Inducible Nitric Oxide Synthase Inhibition of Weibel-Palade Body Release in Cardiac Transplant Rejection

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Background—Inducible nitric oxide synthase (iNOS, or NOS2) reduces the severity of accelerated graft arteriosclerosis (AGA) in transplanted organs, although the precise mechanism is unclear.

Methods and Results—We transplanted wild-type murine hearts into either wild-type or NOS2-null recipient mice; we then measured cardiac allograft survival and analyzed tissue sections by immunohistochemistry. We have confirmed that NOS2 increases cardiac allograft survival. We now show that there is less inflammation of cardiac allografts in wild-type hosts than in NOS2-null hosts. Furthermore, staining for von Willebrand factor reveals that the presence of NOS2 is correlated with the presence of Weibel-Palade bodies inside endothelial cells, whereas the absence of NOS2 is correlated with the release of Weibel-Palade bodies.

Conclusions—Weibel-Palade bodies contain mediators that promote thrombosis and inflammation. Therefore, nitric oxide (NO) may stabilize the vessel wall and prevent endothelial activation in part by inhibiting the release of the contents of Weibel-Palade bodies. Prevention of Weibel-Palade body release might be a mechanism by which NO protects the vessel wall from inflammatory disorders such as atherosclerosis or graft arteriosclerosis. (Circulation. 2001;104:2369-2375.)

Key Words: selectins | rejection | transplantation | grafting | arteriosclerosis

Inducible nitric oxide synthase (iNOS, or NOS2) plays an important role in modulating both acute and chronic allograft rejection. In the acute setting, NO or a derivative of NO is detrimental to the transplanted organ. Heterotopic cardiac allografts express NOS2 protein early after transplantation in a variety of animals, including mice and rats.1–4 In the acutely rejecting heart, NOS2 is expressed by mononuclear inflammatory cells and cardiac myocytes in cardiac allografts.3,5 NOS2 expression is associated with contractile dysfunction in rodent and human cardiac allografts.5,6 NOS2 inhibitors restore contractile function, reduce inflammatory infiltrates, and increase graft survival.2,8–11 Furthermore, NOS2 expression leads to inhibition of myogenic tone in allograft arteries.4 Finally, cardiac allografts transplanted into mice lacking the NOS2 alleles initially have less inflammation and less myocyte damage than allografts transplanted into wild-type mice.12,13 Thus, in the acute setting, NOS2 and NO may exacerbate acute rejection.12

In the chronic setting, however, NOS2 may protect the allograft. The chief cause of long-term graft failure is accelerated graft vasculopathy (AGA), also called chronic rejection or transplant vasculopathy.14,15 AGA is characterized by a diffuse, concentric neointima in graft arteries that gradually obliterates the blood supply of the transplanted organ. Heterotopic cardiac allografts express NOS2 protein late after transplantation in rodent models of AGA.3 During chronic rejection, NOS2 is expressed not only in mononuclear inflammatory cells in the interstitium but also in mononuclear inflammatory cells and smooth muscle cells in the vessel wall.3 Humans express NOS2 in macrophages and smooth muscle cells in coronary arteries of orthotopic cardiac allografts as well.16,17 Several experimental approaches have shown that NOS2 inhibits development of the neointima in allografts. First, inhibitors of NOS exacerbate AGA.18 Second, hearts transplanted into NOS2-null mice develop more severe AGA than hearts transplanted into wild-type mice.12,19 Finally, delivery of an NOS2 transgene into transplanted aortas reduces the neointima of AGA.18 These studies demonstrate that NOS2 inhibits AGA.

The mechanisms by which NOS2 and NO inhibit the formation of neointima during AGA are unclear. NO produced by NOS2 can modulate the development of T helper lymphocytes.20,21 NO can also regulate the immune response during chronic rejection of cardiac allografts.19 In addition, NO can also inhibit the expression of endothelial inflammatory mediators such as vascular cell adhesion molecule and P-selectin.22–24

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We hypothesized that NO derived from NOS2 protects the cardiac allograft from vascular inflammation in part by modulating endothelial expression of inflammatory mediators. Examining cardiac allografts in wild-type and NOS2-null recipient mice, we found that Weibel-Palade bodies are preserved in endothelial cells of allografts in wild-type mice but are absent in endothelial cells of allografts in NOS2-null mice. This suggests that NO might protect the allograft in part by inhibiting Weibel-Palade body release and thereby reducing inflammation triggered by endothelial cells.\textsuperscript{25}

**Methods**

**Reagents**

Antibodies to NOS2 were purchased from Transduction Laboratories. Antibody to von Willebrand factor (vWF) was purchased from Dako. Antibody to CD4 (GK1.5) was prepared from a hybridoma from the ATCC. All mice were from Jackson Laboratories, Bar Harbor, Me.

**Cardiac Allograft Transplantation**

Heterotopic cardiac transplantation was performed with wild-type B10.A (H-2\textsuperscript{a}) donors and C57BL/6 (H-2\textsuperscript{b}) recipients that were either wild-type or NOS2-null. NOS2-null mice were backcrossed 10 generations with C57BL/6 mice at the Jackson Laboratories. Heterotopic cardiac transplantation was performed as previously described.\textsuperscript{26} Briefly, the donor aorta was anastomosed to the recipient abdominal aorta, and the donor pulmonary artery was anastomosed to the recipient inferior vena cava. Antibody to CD4 was given intraperitoneally on days 1 and 3. Graft function was monitored by abdominal palpation. Graft rejection was defined as total cessation of contractions.

**Immunohistochemistry**

Full cross sections of cardiac grafts obtained at the time of death were fixed in 70% ethanol, embedded in paraffin, sectioned at 7 \textmu m, and stained with hematoxylin and eosin for morphology. Additional sections were stained by a standard immunoperoxidase technique of avidin–biotinylated enzyme complex (ABC) for the expression of vWF with a purified polyclonal rabbit antibody to vWF.

**Immunofluorescence**

To double-stain samples for endothelial cells and vWF, FITC-labeled anti-mouse platelet and endothelial cell adhesion molecule (PECAM) (CD31) clone MEC 13.3 (PharMingen) was used to stain endothelial cells, together with rabbit antibody to vWF (Dako) and rhodamine red–labeled F(ab\textsuperscript{‘}) goat anti-rabbit IgG (Jackson Immunoresearch) to stain vWF.

**Electron Microscopy**

Thin samples from all 4 quadrants of the transplanted heart were immediately fixed in 2.5% glutaraldehyde, washed in 0.1 mol/L cacodylate buffer (3 times for 5 minutes), and subjected to postfixation with 1% osmium tetroxide in 0.1 mol/L cacodylate for 1 hour, as described previously.\textsuperscript{27} Formvar-coated copper grids were stained with 2% uranyl acetate for 30 minutes, followed by subsequent staining with 0.02% lead citrate for 3 minutes, and imaged with a Philips CM 120 transmission electron microscope.

**Histological Quantification**

The severity of inflammation in cardiac allografts and the location of vWF were determined by 2 blinded pathologists. Inflammatory infiltrates were graded on a scale of 0 to 3. Interstitial infiltrates were scored on the basis of the area of myocardium involved: focal infiltrates were scored as 1, diffuse infiltrates involving <50% of the cross section were scored as 2, and diffuse infiltrates involving >50% of the cross section were scored as 3. Each component of the arterial vessels (intima, media, and perivascular space) were scored independently by the same criteria: 1 represented 1 to 2 vessels involved, 2 represented <50% of the vessels involved, and 3 represented >50% of the vessels involved.

**Statistics**

Data are described as mean±SD. Significance was measured by \textit{t} test of 2 samples, assuming equal variances.

**Results**

**NOS2 Increases Survival of the Cardiac Allograft**

We transplanted hearts from wild-type B10.A donor mice into either wild-type or NOS2-null C57BL/6 recipient mice. The donor heart was transplanted into the recipient’s abdomen, and the great vessels were anastomosed. Antibody to CD4 was injected intraperitoneally into recipients 1 and 3 days after transplantation for immunosuppression.

Cardiac allografts survive much longer in wild-type recipients than in NOS2-null recipients (Figure 1). One month after transplantation, 90% of the cardiac allografts in wild-type recipients were beating vigorously, but all of the cardiac allografts in the NOS2-null hosts were either completely rejected or near rejection. (As a control, some wild-type recipients were not immunosuppressed with antibody to CD4, and n=18 wild-type not immunosuppressed, n=11 wild-type immunosuppressed with antibody to CD4, and n=12 NOS2-null immunosuppressed with antibody to CD4.) These data confirm the work of others that shows that NOS2 improves long-term cardiac allograft survival.\textsuperscript{10,19}

**NOS2 Decreases Inflammation in the Cardiac Allograft**

We next explored the mechanism by which NOS2 protects cardiac allografts 30 days after transplantation. Others have shown that NO in certain circumstances can reduce or modulate inflammation.\textsuperscript{20,21,28,29} Accordingly, we examined cardiac allografts from wild-type and NOS2-null hosts 30 days after transplantation. Transplanted hearts were excised, fixed, sectioned, and stained with hematoxylin and eosin.
Minimal inflammation is seen in cardiac allografts in wild-type recipients (Figure 2A). In contrast, marked inflammatory infiltrates are present in the cardiac allografts in NOS2-null recipients (Figure 2B). These infiltrates are composed of mononuclear cells in the intima, the media, the perivascular region, and the interstitium of the cardiac allograft in NOS2-null hosts. Inflammation involves both large vessels (asterisks in Figure 2) and small vessels (arrows in Figure 2).

To quantify the differences in allograft inflammation between wild-type hosts and NOS2-null hosts, 2 pathologists who did not know the identity of the sections graded the location and severity of inflammation of sections of transplanted hearts (see Methods). The inflammatory infiltrate is more pronounced in the cardiac allografts in NOS2-null recipients than wild-type recipients (Figure 3).

These data suggest that NOS2 is associated with less inflammation after cardiac transplantation. The data do not show, however, that NOS2 and NO directly cause less inflammation.

**NOS2 Decreases Weibel-Palade Body Release**

NOS2 could modulate inflammation in the cardiac allograft by several mechanisms. One possibility is that NOS2 and NO could reduce inflammation by inhibiting endothelial cell activation, thereby reducing inflammatory cell attachment to the vessel wall and infiltration into the allograft.22–24,30 One hallmark of endothelial cell activation is the release of Weibel-Palade bodies, endothelial cell granules that contain vWF, P-selectin, and interleukin-8 (IL-8).31–34

To explore the association of NOS2 and endothelial cell activation, we stained sections of cardiac allografts from wild-type and NOS2-null recipients with antibody to vWF. Weibel-Palade bodies containing vWF are visible as granules inside endothelial cells in cardiac allograft vessels in wild-type hosts (Figure 4A through 4C). In contrast, Weibel-Palade bodies are decreased or absent in the endothelial cells of cardiac allografts in NOS2-null recipients (Figure 4D through 4F). Furthermore, in many of these vessels in NOS2-null hosts, vWF is detected in the subendothelial cell matrix of vessels in NOS2-null recipients (Figure 4F). The location of vWF in the subendothelial space is significant, because it shows that vWF has been released in large amounts from endothelial cells from the basolateral surface and has been trapped within the vessel wall.35 Decrease of Weibel-Palade bodies in endothelial cells and the appearance of vWF in the subendothelial space of NOS2-null hosts suggests that the lack of NOS2 is associated with enhanced Weibel-Palade body secretion, not diminished synthesis of Weibel-Palade bodies.

To quantify the reduced number of Weibel-Palade bodies from cardiac allografts in NOS2-null donors, 2 pathologists graded the intensity and location of staining for vWF in cardiac allograft sections, without knowing the identity of the sections (Methods). vWF is present in the endothelial cells of cardiac allografts in wild-type recipients, and very little vWF is present in the subendothelial space (Figure 5). In contrast,
vWF is present primarily in the subendothelial space of grafts in NOS2-null mice.

We next used immunofluorescent microscopy to localize vWF. Sections from transplanted hearts were stained simultaneously with antibody to the endothelial cell marker PECAM (CD31) and with antibody to vWF (Figure 6). Immunofluorescent microscopy shows that Weibel-Palade bodies are present as red punctate granules within cardiac allograft vessels in wild-type hosts (Figure 6B). These Weibel-Palade bodies are within endothelial cells, as shown by merging images of endothelial cells (green) and vWF (red) (Figure 6C). Punctate Weibel-Palade bodies are absent from endothelial cells in cardiac allografts in NOS2-null hosts, however, and vWF staining is more diffuse (Figure 6E). Furthermore, vWF is located in the subendothelial space of allograft vessels in NOS-null hosts, as demonstrated by vWF (red) localized below endothelial cells (green) (Figure 6F). These data confirm that in the absence of NOS2, Weibel-Palade bodies are decreased in endothelial cells, and vWF is deposited in the subendothelial space.

Electron microscopy was performed to confirm that endothelial cells in cardiac allografts contain Weibel-Palade bodies. Weibel-Palade bodies are visible within endothelial cells as electron-dense granules that lack internal membranes (Figure 7).

Our data show that Weibel-Palade bodies are present in quiescent endothelial cells in allografts in wild-type hosts, but Weibel-Palade bodies are released in activated endothelial cells in allografts in NOS2-null hosts. These data suggest that NO is associated with a decrease in Weibel-Palade body release but do not show that NO directly inhibits Weibel-Palade body release.

Discussion

Summary

The major finding of this study is that the lack of NOS2 is associated with the release of Weibel-Palade bodies from the
endothelium of cardiac allografts and with an increased inflammatory infiltrate. These data suggest that NO inhibits the release of Weibel-Palade bodies from endothelial cells, thereby reducing inflammatory cell attachment to and infiltration into the cardiac allograft. Inhibition of Weibel-Palade body release may explain one of the mechanisms by which NO exerts its protective effects on the vasculature.

NOS2 Inhibits AGA

Our data support the work of others showing that NOS2 and NO are important mediators of cardiac allograft rejection. During acute rejection, NO is detrimental to the cardiac allograft: NOS2 is expressed in cardiac myocytes of the allograft and in infiltrating mononuclear cells; inhibitors of NOS2 increase graft survival; and cardiac allografts transplanted into NOS2-null mice have more inflammation than those transplanted into wild-type mice. During chronic rejection, however, NOS2 is beneficial to the cardiac allograft: delivery of an NOS2 transgene via adenoviral vector decreases the neointima of vessels during chronic rejection, and the neointima of allograft coronary arteries is decreased when the allograft is transplanted into wild-type recipients compared with NOS2-null recipients. Our data confirm that NOS2 is an allele that protects cardiac allografts from AGA.

Potential Mechanisms of NOS2 Inhibition of AGA

NOS2 and NO may inhibit AGA by many mechanisms. NO might inhibit AGA by blocking smooth muscle cell formation of the neointima, because NO inhibits smooth muscle cell proliferation and migration in vitro (reviewed by Forstermann et al., Luscher, and Scott-Burden et al.), and NOS2 expression correlates with a decrease in smooth muscle cells in the neointima of allograft coronary arteries. NOS2 might also inhibit AGA by inhibiting the migration, proliferation, or differentiation of immune cells. Furthermore, NOS2 might protect the cardiac allograft by inhibiting apoptosis. Finally, NOS2 could protect the cardiac allograft by inhibiting endothelial cell activation: NO can inhibit the expression of vascular cell adhesion molecule, intercellular adhesion molecule-1, E-selectin, IL-6, and IL-8. Our studies, however, suggest another mechanism by which NOS2 reduces AGA in cardiac allografts. NO may inhibit the release of Weibel-Palade bodies from endothelial cells.

Weibel-Palade Bodies and Endothelial Activation

Weibel-Palade bodies are endothelial cell granules that are normally located under the plasma membrane of resting endothelial cells. Weibel-Palade bodies contain P-selectin, vWF, and CD63; during endothelial inflammation, Weibel-Palade bodies contain IL-8 as well. Various stimuli, such as thrombin, complement, or hypoxia, can trigger Weibel-Palade bodies to release their contents. P-selectin expressed on the surface of endothelial cells facilitates neutrophil attachment to the vessel wall. IL-8 not only attracts neutrophils but also activates them to release inflammatory mediators. vWF promotes platelet aggregation. Thus, Weibel-Palade body release is a form of endothelial cell activation that can lead to platelet and neutrophil attachment to the vessel intima.
NO, which in turn can inhibit Weibel-Palade body release and vascular inflammation and thus inhibit AGA.

Our results show an association between NOS2 and Weibel-Palade body stability: during vascular inflammation, the absence of NOS2 correlates with Weibel-Palade body release. Studies are under way to determine whether or not NO plays a direct role in inhibiting Weibel-Palade body release. Because the contents of these granules can lead to thrombosis and inflammation, our data suggest that NO can play a role in preventing endothelial cell activation. This action of NO is another mechanism by which NO might protect the vessel wall during inflammatory states such as atherosclerosis or transplant vasculopathy.

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**Potential Mechanism of NO Inhibition of Weibel-Palade Body Release**

The mechanism by which NO might inhibit Weibel-Palade body release is unclear, because the signal transduction pathways leading to Weibel-Palade body release are not yet defined. Several studies show that intracellular calcium levels rise before Weibel-Palade body release, and NO may interfere with endothelial calcium channels. Small GTP-binding proteins may also play a role in Weibel-Palade body release, and NO may target these proteins. The precise targets of NO within the Weibel-Palade body signaling pathway are unknown.

**NO, Weibel-Palade Bodies, and AGA**

During chronic rejection, many factors are released that can stimulate endothelial cells to release the contents of Weibel-Palade bodies, promoting vascular inflammation and AGA. Host immune cells that infiltrate allograft vessels, however, can be stimulated by cytokines to express NOS2 and produce NO, which in turn can inhibit Weibel-Palade body release and vascular inflammation and thus inhibit AGA.


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