Nitric oxide (NO) stimulates soluble guanylyl cyclase (sGC) levels. It is a currently prevailing concept that NO inhibits platelet activation. This concept, however, does not fully explain why platelet agonists stimulate NO production. Here we show that a major platelet NO synthase (NOS) isoform, NOS3, plays a stimulatory role in platelet secretion and aggregation induced by low doses of platelet agonists. Furthermore, we show that NOS3 promotes thrombosis in vivo. The stimulatory role of NOS is mediated by soluble guanylyl cyclase and results from a cGMP-dependent stimulation of platelet granule secretion. These findings delineate a novel signaling pathway in which agonists sequentially activate NOS3, elevate cGMP, and induce platelet secretion and aggregation. Our data also suggest that NO plays a biphasic role in platelet activation, a stimulatory role at low NO concentrations and an inhibitory role at high NO concentrations.

Development of thrombotic diseases involves the injury or dysfunction of the blood vessel wall and activation of blood platelets (1). Upon exposure to agonists such as thrombin, ADP, collagen, and von Willebrand factor (vWF), platelets become “activated” and aggregate to form primary thrombi (1, 2). Activated platelets secrete large quantities of ADP, serotonin, and other factors that amplify platelet activation and stabilize platelet aggregates (3). Activated platelets also secrete pro-coagulation, pro-inflammatory, and growth factors (3–5). Thus, platelet activation plays a major role not only in acute arterial thrombosis but also in the development of chronic vascular diseases, such as atherosclerosis, which in turn causes thrombosis (1, 6).

A major advance in the field of vascular biology in the last century was the discovery of the vessel dilator, nitric oxide (NO) (7–9). NO is a short-lived messenger molecule synthesized from L-arginine by a family of enzymes known as nitric-oxide synthases (NOS). Three isoforms of NOS enzymes are known (10–12): NOS1 (neuronal NOS), NOS2 (inducible NOS), and NOS3 (endothelial NOS). NOS3 is the major isoform known to be expressed in platelets (13). One of the major functions of NO is to stimulate soluble guanylyl cyclase (sGC) and increase the synthesis of cyclic guanosine monophosphate (cGMP) that serves as a secondary messenger regulating the function of cGMP-dependent protein kinase (PKG), cGMP-dependent ion channels, and cGMP-regulated phosphodiesterases (7). High concentrations of NO can also chemically modify (nitrosylation and nitration) proteins and, thus, affect cell functions in a cGMP-independent manner (7, 14–16). NO is involved in diverse processes, such as smooth muscle relaxation, neurotransmission, immune responses, and inflammation (7). It has been a prevailing concept that NO, by elevating intracellular cGMP, inhibits platelet activation (8). This concept is supported by data that high concentrations of NO donor compounds inhibit platelet activation (17–19). However, the concept that NO inhibits platelet activation does not fully explain why endogenous platelet NO production is stimulated by platelet agonists (20, 21). Thus, the physiological role of endogenous platelet NO synthesis by NOS during platelet activation has been unclear. In this study we show that the major NOS isoform expressed in platelets, NOS3, in fact plays a stimulatory role in low dose agonist-induced platelet activation and promotes in vivo thrombotic response in an injury-induced arterial thrombosis model. The stimulatory roles of NOS3 are dependent on sGC activation and elevation of cGMP. Furthermore, we found that the NOS3-sGC pathway stimulates aggregation-dependent platelet secretion of granule contents, which is required for the stimulatory roles of the NOS3-sGC pathway in platelet activation. Thus, our data provide an important revision to the current concept of NO signaling in platelets. We propose a new concept that NO plays a biphasic role in platelet activation; low concentrations of NO (such as that produced by platelet NOS3) promote platelet secretion and aggregation, but high concentrations of NO, as previously reported, inhibit platelet activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Soluble GC inhibitor 1H-1(2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), PKG activator 8-(4-chlorophenylthio)-guanosine 3’,5’-cyclic monophosphate (8-pCPT-cGMP), nitric oxide donor sodium nitroprusside (SNP), NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA), and thromboxane A2 analog U66619 were from Calbiochem. FeCl₃, o-phthalaldehyde, ADP, Nω-nitro-L-arginine methyl ester (L-NNAME), the inactive stereoisomer of L-NNAME, D-NNAME, and NOS substrate L-arginine were from Sigma. Human α-thrombin was purchased from Enzyme Research Laboratories, South Bend, IN. Luciferin/luciferase reagent and collagen were purchased from Chronolog, Havertown, PA. NOS3-deficient mice back-crossed for more than 10 generations to C57BL background, and C57BL/6 control mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and colonies were maintained at the University of Illinois Biological Resources Laboratory. Some experiments were performed using littermates obtained from heterozygotes breeding, with similar results.

**Preparation of Washed Platelets**—Fresh blood from healthy volunteers was anticoagulated with one-seventh volume of acid citrate dextrose (ACD) as described previously (22). For the preparation of mouse platelets, 6–8-week-old mice of either sex were anesthetized with an intraperitoneal injection of pentobarbital, and blood was drawn from the inferior vena cava (22). Blood from 5–6 mice of either genotype was
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The study investigated the role of Nitric-Oxide Synthase 3 (NOS3) in platelet activation. NOS3-deficient platelets showed reduced and unstable platelet aggregation compared with wild type platelets in response to low concentrations of thrombin. NO synthesis by NOS3 plays an important role in promoting and stabilizing low dose thrombin-induced platelet aggregation. To examine the effect of NOS3 in platelet activation induced by other agonists, platelets were exposed to thromboxane A2 analog U46619 or collagen. Similarly, NOS3-deficient platelets showed a reduced aggregation response to low doses of U46619 (Fig. 2A) and collagen (Fig. 2E). These data indicate that NOS3 is important in low dose agonist-induced platelet aggregation.

RESULTS

NOS3 Knock-out Inhibits Low Dose Agonist-induced Platelet Aggregation in Mouse Platelets—Fig. 1A shows that NOS3 protein is present in wild type mouse platelets but is deficient in NOS3−/− mouse platelets. To investigate the role of NOS3 in platelet activation, washed wild type or NOS3-deficient mouse platelets (26) suspended in modified Tyrode solution were exposed to platelet agonists. NOS3-deficient platelets showed reduced and unstable platelet aggregation compared with wild type platelets in response to low concentrations of thrombin (at 0.017 units/ml thrombin, p < 0.01, n = 5) (Fig. 1, A and D). When exposed to a sub-threshold concentration of thrombin (0.0125 units/ml), wild type platelets only partially aggregated under the same buffer conditions as above but fully aggregated when a physiological concentration of l-arginine (a substrate for NOS (27)) was added, suggesting that l-arginine increased the sensitivity of wild type platelets to thrombin (Fig. 1, B and E). In contrast, the enhancing effect of l-arginine was absent in NOS3-deficient platelets (Fig. 1, B and E). Thus, NO synthesis by NOS3 plays an important role in promoting and stabilizing low dose thrombin-induced platelet aggregation. To examine the effect of NOS3 in platelet activation induced by other agonists, platelets were exposed to the thromboxane A2 analog U46619 or collagen. Similarly, NOS3-deficient platelets showed a reduced aggregation response to low doses of U46619 (Fig. 2A) and collagen (Fig. 2E). These data indicate that NOS3 is important in low dose agonist-induced platelet aggregation.

NOS3 Mediates Dense Granule Secretion—The reduced platelet aggregation in NOS3-deficient platelets was observed at low concentrations of agonists and was characteristic of defects in the secretion-dependent second wave of platelet aggregation (22). Thus, we also examined if NOS3 knock-out affected platelet secretion using a real time luciferase-luciferase assay of secreted dense granule ATP. Platelet secretion induced by low dose thrombin was significantly reduced in NOS3-deficient platelets (Fig. 1, B and F). Thromboxane A2 induced two waves of platelet secretion as we reported previously (22) (Fig. 2, A and B). The first wave secretion was aggregation-independent, but the second wave was aggregation-dependent. The aggregation-dependent second wave platelet secretion in response to low dose U46619 was absent in NOS3 knock-out mouse platelets (Fig. 2A). Also, low dose collagen-induced platelet secretion was inhibited in NOS3 knock-out platelets (Fig. 2E). At higher concentrations of thrombin (Fig. 1, C and G), U46619 (Fig. 2, B and D), or collagen (Fig. 2, F and H) platelet secretion was still significantly reduced by NOS3 deficiency, although the remaining secretion appeared to be sufficient to induce the second wave of platelet aggregation. To exclude the possibility that reduced response of NOS3−/− platelets was due to an altered rate of secretion, we analyzed the kinetics of secretion, and found that NOS3 deficiency had no significant effect on the rate of secretion (22). The results suggest that NOS3 mediates dense granule secretion in platelets.
platelets was due to the "exhausted platelets" effect caused by prior platelet activation and secretion during preparation, we determined the total granule contents of wild type control as well as NOS3−/− platelets. The amounts of total ATP were not significantly different between the platelets from wild type and NOS3−/− mice (Fig. 3A). The amounts of dense granule-specific constituent serotonin were also similar between wild type and NOS3-deficient platelets (Fig. 3B). Additionally, platelet lysates contained comparable amounts of α-granule protein, VWF (Fig. 3C), and thrombospondin (data not shown). Therefore, NOS3 knock-out platelets carry normal levels of granule cargo but are defective in agonist-stimulated secretion.

Thus, it appears that NOS3 deficiency primarily affects platelet secretion. Insufficient secretion consequently abolishes or reduces the second wave of platelet aggregation and stability of aggregates. These data explain why NOS3 knock-out only affects platelet aggregation induced by low dose agonists. These data also suggest that NOS3 mediates an important (but not the only) platelet secretion signaling pathway.

**NOS3 Promotes in Vivo Thrombosis**—NOS3−/− mice are hypertensive, suggesting a strong vasoconstrictive effect of NOS3 deficiency. However, despite these strong vascular effects of NOS3 knock-out that favor in vivo hemostasis, we found no statistically significant difference in tail bleeding time between wild type (n = 29) and NOS3 knock-out (n = 33) littermate mice generated by heterozygous breeding (p = 0.5919). Rather, NOS3 knock-out mice showed a tendency of increased re-bleeding after initial unstable cessation of bleeding (data not shown).
To determine the in vivo physiological relevance of the role of NOS3 in promoting platelet secretion and aggregation, we compared in vivo thrombosis of wild type and NOS3−/− mice using the FeCl3-injured carotid artery thrombosis model. FeCl3-induced arterial thrombi are known to be platelet-rich thrombi. The time to the formation of stable thrombus in NOS3−/− mice is significantly prolonged compared with wild type mice (p = 0.03, n = 20 for NOS3−/− mice, n = 21 for wild type controls) (Fig. 4). These data suggest that NOS3 participates in thrombus formation and stability in vivo, which is in agreement with the observed stimulatory role of NOS3 in platelet activation.

Effects of NOS Inhibitors on Human Platelet Secretion and Aggregation—To determine whether NOS is important in human platelet aggregation and secretion, washed human platelets were preincubated with NOS inhibitors L-NAME or L-N-monomethyl-L-arginine (L-NMMA), then stimulated with low doses of thrombin. Both inhibitors reduced platelet aggregation (Fig. 5, A and E). The inhibitory effect of L-NAME was specific for the biologically relevant L form of the compound, since aggregation was not affected by D-NAME (Fig. 5, D). Furthermore, NOS substrate L-arginine significantly enhanced low dose thrombin-induced platelet aggregation (Fig. 5, F). Similar to the results in

FIGURE 2. Stimulatory roles of NOS3 in platelet activation induced by low dose U46619 and collagen. A and B, concomitantly recorded aggregation and secretion of NOS3+/+ and NOS3−/− platelets stimulated with 250 nM (A) or 500 nM U46619 (B). C and D, quantitative data of aggregation (C) and secretion (D) data from three experiments as shown in A and B, respectively. E and F, platelet aggregation and secretion induced by 0.5 μg/ml (E) or 1 μg/ml (F) collagen. G and H, quantitative data from three different experiments as shown in E and F, respectively. Note that at higher agonist concentrations secretion is still significantly reduced in NOS3−/− platelets.

FIGURE 3. Granule contents of wild type and NOS3 knock-out platelets. The total amounts of dense granule contents, ATP (A) and serotonin (B), in NOS3+/+ and NOS3−/− platelets were determined as described under “Experimental Procedures” (mean ± S.E., n = 3). C, a representative immunoblot (IB) showing that the total VWF (α granule constituent) levels are similar between NOS3+/+ (+/) and NOS3−/− (−/) platelets.

FIGURE 3. Granule contents of wild type and NOS3 knock-out platelets. The total amounts of dense granule contents, ATP (A) and serotonin (B), in NOS3+/+ and NOS3−/− platelets were determined as described under “Experimental Procedures” (mean ± S.E., n = 3). C, a representative immunoblot (IB) showing that the total VWF (α granule constituent) levels are similar between NOS3+/+ (+/) and NOS3−/− (−/) platelets.
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**FIGURE 4.** NOS3 deficiency delays formation of stable occlusive thrombi in vivo. FeCl3-induced carotid artery injury was performed, and time to occlusive thrombosis was recorded as described under "Experimental Procedures." The occlusion time of each mouse is shown as circles (NOS3+/+, n = 21) and triangles (NOS3−/−, n = 20). The bars represent the mean occlusion time ± S.D. (249.8 ± 101.7 s for NOS3+/+ and 327.4 ± 118.4 s for NOS3−/−; p = 0.029).

platelets from NOS3-deficient mice, L-NAME significantly inhibited dense granule release in human platelets (Fig. 5, A and C), whereas L-arginine significantly enhanced platelet secretion (Fig. 5F).

One of the major substances secreted from dense granules is ADP. ADP is known to stimulate integrin activation and amplify platelet aggregation. Thus, it is possible that inhibition of ADP secretion is primarily responsible for the decreased platelet aggregation in NOS inhibitor-treated platelets. Indeed, supplementation with a low concentration of ADP, insufficient to induce aggregation on its own, reversed the inhibitory effects of L-NAME (Fig. 6). Thus, NOS plays an important role in mediating platelet secretion of dense granule contents (mainly ADP), which induces the second wave of platelet aggregation.

**DISCUSSION**

The data described in this study provide evidence for a major revision of the currently accepted concept of NO signaling in platelets. Our data support a new concept that NOS3-mediated low level NO production in platelets stimulates platelet secretion and, thus, amplifies and stabilizes platelet aggregation.

The conclusion that endogenous NO production by NOS3 promotes platelet activation is supported by data that 1) NOS3 knock-out mouse platelets showed reduced platelet secretion and aggregation in response to low concentrations of thrombin, U46619, and collagen, 2) NOS3 knock-out mice showed reduced ability to form occlusive arterial thrombus in an *in vivo* thrombosis model, 3) NOS inhibitors reduced platelet secretion and aggregation, and 4) a NOS substrate, L-arginine, that enhances NO production promotes platelet secretion and aggregation. These data appear to contradict previous results that NO plays an inhibitory role in platelet activation (17, 18, 34). However, previous studies were performed under conditions of high NO concentrations. For example, the inhibitory effects of NO donors on platelet activation require relatively high concentrations of NO donors (micromolar levels), which cause a 100–200-fold elevation of intra-platelet cGMP levels (18). In contrast, we show that physiologically low levels of NO produced endogenously by NOS3 or exogenous NO donor SNP at concentrations comparable with endogenous NO production have a stimulatory effect on platelet activation. Furthermore, we show that a major mechanism for the stimulatory role of NOS3 is the NO- and cGMP-dependent activation of aggregation-dependent platelet secretion that amplifies and stabilizes platelet aggregation and thrombus formation. Thus, the *in vitro* stimulatory role of NOS3 is manifested mainly when platelets were stimulated by low concentrations of platelet agonists, which require secretion for the full scale platelet aggregation. In contrast, some previous studies were performed under the condition where platelets responded poorly to the low concentrations of platelet agonists and were characteristic of a lack of the secretion-dependent second wave platelet aggregation (35). Therefore, combined with previous find-
ingle NO inhibitors and the NOS substrate L-arginine on human platelet aggregation and secretion. A, thrombin-induced aggregation and secretion of platelets pretreated with or without L-NAME. B and C, quantitative data of the effects of L-NAME on platelet aggregation (p < 0.0001, n = 7) (B) and secretion (p = 0.0135, n = 5) (C). D and E, representative traces of aggregation of platelets pretreated with L-NAME or D-NAME (p < 0.01, n = 3) (D) or platelets pretreated with or without a different NOS inhibitor, Nω-monomethyl-L-arginine (L-NMMA) (p = 0.05, n = 3) (E). F, aggregation and ATP secretion of human platelets in response to thrombin in the presence (Arg) or absence of 100 μM L-arginine (p < 0.0001, n = 3).

FIGURE 6. The reversal effect of ADP on L-NAME-induced inhibition of platelet aggregation. Washed human platelets preincubated with L-NAME or buffer (Control) were stimulated with a low dose of thrombin. L-NAME-treated platelets were also stimulated with thrombin followed by the addition of a sub-threshold concentration of ADP (500 nM) (in washed platelets ADP is not sufficient to induce platelet secretion or induce aggregation without exogenous fibrinogen).
effects on platelets via distinct downstream signaling pathways. This new concept helps explain previous controversies on the roles of the NO-cGMP pathway in platelets and is important in further understanding the physiological roles of the NO-cGMP pathway in the vascular system.

There are two types of platelet secretion. So called "strong agonists" such as collagen and thrombin at high concentrations induce "aggregation-independent secretion." The "weak" agonists such as ADP or low concentrations of strong agonists induce integrin-dependent platelet aggregation and require integrin "outside-in" signaling to induce "aggregation-dependent platelet secretion." The thromboxane A2 analog U46619 induces a unique two-wave secretion, the first wave aggregation-independent secretion that precedes the first wave platelet aggregation and the second wave aggregation-dependent secretion that
induces the second wave platelet aggregation (22). NOS3 knock-out and NOS inhibitors inhibited the second wave aggregation-dependent secretion and the subsequent second wave platelet aggregation (Fig. 2), indicating that the primary role for NOS3 is to stimulate aggregation-dependent platelet secretion. This is consistent with our data on the role of PKG in platelet secretion, indicating NOS3 is upstream of cGMP elevation and PKG activation in stimulating platelet secretion (30).

Interestingly, a previous study indicated a role for NOS3 in insulin-induced secretion of ATP and other vasodilator compounds. However, insulin is not known to stimulate platelet activation, and thus, it was postulated that insulin-induced secretion rather than being a proaggregatory signal, results in the release of potent vasodilator compounds (39). Thus, our study establishes an important role for NOS3 in platelet secretion during platelet activation. Because it is known that aggregation-dependent platelet secretion serves to amplify platelet activation induced by low concentrations of agonists and to stabilize platelet aggregates, our data explain why NOS3 and PKG are required only in platelet aggregation induced by low concentrations of platelet agonists. It is known that circulating platelets are often exposed to low level stimulation such as high shear stress, adrenaline, low levels of coagulation, and inflammatory factors such as thrombin and platelet-activating factors that are produced in response to minor vascular injury and inflammation. Thus, it is possible that NOS3-generated NO serves to increase the sensitivity of platelets in response to vascular injuries and to stabilize thrombi at sites of vascular injury. Indeed, NOS3-deficient mice had prolonged time to occlusion in carotid artery injury model compared with wild type controls. Therefore, NOS3 plays a physiologically relevant role in promoting in vivo thrombosis.

Platelets not only secrete agonists, receptors, and pro-coagulant factors to amplify and stabilize primary thrombus but also secrete inflammatory and growth factors (such as platelet-derived growth factor (5) and cytokines (4)). These factors play important physiological roles in vascular remodeling and wound repair and important pathological roles in vascular inflammation and in the development of atherosclerosis. In this respect it is interesting to note that despite the belief that NO is anti-atherosclerotic, recent studies showed that NOS3 promoted the development of atherosclerosis (40, 41), as NOS3 knock-out mice are less prone to atherosclerosis, whereas overexpression of NOS3 in mice promotes atherosclerosis. Thus, the finding that NOS3 plays an important role in stimulating platelet secretion provides a potential mechanism for the role of NOS3 in promoting the development of atherosclerosis.

The currently accepted concept that NO inhibits platelet activation contains intrinsic contradictions. Although high concentrations of NO donors inhibit platelet activation, endogenous NO production in platelets occurs only when platelets are stimulated with various platelet agonists. Furthermore, potential NOS3 activation signals suggested in other cell types, namely, calcium elevation and the phosphoinositide 3-kinase-Akt pathway (42), all play roles in stimulating platelet activation but not in inhibiting platelet activation (22, 43–46). Thus, our data that NO in fact plays biphasic roles in platelet activation provide an explanation for this apparent contradiction. It is important to note that we have recently found that phosphoinositide 3-kinase, like NOS3, plays a critical role in aggregation-dependent platelet secretion and secretion-dependent second wave platelet aggregation (22). Others have reported a stimulatory role for Akt in platelet secretion and aggregation (45, 46). Also, calcium elevation is induced by platelet agonists and aggregation-dependent integrin outside-in signaling (47). Thus, it would be logical to hypothesize a novel platelet secretion and activation pathway in which agonist-induced calcium elevation and/or a possible agonist-induced...
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activation of the phosphoinositide 3-kinase signaling pathway induce NOS3 activation, which subsequently activates the cGMP-PKG pathway. The NOS3-cGMP-PKG pathway coordinates with integrin outside-in signaling, inducing platelet secretion and the second wave of platelet activation. Because the NOS3-cGMP-PKG pathway is important in promoting platelet secretion and aggregation under low agonist conditions, this pathway is also likely to play a major role during minor or chronic vascular injury and, thus, in the development of atherosclerosis.

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