women by vein puncture into sodium heparin tubes for measurement of COX-2 protein. In addition, blood samples were obtained from an additional 10 normal pregnant women to determine the role of COX-2 in neutrophil IL-8 production. Two tubes of blood were collected from each patient. Preeclampsia was defined as sustained blood pressure of $\geq 140/90$ mmHg with readings at least 6 hours apart and proteinuria (300mg/24 hr or $\geq 1+$ urine dipstick). Exclusion criteria included chorioamnionitis, maternal infection, lupus, active STDs, diabetes, smoking and labor because these conditions might impact inflammation. Demographic data are presented in Table 1 for the patients used for measurement of neutrophil COX-2. This study was approved by the Office of Research Subjects Protection, Virginia Commonwealth University.

Neutrophil Isolation and Incubation

The blood was processed in the lab within one hour following sample collection. Neutrophils were isolated by dual Histopaque density gradient centrifugation (Sigma Chemical Company, St. Louis, MO). Neutrophils were aspirated from the Histopaque-1077/Histopaque-1119 interface and washed with phosphate buffered saline (PBS), followed by centrifugation for 10 minutes at $200 \times g$. To lyse contaminating red blood cells, the cell pellet was suspended in ice-cold double distilled water (3 mL) for 30 seconds with agitation on a vortex mixer. Ice-cold 0.6 M potassium chloride (1 mL) was then added to restore tonicity. The cells were pelleted, the supernatant was discarded, and the cells were resuspended in Hank's Balanced Salt Solution (HBSS, no Mg^{++} , no Ca^{++}) (Gibco, Invitrogen, Grand Island, NY). For measurement of COX-2 protein, cells were counted using an hemocytometer, centrifuged and resuspended in 500 μ L TNE buffer (10mM Tris, pH 8.0, 1% NP-40, 0.15M NaCl, 1mM EDTA) and frozen at -20°C until assay. For measurement of IL-8 production, cells were counted, centrifuged and diluted in Medium 199 (Gibco, Invitrogen) to a concentration of 1 million cells per mL and incubated overnight with treatments. An average of 15.4 ± 4.9 million neutrophils were obtained from normal nonpregnant women, 32.9 ± 8.5 million from normal pregnant women, and 24.8 ± 4.9 million from preeclamptic women.

Purity of neutrophils was assessed by immunohistochemical staining using a mouse IgM anti-human monoclonal antibody specific for CD66b, a neutrophil antigen, as previously described^{14, 15}. CD66b was purchased from BD Bioscience, San Diego, CA. Zymed SuperPicture Kit (Invitrogen, Carlsbad, CA) was used for staining.

COX-2 and IL-8 Assays

For neutrophil COX-2 protein expression, neutrophils in TNE buffer were thawed and diluted with additional TNE buffer to a concentration of 10 million cells per mL. Cells were lysed by sonication and samples were centrifuged for 5 min at $1300 \times g$. COX-2 protein was measured in 100 μ L of the supernatant by a specific COX-2 ELISA Kit (Zymed Laboratories, Inc., South San Francisco, CA). For neutrophil production of IL-8, 1 million cells were incubated in 1 mL of Medium 199 overnight at 37°C on a rotating shaker. Treatments, run in duplicate, were medium control, arachidonic acid (AA, 100 μ M), a physiologic activator of neutrophils, and AA + NS398 (20 μ M), a specific COX-2 inhibitor. Media were collected after 4 hours and 18 hours of incubation. Media concentrations of IL-8 were measured by ELISA using commercially available reagents (R&D Systems) as previously described¹⁷.

Statistical Analysis

Neutrophil COX-2 protein data were analyzed by Kruskal-Wallis test with Dunn's multiple comparison test, neutrophil IL-8 production by repeated measures analysis of variance with Tukey's multiple comparison test, and patient demographic data by oneway analysis of variance or t-test. A statistical computer software program was used for analysis (GraphPad Prism 4.0 for Macintosh, GraphPad Software, San Diego, CA). A probability level of $P < 0.05$ was considered to be statistically significant.

RESULTS

There were no significant differences among patient groups used for neutrophil COX-2 expression for age, BMI, or gestational age at sample collection (Table 1). Preeclamptic patients had significantly higher systolic and diastolic blood pressures and lower infant birth weights ($P < 0.001$).

Neutrophils from preeclamptic women expressed significantly more COX-2 than neutrophils from normal pregnant or normal nonpregnant women (18.6 ± 4.3 vs. 5.6 ± 2.0 vs. 2.7 ± 0.7 ng/million cells, respectively, $P < 0.01$) (Fig. 1).

Neutrophils incubated in Medium 199 produced IL-8 in a linear manner reaching a concentration of $1,142 \pm 509$ pg/mL after 18 hours of incubation (Fig. 2). Activation of neutrophils with AA resulted in a significant increase in IL-8 production at 4 hours and at 18 hours of incubation as compared to control (Fig. 3). Treatment of neutrophils with NS398 completely inhibited the ability of AA to stimulate IL-8.

DISCUSSION

In this study we report that neutrophil expression of COX-2 is significantly higher in preeclamptic women than in normal pregnant women or normal nonpregnant women. Low neutrophil expression of COX-2 in normal nonpregnant women and normal pregnant women may have resulted from the isolation procedure, although some COX-2 expression might be expected in normal pregnant women because other investigators have reported moderate activation of neutrophils in normal pregnancy^{5, 18}. Although preeclamptic patients delivered on average at an earlier gestational age and had lower weight infants, neither gestational age nor restricted fetal growth could explain elevated levels of COX-2 in preeclampsia. Only 2 of

the preeclamptic patients had IUGR fetuses and 3 of the preeclamptic patients delivered at term. There was no obvious effect of gestational age or IUGR on the data.

We also show that COX-2 mediates IL-8 production by neutrophils obtained from normal pregnant women when they are activated by arachidonic acid. We used arachidonic acid to activate the neutrophils because it is a physiologic activator and levels of arachidonic acid are elevated in women with preeclampsia^{10, 11}. Previously, we provided evidence that COX-2 mediates neutrophil production of thromboxane, superoxide and TNF α ^{19, 20}. Here we show that COX-2 also mediates production of IL-8. Production of IL-8 by neutrophils that have infiltrated the vasculature in women with preeclampsia^{14, 15} would provide a chemotactic gradient to attract more neutrophils to infiltrate. These data demonstrate that COX-2 plays a pivotal role in the release of inflammatory substances from activated neutrophils in pregnancy.

The mechanism whereby COX-2 mediates neutrophil production of IL-8 remains to be defined, but may involve interactions between NF- κ B and COX-2 metabolites. IL-8 is one of the inflammatory genes regulated by NF- κ B, and COX metabolites enhance NF- κ B dependent transcription^{21–23}. Neutrophil thromboxane is one of the COX-2 metabolites important in neutrophil activation and production of inflammatory mediators^{19, 20}.

There is increasing evidence that inflammation with expression of COX-2 plays a key role in preeclampsia, so increased neutrophil expression of COX-2 is an important finding regarding the pathophysiology of preeclampsia. Increased expression of COX-2 appears to be wide spread. COX-2 is increased in placentas of women with preeclampsia where its expression is related to increased production of thromboxane^{24, 25}. Placentas obtained from preeclamptic women produce more thromboxane and less prostacyclin than placentas obtained from normal pregnant women²⁶. Thromboxane is a potent vasoconstrictor, whereas prostacyclin is a potent vasodilator, so this imbalance contributes to reduced uteroplacental blood flow in preeclampsia.

Recently we found evidence for inflammation and increased expression of COX-2 in the maternal systemic circulation and vasculature of preeclamptic women. We showed extensive infiltration of neutrophils, but not lymphocytes or monocytes, into the systemic vasculature of women with preeclampsia^{14, 15, 27}. Neutrophils were flattened and adhered on the endothelium and infiltrated into the intimal space, the space between the endothelium and vascular smooth muscle. Neutrophils release toxic substances, such as TNF α , reactive oxygen species (ROS), myeloperoxidase, matrix metalloproteinase 8 and thromboxane, which can cause inflammation. Consistent with this finding, neutrophil infiltration was associated with markers of inflammation in the endothelium and vascular smooth muscle. NF- κ B was activated and there was increased expression of intercellular adhesion molecule-1 (ICAM-1), IL-8 and COX-2^{14, 15}. Vascular infiltration of neutrophils and release of ROS may also contribute to hypertension in preeclampsia. We recently found that ROS significantly enhanced vascular reactivity of human omental vessels to angiotensin II via activation of the RhoA kinase pathway²⁸.

In summary, these data are the first to show that COX-2 is expressed in neutrophils of preeclamptic women, and that COX-2 mediates neutrophil production of the potent chemotactic chemokine, IL-8. These data suggest that COX-2 may play an important role in the pathophysiology of preeclampsia.

Acknowledgments

Supported by a grant to SWW from the National Institutes of Health (HL069851)

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Figure 1. Neutrophils obtained from preeclamptic women (PE, n =14) expressed significantly more COX-2 than neutrophils obtained from normal pregnant women (NP, n =14) or normal nonpregnant women (NNP, n = 5). Data are mean $\pm$ SE, ** P < 0.01 as compared to NP or NNP.

Figure 2. Release of IL-8 into media after 4 hours and 18 hours of incubation of neutrophils obtained from normal pregnant women. Neutrophils were activated with arachidonic acid (AA, 100 μ M). AA significantly stimulated neutrophil production of IL-8 at both 4 hours and 18 hours as compared to control. NS398, a specific COX-2 inhibitor, significantly blocked the ability of AA to stimulate IL-8 production. Data are mean $\pm$ SE, n = 10. **P < 0.01 as compared to control or AA + NS398.

TABLE 1

Patient Demographic Information

	NNP (n = 5)	NP (n = 14)	PE (n = 14)
Age, years	27.2 ± 7.9	27.8 ± 6.1	22.1 ± 5.1
Pre-pregnancy BMI	21.5 ± 2.5	25.0 ± 5.6	25.3 ± 5.4
Systolic BP, mmHg	102 ± 9	124 ± 12	164 ± 22 ^a
Diastolic BP, mmHg	68 ± 8	70 ± 8	99 ± 12 ^a
Proteinuria, mg/24 hrs	NA	ND	522 ± 321
Dipstick	NA	ND	3.2 ± 1.3
Parity Primiparous Multiparous	NA NA	5 9	10 4
Gestational age at sample collection, weeks	NA	37.4 ± 5.4	34.0 ± 4.3
Infant birth weight, gms	NA	3658 ± 258	2087 ± 767 ^b

Data are mean ± SD

NA – not applicable, ND – not determined

^aP<0.001 as compared to NNP and NP, one-way ANOVA, Tukey's multiple comparison test^bP<0.001, t-test