2011

Inducible Nitric Oxide Synthase Provides Protection Against Injury-Induced Thrombosis in Female Mice

Rita K. Upmacis

Hao Shen

Lea Esther S. Benguigui

Brian D. Lamon

Ruba S. Deeb

See next page for additional authors

Authors

Rita K. Upmacis, Hao Shen, Lea Esther S. Benguigui, Brian D. Lamon, Ruba S. Deeb, Katherine A. Hajjar, and David P. Hajjar

Inducible Nitric Oxide Synthase Provides Protection Against Injury-Induced Thrombosis in Female Mice

Rita K. Upmacis[1]*, Hao Shen[1], Lea Esther S. Benguigui[1], Brian D. Lamon[1], Ruba S. Deeb[1],
Katherine A. Hajjar[2] and David P. Hajjar[1]

[1]Center of Vascular Biology, Department of Pathology and Laboratory Medicine and [2]Department of Cell and Developmental Biology, Weill Cornell Medical College, Cornell University, 1300 York Avenue, New York, NY 10065.

* Author contact details: Rita, K. Upmacis, phone (212) 746-6469, fax (212) 746-8789, e-mail rupmacis@pace.edu.

Running Title: Beneficial Role of iNOS in Thrombosis
Keywords: Ferric chloride, Eicosanoids, Estrogen

Abstract

Nitric oxide (NO) is an important vasoactive molecule produced by three NO synthase (NOS) enzymes: neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS). While eNOS contributes to blood vessel dilation that is generally thought to protect against the development of hypertension, iNOS has been primarily implicated as a disease-promoting isoform leading to protein-bound 3-nitrotyrosine formation in aortic lesions and select organs during atherogenesis. Despite this, iNOS may also play a physiological role, *via* the modulation of cyclooxygenase and thromboregulatory eicosanoid production. Herein, we examined the role of iNOS in a murine model of thrombosis. Blood flow was measured in carotid arteries of male and female wild-type (WT) and iNOS-deficient mice following ferric chloride-induced thrombosis. Female WT mice were less susceptible to thrombotic occlusion than male counterparts, but this protection was lost upon iNOS deletion. In contrast, male mice (with and without iNOS deletion) were equally susceptible to thrombosis. The protective effect that iNOS affords female WT mice was not associated with a change in the balance of thromboxane A_2 (TxA_2) and antithrombotic prostacyclin (PGI_2). Our findings, however, suggest that iNOS generates a protective source of NO in female WT mice that attenuates the effects of vascular injury. Thus, although iNOS is likely detrimental during atherogenesis, physiological iNOS levels may play a protective role in preventing thrombotic occlusion, a phenomenon that may be enhanced in female mice.

Introduction

Thrombotic events, such as those associated with deep vein thrombosis, ischemic stroke and acute coronary syndrome, affect 20 million people worldwide each year[1]. To date, certain risk factors and acquired or inherited states have been uncovered that lead to an altered hemostatic equilibrium or vascular injury[2]. Interestingly, men and women are not equally susceptible to vascular injury. For instance, the risk of stroke in women under age 55 is lower than in men[3]. While the protective effect in females may be ascribed to reproductive hormones, the use of estrogen in preventing or treating vascular injury is controversial. Thus, a knowledge gap persists concerning the mechanisms underlying the gender effect on thrombotic events[4, 5].

The study of thrombosis has been greatly enhanced by the availability of *in vivo* models coupled with modern intravital techniques[6]. An experimental model of arterial damage can be induced by chemical injury using ferric chloride ($FeCl_3$) as first demonstrated by Kurz et al, who noted that a loss of blood flow during the thrombotic event preceded a drop in temperature[7]. The morphology of the resultant thrombi from $FeCl_3$-induced thrombus formation is similar to that found in human acute coronary syndromes, revealing a disrupted endothelial surface with thrombi consisting of platelets, red blood cells, and leukocytes packed in a fibrin mesh[7, 8]. Thus, the $FeCl_3$-induced vascular injury model represents a valid, reproducible method that can be used to increase our understanding of the complex interplay of components in thrombus formation[9].

Nitric oxide (NO), a signaling molecule associated with various physiological functions, is an important regulator of vasomotor tone and platelet aggregation. NO is produced by a family of NO synthases (NOS), primarily associated with endothelial cells (eNOS), neurons (nNOS) and inflammation (iNOS). Both eNOS and nNOS are constitutively expressed, whereas iNOS expression is induced in macrophages and smooth muscle cells by proinflammatory cytokines during inflammation and atherosclerosis[10, 11]. Unlike nNOS and eNOS, iNOS produces micromolar amounts of NO continuously and mediates cytotoxicity against invading pathogens[12, 13]. The eNOS and nNOS isoforms are considered anti-atherogenic[14-16], while iNOS has been implicated in the etiology of disease[17, 18], causing increased protein tyrosine nitration and tetrahydrobiopterin oxidation in organs during atherosclerosis[19, 20]. Paradoxically, nitrogen oxide (NO_x) species, such as peroxynitrite, may confer protection and play an important role in preconditioning against ischemic injury[21]. Despite potentially detrimental effects in pathophysiology, iNOS displays important physiological functions in some cell types. For instance, iNOS is present in renal medullary cells[22], preosteoclasts[23] and in the brainstem (rostral ventrolateral medulla) where iNOS-derived NO inhibits the sympathetic nervous system *via* a $GABA_A$-receptor dependent pathway[24, 25]. Notably, disruption of the iNOS gene leads to an increased heart rate and a concomitant rise in the low frequency power of heart rate variability that is consistent with increased cardiac sympathetic activity[26].

An indirect manner in which iNOS may modulate vasoregulatory pathways, involves its interaction with cyclooxygenase (COX), which gives rise to prostanoids that

are vasodilatory (*i.e.* prostacyclin, PGI_2) and vasoconstrictive (*i.e.* thromboxane A_2, TxA_2). NO or NO_x species may activate or inhibit arachidonic acid metabolism[27]. More recently, iNOS has been implicated in suppressing COX-2 induction[28]. The effect of iNOS deletion on COX-dependent eicosanoid formation has previously been investigated and was found to alter urinary prostaglandin E_2 (PGE_2) and isoprostane formation, as well as serum thromboxane B_2 (TxB_2)[29]. Another indirect effect of iNOS involves its interaction with the estrogen pathway. The estrogens are steroid hormones that function primarily in female reproductive physiology, but have important effects in both males and females[30]. Atheroprotective effects in females were initially ascribed to estrogen[31], but were contradicted by later clinical studies[32-34]. The increased risk for coronary heart disease revealed by these later clinical studies may only apply to elderly women[35]. Despite conflicting clinical results, estrogen has positive effects in upregulating prostacyclin and conferring atheroprotection in female mice[36] and in preventing the vascular injury response[37, 38]. Furthermore, estrogen has been shown to attenuate vasoconstriction by a pathway involving iNOS[39].

While the iNOS, eicosanoid and estrogen pathways independently contribute to vasoregulatory function, it is not clear whether the mutual interplay of these systems significantly impacts thrombotic events. To this end, we tested the hypothesis that a deficiency in iNOS-derived NO elicits an increase in TxA_2 production with a resultant elevated propensity for thrombotic occlusion. Results from our study reveal evidence for a role of iNOS in providing protection against $FeCl_3$-induced thrombosis in female mice.

Materials and Methods

Reagents: $FeCl_3.6H_2O$ and citrate-dextrose solution (ACD; containing 22.0 g/L citric acid, trisodium salt, dihydrate, 7.3 g/L citric acid, anhydrous; and 24.5 g/L D-(+)-glucose) were obtained from Sigma. Avertin (2,2,2 tribromoethanol; 97%), from Sigma-Aldrich, was prepared in 2-butanol (125 mg in 125 μl) by vortexing for several min in a foil-wrapped tube, followed by further dilution with sterile saline (6 ml). The working solution was refrigerated in a dark sealed bottle and used within a two-week period.

Mice: The animal protocol used in these studies was reviewed and approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee. Male WT and $iNOS^{-/-}$ mice and female WT and $iNOS^{-/-}$ mice were used in this study (6 months in age, fed a regular chow diet). The animals were obtained from Jackson Laboratories and bred to generate subsequent generations in our animal facility. The body weights of male WT (n = 15) and $iNOS^{-/-}$ (n = 15) mice were not significantly different (31.8 ± 0.5 g and 31.0 ± 0.4 g, respectively). The body weights of female WT (n = 9) and $iNOS^{-/-}$ (n = 10) mice were also not significantly different (23.0 ± 0.4 g and 23.9 ± 0.5 g, respectively). Immediately prior to $FeCl_3$-induced carotid artery injury, urine was collected from individual mice. Urine was collected over 24 h, with and without the preservative butylated hydroxytoluene (BHT, 0.005 %) by housing individual mice in metabolic cages. Following $FeCl_3$-induced injury, blood was drawn for isolation of plasma. Hearts were isolated and weighed from euthanized mice. Interestingly, female $iNOS^{-/-}$ mouse hearts were significantly heavier than hearts isolated from female WT mice (0.116 ± 0.003 g and 0.104 ± 0.002 g, respectively; n = 9 - 10). Heart/body

weight ratios were calculated to be 0.00454 ± 0.00011 for female WT mice, n = 9; 0.00484 ± 0.00007 for female iNOS$^{-/-}$ mice, n = 10). In contrast, male WT and iNOS$^{-/-}$ heart weights normalized to body weight were not significantly different (heart/body weight = 0.00513 ± 0.00014 for male WT mice, n = 15; 0.00497 ± 0.00014 for male iNOS$^{-/-}$ mice, n = 15).

$FeCl_3$ Solutions*:* $FeCl_3$ solutions were prepared by dissolving 10% $FeCl_3.6H_2O$ in deionized water. In our experience, we obtained reproducible and consistent results with the $FeCl_3$ model of carotid artery injury using aged $FeCl_3$ solutions (> 5 days old). **Figure 1a** illustrates changes in the UV/Vis spectrum of $FeCl_3$ (a 1:1000 dilution of a 10% $FeCl_3.6H_2O$ solution), using a Perkin Elmer Lambda 20 spectrophotometer, at 15 min, 60 min and 5 days after dissolving in water. At 5 months, the spectrum was similar to that obtained at 5 days (data not shown), indicating that no further significant changes occur in this region of the UV/Vis spectrum. The changes in the UV/Vis spectrum are likely associated with hydrolysis, giving rise to hydroxide and ultimately iron oxide complexes[40].

$FeCl_3$ model of carotid artery injury: Male and female WT and iNOS$^{-/-}$ mice were anesthetized with avertin (150 – 200 mg/kg). The left carotid artery was exposed by surgical incision and careful removal of surrounding tissue. Two silk threads, approximately 3 - 4 mm apart, were used to lift the artery onto a miniature ultrasound flow probe (0.5VB; Transonic Systems, Ithaca, New York, USA). A baseline measurement of blood flow was obtained for 3 - 5 min using a Transonic T106

flowmeter and WinDaq data acquisition software (DataQ Instruments, Akron, OH). The ultrasound probe was removed and, using small forceps, a piece of filter paper (Whatman No. 1; 2 x 1 mm) soaked in aged 10% $FeCl_3.6H_2O$ was placed on the carotid artery. After 1 min, the filter paper was removed and the ultrasound probe was once again positioned underneath the artery, and blood flow monitored for 30 min. **Figure 1b** shows representative traces of a case in which no occlusion (top trace) and total occlusion (bottom trace), *i.e.* loss of blood flow, were observed. Results at different time-points are reported as a mean percent change from the initial baseline value (established immediately prior to $FeCl_3$ injury) and also as the mean total time in which occlusion was observed over the 30 min period that blood flow was monitored.

Clot lysis studies: Plasma clotting was examined using a protocol described previously[41]. Briefly, whole blood was collected by cardiac puncture using a syringe coated with citrate-dextrose solution (50 μl). The whole blood/citrate mixture (9:1 ratio) was expelled into a tube (after removal of the syringe needle to prevent hemolysis), placed on ice (10 min) and centrifuged (17,000 x g at 4°C; 10 min). Plasma aliquots were stored at -80°C. For the clotting and clot lysis study, plasma (15 μl) was mixed with buffer (60 μl; 0.04 M HEPES, pH 7; 0.15M NaCl, 0.01% Tween 80) and deionized water (30 μl) and allowed to equilibrate in a 96-well plate (RT, 5min). To measure the clotting time, a mixture (15 μl total) containing human thrombin (4 μl; 75 NIH U/ml; American Diagnostic), $CaCl_2$ (2 μl; 1M) and deionized water (9 μl) was added to the plasma sample in the 96-well plate. For the measurement of half clot lysis times, a mixture (15 μl total) containing human thrombin (4 μl; 75 NIH U/ml), $CaCl_2$ (2 μl; 1M),

single-chain recombinant tissue plasminogen activator (t-PA; 1.05 μl; 2.86 μM; Genentech Inc.) and deionized water (7.95 μl) was added to plasmin. The absorbance at 405 nm was recorded in 30 s intervals for 30 min at RT using an iMark microplate absorbance reader (Bio-Rad). The half clot lysis time was defined as the time at which the absorbance is halfway between the plateau reached after clotting and the baseline value attained upon complete lysis.

Eicosanoid, nitrite/nitrate and 17β-estradiol analyses: Enzyme immunoassay kits to measure 6-keto-$PGF_{1\alpha}$, 2,3-dinor-6-keto-$PGF_{1\alpha}$, PGE_2 (GE Healthcare), thromboxane (TxB_2; Assay Designs), 8-isoprostane (Oxford Biomedical Research), 11-dehydro-TxB_2, PGE-M, creatinine, nitrite/nitrate and 17β-estradiol (Cayman Chemical) were used according to manufacturer's instructions.

Western blotting of homogenized hearts from WT and iNOS$^{-/-}$ mice: Hearts from WT and iNOS$^{-/-}$ mice (n=3) fed a regular chow diet for 24 weeks were homogenized and proteins in the tissue supernatants were separated via SDS/PAGE (7.5% and 10% polyacrylamide gels) and transferred onto 0.2 μm Immun-Blot PVDF membranes (BioRad, 162-0177). Membranes were then probed and visualized for iNOS at ~130 kDa (polyclonal rabbit iNOS antibody; Santa Cruz, sc-560), COX1 at ~70 kDa (mouse monoclonal antibody; Cayman, 160110), COX2 at ~72 kDa (polyclonal rabbit antibody; Cayman 160126), and for GAPDH at ~37 kDa (polyclonal goat antibody; Santa Cruz, sc-20357) in a manner described previously[19, 42]. Of several iNOS antibodies tested in this application, the best antibody (with regard to reproducible

iNOS detection) also resulted in the appearance of non-specific bands at higher and lower molecular weights than iNOS, an effect that has also been reported previously[43, 44]. These non-specific interactions did not interfere with our assessment and visualization of iNOS protein levels.

Lipopolysaccharide/interferon-γ treatment of ex-vivo tissue: In some experiments, hearts and aortae were exposed *ex-vivo* to lipopolysaccharide (LPS; from *Escherichia coli* 026:B6, 10 μg/mL; Sigma) and recombinant mouse interferon-γ (IFNγ: 100 U/ml; Calbiochem) for 24 h to induce iNOS. Hearts were removed surgically as described above. Aortae were removed surgically from the cardiac origination to the iliac bifurcation. Using a dissecting microscope (SMZ-1B; Nikon), the aortae were cleared of fat, connective tissues, adventitia, and denudation of the endothelial layer was performed by gently scraping with a scalpel. The tissue was dissected into smaller pieces and incubated in Dulbecco's modified Eagle's medium (DMEM) containing LPS/IFNγ. Following incubation for 24 h at 37°C in 5% CO_2 in air, the tissue was homogenized (50 mM Tris buffer, pH 8; 10 mM ethylenediaminetetraacetic acid; 1% Tween 20; 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate and 10 μl/ml protease inhibitor cocktail set III, Calbiochem) and subjected to Western blotting, while the supernatants were analyzed for nitrite/nitrate levels.

Statistical analysis: Results are presented as means SE with significant differences determined by *t*-test or 2-way ANOVA, with $P < 0.05$ defined as statistically significant. Image J (version 1.36b: NIH) was used to quantify Western blot band

densities.

Results

Female WT mice are protected from $FeCl_3$-induced thrombosis compared to female iNOS$^{-/-}$ mice. The response to $FeCl_3$-induced injury in the carotid artery was measured in four groups of mice: female WT and iNOS$^{-/-}$ mice as well as male WT and iNOS$^{-/-}$ mice. **Figure 1c-d** shows the average blood flow measured for female mice and male mice at 0, 5, 10, 20 and 30 min immediately following $FeCl_3$-induced injury. The data indicate that following $FeCl_3$ application, a minimal change in blood flow was observed in female WT mice, but blood flow was impaired in iNOS$^{-/-}$ female mice, indicating significant occlusion. For male mice, there was no difference in the response of WT and iNOS$^{-/-}$ mice, with both groups of mice showing a similar response to that of female iNOS$^{-/-}$ mice. The average amount of thrombotic occlusion for each of the four groups, as measured by the total time that blood flow was blocked (*i.e.* blood flow = 0 ml/min) during 30 min immediately following $FeCl_3$-induced injury, is shown in **Figure 1e**. Female WT mice experienced significantly less thrombotic occlusion compared to female iNOS$^{-/-}$ mice or their male counterparts. Taken together, these data suggest that female WT mice are less susceptible to $FeCl_3$-induced injury than male WT mice, and that this protection is iNOS-dependent. Thus, while iNOS appears to play a protective role in females against vascular injury, the same does not appear to be true for male WT mice.

Plasma clotting and clot lysis times are similar in female and male WT and iNOS$^{-/-}$ mice: It was critical to determine whether fluid-phase components in the coagulation and fibrinolytic pathways are different in female versus male WT mice and

their iNOS$^{-/-}$ counterparts. For this reason, plasma clotting and clot lysis times were measured. **Figure 2** shows that there were no appreciable differences between the groups in the mean plasma clotting times (WT female: 5.2 ± 0.3 min; WT male: 4.2 ± 0.5 min; iNOS$^{-/-}$ female: 4.6 ± 0.6 min; iNOS$^{-/-}$ male: 4.4 ± 0.4 min) or in the times to half-maximal clot lysis (WT female: 7.7 ± 0.7 min; WT male: 7.0 ± 0.6 min; iNOS$^{-/-}$ female: 7.8 ± 0.5 min; iNOS$^{-/-}$ male: 7.3 ± 0.2 min). These data indicate that thrombin-dependent clotting and t-PA-dependent fibrinolytic activities were identical in the different groups of mice.

Female mice produce elevated levels of eicosanoids. Based on the ability of NO and related NO_x species to alter eicosanoid profiles[27], we examined urinary levels of: (a) 6-keto-$PGF_{1\alpha}$, (b) 2,3-dinor 6-keto-$PGF_{1\alpha}$, (c) thromboxane (TxB_2), (d) 11-dehydro-TxB_2, (e) PGE_2 and (f) 8-isoprostane collected from female and male WT and iNOS$^{-/-}$ mice, as shown in **Figure 3**. While no differences were observed between female WT and iNOS$^{-/-}$ mice or male WT and iNOS$^{-/-}$ mice, significant differences were observed between the female and male groups. Notably, female WT and iNOS$^{-/-}$ mice produced significantly higher levels of eicosanoids than their male counterparts. There was no specific gender effect on creatinine production by male or female mice, indicating that the measured disparity in eicosanoid levels between male and female groups is not a result of normalizing the eicosanoid measurements to creatinine levels. Interestingly, despite the fact that urinary eicosanoid production was significantly higher in females compared to males, the ratio of urinary pro-thrombotic TxB_2 and anti-thrombotic 6-keto $PGF_{1\alpha}$ was similar in all groups. Thus, urinary TxB_2:6-keto $PGF_{1\alpha}$

ratios were calculated to be 3.1 ± 0.3 in female WT, 2.6 ± 0.4 in male WT, 2.7 ± 0.3 in female iNOS$^{-/-}$ and 3.0 ± 0.5 in male iNOS$^{-/-}$ mice (n = 10 -12 mice). These data indicate that while gender may affect the extent of eicosanoid production, gender does not influence the balance of pro-thrombotic versus anti-thrombotic eicosanoids.

To investigate whether COX-1, prostacyclin synthase (PGI_2S) and iNOS protein levels were altered in the cardiovascular system, we examined these protein levels in the hearts of female and male WT and iNOS$^{-/-}$ mice (**Figure 4**). Interestingly, iNOS protein was detected in hearts from male and female WT mice but absent, as expected, upon iNOS deletion. Notably, COX-1 and PGI_2S protein levels were elevated in female hearts from WT and iNOS$^{-/-}$ mice compared to males. COX-2 and eNOS protein levels, however, were not significantly altered in the different groups (data not shown). These results demonstrate that iNOS is constitutively expressed under basal conditions in hearts from both male and female WT mice. Furthermore, female mice exhibit increased COX-1 and PGI_2S expression in the heart, as well as increased urinary eicosanoid levels irrespective of iNOS deletion, compared to male mice.

Urinary nitrite/nitrate and estrogen levels are higher in female WT and iNOS$^{-/-}$ mice, respectively, compared to male cohorts. Urinary nitrite and nitrate levels were measured in WT and iNOS$^{-/-}$ male and female mice in order to determine the extent that iNOS contributes to NO-related metabolites in the urine of WT mice. Analysis of urinary levels of nitrite (NO_2^-) and nitrate (NO_3^-) in male and female WT and iNOS$^{-/-}$ mice revealed a significant increase in female WT mice compared to the other

groups (**Figure 5a**). While an increase in urinary levels of nitrite and nitrate might be expected compared to the iNOS$^{-/-}$ cohorts, it is notable that female WT mice produce significantly higher levels than male WT mice. These findings are consistent with an elevation in iNOS-derived NO_x in female mice. Urinary 17β-estradiol levels were also measured in order to determine whether iNOS exerts an influence on this component in either male of female WT mice. Overall, urinary 17β-estradiol levels were significantly higher in female than male mice but, interestingly, significantly increased levels were observed in female iNOS$^{-/-}$ mice compared to female WT cohorts (**Figure 5b)**.

Female mice exhibit increased sensitivity to LPS/IFNγ-induced iNOS and nitrite/nitrate production. Hearts and endothelium-denuded aortae from female and male WT mice were exposed *ex-vivo* to LPS/IFNγ for 24 h to determine whether the capacity to upregulate iNOS protein and NO production was modified in these animals. **Figure 6a** shows that LPS/IFNγ induced higher iNOS protein amounts in female versus male WT hearts. Consistent with this observation, **Figure 6b** demonstrates that LPS/IFNγ induced NO production was higher by endothelium-denuded aortae isolated from female versus male WT mice.

Discussion

Results reported herein demonstrate that female WT mice demonstrate increased protection from vascular injury versus their male counterparts and that iNOS gene deletion results in a loss of this protection in females (**Figure 1)**. Thrombin-dependent clotting times and clot lysis profiles were similar in female and male WT and

iNOS$^{-/-}$ mice, indicating that the protein components in plasma involved in these pathways are not altered (**Figure 2**). Expression of iNOS traditionally plays a role in inflammation and the immune response, and in this regard, iNOS is activated by interferon gamma (IFNγ) and other cytokines[45]. Our results, however, suggest a beneficial role for "constitutive iNOS" or basal levels of iNOS that occur under non-pathological conditions in female mice. NO released from iNOS under these conditions may favorably function to provide vasodilation and prevent platelet aggregation. These are roles traditionally ascribed to eNOS-dependent NO[46].

The function of iNOS has been linked with the COX pathway and for this reason, we measured urinary levels of eicosanoids in female and male WT and iNOS$^{-/-}$ mice. Our present work demonstrates that iNOS deletion did not exert an effect on urinary levels of 6-keto-PGF$_{1\alpha}$, 2,3-dinor 6-keto-PGF$_{1\alpha}$, TxB$_2$, 11-dehydro-TxB$_2$, PGE$_2$, or PGE-M (**Figure 3**). Furthermore, urinary isoprostane levels that are generated by COX-independent mechanisms relying on free-radical driven peroxidation of arachidonic acid were not different between WT and iNOS$^{-/-}$ mice. The data indicate that iNOS-derived NO does not play a role in levels of eicosanoids that are, for the most part, biosynthesized in the kidneys. In contrast to these results, Marnett et al. have previously reported that iNOS-deficient male mice excrete 78% less PGE$_2$ than WT mice[29], suggesting that iNOS-derived NO species (possibly ONOO$^-$) provide a stimulatory affect on COX activity[47, 48]. In this previous study, iNOS-deficient males also produced less urinary isoprostanes than WT mice[29], indicating that iNOS bears some responsibility in producing reactive species that contribute to oxidant stress. Our results

are different to those reported previously, which may be a reflection of the fact that the mice in our experiments were 6 months in age (on a C57BL/6J background) compared to 5-week old male mice (on mixed C57BL/6J and 129Sv/Ev backgrounds) in the earlier study[29]. While no differences were observed within the WT and iNOS$^{-/-}$ cohorts, our results, indicate that female mice produced higher levels of urinary eicosanoids. Indeed, a gender effect has also previously been observed with regard to 17β-estradiol upregulating COX-2 and augmenting PGI_2 production[36, 49]. Furthermore, women and smokers have elevated urinary isoprostane metabolites[50]. In our study, urinary eicosanoid measurements were normalized to creatinine levels, but it should be noted that there were no significant differences in creatinine production by male or female mice, indicating that the difference observed is not a result of the normalization routine. Although a gender effect was noted in eicosanoid production, the ratio of thromboxane to prostacylin remained similar across all groups. Overall, this indicates that the balance of vasodilatory and vasoconstrictive eicosanoids are similar and not influenced by gender.

Western blotting data of homogenized hearts from these animals demonstrate increased levels of COX-1 and PGI_2S enzymes in female compared to male mice, while COX-2 levels were not changed (**Figure 4**). While we observed a gender effect consistent with previous reports, our results indicate a role for constitutive COX-1, rather than COX-2. Interestingly, Duckles et al. have previously noted that 17β-estradiol elevates COX-1 and PGI_2S levels[51, 52]. Furthermore, our study revealed that basal levels of iNOS are present in the hearts of WT mice, which suggests a beneficial

physiological role for a constitutive iNOS protein (**Figure 4**). Indeed, iNOS protein has previously been detected at low levels in rat carotid arteries[53], and we have previously documented that iNOS is present in aortae and hearts of ApoE$^{-/-}$ mice[19, 54]. Furthermore, basal levels of iNOS play a role in the control of heart-rate[26]. Upon, LPS/IFNγ treatment, female WT hearts demonstrated a greater propensity for further iNOS induction over basal levels than male WT hearts (**Figure 6a**).

NO is now well established as a vasodilator and inhibitor of platelet activation[46]. To determine whether increased levels of NO might confer protection against vascular injury, urinary nitrite and nitrate levels were measured in all four groups of mice (**Figure 5a**). A comparison of nitrite and nitrate levels in WT and iNOS$^{-/-}$ mice reveals the contribution of iNOS to these NO_x species. In fact, the difference between male WT and iNOS$^{-/-}$ was not significant, indicating that urinary nitrite and nitrate levels likely originate from other NOS-dependent pathways. This result also correlates with the fact that $FeCl_3$-induced injury occurs to a similar extent in both male WT and iNOS$^{-/-}$ mice (**Figure 1d**). In contrast, significantly greater levels of urinary nitrite and nitrate were found in female WT mice compared to cohorts, highlighting a greater role for iNOS in female WT mice (**Figure 5a**). Furthermore, nitrite/nitrate levels were signficiantly higher in LPS/IFN-γ treated endothelium-denuded aortae isolated from female versus male WT mice, as shown in **Figure 6b**, indicating a greater capacity for iNOS-dependent NO production.

Urinary 17β-estradiol levels were significantly increased in female mice

compared to male mice. However, an unexpected finding was that urinary 17β-estradiol levels were significantly higher in female iNOS$^{-/-}$ mice compared to female WT cohorts (**Figure 5b)**, which suggests a feedback mechanism, whereby the loss of NO by genetic deletion of iNOS drives 17β-estradiol production in a possible attempt to restore loss of iNOS-derived NO. With respect to the cardiovascular system, ERα and ERβ are expressed in vascular endothelial and smooth muscle cells, as well as in myocardial cells, but the differences in the levels of expression of these two receptors based on gender and in vascular disease states is not yet well characterized[55]. Estrogen-regulated genes include PGI_2S, eNOS and iNOS, as well as genes associated with the coagulation and fibrinolysis pathways, such as tissue factor, fibrinogen, and others[39, 55]. Activation of ERα in endothelial cells leads to eNOS-dependent NO generation, possibly *via* a tyrosine kinase or mitogen-activated protein kinase pathway[56]. However, estrogen also targets increased expression of iNOS *via* the ERβ receptor pathway[39] and it has been previously proposed that low basal synthesis of NO by iNOS may well be beneficial to the circulation and for preventing damage during vascular injury or ischemia in the myocardium[57]. Estrogen has been shown to protect against vascular injury in mice bearing genetically disrupted estrogen receptors[37, 39, 55].

In summary, our results indicate that female WT mice are protected from $FeCl_3$-induced thombosis in the carotid artery compared to male WT mice and that this protection is afforded by iNOS-derived NO.

Acknowledgements

These studies were supported, in part, by grants from the NIH (PO1 HL046403 to D.P.H. and K.A.H., and HL042493 and HL090895 to K.A.H.), an award from the Alice Bohmfalk Charitable Trust (to R.K.U.), The Hartwell Foundation award to B.D.L., a Grant-in-Aid from the American Heart Association to R.S.D. (AHA655783T), and the Julia and Seymour Gross Foundation to D.P.H. We are grateful to Dr. Kaili He for assistance with the plasma clotting studies and to Drs. Andrew Nicholson and Domenick Falcone for helpful discussions.

References

1. White GC: Current status of thrombolytics-the need for better, especially safer, agents, Thrombosis Research 2008, 122:S1-S2
2. Feinbloom D, Bauer KA: Assessment of hemostatic risk factors in predicting arterial thrombotic events, Arteriosclerosis Thrombosis and Vascular Biology 2005, 25:2043-2053
3. Calonge N, Petitti DB, DeWitt TG, Gordis L, Gregory KD, Harris R, Isham G, LeFevre ML, Loveland-Cherry C, Marion LN, Moyer VA, Ockene JK, Sawaya GF, Siu AL, Teutsch SM, Yawn BP, Force UPST: Aspirin for the Prevention of Cardiovascular Disease: US Preventive Services Task Force Recommendation Statement, Annals of Internal Medicine 2009, 150:396-W370
4. Miller VM, Duckles SP: Vascular actions of estrogens: Functional implications, Pharmacological Reviews 2008, 60:210-241
5. Evangelista O, McLaughlin MA: Review of Cardiovascular Risk Factors in Women, Gender Medicine 2009, 6:17-36
6. Sachs UJH, Nieswandt B: In vivo thrombus formation in murine models, Circulation Research 2007, 100:979-991
7. Kurz KD, Main BW, Sandusky GE: Rat Model of Arterial Thrombosis Induced by Ferric-Chloride, Thrombosis Research 1990, 60:269-280
8. Farrehi PM, Ozaki CK, Carmeliet P, Fay WP: Regulation of arterial thrombolysis by plasminogen activator inhibitor-1 in mice, Circulation 1998, 97:1002-1008
9. Wang XK, Xu L: An optimized murine model of ferric chloride-induced arterial thrombosis for thrombosis research, Thrombosis Research 2005, 115:95-100
10. MacMicking J, Xie QW, Nathan C: Nitric oxide and macrophage function, Annu. Rev. Immunol. 1997, 15:230-350

11. Buttery LD, Springall DR, Chester AH, Evans TJ, Standfield EN, Parums DV, Yacoub MH, Polak JM: Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite, Lab Invest 1996, 75:77-85

12. Hewett SJ, Muir JK, Lobner D, Symons A, Choi DW: Potentiation of oxygen-glucose deprivation-induced neuronal death after induction of iNOS, Stroke 1996, 27:1586-1591

13. Vodovotz Y, Kwon NS, Pospischil M, Manning J, Paik J, Nathan C: Inactivation of Nitric-Oxide Synthase after Prolonged Incubation of Mouse Macrophages with Ifn-Gamma and Bacterial Lipopolysaccharide, Journal of Immunology 1994, 152:4110-4118

14. Knowles JW, Reddick RL, Jennette JC, Shesely EG, Smithies O, Maeda N: Enhanced atherosclerosis and kidney dysfunction in $eNOS^{-/-}Apoe^{-/-}$ mice are ameliorated by enalapril treatment, J. Clin. Invest. 2000, 105:451-458

15. Hoetten S, Kai H, Widder J, Riehl G, Marx A, Huang PL, Ertl G, Kuhlencordt PJ: Atheroprotective effects of neuronal nitric oxide synthase (nNOS) in Apolipoprotein E knockout (apoE ko) mice, Circulation 2003, 108:40-40

16. Lowenstein CJ: Beneficial effects of neuronal nitric oxide synthase in atherosclerosis, Arterioscler Thromb and Vasc Biol 2006, 26:1417-1418

17. Detmers PA, Hernandez M, Mudgett J, Hassing H, Burton C, Mundt S, Chun S, Fletcher D, Card DJ, Lisnock J, Weikel R, Bergstrom JD, Shevell DE, Hermanowski-Vosatka A, Sparrow CP, Chao YS, Rader DJ, Wright SD, Pure E: Deficiency in inducible nitric oxide synthase results in reduced atherosclerosis in apolipoprotein E-deficient mice, J Immunol 2000, 165:3430-3435

18. Kuhlencordt PJ, Chen J, Han F, Astern J, Huang PL: Genetic deficiency of inducible nitric oxide synthase reduces atherosclerosis and lowers plasma lipid peroxides in apolipoprotein E-knockout mice, Circulation 2001, 103:3099-3104

19. Upmacis RK, Crabtree MJ, Deeb RS, Shen H, Lane PB, Benguigui LES, Maeda N, Hajjar DP, Gross SS: Profound biopterin oxidation and protein tyrosine nitration in tissues of ApoE-null mice on an atherogenic diet: contribution of inducible nitric oxide synthase, American Journal of Physiology-Heart and Circulatory Physiology 2007, 293:H2878-H2887

20. Upmacis RK: Atherosclerosis: A link between lipid intake protein tyrosine nitration, Lipid Insights 2008, 2:75-88

21. Kawano T, Kunz A, Abe T, Girouard H, Anrather J, Zhou P, Iadecola C: iNOS-derived NO and nox2-derived superoxide confer tolerance to excitotoxic brain injury through peroxynitrite, Journal of Cerebral Blood Flow and Metabolism 2007, 27:1453-1462

22. Morrissey JJ, Mccracken R, Kaneto H, Vehaskari M, Montani D, Klahr S: Location of an Inducible Nitric-Oxide Synthase Messenger-Rna in the Normal Kidney, Kidney International 1994, 45:998-1005

23. Brandi ML, Hukkanen M, Umeda T, Moradibidhendi N, Bianchi S, Gross SS, Polak JM: Bidirectional Regulation of Osteoclast Function by Nitric-Oxide Synthase Isoforms, Proceedings of the National Academy of Sciences of the United States of America 1995, 92:2954-2958

24. Chan SHH, Wang LL, Wang SH, Chan JYH: Differential cardiovascular responses to blockade of nNOS or iNOS in rostral ventrolateral medulla of the rat, British Journal of Pharmacology 2001, 133:606-614

25. Chan SHH, Wang LL, Chan JYH: Differential engagements of glutamate and GABA receptors in cardiovascular actions of endogenous nNOS or iNOS at rostral ventrolateral medulla of rats, British Journal of Pharmacology 2003, 138:584-593

26. Mani AR, Ollosson R, Mani Y, Ippolito S, Moore KP: Heart rate dynamics in iNOS knockout mice, Life Sciences 2006, 79:1593-1599

27. Upmacis RK, Deeb RS, Hajjar DP: Oxidative alterations of cyclooxygenase during atherogenesis, Prostaglandins & Other Lipid Mediators 2006, 80:1-14

28. Lamon BD, Upmacis RK, Deeb RS, Koyuncu H, Hajjar DP: Inducible nitric oxide synthase gene deletion exaggerates MAPK-mediated cyclooxygenase-2 induction by inflammatory stimuli, Am. J. Physiol. Heart Circ. Physiol. 2010, in press.:

29. Marnett LJ, Wright TL, Crews BC, Tannenbaum SR, Morrow JD: Regulation of prostaglandin biosynthesis by nitric oxide is revealed by targeted deletion of inducible nitric-oxide synthase, J. Biol. Chem. 2000, 275:13427-13430

30. Kuiper GGJM, Enmark E, PeltoHuikko M, Nilsson S, Gustafsson JA: Cloning of a novel estrogen receptor expressed in rat prostate and ovary, Proceedings of the National Academy of Sciences of the United States of America 1996, 93:5925-5930

31. Grady D, Rubin SM, Petitti DB, Fox CS, Black D, Ettinger B, Ernster VL, Cummings SR: Hormone-Therapy to Prevent Disease and Prolong Life in Postmenopausal Women, Annals of Internal Medicine 1992, 117:1016-1037

32. Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E, Hers: Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women, Jama-Journal of the American Medical Association 1998, 280:605-613

33. Grady D, Herrington D, Bittner V, Blumenthal R, Davidson M, Hlatky M, Hsia J, Hulley S, Herd A, Khan S, Newby LK, Waters D, Vittinghoff E, Wenger N, Grp HR: Cardiovascular disease outcomes during 6.8 years of hormone therapy - Heart and Estrogen/progestin Replacement Study follow-up (HERS II), Jama-Journal of the American Medical Association 2002, 288:49-57

34. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SAA, Howard BV, Johnson KC, Kotchen M, Ockene J: Risks and benefits of estrogen plus progestin in healthy postmenopausal women - Principal results

from the Women's Health Initiative randomized controlled trial, Jama-Journal of the American Medical Association 2002, 288:321-333

35. Rossouw JE, Prentice RL, Manson JE, Wu LL, Barad D, Barnabei VM, Ko M, LaCroix AZ, Margolis KL, Stefanick ML: Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause, Jama-Journal of the American Medical Association 2007, 297:1465-1477

36. Egan KM, Lawson JA, Fries S, Koller B, Rader DJ, Smyth EM, FitzGerald GA: COX-2-derived prostacyclin confers atheroprotection on female mice, Science 2004, 306:1954-1957

37. Iafrati MD, Karas RH, Aronovitz M, Kim S, Sullivan TR, Lubahn DB, ODonnell TF, Korach KS, Mendelsohn ME: Estrogen inhibits the vascular injury response in estrogen receptor alpha-deficient mice, Nature Medicine 1997, 3:545-548

38. Pare G, Krust A, Karas RH, Dupont S, Aronovitz M, Chambon P, Mendelsohn ME: Estrogen receptor-alpha mediates the protective effects of estrogen against vascular injury, Circulation Research 2002, 90:1087-1092

39. Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, Hodgin J, Shaul PW, Thoren P, Smithies O, Gustafsson JA, Mendelsohn ME: Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta, Science 2002, 295:505-508

40. Hellman H, Laitinen RS, Kaila L, Jalonen J, Hietapelto V, Jokela J, Sarpola A, Ramo J: Identification of hydrolysis products of FeCl3 center dot 6H(2)O by ESI-MS, Journal of Mass Spectrometry 2006, 41:1421-1429

41. Nagashima M, Yin ZF, Zhao L, White K, Zhu YH, Lasky N, Halks-Miller M, Broze GJ, Fay WP, Morser J: Thrombin-activatable fibrinolysis inhibitor (TAFI) deficiency is compatible with murine life, Journal of Clinical Investigation 2002, 109:101-110

42. Deeb RS, Shen H, Gamss C, Gavrilova T, Summers BD, Kraemer R, Hao G, Gross SS, Laine M, Maeda N, Hajjar DP, Upmacis RK: Inducible nitric oxide synthase mediates

prostaglandin h2 synthase nitration and suppresses eicosanoid production, Am J Pathol 2006, 168:349-362

43. Wu F, Wilson JX, Tyml K: Ascorbate inhibits iNOS expression and preserves vasoconstrictor responsiveness in skeletal muscle of septic mice, American Journal of Physiology-Regulatory Integrative and Comparative Physiology 2003, 285:R50-R56

44. Scott JA, Weir ML, Wilson SM, Xuan JW, Chambers AF, McCormack DG: Osteopontin inhibits inducible nitric oxide synthase activity in rat vascular tissue, American Journal of Physiology-Heart and Circulatory Physiology 1998, 44:H2258-H2265

45. Kleinert H, Schwarz PM, Forstermann U: Regulation of the expression of inducible nitric oxide synthase, Biological Chemistry 2003, 384:1343-1364

46. Moncada S, Higgs A: Mechanisms of Disease - the L-Arginine Nitric-Oxide Pathway, New England Journal of Medicine 1993, 329:2002-2012

47. Landino LM, Crews BC, Timmons MD, Morrow JD, Marnett LJ: Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis, Proc Natl Acad Sci U S A 1996, 93:15069-15074

48. Upmacis RK, Deeb RS, Hajjar DP: Regulation of prostaglandin H2 synthase activity by nitrogen oxides, Biochemistry 1999, 38:12505-12513

49. Akarasereenont P, Techatraisak K, Thaworn A, Chotewuttakorn S: The induction of cyclooxygenase-2 by 17 beta-estradiol in endothelial cells is mediated through protein kinase C, Inflammation Research 2000, 49:460-465

50. Taylor AW, Bruno RS, Traber MG: Women and smokers have elevated urinary F-2-isoprostane metabolites: A novel extraction and LC-MS methodology, Lipids 2008, 43:925-936

51. Li XD, Geary GG, Gonzales RJ, Krause DN, Duckles SP: Effect of estrogen on cerebrovascular prostaglandins is amplified in mice with dysfunctional NOS, American Journal of Physiology-Heart and Circulatory Physiology 2004, 287:H588-H594

52. Ospina JA, Krause DN, Duckles SP: 17 beta-estradiol increases rat cerebrovascular prostacyclin synthesis by elevating cyclooxygenase-1 and prostacyclin synthase, Stroke 2002, 33:600-605

53. Zhang R, Bai YG, Lin LJ, Bao JX, Zhang YY, Tang H, Cheng JH, Jia GL, Ren XL, Ma J: Blockade of AT(1) receptor partially restores vasoreactivity, NOS expression, and superoxide levels in cerebral and carotid arteries of hindlimb unweighting rats, Journal of Applied Physiology 2009, 106:251-258

54. Palombo F, Shen H, Benguigui LES, Kazarian SG, Upmacis RK: Micro ATR-FTIR spectroscopic imaging of atherosclerosis: an investigation of the contribution of inducible nitric oxide synthase to lesion composition in ApoE-null mice, Analyst 2009, 134:1107-1118

55. Mendelsohn ME, Karas RH: The protective effects of estrogen on the cardiovascular system, New England Journal of Medicine 1999, 340:1801-1811

56. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn RE, Shaul PW: Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen, Journal of Clinical Investigation 1999, 103:401-406

57. Binko J, Majewski H: 17 beta-estradiol reduces vasoconstriction in endothelium-denuded rat aortas through inducible NOS, American Journal of Physiology-Heart and Circulatory Physiology 1998, 43:H853-H859

Figure Captions

Figure 1: Male and female response to $FeCl_3$-induced thrombosis in WT and iNOS$^{-/-}$ mice. (a) UV/Vis spectra monitoring changes in aqueous $FeCl_3$ over time. UV/Vis spectra of $FeCl_3$ (a 1:1000 dilution of a 10% $FeCl_3.6H_2O$ aqueous solution) recorded at 0 min, 15 min, 60 min and 5 days. (b) Representative blood flow traces following $FeCl_3$ application to the carotid artery (1 min) illustrating no occlusion (top trace) and occlusion ~7 min after $FeCl_3$-induced injury (bottom trace). # denotes an artifact introduced upon repositioning the probe. (c) Female WT (n = 10) and iNOS$^{-/-}$ mice (n = 10) and (d) male WT (n = 14) and iNOS$^{-/-}$ mice (n = 15) were subjected to $FeCl_3$-induced thrombosis and blood flow was measured for 30 min following injury. The data represent the average % blood flow measured for the different groups at 0, 5, 10, 20 and 30 min. Note that 100% blood flow represents the maximal blood flow measured during a 5 min period immediately before application of $FeCl_3$. *P < 0.05 compared to female WT mice in both (c) and (d). (e) Total time that carotid is occluded during 30 min immediately following $FeCl_3$-induced injury. The data represent the mean total time that blood flow was blocked during 30 min for the different groups immediately following $FeCl_3$ application. *P < 0.05 for female iNOS$^{-/-}$ mice compared to female WT mice. $^{+}$P < 0.05 for male WT mice compared to female WT mice. All mice were 6 months in age and were fed a regular chow diet.

Figure 2: Plasma clotting and clot lysis times for female and male WT and iNOS$^{-/-}$ mice. Plasma clotting (- t-PA) and clot lysis (+ t-PA) profiles were obtained for (a) female WT, (b) male WT, (c) female iNOS$^{-/-}$ and (d) male iNOS$^{-/-}$ mice as described in the Materials and Methods section. The traces represent an average of n = 3-5 mice for each group.

Figure 3: Urinary levels of eicosanoids in WT and iNOS$^{-/-}$ mice. Urinary levels of (a) 6-keto-$PGF_{1\alpha}$, (b) 2,3 dinor 6-keto-$PGF_{1\alpha}$, (c) TxB_2, (e) 11-dehydro-

TxB_2, (f) PGE_2 and (g) 8-isoprostane were measured in male and female WT and $iNOS^{-/-}$ mice (n = 8 - 12 for each group) and normalized to creatinine. *P < 0.0001 and ∝P < 0.006 between female and male groups. By 2-way ANOVA analysis, the gender effect was significant for all eicosanoids measured (p < 0.0001).

Figure 4: **COX-1 and iNOS protein expression in female and male WT and $iNOS^{-/-}$ mice.** (a) Homogenized hearts from female and male WT and $iNOS^{-/-}$ mice were analyzed by Western blotting for COX-1, PGI_2S, iNOS and GAPDH protein. (b) Western blot band densities were quantified and are represented as either a COX-1/actin or PGI_2S/actin ratio. (n = 3 for each group; *p < 0.05).

Figure 5: **Urinary nitrite/nitrate and 17β-Estradiol levels.** Urinary nitrite/nitrate (a) and 17β-estradiol levels (b) were measured for male and female WT and $iNOS^{-/-}$ mice (n = 9 - 10 for all other groups; *p < 0.05).

Figure 6: **Ex-vivo LPS/IFNγ treatment of tissue from female and male WT mice.** (a) hearts and (b) endothelium-denuded aortae from female and male WT mice were exposed *ex-vivo* to LPS (10 μg/mL) and IFNγ (100 U/mL) for 24 h to induce iNOS, as described in the Materials and Methods section. (a) Hearts were homogenized, subjected to Western blotting and probed for iNOS and actin, and (b) supernatants from endothelium-denuded aortae were analyzed for nitrite/nitrate levels. (n = 3 for all groups; *p < 0.05).

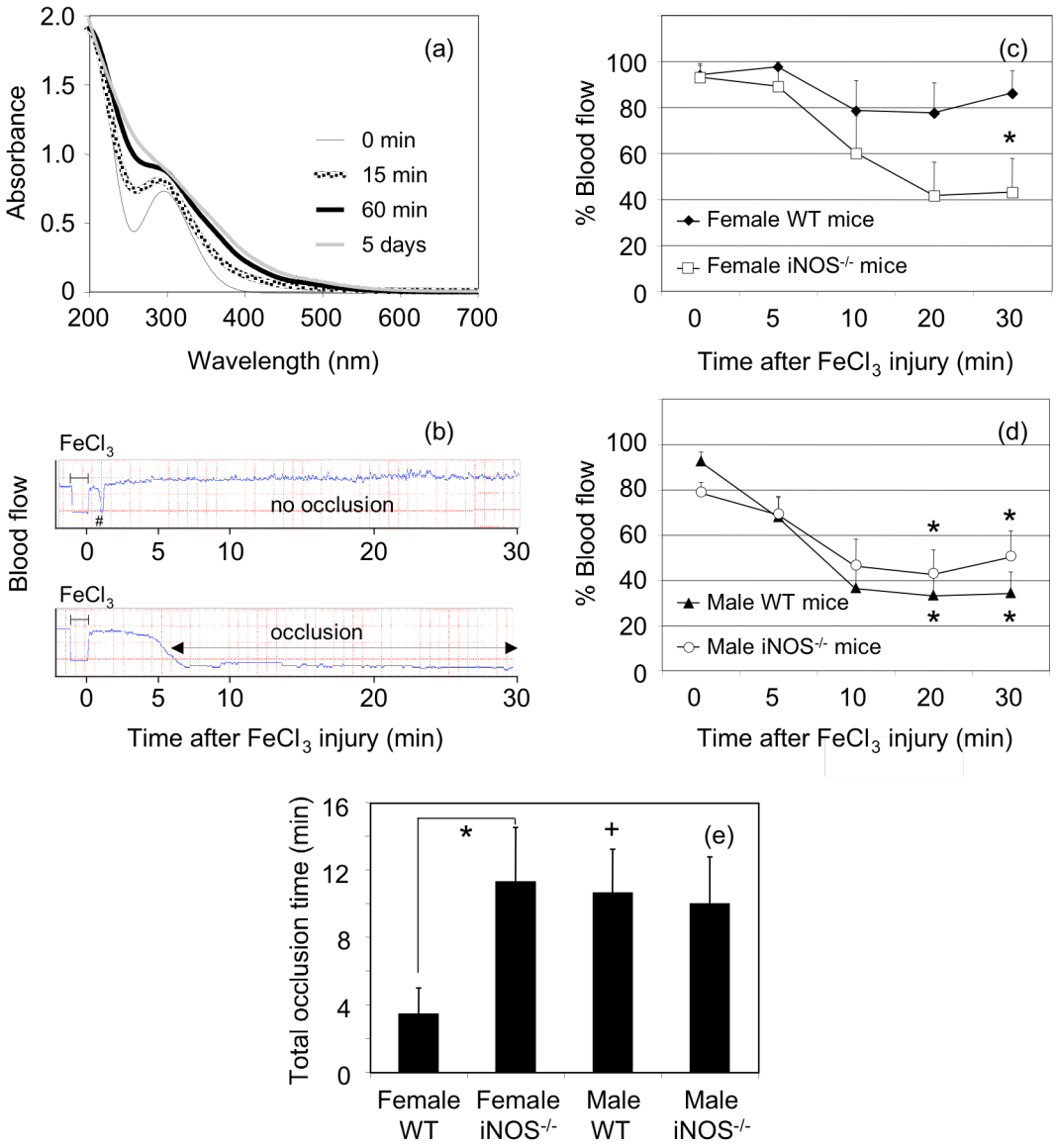

(a)
Absorbance
Wavelength (nm)
0 min
15 min
60 min
5 days
(b)
FeCl3
no occlusion
occlusion
Blood flow
Time after FeCl3 injury (min)
(c)
% Blood flow
Female WT mice
Female iNOS-/- mice
Time after FeCl3 injury (min)
(d)
% Blood flow
Male WT mice
Male iNOS-/- mice
Time after FeCl3 injury (min)
(e)
Total occlusion time (min)
Female WT
Female iNOS-/-
Male WT
Male iNOS-/-

Figure 1

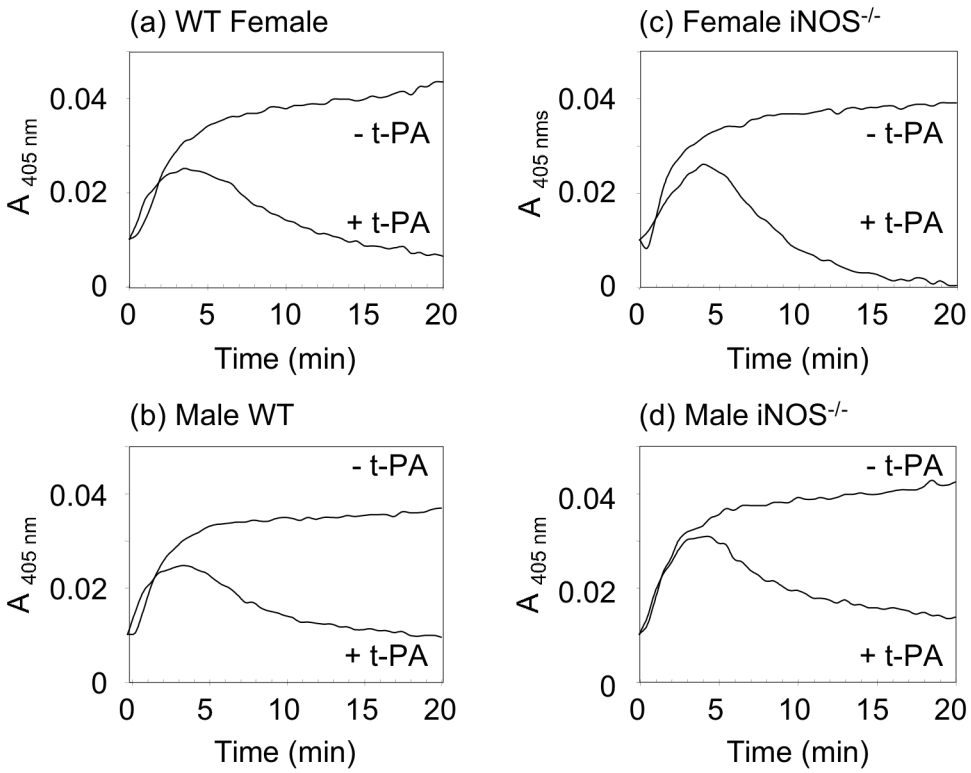

(a) WT Female
(c) Female iNOS-/-
(b) Male WT
(d) Male iNOS-/-
A 405 nm
A 405 nms
0.04
0.02
0
0 5 10 15 20
Time (min)
- t-PA
+ t-PA

Figure 2

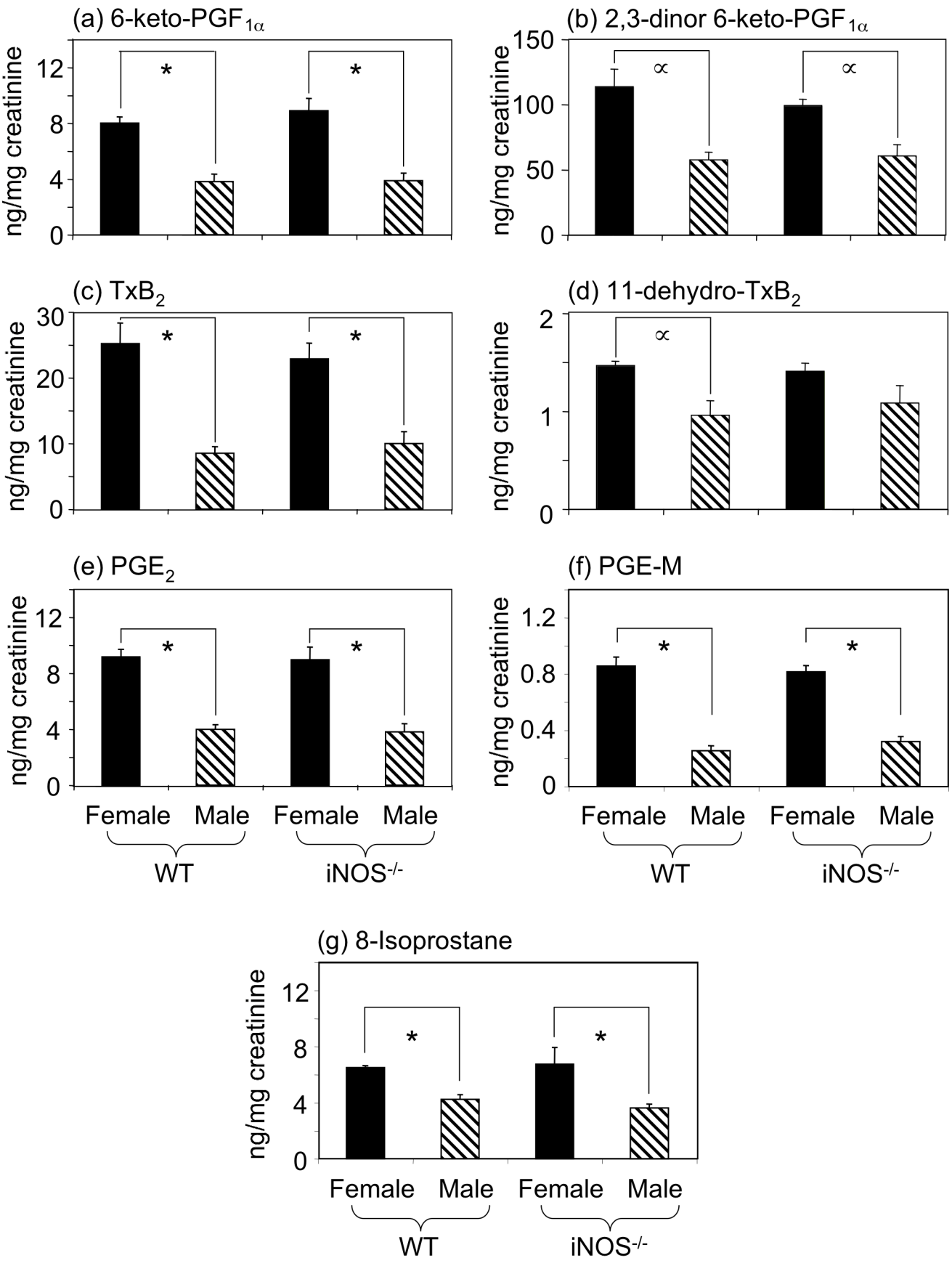
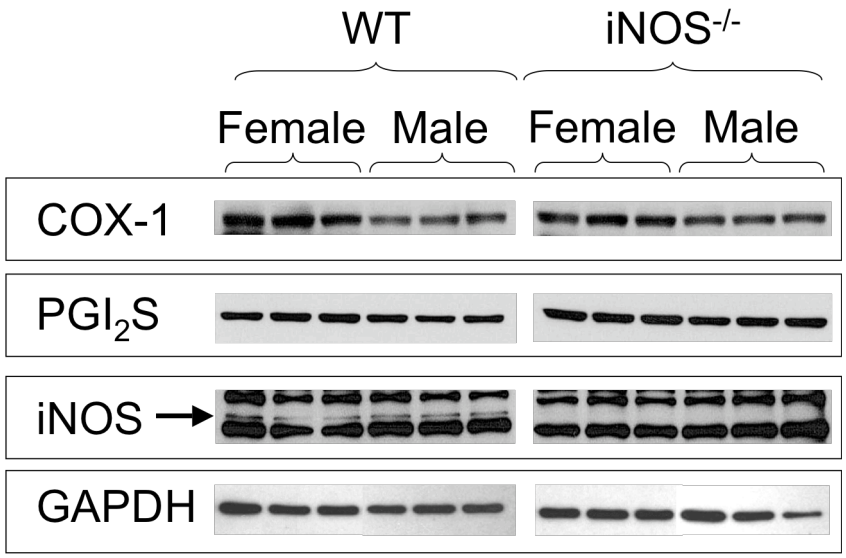

(a) 6-keto-PGF1α
ng/mg creatinine
0
4
8
12
*
*
(b) 2,3-dinor 6-keto-PGF1α
ng/mg creatinine
0
50
100
150
∝
∝
(c) TxB2
ng/mg creatinine
0
10
20
30
*
*
(d) 11-dehydro-TxB2
ng/mg creatinine
0
1
2
∝
(e) PGE2
ng/mg creatinine
0
4
8
12
*
*
(f) PGE-M
ng/mg creatinine
0
0.4
0.8
1.2
*
*
Female
Male
Female
Male
WT
iNOS-/-
(g) 8-Isoprostane
ng/mg creatinine
0
4
8
12
*
*
Female
Male
Female
Male
WT
iNOS-/-

Figure 3

(a)


WT
iNOS-/-
Female
Male
Female
Male
COX-1
PGI2S
iNOS
GAPDH

(b)

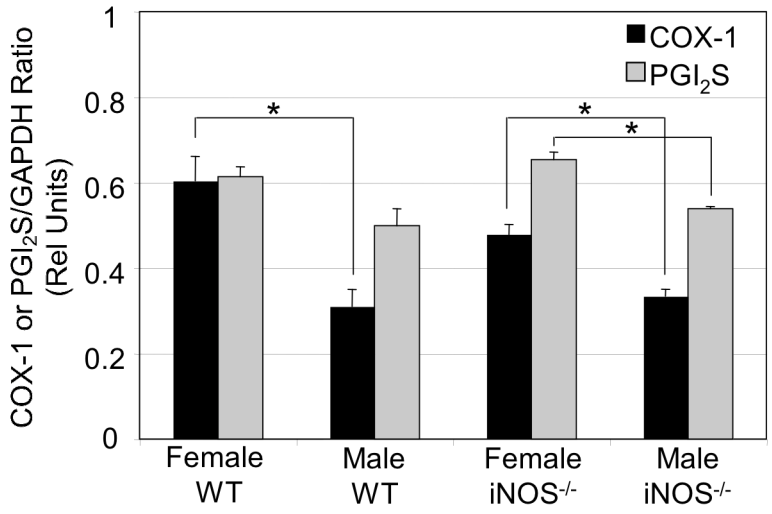

COX-1 or PGI2S/GAPDH Ratio (Rel Units)
1
0.8
0.6
0.4
0.2
0
COX-1
PGI2S
*
*
*
Female WT
Male WT
Female iNOS-/-
Male iNOS-/-

Figure 4

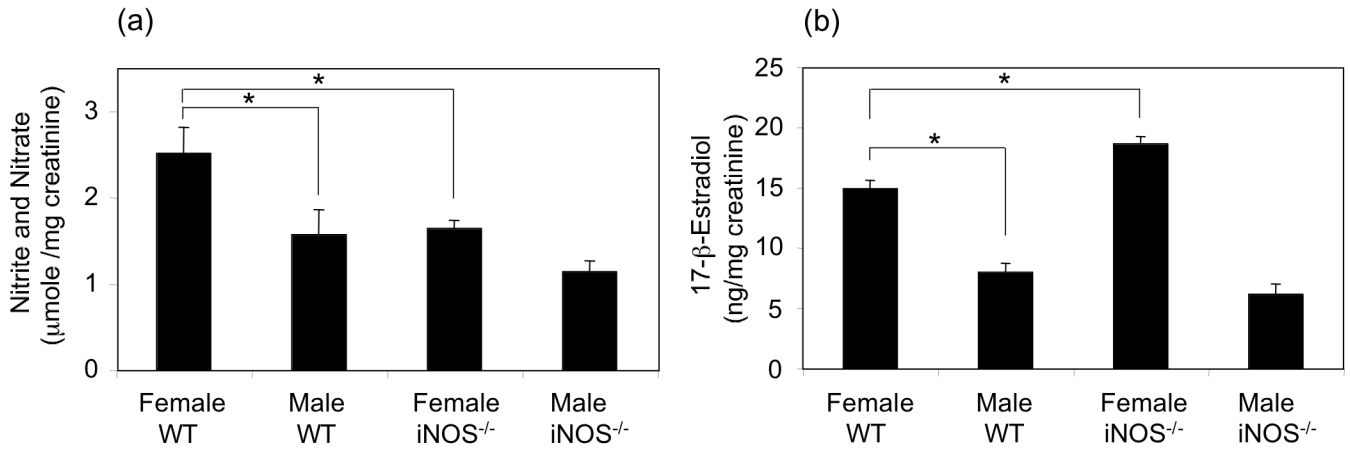

(a)
Nitrite and Nitrate
(μmole /mg creatinine)
0
1
2
3
*
*
Female WT
Male WT
Female iNOS-/-
Male iNOS-/-
(b)
17-β-Estradiol
(ng/mg creatinine)
0
5
10
15
20
25
*
*
Female WT
Male WT
Female iNOS-/-
Male iNOS-/-

Figure 5

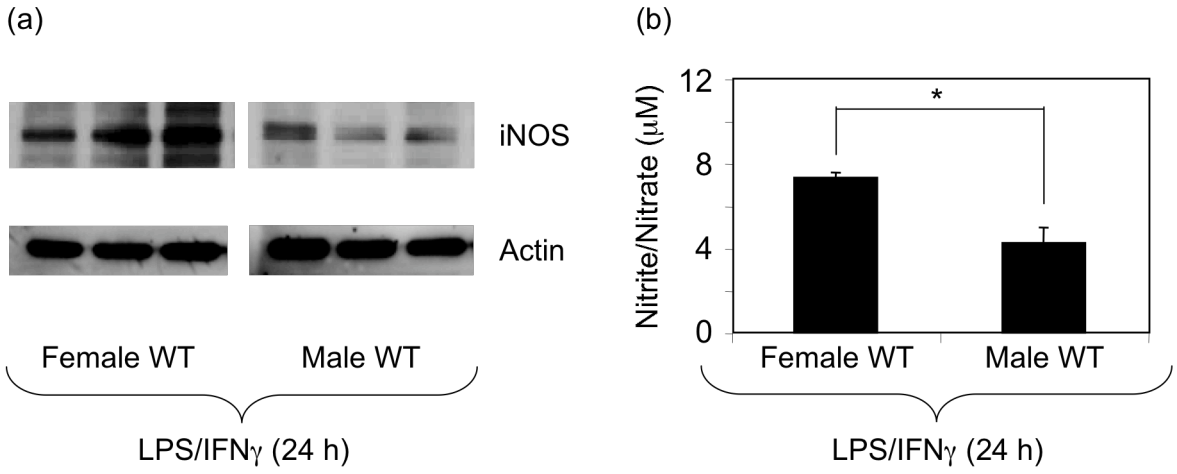

(a)
iNOS
Actin
Female WT
Male WT
LPS/IFNγ (24 h)
(b)
Nitrite/Nitrate (μM)
12
8
4
0
*
Female WT
Male WT
LPS/IFNγ (24 h)

Figure 6