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Ovariectomy enhances renal cortical expression and function of cyclooxygenase-2

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Ovariectomy enhances renal cortical expression and function of cyclooxygenase-2.

Background. Cyclooxygenase-2 (COX-2) inhibitors are used as analgesics in postmenopausal women, who develop edema and require a salt-restricted diet. This study was performed to determine the renal expression of COX-2 and on COX-2–dependent regulation of renal blood flow (RBF) in ovariectomized rats.

Methods. Sprague-Dawley rats were divided into 4 groups: sham-operated rats fed a normal-salt diet (Sh+NS) or a low-salt diet (Sh+LS), and bilaterally ovariectomized rats fed a normal-salt diet (Ox+NS) or a low-salt diet (Ox+LS) ($N = 6$ in each group). Estrogen replacement therapy was performed on other ovariectomized rats. A renal clearance study was performed in anesthetized animals.

Results. Ovariectomy increased renal cortical COX-2 expression independently of dietary salt intake (Sh+NS <Ox+N; Sh+LS <Ox+LS). Inhibition of COX-2 by NS398 reduced the urinary excretion of 6-keto-prostaglandin $F_{1\alpha}$ in all 4 groups, although the reduction was greater in the Ox+LS group than in the Ox+NS and Sh+LS groups, which in turn had a greater reduction than the Sh+NS group. RBF significantly decreased in every group except the Sh+NS group, but no effect on blood pressure, inulin clearance, or urinary sodium excretion was seen. The decrease in RBF was significantly greater in the Ox+LS group than in the Sh+LS and Ox+NS group. The decrease in RBF was dependent on cortical RBF in the Sh+LS and Ox+NS groups, and on both cortical and medullary RBF in the Ox+LS group. Estrogen replacement therapy reversed the ovariectomy-induced changes.

Conclusion. Estrogen-dependent COX-2 expression plays an important role in the RBF regulation in female rats.

Cyclooxygenase (COX) occurs in two isoforms, COX-1 and COX-2, and catalyzes the conversion of arachidonic acid to prostaglandins, which are known to be important physiologic modulators of vascular tone and salt and water homeostasis in the mammalian kidney [1–3]. COX-1 is present at relatively stable levels in the kidneys, whereas COX-2 is constitutively present in the area adjacent to the macula densa cells of the renal cortex and in medullary interstitial cells [4, 5].

Previous studies have demonstrated that the COX-2 in the renal cortex contributes to the regulation of renin synthesis [6] and renal microvascular tone [7], and that its expression is regulated by dietary salt intake [8, 9], angiotensin II [10], corticosteroids [11], and furosemide [12]. However, the effects of ovariectomy on expression and function of renal COX-2 remain unknown, although a recent study revealed that the COX-2 promoter gene contains an estrogen-binding site [13]. Therefore, the present study was performed to determine the effect of ovariectomy and estrogen replacement therapy on renal COX-2 expression and COX-2–dependent regulation of renal blood flow.

For headache, arthralgia, and menstrual pain, nonsteroidal anti-inflammatory drugs (NSAIDs) are used worldwide in hypertensive women [14] who tend to develop edema and require a salt-restricted diet. Although selective COX-2 inhibitors have been developed as NSAIDs with few adverse effects, the results of the present study might suggest caution in clinical use of COX-2 inhibitors in postmenopausal patients and post-ovariectomy patients for any reason, especially when they are on a restricted salt diet.

METHODS

Preparation of animals

Female Sprague-Dawley rats (Charles River Labs, Wilmington, MA, USA) were housed in wire cages, and maintained in a temperature-controlled room on a 12:12-hour light-dark cycle. The animals had free access to water and rat chow consisting of a normal-salt diet (0.4% NaCl; CE-2; Nihon Clea, Tokyo, Japan) or a low-salt diet

Key words: cyclooxygenase-2, ovariectomy, prostaglandins, renal plasma flow, cells of the thick ascending limb of Henle, estrogen replacement therapy.

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(0.02%; T-02378; Nihon Clea). All experimental protocols were approved by the Keio University Animal Care and Use Committee. Rats weighing 175 g to 200 g were anesthetized with sodium pentobarbital (50 mg/kg, IP), and ovariectomy or a sham operation was performed via bilateral flank incisions. The surgically prepared rats were divided into 4 groups: sham-operated rats fed a normal-salt diet (Sh+NS), sham-operated rats fed a low-salt diet (Sh+LS), bilaterally ovariectomized rats fed a normal-salt diet (Ox+NS), and bilaterally ovariectomized rats fed a low-salt diet (Ox+LS).

In the estrogen replacement therapy experiments, pellets containing either 17β-estradiol (0.5 mg per rat with a 21-day release; Innovative Research of America, Sarasota, FL, USA) or placebo (0.5 mg per rat with a 21-day release; Innovative Research of America) were implanted in the subcutaneous tissue of bilaterally ovariectomized rats. The ovariectomized rats in this series of experiments were divided into 4 groups: a placebo-treated group fed a normal-salt diet (Ox+NS+PL); a placebo-treated group fed a low-salt diet (Ox+LS+PL); a 17β-estradiol–treated group fed a normal-salt diet (Ox+NS+ER); and a 17β-estradiol–treated group fed a low-salt diet (Ox+LS+ER). All experiments were performed on day 14 after surgical preparation. Systolic blood pressure measured by tail-cuff plethysmography immediately before the experiments yielded similar values in all 4 groups, with average values in the Sh+NS, Sh+LS, Ox+NS, Ox+LS, Ox+NS+PL, Ox+LS+PL, Ox+NS+ER, and Ox+LS+ER groups of 120 ± 3, 121 ± 2, 119 ± 3, 121 ± 2, 122 ± 2, 123 ± 2, 120 ± 3, and 119 ± 4 mm Hg, respectively ($N = 6$ in each group). The plasma estradiol concentration was determined by radioimmunoassay (RIA), and the values in the sham-operated rats, ovariectomized rats treated with placebo, and ovariectomized rats treated with 17β-estradiol were 28.7 ± 4.5, 6.9 ± 1.2, and 78.4 ± 5.2 pg/mL, respectively ($N = 9$ in each group).

Determination of the plasma renin activity

One mL of blood was extracted from tail vein on the day before the clearance study in the ovariectomized and sham-operated rats fed either low- or normal-salt diet. The plasma was used to determine plasma renin activity (PRA) with commercially available RIA kit, according to the manufacturer's instructions (Renin-Riabead, Dainabot, Tokyo, Japan). PRA was expressed as the rate of angiotensin I formation (ng/mL/h).

Double-immunolabeling of cyclooxygenase-2 and Tamm-horsfall glycoprotein

This technique was performed with a modification of a previously described procedure [15]. Kidney tissue was fixed overnight in 4% phosphate saline-buffered paraformaldehyde, then rinsed in a series of sucrose solutions of increasing concentration and snap-frozen. Dual immunofluorescence (DIF) was performed on 4-μm cryostat sections. The primary antibodies were as follows: rabbit polyclonal anti-COX-2 antibody (1:400) (Cayman Chemical, Ann Arbor, MI, USA) and sheep polyclonal anti-uromucoid antibody (1:400) (Biodesign International, Saco, ME, USA), and the secondary antibodies were rhodamin-conjugated anti-rabbit IgG and fluoroscein isothiocyanate (FITC)-conjugated anti-sheep IgG (1:500) (Chemicon International, Temecula, CA, USA). The DIF sections were examined with a confocal microscope (MRC600; BioRad Laboratories, Hercules, CA, USA). The secondary antibodies had been isolated by immunoaffinity chromatography and absorbed for dual labeling. Control measures included omitting the primary antibody and substituting normal IgG from the same animal for the primary antibody.

Immunohistochemistry

Kidneys harvested from the rats from each group were perfused in situ with saline and fixed in Bouin's solution. The kidneys were then dehydrated with a graded series of ethanol and embedded in paraffin. After deparaffinization and rehydration, sections (4-μm thick) were boiled in citrate buffer in a microwave to unmask antigenicity, immersed in 3% H_2O_2 in methanol to inhibit endogenous peroxidase, and flooded with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) to inhibit nonspecific reactions. Polyclonal rabbit anti-COX-2 antibodies (160106, 160116, and 160126, Cayman Chemical) (1:200–500 dilution) were used as the primary antibodies [16], and a biotinylated polyclonal goat anti-rabbit antibody (1:500 dilution) was applied as the secondary antibody. Immunoreaction was performed with a Vectastain ABC-Elite kit (Vector Laboratories, Burlingame, CA, USA) and visualized with 0.02% 3′,3′-diaminobenzidine tetrahydrochloride as a substrate followed by light counterstaining with hematoxylin. Control staining was performed with normal rabbit IgG.

Western blotting of renal cortex

The renal cortex or medulla was lysed in T-PER™ reagent (Pierce, Rockford, IL, USA) containing 25 μL DTT (1 mol/L) and 250 μL PMSF (100 mmol/L), and after 5-minute centrifugation at 10,000*g*, the supernatant was collected and subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes, and after blocking the blots overnight with PBScontaining 5% bovine albumin and 0.5% Tween 20, they were incubated for 16 hours with polyclonal rabbit anti-COX-2 antibody (160116, Cayman Chemical) (1:1000 dilution). Immunoreactivity was determined by using horseradish

peroxidase–conjugated donkey anti-rabbit antibody and the enhanced chemiluminescence reaction, and the quantitative analyses were performed using Image 1D (Pharmacia, Peapack, NJ, USA).

Renal clearance study

Renal clearance experiments were performed to assess the effect of NS398 (Cayman Chemical) on renal hemodynamics and excretory function. Rats were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg), and placed on a thermoregulated surgical table to maintain rectally monitored body temperature at 37°C. A tracheostomy was performed to ensure a patent airway, and positive-pressure ventilation was maintained with an artificial respirator (model 7025; Ugo Basile, Comerio, Italy) at a rate of 60 strokes/min and a stroke volume of 10 mL/kg body weight. A PE-60 catheter was inserted into jugular vein to infuse solutions and pentobarbital sodium as needed to maintain an appropriate level of anesthesia. The right femoral artery was cannulated, and the cannula was connected to a computer system (MacLab/8s, AD Instruments, Nagoya, Japan) via a pressure transducer to monitor arterial pressure. The right carotid artery was also cannulated to provide separate access for intra-arterial bolus doses of NS398.

The left kidney was exposed by a flank incision, isolated from surrounding tissue, and placed in a Lucite cup to keep it stable. Two needle flow probes (500-μm diameter) connected to a laser-Doppler flowmeter (Flo-C1-Twin; Omegawave, Tokyo, Japan) were inserted into the kidney mass to a depth of 1 mm and 4 mm, respectively, to position their tips in the superficial cortex and the mid-medullary region, respectively, and measure relative changes in renal cortical blood flow (CBF) and medullary blood flow (MBF). The bladder was catheterized with PE-10 tubing via a suprapubic incision for urine collection. During surgery, isotonic saline solution containing 6% albumin (bovine; Sigma Chemical Co., St. Louis, MO, USA) was infused at a rate of 20 μL/min. After surgery, the intravenous infusion was switched to isotonic saline solution containing 1% albumin, 1.5% *p*-aminohippurate sodium (PAH; Merck, Sharp & Dohme, Hoddesdon, UK), and 7.5% inulin (Inutest; Laevosan, Linz, Austria), and infused at the same rate. A 90-minute equilibration period was provided after the completion of surgery.

The experimental protocol consisted of three 30-minute clearance periods to assess renal function. The rats were given intra-arterial bolus injections of increasing doses of NS398 [0, 1, and 10 ng/gram body weight (gbw)] at 40-minute intervals. NS398 is thought to selectively inhibit COX-2, and have no effect on COX-1 activity at the concentrations used in the present study [17]. After a 10-minute delay, a 30-minute experimental clearance period was provided for each dose of NS398, and an arterial blood sample was collected at the midpoint of each 30-minute clearance period to calculate inulin clearance (Cinulin) and PAH clearance (CPAH). At the end of each experiment, the left kidney was removed, stripped of surrounding tissue, blotted dry, and weighed, so that the data could be normalized per gram kidney weight (gkw). The microhematocrit of all arterial blood samples was measured and remained constant throughout the experiment. Inulin and PAH concentrations in blood and urine were measured colorimetrically, and urinary sodium concentrations were determined by flame photometry. Cinulin was used as an index of GFR, and CPAH was used as an index of renal blood flow (RBF) because of the constant hematocrit throughout renal clearance study. All drugs were given in a total volume 100 μL.

Determination of urinary prostaglandin E2 and 6-keto prostaglandin F1α

The urine samples were collected during the first and last 30-minute renal clearance periods (i.e., during 30 minutes after administration of 0 and 10 ng/gbw of NS398, respectively). The urinary concentration of prostaglandin (PG) E2 and 6-keto PGF1α were determined by RIA using a [^{125}I] RIA kit (PerkinElmer Life Sciences, Inc., Boston, MA, USA) after the extraction by conventional methods and subsequent purification on BOND-ELUT Si columns (Analytichem International, Harbor City, CA, USA).

Statistical analysis

Statistical comparisons within a group were made by one-way analysis of variance (ANOVA) for repeated measures followed by the Newman-Keuls post hoc test. Differences between two groups were evaluated by two-way ANOVA for repeated measures combined with the Newman-Keuls post hoc test. A P value of <0.05 was considered significant. Data are reported as mean ±SEM.

RESULTS

Double-immunolabeling of COX-2 and Tamm-horsfall glycoprotein

Figure 1A shows double-immunolabeling with specific antibodies against COX-2 and Tamm-Horsfall glycoprotein, and revealed that COX-2 is present in Tamm-Horsfall–positive cells. Since Tamm-Horsfall glycoprotein is a specific antigenic marker of cells in the thick ascending limb (TAL) of Henle, including macula densa cells, this finding confirmed that COX-2 was localized exclusively in TAL cells.

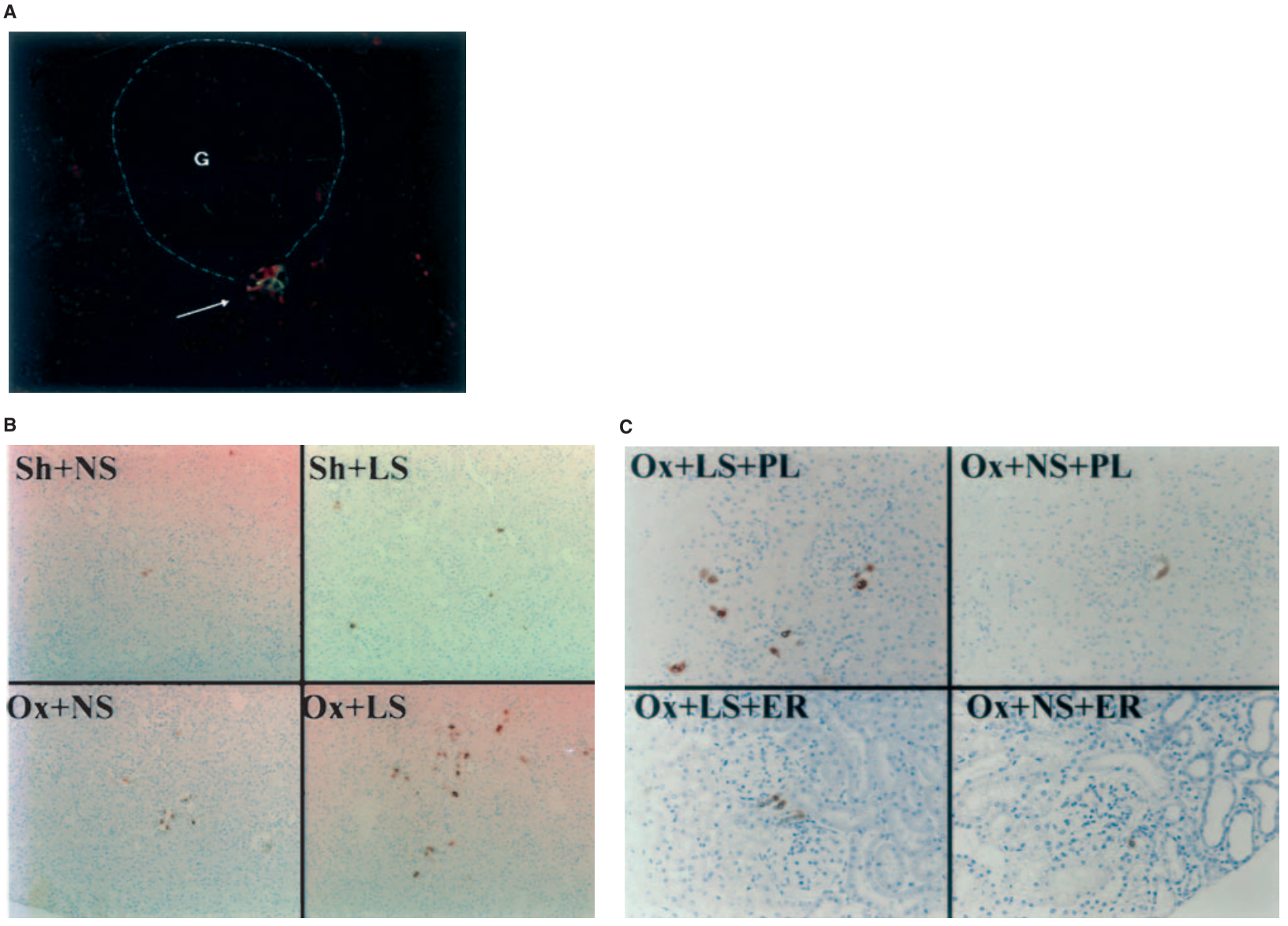

Fig. 1. COX-2 expression. (*A*) Double-immunolabeling with specific antibodies against COX-2 (red) and Tamm-Horsfall glycoprotein (green) in the thick ascending limb of Henle adjacent to glomerulus (G). (*B*) Immunohistochemical staining of COX-2 (magnification, ×100) increased in the following order: sham-operated rats fed a normal-salt diet (Sh+NS), sham-operated rats fed a low-salt diet (Sh+LS), ovariectomized rats fed a normal-salt diet (Ox+NS), and ovariectomized rats fed a low-salt diet (Ox+LS). (*C*) Immunohistochemical staining of COX-2 in ovariectomized rats (magnification, ×100) decreased in the following order: placebo-treated rats fed a low-salt diet (Ox+LS+PL), 17β-estradiol–treated rats fed a low-salt diet (Ox+LS+ER), placebo-treated rats fed a normal-salt diet (Ox+NS+PL), and 17β-estradiol–treated rats fed a normal-salt diet (Ox+NS+ER).

Immunohistochemistry

Figure 1B shows the effects of salt restriction and ovariectomy on expression of COX-2 in TAL cells. COX-2 stained weakly in the macula densa cells in the Sh+NS group, whereas the salt restriction and ovariectomy similarly increased the number and intensity of COX-2–positive macula densa cells in the Sh+LS and Ox+NS groups. In the Ox+LS group, on the other hand, the number of COX-2–positive cells increased not only adjacent to the macula densa, but along the TAL segment as well, and the intensity of the COX-2 staining was also greater than in any of the other three groups.

Figure 1C shows the effects of estrogen replacement therapy on the expression of renal cortical COX-2 in ovariectomized rats fed either a normal- or low-salt diet. In either a normal- or low-salt diet, estradiol administration decreased the number and intensity of COX-2–positive cells increased by ovariectomy.

Western blotting of renal cortex and medulla

Figure 2A shows the results of immunoblotting of microsomes from renal cortex with antiserum specific for COX-2, and they revealed immunoreactrive protein with a molecular mass of −73kD in all four groups. The COX-2 immunoblotting increased with salt restriction, ovariectomy, and both (Sh+NS<Ox+NS = Sh+LS<Ox+LS). Ovariectomy enhanced the COX-2 immunoblotting in both the rats fed the low- and the normal-salt diet. Combined with the immunohistochemistry findings, these results suggest that ovariectomy enhances expression of COX-2 in TAL cells independently of salt intake, although salt restriction significantly enhanced the expression of COX-2.

Figure 2B shows the results of immunoblotting of microsomes from renal medulla with antiserum specific for COX-2, and they revealed immunoreactrive protein with a molecular mass of −73kD in all four groups. Salt restriction decreased the medullary COX-2 immunoblotting in sham-operated rats, whereas salt restriction increased it in ovariectomized rats. Ovariectomy decreased the medullary COX-2 immunoblotting in rats fed a normal-salt diet, but increased it in rats fed a low-salt diet.

Figure 2C shows the effects of estrogen replacement therapy on the COX-2 immunoblotting of microsomes from renal cortex. Estrogen replacement decreased the cortical COX-2 immunoblotting in the ovariectmomized rats fed either a normal- or a low-salt diet. Combined with the immunohistochemical findings, in ovariectomized rats, estrogen replacement appears to inhibit the renal cortical expression of COX-2 independently of dietary salt. Figure 2D shows the effects of estrogen replacement therapy on the medullary COX-2 immunoblotting. Salt restriction increased the medullary COX-2 immunoblotting in ovariectomized rats treated with placebo, whereas

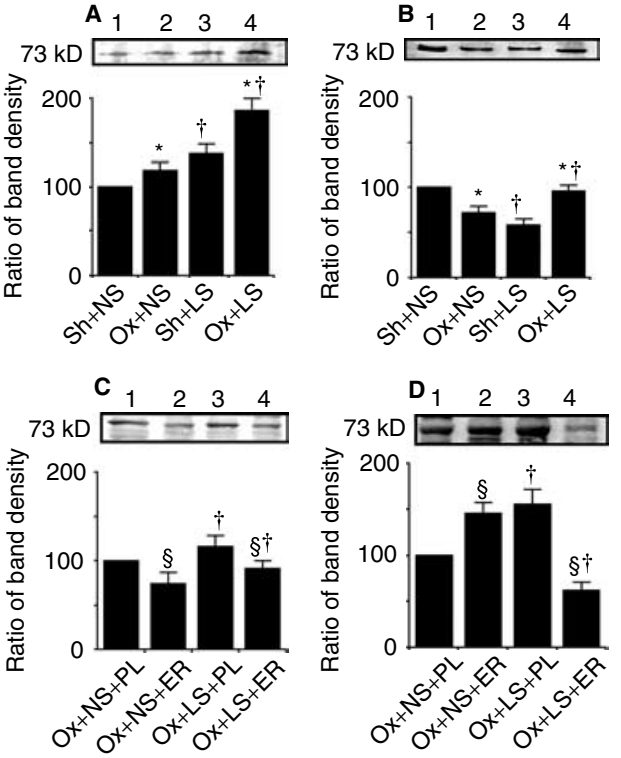

Fig. 2. Representative Western blotting and quantitative analysis of COX-2 protein expression. (*A*) COX-2 protein expression in the renal cortex of sham-operated rats fed a normal-salt diet (Sh+NS, $N = 6$), sham-operated rats fed a low-salt diet (Sh+LS, $N = 6$), ovariectomized rats fed a normal-salt diet (Ox+NS, $N = 6$), and ovariectomized rats fed a low-salt diet (Ox+LS, $N = 6$). (*B*) COX-2 protein expression in the renal medulla of sham-operated rats fed a normal-salt diet (Sh+NS, $N = 6$), sham-operated rats fed a low-salt diet (Sh+LS, $N = 6$), ovariectomized rats fed a normal-salt diet (Ox+NS, $N = 6$), and ovariectomized rats fed a low-salt diet (Ox+LS, $N = 6$). (*C*) COX-2 protein expression in the renal cortex of ovariectomized rats. Placebo-treated rats fed a normal-salt diet (Ox+NS+PL, $N = 6$), estradiol-treated rats fed a normal salt diet (Ox+NS+ER, $N = 6$), placebo-treated rats fed a low salt diet (Ox+LS+PL, $N = 6$), and estradiol-treated rats fed a low salt diet (Ox+LS+ER, $N = 6$). (*D*) COX-2 protein expression in the renal medulla of ovariectomized rats. Placebo-treated rats fed a normal-salt diet (Ox+NS+PL, $N = 6$), estradiol-treated rats fed a normal salt diet (Ox+NS+ER, $N = 6$), placebo-treated rats fed a low salt diet (Ox+LS+PL, $N = 6$), and estradiol-treated rats fed a low salt diet (Ox+LS+ER, $N = 6$). $^*P < 0.05$ for Ox vs. Sh; †$P < 0.05$ for LS vs. NS; §$P < 0.05$ for ER vs. PL.

salt restriction decreased it in ovariectomized rats treated with estrogen. Estrogen replacement therapy increased the medullary COX-2 immunoblotting in ovariectomized rats fed a normal-salt diet, but decreased it in rats fed a low-salt diet.

Plasma renin activity

The average values of PRA in the Sh+NS, Sh+LS, Ox+NS, and Ox+LS groups were 14.6 ± 1.9, 54.2 ± 9.5, 19.6 ± 3.0, and 63.9 ± 9.6, respectively ($N = 4$ in each group). PRA was significantly increased by salt restriction both in ovariectomized and sham-operated rats.

Table 1. Urinary excretion of prostaglandin E2 and 6-keto prostaglandin F1α before and during NS398 treatment

NS398 treatment	Before	During
Prostaglandin E2 (ng/h)		
Sh+NS ($N = 6$)	21.8±3.2	4.5 ± 0.7^{a}
Sh+LS ($N = 6$)	24.6 ± 1.8	5.4 ± 0.6^{a}
Ox+NS ($N = 6$)	35.2 ± 8.6	6.7 ± 1.1^{a}
Ox+LS ($N = 6$)	36.8 ± 5.4^{b}	6.2 ± 1.2^{a}
6-keto prostaglandin F1 α (ng/h)		
Sh+NS ($N = 6$)	2.7 ± 0.6	0.8 ± 0.2^{a}
Sh+LS ($N = 6$)	14.4 ± 1.9^{b}	2.8 ± 0.8^{a}
Ox+NS ($N = 6$)	16.9 ± 1.9^{b}	2.9 ± 0.7^{a}
Ox+LS ($N = 6$)	$35.8 \pm 6.6^{b,c,d}$	1.9 ± 0.8^{a}

Sh+NS, sham-operated rats fed a normal-salt diet; Sh+LS, sham-operated rats fed a low-salt diet; Ox+NS, ovariectomized rats fed a normal-salt diet; Ox+LS, ovariectomized rats fed a low-salt diet. Data are mean ± SEM.

[a] $P < 0.05$ versus before NS398 treatment; [b] $P < 0.05$ vs. Sh+NS; [c] $P < 0.05$ for Ox+LS vs. Sh+LS; [d] $P < 0.05$ for Ox+LS vs. Ox+NS.

Ovariectomy slightly increased PRA without a significance in either the rats fed a normal- or low-salt diet. This result was consistent with previous studies showing that postmenopausal women have higher plasma renin levels than premenopausal women [18].

Urinary prostaglandin excretion

Table 1 shows urinary excretion of PGE2 and 6-keto PGF1α in the Sh+NS, Sh+LS, Ox+NS, and Ox+LS groups before and during COX-2 inhibition with NS398. Urinary excretion of PGE2 was significantly greater in Ox+LS group than in Sh+NS group. Ovariectomy tended to increase urinary excretion of PGE2 in rats fed either the normal- or low-salt diet. The low-salt diet did not affect urinary excretion of PGE2 in either sham-operated or ovariectomized rats. Urinary excretion of 6-keto PGF1α, however, was elevated by ovariectomy or the low-salt diet, and it was further increased by ovariectomy and the low-salt diet in combination. COX-2 inhibition significantly decreased urinary excretion of PGE2 and 6-keto PGF1α in all four groups.

Renal clearance study

Table 2 shows BP, Cinulin, and UNaV in the Sh+NS, Sh+LS, Ox+NS, and Ox+LS groups before and during COX-2 inhibition with NS398. The UNaV values in the Sh+LS and Ox+LS groups were significantly lower than in the Sh+NS and Ox+NS groups, but no significant differences in the basal values of BP or Cinulin were observed among the four groups. NS398 did not affect BP, Cinulin, or UNaV significantly in any of the four groups, although it significantly decreased urinary excretion of prostaglandins. As shown in Figure 3, however, COX-2 inhibition with NS398 significantly decreased CPAH in the Sh+LS, Ox+NS, and Ox+LS groups, but did not change it in the Sh+NS group, even though the basal CPAH level in the four groups was similar. Before, and during treatment with the 1 ng/gbw and 10 ng/gbw doses of NS398, the CPAH values averaged 6.0 ± 0.4, 5.7 ± 0.5, and 5.7 ± 0.5 mL/min/gkw, respectively, in the Sh+NS group, 6.1 ± 0.4, 5.5 ± 0.5, and 4.8 ± 0.5 mL/min/gkw, respectively, in the Sh+LS group, 5.9 ± 0.5, 5.0 ± 0.3, and 4.5 ± 0.3 mL/min/gkw, respectively, in the Ox+NS group, and 6.1 ± 0.4, 4.2 ± 0.3, and 3.6 ± 0.2 mL/min/gkw, respectively, in the Ox+LS group. The magnitude of the

Table 2. Blood pressure, inulin clearance, and urinary sodium excretion before and during NS398 treatment in the Sh+NS, Sh+LS, Ox+NS, and Ox+LS groups

NS398 *ng/g of body weight*	0	1	10
Blood pressure *mm Hg*			
Sh+NS ($N = 6$)	92 ± 7	89 ± 7	89 ± 8
Sh+LS ($N = 6$)	92 ± 5	89 ± 5	88 ± 6
Ox+NS ($N = 6$)	95 ± 5	93 ± 4	91 ± 5
Ox+LS ($N = 6$)	93 ± 4	90 ± 4	87 ± 6
Cinulin *mL/min/g of kidney weight*			
Sh+NS ($N = 6$)	1.52 ± 0.06	1.43 ± 0.11	1.42 ± 0.15
Sh+LS ($N = 6$)	1.48 ± 0.13	1.42 ± 0.09	1.42 ± 0.11
Ox+NS ($N = 6$)	1.48 ± 0.06	1.42 ± 0.10	1.43 ± 0.13
Ox+LS ($N = 6$)	1.50 ± 0.09	1.42 ± 0.10	1.40 ± 0.12
UNaV *mEq/30 min*			
Sh+NS ($N = 6$)	0.145 ± 0.006	0.110 ± 0.018	0.118 ± 0.007
Sh+LS ($N = 6$)	0.057 ± 0.011^{a}	0.055 ± 0.014^{a}	0.045 ± 0.017^{a}
Ox+NS ($N = 6$)	0.151 ± 0.030	0.128 ± 0.048	0.129 ± 0.065
Ox+LS ($N = 6$)	0.037 ± 0.005^{a}	0.047 ± 0.011^{a}	0.052 ± 0.011^{a}

Abbreviations are: Cinulin, inulin clearance; UNaV, urinary sodium excretion. Data are mean ± SEM.

[a] $P < 0.05$ for LS vs. NS in the sham-operated or ovariectomized rats.

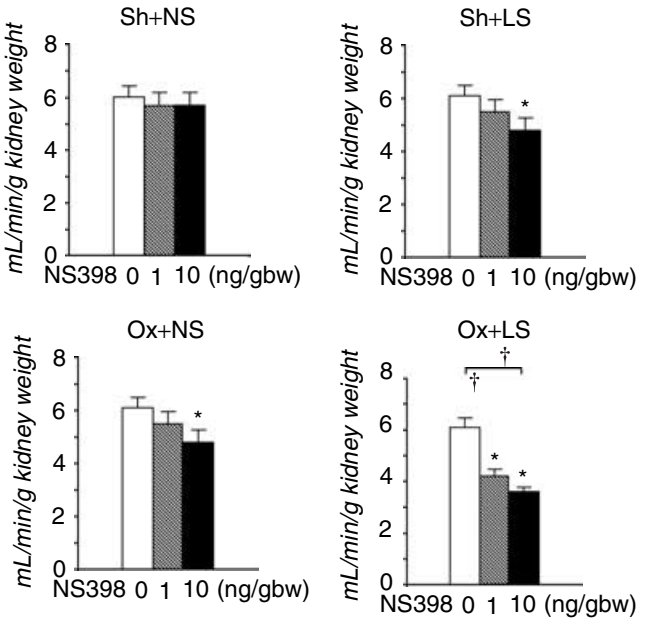

Fig. 3. Effect of COX-2 inhibition with NS398 on *p*-aminohippurate clearance (CPAH) in sham-operated rats fed a normal-salt diet (Sh+NS, $N = 6$), sham-operated rats fed a low-salt diet (Sh+LS, $N = 6$), ovariectomized rats fed a normal-salt diet (Ox+NS, $N = 6$), and ovariectomized rats fed a low-salt diet (Ox+LS, $N = 6$). $^{*}P < 0.05$ vs. 0 ng/g body weight (gbw). $\dagger P < 0.05$ vs. the other groups.

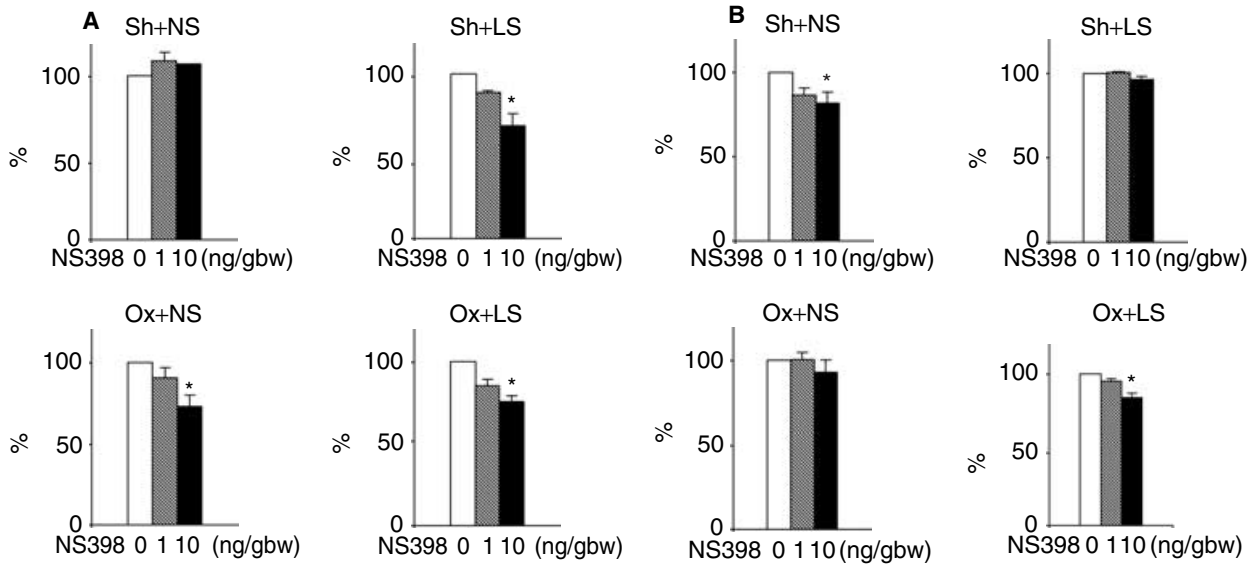

Fig. 4. Changes in cortical blood flow (CBF) and medullary blood flow (MBF) during COX-2 inhibition with NS398. (*A*) CBF during COX-2 inhibitor with NS398 in sham-operated rats fed a normal-salt diet (Sh+NS, $N = 6$), sham-operated rats fed a low-salt diet (Sh+LS, $N = 6$), ovariectomized rats fed a normal-salt diet (Ox+NS, $N = 6$), and ovariectomized rats fed a low-salt diet (Ox+LS, $N = 6$). (*B*) MBF during COX-2 inhibition with NS398 in Sh+NS ($N = 6$), Sh+LS ($N = 6$), Ox+NS ($N = 6$), and Ox+LS ($N = 6$). $^*P < 0.05$ vs. 0 ng/g of body weight (gbw).

decrease in CPAH was significantly greater in the Ox+LS group (2.5 ± 0.4 mL/min/gkw) than in the Sh+LS group (1.3 ± 0.4 mL/min/gkw, $P = 0.0313$) and Ox+NS group (1.4 ± 0.3 mL/min/gkw, $P = 0.0478$). In the Sh+LS group, indomethacin (10 ng/gbw) was administered at the conclusion of renal clearance study. CPAH was significantly decreased to 2.6 ± 0.5 mL/min/gkw. This result suggested that NS398 is selective for COX-2 up to 10 ng/gbw.

Figure 4A shows the changes in CBF during COX-2 inhibition with NS398. In the Sh+NS group, NS398 had no effect on CBF. In the Sh+LS and Ox+NS groups however, the 10 ng/gbw doses of NS398 significantly decreased CBF by 31.7 ± 7.8% and 27.2 ± 7.2%, respectively, and in the Ox+LS group, it significantly decreased CBF by 24.2 ± 3.3%. The NS398-induced decrease in CBF was similar in the Sh+LS, Os+NS, and Ox+LS groups. Figure 4B shows the changes in MBF during COX-2 inhibition with NS398. In the Sh+NS and Ox+LS groups, COX-2 inhibition with 10 ng/gbw NS398 caused a similar decrease in MBF by 18.3 ± 6.6% and 15.8 ± 3.3%, respectively, whereas NS398 did not affect MBF in the Sh+LS and Ox+NS groups. All of the reductions in CBF and MBF induced by the intra-arterial injection of NS398 had a gentle downward slope with no peak, and were sustained for a 30-minute experimental clearance period.

Table 3 shows the BP, Cinulin, and UNaV values in the Ox+NS+PL, Ox+LS+PL, Ox+NS+ER, and Ox+LS+ER groups before and during COX-2 inhibition with NS398. The UNaV values in the PL+LS and ER+LS groups were significantly lower than in the PL+NS and ER+NS groups, but no significant differences between the basal BP or Cinulin values were observed among the four groups. NS398 did not significantly affect BP, Cinulin, or UNaV in any of the four groups. As shown in Figure 5, however, COX-2 inhibition with NS398 significantly decreased CPAH in the Ox+LS+PL, Ox+NS+PL, and Ox+LS+ER groups, but did not change CPAH in the Ox+NS+ER group. The CPAH value before and during treatment with the 1 ng/gbw and 10 ng/gbw doses of NS398 averaged 5.9 ± 0.3, 4.3 ± 0.3, and 3.7 ± 0.2 mL/min/gkw, respectively, in the Ox+LS+PL group, 5.8 ± 0.4, 5.1 ± 0.2, and 4.6 ± 0.2 mL/min/gkw, respectively, in

Table 3. Blood pressure, inulin clearance, and urinary sodium excretion before and during NS398 treatment

NS398 *ng/g of body weight*	0	1	10
Blood pressure *mm Hg*			
Ox+NS+PL ($N = 9$)	94 ± 3	93 ± 3	95 ± 4
Ox+LS+PL ($N = 9$)	95 ± 2	92 ± 3	90 ± 5
Ox+NS+ER ($N = 9$)	95 ± 3	93 ± 3	93 ± 4
Ox+LS+ER ($N = 9$)	92 ± 4	90 ± 4	88 ± 4
Cinulin *mL/min/g of kidney weight*			
Ox+NS+PL ($N = 9$)	1.46 ± 0.04	1.39 ± 0.06	1.31 ± 0.08
Ox+LS+PL ($N = 9$)	1.40 ± 0.08	1.41 ± 0.10	1.33 ± 0.13
Ox+NS+ER ($N = 9$)	1.49 ± 0.03	1.46 ± 0.06	1.41 ± 0.08
Ox+LS+ER ($N = 9$)	1.44 ± 0.09	1.39 ± 0.07	1.34 ± 0.08
UNaV *mEq/30 min*			
Ox+NS+PL ($N = 9$)	0.194 ± 0.028	0.196 ± 0.037	0.202 ± 0.046
Ox+LS+PL ($N = 9$)	0.040 ± 0.007[a]	0.051 ± 0.009[a]	0.054 ± 0.010[a]
Ox+NS+ER ($N = 9$)	0.173 ± 0.008	0.170 ± 0.019	0.154 ± 0.012
Ox+LS+ER ($N = 9$)	0.051 ± 0.008[a]	0.052 ± 0.010[a]	0.047 ± 0.012[a]

Data are mean ± SEM.
[a] $P < 0.05$ for LS vs. NS in the placebo- or 17β-estradiol–treated ovariectomized rats.

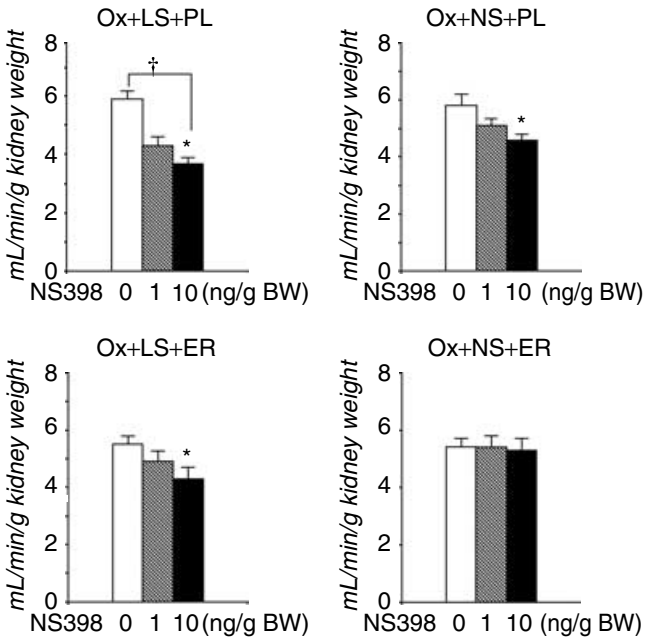

Fig. 5. Effect of COX-2 inhibition with NS398 on *p* -aminohippurate clearance (CPAH) in ovariectomized rats. Placebo-treated rats fed a normal-salt diet (Ox+NS+PL, $N = 9$), placebo- treated rats fed a low-salt diet (Ox+LS+PL, $N = 9$), 17β-estradiol–treated rats fed a normal-salt diet (Ox+NS+ER, $N = 9$), and 17β-estradiol–treated rats fed a low-salt diet (Ox+LS+ER, $N = 9$). $^*P < 0.05$ vs. 0 ng/g body weight (gbw). $\dagger P < 0.05$ vs. the other groups.

the Ox+NS+PL group, 5.5 ± 0.3, 4.9 ± 0.4, and 4.3 ± 0.4 mL/min/gkw, respectively, in the Ox+LS+ER group, and 5.4 ± 0.3, 5.4 ± 0.4, and 5.3 ± 0.4 mL/min/gkw, respectively, in the Ox+NS+ER group. The magnitude of the decrease in CPAH was similar in the Ox+LS+ER group (1.2 ± 0.2 mL/min/gkw) and Ox+NS+PL group (1.2 ± 0.2 mL/min/gkw), but it was significantly greater in the Ox+LS+PL group (2.1 ± 0.2 mL/min/gkw) than in the Ox+LS+ER and Ox+NS+PL groups. NS398 did not alter CPAH in the Ox+NS+ER group.

Figure 6A shows the changes in CBF during COX-2 inhibition with NS398. In the Ox+LS+PL, Ox+LS+ER, and Ox+NS+PL groups, 10 ng/gbw NS398 similarly decreased CBF by 26.9 ± 3.2%, 22.9 ± 2.3%, and 23.8 ± 4.9%, respectively. In the Ox+NS+ER group, however, NS398 had no effect on CBF. Figure 6B shows the changes in MBF during COX-2 inhibition with NS398. In the Ox+LS+PL group, COX-2 inhibition with 10 ng/gbw NS398 significantly decreased MBF by 20.0 ± 3.0%, whereas NS398 did not alter MBF in the Ox+LS+ER, Ox+NS+PL, or Ox+NS+ER groups.

DISCUSSION

The expression of COX-2 was enhanced in the renal cortical TAL cells of ovariectomized rats compared with that in sham-operated rats. Because this enhancement was observed when rats were fed either a low- or normal-salt diet, the ovariectomy appears to have enhanced renal cortical COX-2 expression independently of salt-dependent regulatory mechanisms. In the present study, ovariectomy increased urinary PGE_2 excretion in rats fed a low-salt diet and increased the urinary excretion of 6-keto-$PGF_{1\alpha}$, a breakdown product of unstable PGI_2, in rats fed either a low- or normal-salt diet. The inhibition of COX-2 using NS398 significantly decreased the urinary excretion of 6-keto-$PGF_{1\alpha}$ and PGE_2. The reduction in the urinary excretion of 6-keto-$PGF_{1\alpha}$ was greater in the Ox+LS group than in the Sh+LS or Ox+NS groups, which in turn had a greater reduction than the Sh+NS group. These results suggest that the enhancement of COX-2 expression induced by ovariectomy and low-salt diet stimulates the renal production of PGI_2, a well-known vasodilator in the human kidney, and of PGE_2, which dilates the afferent arterioles via EP_2 receptors, and buffers the renal vasoconstrictor responses that are mediated by the EP_1 and EP_3 receptors [19]. In addition, NS398 significantly decreased the RBF in the Sh+LS, Ox+NS, and Ox+LS groups, in which renal cortical COX-2 expression was enhanced; the reductions in RBF in these three groups were associated with decreases in the urinary excretion of 6-keto-$PGF_{1\alpha}$ and PGE_2. Therefore, the increased expression of renal cortical COX-2 in response to salt restriction and ovariectomy can stimulate the production of vasodilator prostaglandins and contribute to the regulation of renal blood flow.

Under normal conditions, most of the RBF perfuses the renal cortex; thus, the NS398-induced changes in total RBF may be primarily due to changes in renal CBF. In contrast, renal MBF accounts for only 10% of the total RBF [20] when the CBF remains intact, and the reduction in MBF is a negligible part of the overall RBF. This was supported by evidence that that NS398 significantly decreased renal MBF in the Sh+NS group, but did not affect the total RBF. However, the reduction in the total RBF in the Ox+LS group was greater than those observed in the Ox+NS and Sh+LS groups, and the reduction in the CBF in these three groups was similar. Because NS398 significantly decreased the MBF in the Ox+LS group, the decrease in MBF might significantly affect the overall RBF in the Ox+LS group. Thus, once the CBF has been reduced, the contribution of MBF to the overall RBF can increase significantly.

COX-2 is constitutively expressed in renal medullary interstitial cells [21, 22]; since previous studies have demonstrated that salt loading enhances renal medullary COX-2 expression [23], salt restriction may stimulate renal cortical COX-2 expression and inhibit renal medullary COX-2 expression. The latter hypothesis was confirmed by the results of the present study, which showed that salt restriction inhibited renal medullary COX-2 expression in sham-operated rats. In the Sh+LS

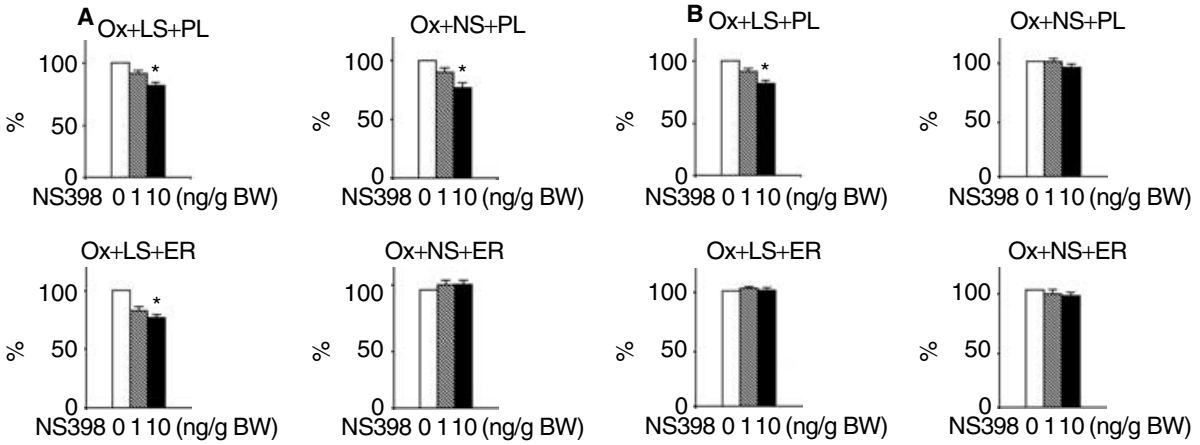

Fig. 6. Changes in cortical blood flow (CBF) and medullary blood flow (MBF) during COX-2 inhibition with NS398 in ovariectomized rats. (*A*) CBF during COX-2 inhibition with NS398 in placebo-treated rats fed a normal-salt diet (Ox+NS+PL, $N = 9$), placebo-treated rats fed a low-salt diet (Ox+LS+PL, $N = 9$), 17β-estradiol–treated rats fed a normal-salt diet (Ox+NS+ER, $N = 9$), and 17β-estradiol–treated rats fed a low-salt diet (Ox+LS+ER, $N = 9$). (*B*) MBF during COX-2 inhibition with NS398 in Ox+NS+PL ($N = 9$), Ox+LS+PL ($N = 9$), Ox+NS+ER ($N = 9$), and Ox+LS+ER ($N = 9$). $^*P < 0.05$ vs. 0 ng/g of body weight (gbw).

group, therefore, COX-2 inhibition did not affect the renal MBF. In addition, ovariectomy reduced renal medullary COX-2 expression in animals fed a normal-salt diet, and increases renal medullary COX-2 expression in animals fed a low-salt diet. Ovariectomy can reduce COX-2 expression in renal medullary interstitial cells, whereas ovariectomy combined with salt restriction can increase COX-2 expression of the TAL cells toward the medulla, thereby accounting for the enhancement of renal medullary COX-2 expression in the Ox+LS group. This hypothesis was supported by the immunohistochemistry findings in the present study, which showed that COX-2 expression of the TAL cells increased toward the renal medulla in ovariectomized rats. Therefore, COX-2 inhibition did not affect renal MBF in the Ox+NS group, but significantly decreased renal MBF in the Ox+LS group.

Previous studies have shown that an acute injection of COX-2 inhibitor does not affect the RBF in euvolemic animals fed a standard diet [24, 25]. These results are consistent with the results of the present study. Also, in previous studies, nimesulide, a selective COX-2 inhibitor, reduced UNaV in animals fed a normal-salt diet, but did not influence UNaV in animals fed a low-salt diet [26]; in the present study, NS398 did not affect UNaV in animals fed either a normal- or a low-salt diet. In these studies, however, the UNaV of the rats fed normal- and low-salt diets averaged 30 to 60 μEq/min and 3 μEq/min, respectively. In the present study, the UNaV of the rats fed a normal-salt diet averaged 5 μEq/min, and was similar to that of the rats fed a low-salt diet in the previous study [26]. Therefore, the normal-salt diet in the present study was closer to the low-salt diet rather than the normal-salt diet in the previous study [26], and NS398 did not influence the UNaV of the rats fed a normal-salt diet in the present study.

COX-2 inhibition elicited an elevation in the filtration fraction, with a decrease in RBF, but no change in GFR, in the Sh+LS, Ox+NS, and Ox+LS groups. These results suggest that the enhancement of COX-2 may influence not only the afferent but also the efferent arterioles. However, previous studies have demonstrated that macula densa COX-2 predominantly influences the afferent arterioles during enhanced tubuloglomerular feedback activity [7]; thus, ovariectomy may alter the efferent arteriolar response. Further studies are needed to clarify the effects of COX-2 on renal microcirculation in ovariectomized rats.

Several studies have demonstrated the cell-specific regulation of COX-2 expression by estrogen. Estrogen upregulates COX-2 expression in endothelial cells of human umbilical vein [27], the uterine stroma and epithelium of the guinea pig [28], the endometrium of the rat [29], and the endometrium [30, 31] and myometrium of ovine [30], but down-regulates it in the chondrocytes [32] and epithelial cells [33] of bovines. In the present study, estrogen replacement decreased the enhanced COX-2 expression and improved the COX-2–dependent regulation of RBF in the kidneys of ovariectomized rats. Because this decrease was observed when rats were fed either a low- or normal-salt diet, estrogen replacement appears to inhibit renal cortical COX-2 expression independently of salt diet.

Although the mechanism of renal COX-2 regulation by estrogen has not been clearly defined, several possible mechanisms have been proposed. A recent study

revealed that the COX-2 promoter gene contains an estrogen-binding site [13], suggesting that estrogen may directly affect expression of the COX-2. Previous studies demonstrated that estrogen up-regulates the expression of endothelial nitric oxide synthesis (eNOS) and neuronal nitric oxide synthesis (nNOS), but down-regulates expression of inducible nitric oxide synthesis (iNOS) [34, 35]. Since nNOS-derived NO stimulates COX-2 activity in the kidneys [7], estrogen may influence renal COX-2 expression through stimulation of the NO systems. Moreover, a recent study showed that estrogen up-regulates renal Ang II type 2 receptors [36], and since Ang II type 1 receptors inhibit renal COX-2 expression [37], the Ang II type 2 receptors enhanced by estrogen may antagonize the inhibitory effect of Ang II type 1 receptors on renal COX-2 expression. A previous work has shown that estradiol stimulates adrenal activity [38–40], and since adrenalectomy enhances renal COX-2 expression, and the enhancement is reversed by replacement therapy with either corticosterone or deoxycorticosterone [41], estrogen may also enhance renal COX-2 expression by stimulating glucocorticoid receptor-mediated function. Thus, NO, Ang II, or glucocorticoid hormones may significantly contribute to the mechanism of up-regulation of renal COX-2 by ovariectomy, but further study is required to clarify the mechanisms.

CONCLUSION

Ovariectomy enhances the expression of COX-2 adjacent to the macula densa and along the TAL segment through a mechanism that is independent of salt intake. This enhancement was reversed by estrogen replacement therapy. Therefore, estrogen-dependent COX-2 expression plays an important role in the regulation of renal blood flow in female rats.

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