Nitric Oxide Inhibition of Endothelin-1 Release in the Vasculature
In Vivo Relevance of In Vitro Findings

Robert M. Rapoport

Online Data Supplement

A key function of endothelial derived nitric oxide (NO) is to limit the constrictor capacity of endothelin (ET)-1. Thus, an imbalance between NO and ET-1 can result in ET-1-dependent increased vascular tone. Indeed, the importance of sustained, NO inhibition of the ET-1 drive to increase tone is exemplified in vivo by the rapid, ET-1-mediated arterial pressure elevation after acute NO synthase (NOS) inhibition. Moreover, the ET-1 pressor effect after NOS inhibition is independent of other pressor systems and, thus, reflects the relative importance of NO suppression of this ET-1 drive.

Two, nonmutually exclusive mechanisms thought to underlie the ET-1 dependency of the increased vascular tone responsible for the NOS inhibitor-induced pressure elevation are (1) NO inhibition of constriction to endogenously released ET-1. This mechanism is essentially supported by demonstrations that NOS inhibitor enhanced the constrictor effects of exogenous ET-1 in several in vivo and ex vivo preparations. Thus, an assumption underlying these observations is that the constrictor actions of exogenous and endogenous ET-1 are similarly affected by NO; (2) NO inhibition of ET-1 release. Although the detailed mechanism underlying the NO inhibition of acute ET-1 release has not been clearly established, possibilities include decreased conversion of the immediate precursor to ET-1, big ET-1, to ET-1 and inhibition of pathways involved in the exocytosis of Weibel–Palade bodies; endothelial granules that store ET-1. However, it should be noted that in vivo findings that actually demonstrate the fundamental phenomenon of NO inhibition of ET-1 release are generally lacking. Thus, studies purported to demonstrate NO inhibition of ET-1 release have relied largely on isolated vessels and cultured endothelium, as described herein.

This review assessed these in vitro findings, with a specific focus toward elucidating the mechanism underlying the NO-mediated negative regulation of ET-1-dependent increased arterial pressure, as demonstrated after acute NOS inhibitor in vivo. Findings referred to are mainly those of ET-1 release rather than contractility (eg, those in which ET receptor antagonist prevented the NOS inhibitor-induced decreased flow/increased pressure/tension). The rationale for this emphasis is that findings based on contractility could reflect NOS inhibitor enhancement of ET-1 constriction rather than increased ET-1 release. Furthermore, we also focus on whether laminar shear stress influences the manifestation of NO inhibition of ET-1 release. That is, the endothelium of arteries that are anatomically linear is subjected to high levels of shear stress while, for example, at bifurcated arterial locations is subjected to low levels of shear stress, regions associated with atherosclerosis.

Acute NOS Inhibition/No Donors and ET-1 Release

Arteries

In rat heart perfused at constant flow, increased amounts of ET-1 were detected in the coronary effluent of samples collected for the final 5 to 15 minutes of a 1.5-hour perfusion with NOS inhibitor (Figure). The increased ET-1 release qualitatively correlated with the NOS inhibitor-elevated perfusion pressure because maximal pressure occurred after 1-hour perfusion with the inhibitor. Also, ET receptor antagonist prevented the NO inhibitor-elevated pressure. The NO donor, S-nitroso-N-acetyl-penicillamine, decreased ET-1 release below basal levels and increased coronary flow.

In possible contrast, in rat heart perfused at constant pressure, increased amounts of ET-1 were not detected in coronary effluent after NOS inhibitor perfusion for 15 minutes (Figure). Because perfusion with NOS inhibitor for 15 minutes caused maximal decreased coronary flow and, additionally, the decreased flow was prevented by ET receptor antagonist, it was suggested that NOS inhibitor-induced decreased ET-1-dependent flow resulted from potentiation of vasoconstriction to basally released ET-1.
Investigations of ET-1 release in isolated arteries were largely limited to static preparations (ie, nonperfused and unstretched vessels; Figure). In porcine aorta, even prolonged exposure (4 and 9 hours) to NOS inhibitor failed to increase ET-1 release.27,28 NOS inhibitor for 35 minutes also failed to increase ET-1 release in rat aorta.30 In bovine aorta, NOS inhibitor caused a nonstatistically significant increase in ET-1 release and, moreover, the increase was observed after 4.3-hour NOS inhibitor exposure.29

In the one study performed in an isolated perfused preparation in which ET-1 release was measured, the rat mesenteric arterial bed, pretreatment with combined NOS inhibitor, methylene blue (inhibitor of NO activation of guanylate cyclase), tetraethylammonium (nonselective K+-channel blocker), and indomethacin (cyclooxygenase inhibitor) increased ET-1 release at a flow rate of 30 mL/min but not at 5 mL/min, as assayed in perfusate recirculated through the vascular bed for 2 hours31 (Figure). An increase in perfusion pressure was also elicited by these combined agents at 20 to 30 mL/min, but not at 5 to 10 mL/min, and the increased pressure was prevented by combined selective ET_a plus ET_b receptor antagonists.31 The release of NO, as detected by nitrite, at 30 mL/min was 2.5× greater than at 5 mL/min.31

**Conclusion**

It is difficult to conclude whether NOS inhibitors release ET-1 in isolated heart because of the inconsistent findings,18,20 as well as the few studies performed. Whether the inconsistent findings reflect experimental conditions, that is, constant flow20 versus constant pressure,18 remains a possibility. Another complication is the disposition of released ET-1 because detection may be difficult because of the abluminal release of ET-132 and clearance of ET-1 by ETB receptors.33 Also, whether the ET-1 released by NOS inhibitor18 occurs exclusively from the endothelium is not entirely clear. Along these lines, it was suggested that ET-1 release from cardiac myocytes was responsible for the ET-1 dependency of phenylephrine-induced contraction of epicardial coronary vessels in vivo.34
Also, the inability of NOS inhibitor to increase ET-1 release in static arterial preparations does not support the suggestion that NO acts as a negative regulator of basal ET-1 release. However, the finding that NOS inhibitor plus methylene blue, albeit also only investigated in combination with tetraethylammonium and indomethacin, elicits flow- and possibly pressure-dependent ET-1 release suggests that the activation of the pathway whereby NO inhibits ET-1 release requires underlying shear and possibly pressure-related stress (Figures S1 and S2 in the online-only Data Supplement). Of interest is whether the increased ET-1 release observed after 2-hour exposure to combined agents would also be detected (1) with only NOS inhibitor; (2) at earlier time points, thereby demonstrating greater relevancy to the maintained increase in arterial pressure that occurs within 10 minutes of NOS inhibitor in vivo; (3) with NOS inhibitor added during the maintained elevated flow. Indeed, NOS inhibitor prevented, but not reversed, the decreased ET-1 release caused by increased shear stress in cultured endothelium (Figure; see section Endothelium: Static shear stress: Long duration: High level of this article), and (4) in the absence of the elevated pressure associated with the increased flow, thereby addressing whether the increased flow was sufficient to increase ET-1 release in the presence of NOS inhibitor.

Endothelium: Static

Increased basal ET-1 release by NOS inhibitor was observed in only 2 of 9 studies with static cultures (Figure). Furthermore, in these 2 studies, the increased ET-1 release was determined after prolonged incubation with NOS inhibitor (ie, 637 and 24 hours). Thus, the observed ET-1 release is not reflective of possible acute negative regulation of ET-1 release by NO.

Other studies failed to demonstrate increased ET-1 release after extended incubation (3–24 hours) with NOS inhibitor (27,35,36–41) (Figure). Importantly, the general inability of NOS inhibitor to increase ET-1 release does not seem to be because of the failure of NOS inhibitor to decrease NO because NO levels were decreased by 637 but did not increase ET-1 release (above; Figure S1).

The effect of NO inactivation on basal ET-1 release was also examined with oxyhemoglobin, which binds NO (28,39,42,43) (Figure). Although exposure to oxyhemoglobin for 1 to 24 hours increased ET-1 release, it is likely that the increased release was unrelated to NO inactivation because parallel experiments demonstrated that (1) NOS inhibitor failed to increase ET-1 release and (2) methemoglobin, which does not bind NO, also increased ET-1 release (above; Figure S1). In addition, oxyhemoglobin for 24 hours actually decreased ET-1 release in cerebrovascular endothelium (23).

Corollary studies were performed in which the effects of NO donors on ET-1 release were examined. Although 4-hour incubation with 50 μmol/L nitroglycerin and 50 μmol/L sodium nitroprusside decreased ET-1 release, the decrease was minimal. Indeed, caution was suggested in the mechanistic interpretation of these findings because of the relatively high NO donor concentrations required to decrease ET-1 release (above; Figure S1). In addition, 50 μmol/L NOR 3 [(±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexanamide] failed to decrease ET-1 release after 3 hours although release was decreased by 50% after 6 hours. The lack of decreased ET-1 release after NOR 3 for 3 hours was not because of the inability of NOR 3 to elevate cyclic GMP levels because 50 μmol/L NOR 3 for 10 minutes increased cyclic GMP levels 5-fold. In addition, 0.2 mmol/L S-nitro-N-acetyl-dl-penicillamine and SIN-1 did not decrease ET-1 release after 2 hours even though cyclic GMP levels were elevated 3 to 4 fold within 10 minutes. Similarly, 0.5 mmol/L DETA NONOate decreased ET-1 release after 24 hours but not after 12 hours. Also, although spermine-NO and S-nitroso-glutathione decreased ET-1 release, 1 mmol/L sodium nitroprusside failed to decrease ET-1 release, despite elevating cyclic GMP levels. In another report, although 50 μmol/L FK409 decreased ET-1 release by ≈25%, the duration of FK409 exposure was between 3 and 12 hours (specific duration not specified).

Conclusion

The inability of acute (1) NOS inhibition to increase ET-1 release (above), even though lowering NO and (2) NO donor challenge to decrease ET-1 release, even though activating guanylate cyclase (above), suggests that under static conditions the endothelium is dysfunctional with respect to the negative coupling of NO to ET-1 release (Figures S1 and S2).

Endothelium: Stress: Pressure/Cyclic Stretch

Pressure for 8 hours at 80 mm Hg, conditions that reflect those under which NOS inhibition administration causes ET-1–dependent pressure elevation, actually increased ET-1 release 1.3-fold. NO formation was decreased 25% by pressure. Furthermore, despite that at 80 mm Hg exposure to NOS inhibitor lowered NO formation to basal levels (absence of pressure), pressure-induced ET-1 release remained unchanged (above; Figure S1).

Cyclic stretch also increased ET-1 release, with both a rapid (20 minutes) and delayed (hours) time course, and increased ET-1 gene expression. NO formation was also increased by cyclic stretch, as detected by increased endothelial cyclic GMP levels. The effect of NOS inhibition on cyclic stretch–induced ET-1 release has not been investigated (to our knowledge).

Conclusion

The inability of NOS inhibitor to enhance ET-1 release at a physiological pressure level does not support the involvement of pressure per se in possible increased ET-1 release related to the NOS inhibitor-mediated, ET-1–dependent plateau pressure elevation. However, it should also be considered that pressure-related stress may contribute to the coupling of NO to decreased ET-1 release (Figure S2).

Endothelium: Stress: Laminar Shear Stress: Short Duration

Laminar shear stress of short duration at a high level increased ET-1 release (Figure). Although NOS inhibitor pretreatment increased ET-1 release at this shear stress condition, as well as at low-level short duration shear stress, it is not clear whether the inhibition actually enhanced underlying increased ET-1 release because ET-1 release was not measured under static conditions (above; Figure). Moreover, this
study used the immortalized EA hy.926 cell line, raising the possibility that relevant regulatory pathways may be altered. In this regard, cyclic stretch decreased and increased NOS expression in EA hy.926 and bovine aorta endothelial cells, respectively.

Somewhat surprisingly, the increased ET-1 release in response to high-level short duration shear stress (Figure S1) occurred, despite that NO release/NOS activity was also increased. Low-level short duration shear stress also increased NO release, although extremely low shear stress (0.105 dyne/cm²) decreased NO release.

**Endothelium: Stress: Laminar Shear Stress: Long Duration: Low Level**

Low level of laminar shear stress for long duration represents conditions associated with arterial sites prone to atherosclerosis. Hours-long (6 and 16 hours) shear stress at 1.8 dynes/cm² increased ET-1 release (Figure; Figure S1). However, a nonsignificant decrease in ET-1 release at 2 dynes/cm² for 6 hours was also reported.

Shear stress at 2 dynes/cm² for 6 hours tended to increase NO release (based on 3H-citrulline formation from 'H-arginine; Figure; Figure S1). It is not known (to our knowledge) whether NOS inhibitor alters ET-1 release under these conditions of shear stress.

**Conclusion**

Conclusions about the relationship between NO and ET-1 release at low-level long duration shear stress are difficult to derive because only 2 studies (to our knowledge) have been performed and, moreover, one study reported increased ET-1 release whereas the other study failed to demonstrate an increase. It is of interest to note that the latter study also failed to demonstrate decreased ET-1 release at 12 dynes/cm² for 6 hours, which contrasts with the substantial decrease in ET-1 release at similar shear stress conditions reported by others (below). Thus, we tentatively conclude that low-level long duration shear stress increased ET-1 release (Figure S1). Indeed, this increased ET-1 release occurred, despite the increased NO release (Figure S1). Similarly, NO and ET-1 release also increased with high- and low-level short duration shear stress (see Endothelium: Stress: Laminar Shear Stress: Short Duration: Section of this article).

Although increases in both NO and ET-1 release with low-level long duration shear stress, as well as at low- and high-level short duration shear stress, suggest a lack of coupling between NO-ET-1, this negative coupling still remains a possibility in the absence of reports on the effects of NOS inhibitor at these conditions. Thus, at sites prone to atherosclerosis, possible increased ET-1 release by NOS inhibitor could contribute to the ET-1–dependent increased vascular tone underlying the NOS inhibitor-mediated pressor effect.

**Endothelium: Stress: Laminar Shear Stress: Long Duration: High Level**

High level of laminar shear stress for long duration represents conditions under which the endothelium of arteries at nonbi-furcated locations are exposed. Hours-long shear stress at 10 to 25 dynes/cm² decreased ET-1 release (Figures S1 and S2) although a lack of effect was reported under similar shear stress conditions. These conditions also increase NO release/activate NOS.

Nω-nitro-L-arginine (L-NNA; 0.4 mmol/L) failed to reverse the decreased ET-1 release that resulted from 25 dynes/cm² shear stress for 3.2 hours (Figure; Figure S1). This complete lack of reversal was unexpected because 0.4 mmol/L L-NNA reversed the shear stress–elevated cellular levels of cyclic GMP by 40%. Moreover, pretreatment with 0.3 and 0.4 mmol/L L-NNA, as well as methylene blue, prevented the decreased ET-1 release that resulted from 10 dynes/cm² shear stress for 12 hours. Also, 0.5 mmol/L Nω-nitro-L-arginine methyl ester partially prevented (by 65%) the decreased ET-1 release that resulted from 15 dynes/cm² shear stress for 24 hours, although 0.3 mmol/L Nω-nitro-L-arginine methyl ester was without effect (Figure). The lower relative efficacy of Nω-nitro-L-arginine methyl ester at prevention of shear stress–induced decreased ET-1 release may reflect the greater relative amount of shear stress in this latter study. Pretreatment with 0.3 mmol/L L-NNA also abolished the increased NO (3H-citrulline) release that resulted from 8 dynes/cm² shear stress for 6 hours.

**Conclusion**

At higher levels of laminar shear stress of long duration, a negative coupling between NO and ET-1 release is suggested by the associated decreased NO and increased ET-1 release (Figure; Figure S1). However, although NOS inhibitor prevented the lowered ET-1 release, the inhibitor did not reverse the lowered release. One interpretation of these findings is that NO and ET-1 release remain uncoupled (Figure S2). Indeed, the ability of NOS inhibitor before but not during shear stress to prevent lowered ET-1 release could suggest that shear stress initiates the negative coupling of NO to decrease ET-1 release, but this regulatory mechanism is not maintained. Clearly, this explanation is incompatible with possible increased ET-1 release in response to NOS inhibitor in vivo.

Alternatively, we speculate that the inability of NOS inhibitor to reverse the lowered ET-1 release associated with high levels of laminar shear stress may result from (1) NO release at a level in excess of that required to lower ET-1 release and (2) the inability of NOS inhibitor to cause a great enough reduction of these high levels of NO release such that ET-1 release becomes regulated by NO (Figure S1). In support of this possibility are the combined observations that phorbol ester (protein kinase C activator) reversed shear stress–increased NO release (as determined by lowered cyclic GMP levels) although to a lesser magnitude than L-NNA (reported as data not shown by Kuchan and Frangos). Indeed, phorbol ester–lowered NO release is consistent protein kinase C negative regulation of NO activity, (2) did not reverse the lowered ET-1 levels, but (3) in the presence of NOS inhibitor, reversed shear stress–lowered ET-1 release. Therefore, combined phorbol ester and NOS inhibitor may have lowered NO release to a level where ET-1 release could then be regulated (Figure S1). Thus, it should be considered whether stress conditions other than high-level long duration laminar
shear stress, which would more accurately reflect those present in vivo, allows NOS inhibitor to reverse the associated lowered ET-1 release.

Implications

We presently speculate that the more relevant physiological pulsatile shear stress, which varies depending on the vessel, rather than laminar shear stress, represents a condition in which NO is not released in excess with respect to the negative regulation of ET-1 release (Figures S1 and S2). In support of this possibility are the findings that (1) short duration of oscillatory pulsatile flow at both 15 and 30 dynes/cm², but not at steady laminar flow at 30 dynes/cm², increased reactive oxygen species, and reactive oxygen species inactivate NO; (2) in subendocardial and subepicardial porcine arteries, oscillatory flow accompanying laminar flow resulted in increased superoxide anion, which lowered NO release; (3) laminar shear stress caused greater NO release when compared with oscillatory shear stress; and (4) endothelial NOS levels were reduced after oscillatory when compared with laminar shear stress (shear stress effects determined in the presence of pressure). It should be noted, however, that one report demonstrated that oscillatory shear stress increased NO release.

Consistent with the pulsatile/oscillatory shear stress–lowered NO release, laminar shear stress caused greater ET-1 release than oscillatory shear stress, and oscillatory shear stress transiently upregulated ET-1 expression (as determined in the presence of pressure). Furthermore, the in vivo relevance of these in vitro findings is borne out by observations that (1) shear stress accompanied by increased amplitude of pulsation decreased endogenous NO-mediated dilation of the iliac artery in anesthetized pig and (2) pulsatile shear stress caused endogenous ET-1– and endothelium-dependent constriction of the iliac artery in anesthetized dog and pig. Indeed, these in vivo studies suggest a role for NO inhibition of ET-1 release under physiological conditions in the moment-to-moment regulation of vascular resistance. Overall, therefore, physiological pulsatile shear stress may uncover NO-mediated negative feedback in vitro through its ability to approximate a lower level of NO release present in vivo (Figures S1 and S2).

Although this review focuses on the acute NO inhibition of ET-1 release, as exemplified by the rapid, ET-1–dependent pressor effect after NOS inhibitor, the implications of the present conclusions extend beyond vascular pathophysiology in which there can be a relatively acute increase in the endogenous NOS inhibitor, asymmetrical dimethylarginine, to those in which there is lowered NO, possibly as the result of the continuous formation of asymmetrical dimethylarginine and reactive oxygen species. Thus, incremental NO reduction results in incremental ET-1 release, which contributes to several cardiovascular diseases. Importantly, accurate modeling of the conditions required for endogenous NO-mediated decreased ET-1 release will allow further relevant investigation into potential interventions that reverse/ prevent the disruption of this negative regulatory pathway and, thus, eventual therapeutic modalities.

Acknowledgments

We thank Geoffrey Liebrandt (Veterans Affairs Medical Center, Cincinnati, OH) for the illustrations.

Sources of Funding

This study was supported by a grant from the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

Disclosures

None.

References


Nitric Oxide Inhibition of Endothelin-1 Release in the Vasculature: In Vivo Relevance of In Vitro Findings
Robert M. Rapoport

Hypertension. 2014;64:908-914; originally published online August 18, 2014;
doi: 10.1161/HYPERTENSIONAHA.114.03837
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/64/5/908

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2014/08/18/HYPERTENSIONAHA.114.03837.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Nitric oxide inhibition of endothelin-1 release in the vasculature: *in vivo* relevance of *in vitro* findings

Robert M. Rapoport

Research Service, Veterans Affairs Medical Center, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, PO Box 670575, Cincinnati, OH 45267-0575

Short title: Nitric oxide and endothelin-1 release

*Correspondence to Robert M. Rapoport, PhD, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, PO Box 670575, Cincinnati, OH 45267-0575. FAX 513-558-1169; Telephone 513-861-3100x4337; Email robert.rapoport@uc.edu
Figure S1. Effects of NOS inhibitor on endothelial NO and ET-1 release. A) NO and B) ET-1 release under static conditions and increasing amounts of shear stress for long duration in the absence (solid curved lines) and presence (X) of NOS inhibitor (the paucity of “X’s” reflects the lack of findings). Horizontal dashed lines (a) are shown for reference and indicate NO (A) and ET-1 release (B) under static conditions, as indicated on the Y axis intercept. Vertical dotted lines (b) serve as references for the relative corresponding NO (A) and ET-1 release (B). Vertical dotted-dashed lines (c) refer to the level of shear stress below which lowered NO release (A) reverses shear stress-lowered ET-1 release (B; present speculation). Solid arrows (d) indicate the reversal of shear stress-increased and decreased NO (A) and ET-1 release (B), respectively, by NOS inhibitor (NOS-I) plus phorbol ester and by NOS inhibitor plus physiologic pulsatile shear stress (present speculation). See text for details.
Figure S2. General working scheme of endothelial coupling between NO and decreased ET-1 release as determined by NOS inhibition. NO uncoupled (dashed line) and coupled (solid line) to decreased ET-1 release and the relative amount of endogenous NO (circle) and ET-1 release (square) are shown. Static conditions: NOS inhibitor (NOS-I) lowers NO but fails to increase ET-1 release, indicative of NO uncoupled to decreased ET-1 release. Laminar shear stress: increased and decreased ET-1 release, respectively, as compared to static conditions. NOS-I partially reverses the increased NO but fails to reverse the lowered ET-1 release, indicative of NO uncoupled to decreased ET-1 release. Physiologic pulsatile shear stress: partially lowered NO release while ET-1 release remains unchanged as compared to laminar shear stress. NOS-I reverses the increased NO and decreased ET-1 release, respectively, indicative of NO coupled to decreased ET-1 release. See text for details.

ACKNOWLEDGEMENTS

We thank Geoffrey Liebrandt (VAMC, Cincinnati, OH) for the illustrations.
SOURCES OF FUNDING

Supported by a grant from the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

DISCLOSURES

None