Nitric oxide deficiency promotes vascular side-effects of cyclooxygenase inhibitors in vivo.

**Short title:** Nitric oxide prevents vascular NSAID side-effects.


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The cardiovascular safety of COX-2 selective and non-selective non-steroidal anti-inflammatory drugs (NSAIDs) has recently been called into question. The factors that predispose to adverse events by NSAIDs are unknown. Since patients with arthritis have decreased nitric oxide (NO) bioavailability, the in vivo effects of NSAIDs on murine vascular tone and platelet activity in the presence/absence of NO were examined. Here we show that acute hypertensive and pro-thrombotic activities of the COX-2 selective inhibitor celecoxib are revealed only after in vivo inhibition of NO generation. The non-selective NSAID indomethacin was hypertensive but anti-thrombotic when NO was absent. In vitro myography of aortic rings confirmed that vasoconstriction required inhibition of both NOS and COX-2, and was abolished by supplementation with exogenous NO. These data indicate that NO suppresses vascular side-effects of NSAIDs, suggesting that risk will be greatest in patients with impaired vascular function associated with decreased NO bioavailability.
Introduction

Recently, concerns were raised over elevated cardiovascular risks following administration of selective COX-2 inhibitors and non-selective NSAIDs \(^1\)-\(^9\). However, factors that interact with COX and modulate risk of adverse events are currently unknown. Prostacyclin (PGI) synthesis is elevated in patients with cardiovascular disease and arthritis\(^{10-13}\). Also, decreased large vessel NO bioactivity is observed \(^{11,14-18}\). Indeed, due to the lack of NO, it is possible PGI may play an even more important role in maintaining vascular homeostasis and preventing adverse events in these groups than in healthy subjects. This led us to hypothesize that the ability of NSAIDs to mediate undesirable vascular events would be revealed or magnified in the absence of NO. In support, previous studies have found multiple complex interactions between NO and COX, including studies showing that NO inhibition can alter PGI signaling, consistent with this hypothesis\(^{19-23}\). In this study, we examined acute effects of NSAID administration in healthy mice in vivo, with/without simultaneous NO blockade, specifically to examine whether NO influenced the ability of NSAIDs to mediate vascular side-effects. The results suggest that NO bioactivity may be a determinant of susceptibility to adverse events of NSAIDs in patients with inflammatory diseases.
Study Design

**Animal studies** All animal experiments were performed in accordance to the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. Disruption of the *Ptgs2* gene was originally carried out in AB2.1 (129) embryonic stem cells by homologous recombination as previously described.

**Isometric tension functional studies.** Male mice (10 - 12 wks old) were sacrificed by cervical dislocation. The thoracic aorta was dissected, cut into rings (2 - 3 mm) and suspended in an isometric tension myograph (DMT, Aarhuis, Denmark) containing Krebs buffer at 37 °C and gassed with 5 % CO₂/95 % O₂. Cumulative concentration-response curve to phenylephrine (1 nM – 1 uM) or acetylcholine (1 nM – 10 uM) were constructed, with/without 300 uM L-nitroarginine-methyl ester (L-NAME), 30 uM diethylenetriamineNONOate (DETA NONOate), 10 uM celecoxib, 10 uM indomethacin or 100 uM aspirin. In some experiments, endothelium was removed by gentle rubbing before myography. Responses were expressed as percentage baseline tension (vasoconstriction) or contracted tension (vasodilation). Responses from 3 - 4 rings of each animal were combined to produce an average.

**Hypertension.** Male 10-12 wk-old wild type C57BL/6 mice were administered L-NAME (100 mg.kg⁻¹.day⁻¹ in drinking water) with/without celecoxib (400 mg.kg⁻¹.day⁻¹ in chow) or indomethacin (6 mg/L in drinking water). Systolic blood pressure was monitored daily for 3 days pre-drug administration (training) and 6 days post-drug administration via tail cuff plethysmography (World Precision...
Instruments, UK) in un-anesthetized mice.

**Whole blood FACS analysis of platelet P-selectin expression.** Mice were sacrificed at day 3 post-drug administration, and whole blood collected as described\textsuperscript{26}. 5 ul of antibody (anti-P-selectin-FITC, Emfret Analytics (Heidelberg, Germany), anti-mouse $\alpha$IIb-FITC or rat IgG\textsubscript{1}-FITC, Santa Cruz Biotechnology (Santa Cruz, CA)) was added to 26 ul diluted blood and incubated 15 min at room temperature, before FACS analysis. Platelets were identified based on forward and side-scatter characteristics and $\alpha$IIb expression, then P-selectin expression determined on the gated $\alpha$IIb-positive platelet population\textsuperscript{26}.

**Immunohistochemistry of COX-2.** Aortic ring sections (10 um) were methanol fixed, permeabilized using 0.1 % (w/v) Triton X-100/PBS, blocked using 1 % (w/v) bovine serum albumin/PBS. COX-2 was visualized using goat anti-COX-2 (Santa Cruz, CA) and anti-goat IgG-Alexa 568. Negative controls utilized equivalent concentrations of isotype control IgG. Images were acquired using a 10x air lens, with excitation at 568 nM and emission 595/35 nM.

**GC/MS determination of TX and PGI metabolites in urine.** Mice were administered celecoxib or L-NAME (doses as above) with 24-hr urine collections on day 3. Metabolites were quantified using a precise and accurate gas chromatographic mass spectrometric/stable isotope dilution method\textsuperscript{27}. 
Results and Discussion.

**Celecoxib/indomethacin mediate vasoconstriction in vivo, when NO generation is inhibited.**

Since elevated blood pressure has been reported as a side-effect of NSAIDs, even as early as a month for some selective COX-2 inhibitors, we determined the effect of COX inhibitors with/without NO blockade *in vivo* in healthy mice. Oral celecoxib (400 mg kg$^{-1}$.day$^{-1}$) reduced urinary excretion of the COX-2-derived prostacyclin (PGI) metabolite 2,3-dinor-6-KetoPGF$_{1\alpha}$ by 71% by C57BL/6 mice, while having no effect on the COX-1-derived thromboxane metabolite 11-dehydroTxB$_{2}$ (Fig. 1A,B), confirming selective inhibition of COX-2 at this dose$^{29,30}$. There was no effect of L-NAME alone on excretion of either metabolite. This indicates that NO does not directly modulate COX-1 or –2 turnover in the vasculature. However, L-NAME appeared to partially blunt the inhibitory effect of celecoxib on COX-2, through an unknown mechanism. Celecoxib alone did not alter systolic blood pressure (BP) (Fig. 1 C). In contrast, L-NAME increased BP acutely, peaking at day 1 (Fig. 1 C). However when L-NAME was given together with celecoxib, the resulting BP elevation was significantly higher and more prolonged than for L-NAME alone (Fig. 1 C). This indicates that the removal of NO reveals a pro-hypertensive action of celecoxib, which is not detected in healthy mice. Next, BP was measured following indomethacin administration at a dose that blocks excretion of both TX and PGI urinary metabolites by over 70%$^{31}$. Indomethacin did not raise BP, but in combination with L-NAME, the resulting elevation was significantly higher and more prolonged than for L-NAME alone (Fig. 1 D). Importantly, BP elevations for celecoxib/L-NAME or indomethacin/L-NAME were not significantly different, indicating that both drugs had identical effects on tone. These elevations in BP were acute and peaked at day 3, then decreased thereafter. These are otherwise healthy mice, and so may overcome the hypertensive effects of NSAIDs over a
period of days, by inducing alternative vasorelaxant pathways (e.g. endothelium derived
hyperpolarizing factor). These studies show that celecoxib/indomethacin are without effect on basal
blood pressure in healthy mice, however they significantly increase the vasoconstriction mediated by
inhibition of vascular NO generation.

Myography studies demonstrate that COX-2 inhibition is vasoconstrictive in the absence of NO.

To determine whether effects of NSAIDs were due to COX inhibition, in vitro studies
examined agonist-induced constriction and relaxation in isolated murine aortic rings. For all NSAIDs,
a significant enhancement of phenylephrine-induced constriction was found, but only with L-NAME
present (Fig. 1, E-G). Similarly, aortic rings from COX-2<sup>−/−</sup> mice also constricted significantly more
than wild-type when NO generation was blocked (Fig. 1 H). In separate experiments, it was found
that incubation of aortic rings with a more potent NOS inhibitor, S-methyl-L-thiocitrulline (SMTC)<sup>32</sup>
led to a spontaneous and immediate increase in tone, but only when celecoxib was present (Fig 1 I).
Addition of an NO donor (DETA NONOate), generating NO at low levels that inhibited L-NAME-
induced vasoconstriction, totally abolished the vaso-constrictive effect of celecoxib. Indeed, in the
presence of exogenous NO, celecoxib mediated some vasorelaxation (Fig 1 J). Although we do not
know the molecular mechanism for this, it may include generation of vasoconstrictive prostanoids,
such as PGF<sub>2α</sub>, with suppression of prostacyclin bioactivity by NO<sup>33</sup>. Importantly, it suggests the
therapeutic potential of prescribing NO donors, or NO-donating NSAID prodrugs in situations where
NO bioactivity is compromised. Finally, all NSAIDs increased the inhibitory activity of L-NAME on
acetylcholine-induced relaxation (Fig. 2 A-C), while having little/no effect in its absence. The vascular
expression of COX-2 was examined using immunohistochemistry and myography of endothelium-
denuded rings. Predominant staining of COX-2 in endothelium (although some appears to also be
localized to smooth muscle) and attenuation of the vasoconstrictive effect of celecoxib following endothelial-removal indicate that COX-2 expression in aorta is largely endothelial (Fig 2 D-F). In contrast, COX-1 expression was not detected in aorta of wild-type mice (not shown). In aggregate, these data indicate that the vasoconstrictive effects of NSAIDS revealed in the absence of NO most likely result from their action as COX-2 inhibitors.

**Inhibition of NO leads to platelet regulatory effects of NSAIDs in vivo**

Since myocardial infarction is a side-effect of selective COX-2 inhibition, the effects of NSAIDs on *in vivo* platelet function, with/without NO inhibition were examined, by FACS analysis of whole blood platelet P-selectin. Since there is variability between days (e.g. due to sampling variations), each experiment utilized separate controls, conducted at the same time. Platelet P-selectin expression was not significantly altered from basal following 3 day L-NAME or celecoxib administration (Fig. 2 G-I). In contrast, co-administration of L-NAME with celecoxib caused a 19% elevation of P-selectin-expressing platelets (Fig. 2 H,I). This indicates that PGI inhibition promotes platelet activity *in vivo*, but only in the absence of NO. Similar to celecoxib, indomethacin administration was without significant effect on P-selectin expression (Fig. 2 J). However, when co-administered with L-NAME, P-selectin expression was undetectable (Fig 2 J). These data reveal a difference between the effects of selective COX-2 inhibitors *versus* non-selective NSAIDS in regulation of *in vivo* platelet activation in the absence of NO. Specifically, celecoxib slightly increases platelet P-selectin expression while indomethacin inhibits.

*NO regulates bioactivity of NSAIDs; implications their use in patients with compromised NO.*
Herein, we found that NO bioavailability regulates the vascular effects of COX-2 selective and non-selective NSAIDs in healthy mice. This idea is supported by previous studies using eNOS-deficient mice showing that COX-2 prostaglandins compensate for loss of NO in regulating coronary hemodynamics and flow-induced dilatation \textit{in vivo}^{34,35}. The data indicates that a balance of NO and prostaglandins is required to maintain BP and platelet function at optimum levels \textit{in vivo}, and that altering this is undesirable. These data have implications for our understanding of PGI bioactivity in patients with arthritis and vascular disease who typically show 2-fold elevations in PGI, along with decreased large vessel NO bioactivity \textit{in vivo}^{10-19}. Specifically, the biological role of PGI in regulating vessel tone and platelet function may be enhanced in these groups (Scheme 1). As a direct result, the risk of cardiovascular events would be greater on administration of NSAIDs to individuals with defects in vascular NO production. In summary, our study identifies an interaction between NO and COX that influences the risk of NSAID vascular side-effects and may offer a strategy for reducing this, based on simultaneous elevation of NO levels using NO donors or NO-donating NSAID prodrugs.
References


Figure Legends

Figure 1. Systolic blood pressure and vasoconstriction is significantly elevated in mice given both L-NAME and NSAIDs.  
Panel A. In vivo inhibition of COX-2 using celecoxib. 24-hr urine samples were obtained from mice administered celecoxib +/- L-NAME for 3 days and analyzed using GCMS for PGI metabolite (2,3-Dinor-6-KetoPGF1α) urinary excretion.  
Panel B. No inhibition of COX-1 in vivo using celecoxib. 24-hr urine samples were obtained as for Panel A and analyzed using GCMS for TX urinary metabolite (1-DehydroTxB2) urinary excretion (for both panels, n = 3 – 4 cages per group with 3 mice per cage, mean ± SEM).  
Panel C. Blood pressure elevations following celecoxib and L-NAME administration. 10-12 week old male C57BL/6 mice were administered L-NAME and/or celecoxib. Systolic blood pressure was monitored daily. □, control mice; ○, L-NAME; △, celecoxib; ▲, celecoxib and L-NAME  
Panel D. Blood pressure elevations following indomethacin and L-NAME administration. 10-12 week old male C57BL/6 mice were administered L-NAME and/or indomethacin. Systolic blood pressure was monitored daily. □, control mice; ○, L-NAME; △, indo; ▲, indo and L-NAME (for all blood pressure determinations, n = 5 animals per group, mean ± S.E.M., ★ p < 0.05 c.f. day 0; using ANOVA test with Dunnett’s Post-Hoc Test to isolate differences).  
Panel E. Effect of celecoxib on in vitro constriction dose response. Aortic ring constriction responses to phenylephrine were determined (n = 5 - 9 per group). Control (□), L-NAME (300 uM; ○), celecoxib (10 uM; △) or L-NAME (300 uM) and celecoxib (10 uM; ▲).  
Panel F. Effect of indomethacin on constriction dose response. Aortic ring constriction responses to phenylephrine were determined (n = 5 - 9 per group). Control (□), L-NAME (300 uM; ○), indomethacin (10 uM; △) or L-NAME (300 uM) and indomethacin (10 uM; ▲)  
Panel G. Effect of aspirin on constriction dose response. Aortic ring constriction responses to phenylephrine were determined (n = 5 - 9 per group). Control (□), L-
NAME (300 uM; ○), indomethacin (10 uM; △) or L-NAME (300 uM) and aspirin (100 uM; ▲).

*Panel H. Effect of COX-2 deletion on constriction dose response.* Aortic ring constriction responses to phenylephrine were determined (n = 5 - 9 per group). WT (■), COX2−/− (○), WT + L-NAME (300 uM; △) and COX-2−/− + L-NAME (▲; 300 uM).

*Panel I. Aortic rings spontaneously constrict in the presence of SMTC and celecoxib.* Aortic ring tone was determined using myography after 15 min incubation (n = 4 - 6 per group) with SMTC (100 uM) with/without celecoxib (10 uM). Inset shows representative trace from myograph.

*Panel J. Exogenous NO abolishes the vasoconstrictive effect of celecoxib.* Aortic ring constriction responses to phenylephrine were determined (n = 6 per group). WT + L-NAME (300 uM) + DETA NONOate (30 uM; ▼) WT + L-NAME + DETA NONOate + celecoxib (10 uM; △). For all aortic ring myography, data are expressed as mean ± S.E.M. ★ p < 0.05 c.f. WT group, using 2-way ANOVA to isolate differences between groups.

**Figure 2.** NSAIDs enhance the inhibitory effect of L-NAME on aortic ring relaxation to acetylcholine, and platelet P-selectin expression is significantly elevated in mice given both L-NAME and celecoxib, but decreased following L-NAME and indomethacin.

*Panel A. Effect of celecoxib on L-NAME inhibition of relaxation.* Aortic ring relaxation responses to acetylcholine were determined. Control (■), L-NAME (300 uM; ○), celecoxib (10 uM; △) or L-NAME (300 uM) and celecoxib (10 uM; ▲).

*Panel B. Effect of indomethacin on L-NAME inhibition of relaxation.* Aortic ring relaxation responses to acetylcholine were determined. Control (■), L-NAME (300 uM; ○), indomethacin (10 uM; △) or L-NAME (300 uM) and indomethacin (10 uM; ▲).

*Panel C. Effect of aspirin on L-NAME inhibition of relaxation.* Aortic ring relaxation responses to acetylcholine were determined. Control (■), L-NAME (300 uM; ○), aspirin (10 uM; △) or L-NAME (300 uM) and aspirin (10 uM; ▲). ★p<0.05 c.f. wild-type group, using 2-way ANOVA to isolate differences.
between groups. For all experiments, n = 5 – 9 per group, mean ± S.E.M. **Panel D. Effect of celecoxib on constriction of endothelium-denuded rings.** Aortic ring constriction responses to phenylephrine were determined (n = 5 - 9 per group). WT denuded (■), WT denuded + celecoxib (▲; 10 uM).

**Panel E. Effect of celecoxib on relaxation of endothelium-denuded rings.** Aortic ring relaxation responses to acetylcholine were determined (n = 5 - 9 per group). WT denuded (▲), WT denuded + celecoxib (10 uM; ▼). **Panel F. Immunohistochemistry of COX-2 in aortic rings** Rings were stained using anti-COX-2 (i) or isotype control IgG (ii). Inset shows corresponding transmission images.

**Panel G. Identification of platelets in murine whole blood.** Mice were administered L-NAME and/or indomethacin or celecoxib for 3 days. Platelets were gated in mouse whole blood by FACS analysis using anti-mouse αIIβ-FITC (shown as R1). **Panel H. Representative data showing P-selectin expression following 3 days administration with celecoxib and L-NAME.** Platelets identified by αIIβ expression as in Panel G were analysed for P-selectin expression using anti-mouse P-selectin-FITC, or isotype rat anti-IgG-FITC. **Panel I. Platelet P-selectin expression is significantly increased on day 3 following co-administration of celecoxib and L-NAME.** % P-selectin expressing cells were defined as those in M1, shown on Panel B with subtraction of % isotype values from all samples. **Panel J. Platelet P-selectin expression is significantly decreased on day 3 following co-administration of indomethacin and L-NAME.** % P-selectin expressing cells were defined as those in M1, shown on Panel B with subtraction of % isotype values from all samples (for both panels, n = 5, mean ± SEM, ★ p < 0.05 c.f control using Students 2-tailed t-test).

**Scheme 1. COX-2 inhibition potentiates vasoconstriction in the absence of NO.** Healthy large vessels maintain tone predominantly using NO. If NO is depleted, COX-2-derived prostanoids (e.g. PGI) compensate. Then, inhibition of COX-2 leads to constriction since PGI synthesis is blocked, and
may predispose to cardiovascular side effects. Repletion with NO prevents the increase in tone mediated by COX-2 blockade.
Figure 1
Healthy vessel

Deplete NO

COX-2 inhibition

COX-2 inhibition

Restore NO

Scheme 1
Nitric oxide deficiency promotes vascular side-effects of cyclooxygenase inhibitors \textit{in vivo}

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