Induction of platelet formation from megakaryocytoid cells by nitric oxide

Elisabeth Battinelli*†, Scott R. Willoughby*†, Thomas Foxall‡, C. Robert Valeri†§, and Joseph Loscalzo†¶

†Whitaker Cardiovascular Institute and Evans Department of Medicine, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118; §Naval Blood Research Laboratory, 615 Albany Street, Boston, MA 02118; and ‡University of New Hampshire, Department of Animal and Nutritional Science, Kendall Hall, Durham, NH 03824

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Although the growth factors that regulate megakaryocytopoiesis are well known, the molecular determinants of platelet formation from mature megakaryocytes remain poorly understood. Morphological changes in megakaryocytes associated with platelet formation and removal of senescent megakaryocytes are suggestive of an apoptotic process. Previously, we have established that nitric oxide (NO) can induce apoptosis in megakaryocytoid cell lines. To determine whether there is an association between NO-induced apoptosis and platelet production, we exposed Meg-01 cells to *S*-nitrosoglutathione (GSNO) with or without thrombopoeitin (TPO) pretreatment and used flow cytometry and electron microscopy to assess platelet-sized particle formation. Meg-01 cells treated with TPO alone produced few platelet-sized particles (<3% of total counts), whereas treatment with GSNO alone produced a significant percentage of platelet-sized particles (22 ± 4% of total counts); when combined with TPO pretreatment, however, GSNO led to a marked increase in platelet-sized particle production (48 ± 3% of total counts). Electron microscopy confirmed that Meg-01 cells treated with TPO and GSNO yielded platelet-sized particles with morphological features specific for platelet forms. The platelet-sized particle population appears to be functional, because addition of calcium, fibrinogen, and thrombin receptor-activating peptide led to aggregation. These results demonstrate that NO facilitates platelet production, thereby establishing the essential role of NO in megakaryocyte development and thrombopoiesis.

thrombopoiesis | apoptosis | guanylyl cyclase

The mechanism by which platelets are produced from megakaryocytes (thrombocytopoiesis) is not well understood. Megakaryocytes differentiate from pluripotent hematopoietic stem cells in a process orchestrated by a series of growth factors that regulate distinct stages of megakaryocyte maturation. These stages include proliferation of progenitor cells, nuclear polyploidization (via endomitosis), cytoplasmic maturation, and, ultimately, proplatelet formation and platelet release (1). Cytoplasmic maturation is characterized by the formation of platelet-specific granules and the development of a demarcation membrane system that delineates the platelet fields in the cytoplasm. In recent years, a theory of proplatelet development has emerged in which long cytoplasmic exvaginations form from the mature megakaryocyte cytoplasm in the terminal phase of megakaryocyte development. These proplatelets insinuate between bone marrow sinusoidal cells to enter the circulation (2). Circulatory shear force within the marrow or possibly in the pulmonary circulation results in the fragmentation of these proplatelets, thereby releasing platelets into the circulation (3, 4). Moreover, Italiano and colleagues have recently demonstrated that the number of platelets released per megakaryocyte is enhanced by coordinated bending and bifurcation of proplatelet processes (5).

The recently discovered growth factor thrombopoeitin (TPO) is essential for megakaryocyte proliferation and maturation. Its role in platelet formation, however, is unclear. Nagahisa *et al.* recently showed that TPO induces morphological changes in megakaryocytes resulting in the formation of lengthy, beaded cytoplasmic processes that are similar in appearance to the cytoplasmic projections of the proplatelet (6). Others, however, have shown that, although TPO is essential for megakaryocyte production and maturation, it plays only an indirect role in proplatelet formation (7, 8). A secondary role of TPO in platelet formation has been supported by the phenotype of mice in which the gene for TPO has been deleted. These TPO-null mice are only marginally thrombocytopenic yet are able to produce platelets, albeit at a reduced rate (15%) (9). This observation suggests that other yet undiscovered factors may be critical for platelet formation.

Previously, we have reported that exogenous and endogenous forms of nitric oxide (NO) can induce apoptosis in megakaryocytes (10). To attempt to establish an association between NO-induced apoptosis and platelet formation, we explored the role of NO in the terminal stages of megakaryocytopoiesis, including proplatelet formation, platelet release, and ultimately megakaryocyte death. In this paper, we show that treatment with NO can lead to platelet-sized particle production from the human megakaryocytoid cell line Meg-01, as measured by flow cytometry and viewed by scanning and transmission electron microscopy. This response can be augmented by factors that increase the maturation stage of the megakaryocytes, including treatment with TPO and/or cell synchronization. Meg-01 cells from which NO induced production of functional platelet-sized particles were also found to become apoptotic in the process. These results demonstrate that NO facilitates platelet production during the final stages of megakaryocytopoeisis.

Methods

Cell Culture. The properties of the human megakaryocytoblastic cell line Meg-01 have been described in detail previously (11). Meg-01 cells are grown in RPMI 1640 medium (GIBCO) supplemented with 10% FBS (GIBCO) at 37°C in a humidified atmosphere of 5% CO_2. For cell maturation experiments, cells were exposed to 100 ng/ml of TPO, a generous gift of Amgen Biologicals. For cell synchronization experiments, the cells were grown without FBS for 24 h.

Nitrite/Nitrate Assay. Nitrate was converted to nitrite by incubation with nitrate reductase (Sigma) in the presence of NADPH (Sigma). Total nitrite was then measured by the Griess reaction (12).

Flow Cytometry. Samples were fixed by adding 0.3% formaldehyde [final concentration, ultrapure methanol-free formaldehyde (Polysciences)] for 30 min at room temperature diluted

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Abbreviations: GSNO, *S*-nitrogoglutathione; TPO, thrombopoeitin; TRAP, thrombin receptor-activating peptide; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase.

*E.B. and S.R.W. contributed equally to this work.

¶To whom reprint requests should be addressed. E-mail: jloscalz@bu.edu.

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with PBS buffer and stored at 4°C until labeled with monoclonal antibody for flow cytometry. Aliquots of this preparation were incubated with FITC-conjugated anti-GPIIIa monoclonal antibody (CD41, Dako) at a near-saturating concentration for 20 min at room temperature. Samples were then analyzed in a Coulter EPICS XL flow cytometer. DNA check flow cytometry beads purchased from Coulter were used for daily instrument calibration. Appropriate color compensation was set for FITC fluorescence and phycoerythrin fluorescence by using 525- and 575-nm band pass filters, respectively. All data were saved in flow cytometry listmode files and analyzed by using Coulter ELITE software, Ver. 2.21. Platelet-sized particles were identified by characteristic log forward light scatter. Only those particles that bound the anti-GPIIIa-FITC antibody (CD41 FITC-conjugated antibody) (Dako, Denmark) were analyzed (by gating the fluorescence signal).

Annexin V Labeling. Annexin V labeling was monitored by using the Apoptosis Detection Kit manufactured by R & D Systems. Briefly, cells were washed with cold PBS twice and resuspended in a binding buffer provided by the manufacturer. Fluorescein-labeled annexin V and propidium iodide were added to the cells. The cells were then analyzed immediately by flow cytometry by using the Coulter EPICS cytometer, which is equipped with a single laser emitting excitation at 488 nm. The annexin V-FITC-generated signal was detected in the FITC signal detector (FL1), whereas the propidium iodide signal was detected by the phycoerythrin emission signal detector (FL2).

Aggregation. Putative culture-derived platelets (platelet-sized particles) were isolated by centrifuging the cell suspensions at 120 × *g* and collecting the supernatant from treated and untreated cells. To each tube containing the platelet-sized particles in the supernatant, aggregation was assayed by adding 2.5 mM $CaCl_2$, 1 mM thrombin receptor-activating peptide (TRAP), and 1 mg/ml fibrinogen. The samples were incubated at room temperature for 10 min and then analyzed for GPIIIa by using flow cytometry as described above to identify a size shift.

Scanning Electron Microscopy. Cultured cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate, pH 7.2, for 4 h and then gently filtered onto Nucleopore filters (Millipore). The filters were rinsed with cacodylate buffer (0.1 M cacodylate, pH 7.2) twice under vacuum, then fixed with 1% osmium tetroxide for 1 h and dehydrated through a graded series of ethanol concentrations. Hexamethyldisilizane was then added, and the samples were air-dried. The filters were mounted onto aluminum stubs and coated with gold palladium alloy for viewing in a AMR scanning electron microscope (Bedford, MA).

Transmission Electron Microscopy. Cultured cells were harvested and pelleted by centrifugation. The pellets were fixed with 3% glutaraldehyde (Polysciences) in cacodylate buffer (0.1 M, pH 7.2) for 4 h at room temperature. The pellet was gently rinsed twice with buffer before a secondary fixation in 1% osmium tetroxide (Polysciences) in cacodylate buffer at 4°C for 1 h. The cells were dehydrated through a graded series of ethanol concentrations and embedded in Poly/Bed 812 resin (Polysciences) with propylene oxide as an intermediate solvent. Thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100S transmission electron microscope. Results were photodocumented.

Collection of Platelet-Rich Plasma from Mice. After withdrawal of blood samples from the aortae of euthanized mice into syringes containing 10% of 90 mM trisodium citrate to prevent coagulation, blood was carefully placed in a polypropylene tube (17 × 119 mm) and centrifuged at 190 × *g* to prepare platelet-rich plasma. The platelet-rich plasma was collected and platelet counts determined by using a Coulter counter, model Z.

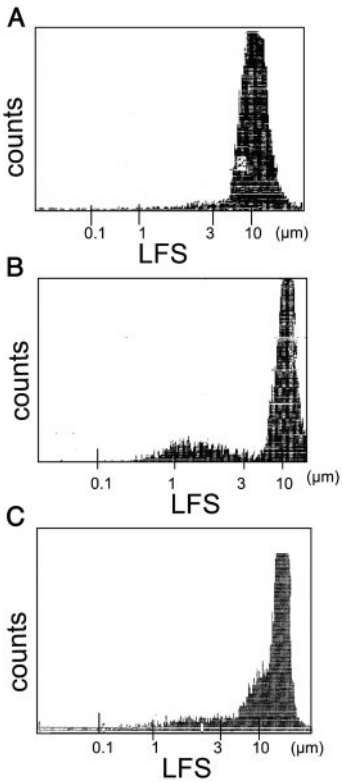

Fig. 1. Meg-01 cells treated with GSNO. Meg-01 cells were incubated with FITC-conjugated anti-GPIIIa antibody and analyzed by flow cytometry. Data are expressed as a number of events (counts) vs. particle size (LFS, log forward scatter); only those particles that bound the anti-GPIIIa antibody were analyzed. (*A*) Untreated Meg-01 cells; (*B*) Meg-01 cells treated with 100 μM GSNO for 2 h showing GPIIIa-positive Meg-01 cells in addition to a population of platelet-sized particles; (1–3 μm) and (*C*) Meg-01 cells treated with 10 ng/ml of IL-1β, 10 ng/ml of tumor necrosis factor-α, 10 ng/ml of IFN-γ, and endotoxin for 16 h to induce iNOS showing a population of GPIIIa-positive Meg-01 cells in addition to a population of platelet-sized particles.

cGMP Analysis. Cells were collected by centrifugation, and 6% trichloroacetic acid was added for precipitation. The cGMP was extracted four times by using distilled water–saturated ether. For cGMP measurements, a standard ELISA assay (Caymen Chemicals, Ann Arbor, MI) was used.

Results

NO Induction of Platelet-Sized Particle Formation. To determine whether treatment with the NO donor GSNO led to the production of platelet-sized particles from mature megakaryocytoid cells, the megakaryocyte cell line Meg-01 was incubated with 100 μM GSNO for 2 h. After this incubation period, an aliquot of the cell suspension was removed and labeled with a monoclonal antibody against the platelet cell-surface marker GPIIIa. Flow cytometry measurements showed that treatment with GSNO led to the formation of a population of GPIIIa-positive particles that were smaller in size than megakaryocytes and similar in size to platelets (Fig. 1*B*). This same population was not observed in control cells not incubated with GSNO (Fig. 1*A*). The percentage of GPIIIa-positive platelet-sized particles (as function of total particles counted) observed after treatment of the Meg-01 cells with the NO donor was 22 ± 4%, with untreated cells showing no significant formation of platelet-sized particles (<1%) (Fig. 1*A*).

Cytokine Induction of Inducible Nitric Oxide Synthase (iNOS) in Meg-01 Cells and Platelet-Sized Particle Formation. Previously, we showed that incubation of megakaryocytes with interleukin–1β (IL-1β), tumor necrosis factor-α, IFN-γ, and endotoxin leads to the induction of iNOS in megakaryocytoid cells (10). We next analyzed whether treatment with these cytokines to induce iNOS would result in NO production that could lead to the formation of platelet-sized particles. A small percentage of platelet-sized particles was evident after cytokine stimulation of Meg-01 iNOS (8.4%) (Fig. 1*C*), an effect that was not evident in cells not treated with the cytokines.

Fibroblast Coculture and NO-Induced Platelet Production. In an attempt to determine whether stromal cells present in the bone marrow milieu of megakaryocytes may be a source of NO that promotes thrombopoiesis, we cocultured Meg-01 cells with a representative stromal cell, the fibroblast. Fibroblasts that have previously been shown to produce NO on cytokine induction of iNOS were grown in the upper chamber of a two-chamber well. After overnight incubation with 10 ng/ml IL-1β, 10 ng/ml tumor necrosis factor-α, and 10 ng/ml IFN-γ to induce iNOS, the fibroblast medium was changed, and these NO-producing cells were then incubated in a co-well in which the lower chamber contained Meg-01 cells. Meg-01 cells exposed to fibroblasts in which iNOS was induced exhibited formation of platelet-sized particles (8.9%). Platelet-sized particles were not detected in Meg-01 cell cultures exposed to uninduced fibroblasts, nor were platelet-sized particles produced in cultures treated with the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) in conjunction with cytokines (data not shown).

Thrombopoietin and NO-Induced Platelet Formation. The megakaryocyte growth factor thrombopoeitin (TPO) is essential for the maturation and growth of the megakaryocyte. To determine whether the presence of TPO augmented the effects of NO, Meg-01 cells were grown in the presence of 100 ng/ml of TPO for 72 h before addition of GSNO. After incubation with TPO, medium was removed, and the cells were washed with PBS and then resuspended in fresh RPMI 1640 medium without the addition of TPO. These cells were then incubated with 100 μM GSNO for 2 h and the cell suspension labeled with the anti-GPIIIa antibody for analysis by flow cytometry. In comparison to GSNO treatment alone (Fig. 1*B*), preincubation with TPO followed by GSNO treatment led to an almost 2-fold increase in the number of platelet-sized particles, i.e., 48 $\pm$ 3%, and more than a 10-fold increase in comparison to cells treated with TPO alone, i.e., 3 $\pm$ 1% (Fig. 2 *A* and *B*). In coculture experiments, preincubation of Meg-01 cells with TPO also increased the percentage of platelet-sized particles produced when the Meg-01 cells were incubated with fibroblasts induced to express iNOS (23%) (Fig. 2*C*).

To demonstrate that the fibroblasts were producing NO and that this NO was accessible to the Meg-01 cells in the lower chamber, the oxidative byproducts of NO, nitrite and nitrate, were assayed in the medium of the lower chamber. Meg-01-conditioned medium contained 6 μM/mg/ml of protein total nitrite and nitrate (NO_x), which was significantly greater than that measured in the medium collected from cells cocultured with uninduced fibroblasts (0.6 μM/mg/ml of protein).

Cell Synchronization and Platelet-Sized Particle Formation. To create a clonally mature homogeneous population of Meg-01 cells in which to study the process of platelet formation by NO, cells were grown for 24 h in RPMI 1640 medium without FBS. After the starvation period, cells were collected, washed with PBS, and fed with new RPMI 1640 that was supplemented with both 10% FBS with or without the addition of 100 ng/ml TPO before treatment with GSNO. Cell synchronization in the presence or absence of TPO led to the formation of GPIIIa-positive platelet-sized particles after

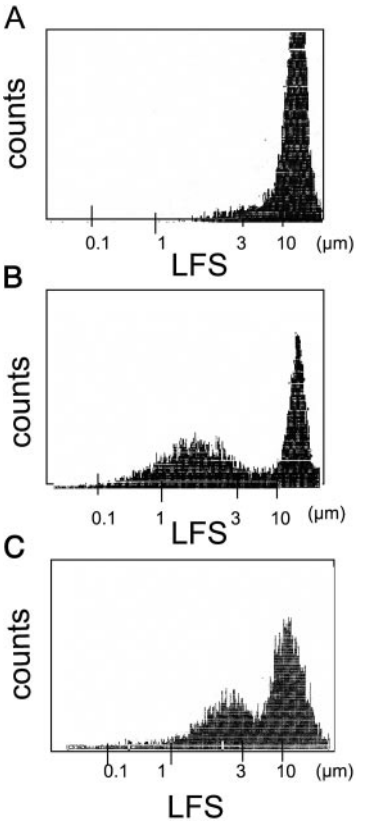

Fig. 2. TPO pretreatment and GSNO-induced platelet-sized particle formation from Meg-01 cells. Data are expressed as a number of events (counts) vs. particle size (LFS, log forward scatter); only those particles that bound the anti-GPIIIa antibody were analyzed. (*A*) Meg-01 cells were pretreated with 100 ng/ml TPO for 72 h. Cells were labeled with FITC-conjugated GPIIIa-antibody and analyzed by flow cytometry. (*B*) Meg-01 cells were pretreated with 100 ng/ml TPO for 72 h before addition of 100 μM GSNO for 2 h. An abundant population of GPIIIa-positive platelet-sized particles is visible in addition to the Meg-01 cells. (*C*) Meg-01 cells were pretreated with TPO and grown in coculture with fibroblasts induced to express iNOS. A population of GPIIIa-positive Meg-01 cells is noted in addition to the large population of platelet-sized particles.

treatment with GSNO. In the synchronized cell cultures treated with TPO for 72 h before addition of GSNO for 2 h, the platelet-sized particle yield was 62%. Fig. 3 compares the extent of platelet-sized particle formation by the different treatment regimens.

Functional Responses of Platelet-Sized Particles. To determine whether the platelet-sized particles observed by flow cytometry were functional platelets, aggregation was measured by means of flow cytometry. Meg-01 cells were treated with GSNO with or without TPO pretreatment, and the platelet-sized particles were collected by centrifugation at 120 $\times$ *g* for 10 min to remove remaining megakaryocytes. The platelet-sized particle supernatant was then exposed to calcium, fibrinogen, and TRAP to attempt to induce aggregation. In treated cell cultures, aggregation was evident by the shift to the right of the platelet-sized particles (Fig. 4 *A* and *B*). Cells treated with TPO before GSNO also showed a shift to the right (Fig. 4 *C* and *D*), as did synchronized cells that were pretreated with TPO before GSNO exposure (Fig. 4 *E* and *F*). This shift in particle size was not observed in the supernatant of untreated Meg-01 cells (data not shown).

Platelet Counts in iNOS (−/−) and Endothelial Nitric Oxide Synthase (eNOS) (−/−) Mice. We collected blood from the aortae of age-matched wild-type (+/+), iNOS (−/−), and eNOS (−/−)

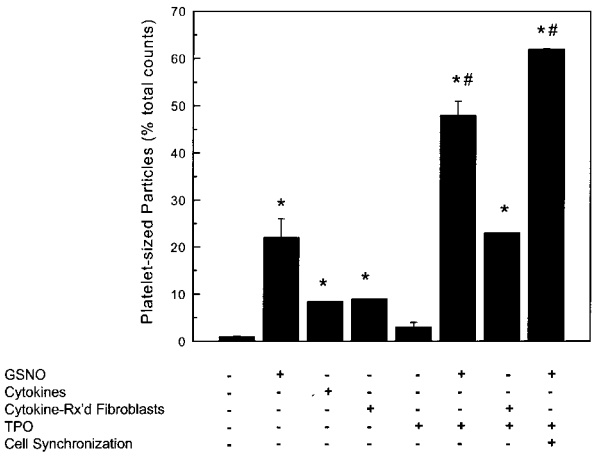

Fig. 3. Comparison of treatments used to induce platelet-sized particle formation. Meg-01 cells were treated with 100 μM GSNO for 2 h; treated with cytokines (10 ng/ml of IL-1β, 10 ng/ml of tumor necrosis factor-α, 10 ng/ml of INF-γ, and endotoxin) for 16 h before no additional treatment or GSNO treatment; exposed to cytokine-treated cocultured fibroblasts; treated with 100 ng/ml of TPO for 72 h before no additional treatment, treatment with GSNO, or exposure to cytokine-treated cocultured fibroblasts; or grown in media without FBS for 24 h to induce cell synchronization before treatment with TPO and then GSNO. Each bar represents the results of two to five experiments each performed in duplicate. *, $P < 0.02$ compared with untreated control; #, $P < 0.03$ compared with GSNO or TPO treatment alone.

mice and measured the platelet counts in platelet-rich plasma. In 17-week-old knockout mice, we found a 50% decrease in platelet count in iNOS (−/−) in comparison to strain- and age-matched

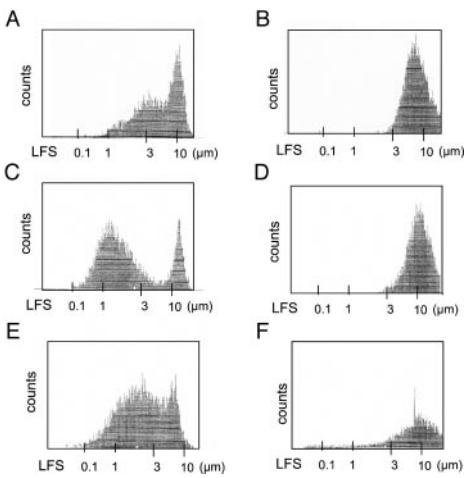

Fig. 4. Aggregation of culture-derived platelet-sized particles. Data are expressed as a number of events (counts) vs. particle size (LFS, log forward scatter); only those particles that bound the anti-GPIIIa antibody were analyzed. (*A*) Culture supernatant of Meg-01 cells treated with GSNO for 2 h collected by centrifugation at 120 × *g* showing a decreased percentage of megakaryocytoid cells in the culture; (*B*) culture supernatant incubated with 2.5 mM $CaCl_2$, 1 mg/ml fibrinogen, and 1 mM TRAP showing a shift to the right (larger size) indicating aggregation; (*C*) culture supernatant of Meg-01 cells treated 100 ng/ml TPO for 72 h before treatment with GSNO for 2 h was collected by centrifugation showing decreased percentage of megakaryocytes in the culture; (*D*) TPO pretreated culture supernatant incubated with TRAP, calcium, and fibrinogen showing a shift to the right (larger size) indicating aggregation; (*E*) culture supernatant of Meg-01 cells synchronized and treated with 100 ng/ml TPO for 72 h before treatment with GSNO for 2 h was collected by centrifugation showing decreased percentage of megakaryocytes in the culture; (*F*) synchronized and TPO-treated culture supernatant incubated with TRAP, calcium, and fibrinogen showing a shift to the right (larger size) indicating aggregation.

Table 1. Platelet count and NOS isoforms

Group	Platelet count,/μl	Number of animals
Wild type (+/+)	569,000 ± 400	7
iNOS (−/−)	320,000 ± 700	10
eNOS (−/−)	540,000 ± 600	3

control animals and eNOS (−/−) animals: the iNOS null mice (−/−) had an average platelet count of 320,000 ± 700/μl, whereas the wild-type animals had an average platelet count of 569,000 ± 400/μl, a highly statistically significant difference ($P = 0.00325$). No difference was noted between the platelet counts of the eNOS (−/−) mice (540,000 ± 600/μl) and wild-type mice (Table 1).

Platelet-Sized Particles and Apoptotic Bodies. As we showed previously that NO can induce apoptosis in megakaryocytes (10), we sought to establish that NO-induced platelet-sized particles were distinct in size from the apoptotic bodies that are remnants of senescent Meg-01 cells. To measure the formation of apoptotic bodies after treatment of Meg-01 cells with NO, we used the phosphatidylserine label, annexin V. Meg-01 cells were treated with 100 μM GSNO, washed and stained by using FITC-conjugated annexin V antibody, then analyzed by flow cytometry. Treatment with 100 μM GSNO led to the formation of a population of particles distinct in size from platelet-sized particles (≈0.1 μM diameter; data not shown). Because these bodies were annexin V-positive, they may represent the remnants of the apoptotic process. Importantly, these apoptotic bodies stained positive with propidium iodide, whereas the platelet-sized particles (not containing DNA) did not.

Scanning Electron Microscopy. Scanning electron microscopy of Meg-01 cells showed clear signs of platelet production (Fig. 5). Cell surfaces of megakaryocytes revealed pseudopod formation and membrane blebbing, a precursor of platelet production. On

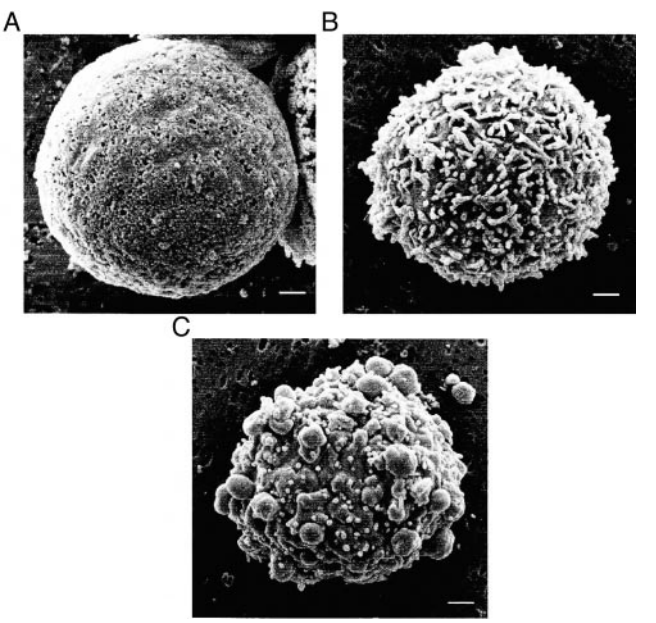

Fig. 5. Classification of Meg-01 cell maturation by scanning electron microscopy. (*A*) Meg-01 cells with no pseudopod formation or membrane blebbing (Stage 1); (*B*) Meg-01 cells with only pseudopod formation (Stage 2); and (*C*) Meg-01 cells with pseudopod formation, extensive membrane blebbing and distinctive platelet-sized particles (Stage 3). Size marker = 1 μm.

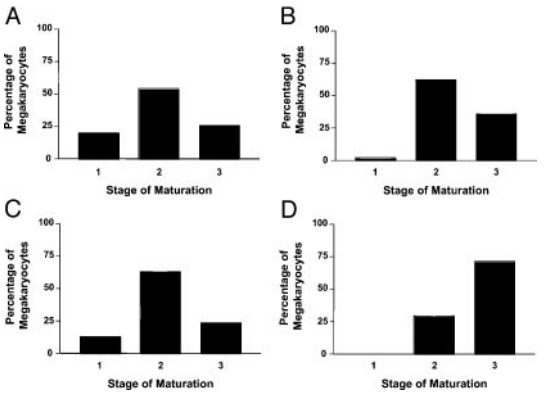

Fig. 6. Stage of maturation of Meg-01 cells. (*A*) Untreated Meg-01 cells; (*B*) Meg-01 cells treated with 100 μM GSNO for 2 h; (*C*) Meg-01 cells treated with TPO for 72 h; and (*D*) Meg-01 cells pretreated with TPO for 72 h before treatment with 100 μM GSNO for 2 h. Cells were scored by an independent reviewer blinded to treatment group according to the classification in Fig. 6. Number of cells counted per group was 100–150.

the basis of a previous classification (13), the megakaryocytes could be grouped into three distinct stages: stage 1, immature (Fig. 5*A*); stage 2, pseudopod formation (Fig. 5*B*); and stage 3, membrane blebbing and platelet-sized particle formation (Fig. 5*C*). Histogram analysis of cell forms revealed that Meg-01 cells exposed to GSNO in combination with TPO (Fig. 6*D*) demonstrated characteristics of maturation and platelet formation in comparison to untreated cells (Fig. 6*A*) or cells treated with GSNO alone (Fig. 6*B*) or TPO alone (Fig. 6*C*).

Transmission Electron Microscopy. To examine the morphology of cells in greater detail, transmission electron microscopy was performed. Meg-01 cells pretreated with TPO and GSNO demonstrated features characteristic of platelet production, including pseudopod extensions, vacuolization, demarcation membrane formation, membrane blebbing, and platelet-size particle formation (Fig. 7). Furthermore, the resulting platelet-sized particles contained dense and α-granules, as well as a dense cannalicular system.

cGMP Analysis. To determine whether NO-mediated platelet-sized particle production is associated with an established mediator of NO's bioactivity, cGMP measurements were determined in cell lysates from Meg-01 cells treated with 100 μM GSNO for varying times. As early as 5 minutes after treatment, levels of cGMP were increased over the level found in untreated cells, and this cyclic nucleotide continued to increase during the first 30 min of treatment. Interestingly, TPO completely suppressed the increase in cGMP activity seen in cells treated with GSNO for 30 min (Table 2).

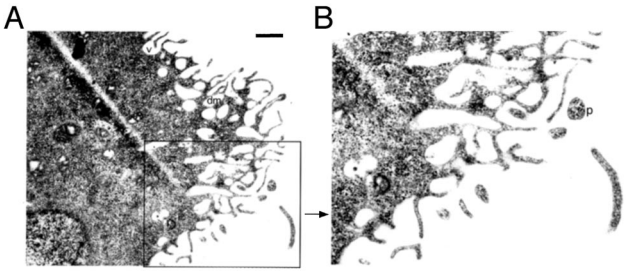

Fig. 7. Transmission electron micrograph of Meg-01 cell. (*A*) Representative Meg-01 cell showing membrane vacuolization, demarcation membrane formation, and membrane blebbing (size marker 5 μm). (*B*) Higher magnification showing platelet-sized particles (p) with dense and α-granules.

Table 2. Cyclic GMP and GSNO in Meg-01 cells

Treatment group	pmol cGMP/mg protein ($n = 2$)
Untreated	1.3
5 min, 100 μM GSNO	263.1
10 min, 100 μM GSNO	461.2
20 min, 100 μM GSNO	472.9
30 min, 100 μM GSNO	611.3
30 min, 100 μM GSNO in presence of TPO	0.96

Discussion

We have established that treatment with GSNO, cytokine induction of megakaryocyte NO production, or cytokine induction of fibroblast NO production can all lead to platelet formation from a megakaryocytoid line. Platelet formation is further enhanced by factors that stimulate megakaryocyte maturation, including TPO and cell synchronization. The platelet-sized particles that are produced appear to be functional in that they aggregate in response to agonists. Mice in which the iNOS gene has been deleted have one-half the platelet count of wild-type and eNOS ($-/-$) mice, supporting the importance of endogenous iNOS-derived NO in thrombocytopoiesis.

Two recent animal studies have suggested that NO may play a role in platelet production. Molnar *et al.* treated rats with prolonged blockade of NO synthases by using a continuous i.v. infusion of the NOS inhibitor, L-nitroarginine. In these animals, the platelet count was found to be 50% lower than that of the control animals infused with saline (14). In a second study, Nagase *et al.* observed that Wistar rats treated with L-NAME also had a significant reduction in platelet count (15). These two studies and the data presented here in iNOS ($-/-$) mice suggest that inhibition or absence of iNOS-derived NO is associated with decreased platelet production leading to reduced circulating platelet counts.

On the basis of our results, we hypothesize that NO acts in the terminal stages of megakaryocytopoiesis, specifically at the stage of platelet release. Previously, it has been shown that TPO acts as an essential maturation factor before the formation of proplatelets from megakaryocytes, whereas NF-E2 is an essential transcription factor activated in the proplatelet stage. NF-E2 is a heterodimeric protein belonging to the basic leucine zipper family. When Shivdasani *et al.* generated a mouse with targeted disruption of the gene for the p45 subunit of the transcription factor, they observed the mice sustained a high mortality rate from hemorrhage secondary to the absence of circulating platelets (16). Although numerous megakaryocytes were found in the bone marrow, they had not undergone the process of cytoplasmic

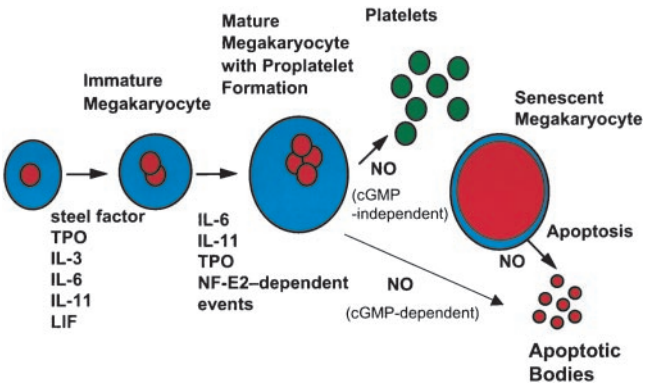

Fig. 8. Determinants of megakaryocytopoiesis and thrombopoiesis. LIF, lymphocyte inhibitory factor.

maturation demonstrated by the finding that the cytoplasm did not contain a demarcation membrane system, nor were platelet granules formed (17). In an attempt to rescue the mice from thrombocytopenia, TPO was given with the hope that its effects would result in increased platelet formation. Although the megakaryocytes were able to respond to TPO by increasing in size and ploidy, they were still not able to produce platelets (18).

Our data suggest that the role of NO is downstream of both TPO and transcriptional regulation by NF-E2 since NF-E2 is constitutively active in this immortalized cell line (data not shown). In Meg-01 cells, TPO alone produced few platelets, yet it appears that exposure to TPO before addition of NO enhanced platelet production. We hypothesize that TPO prepares the Meg-01 cells for platelet production by facilitating maturation and terminal differentiation. Our results also suggest that NO functions independently of NF-E2 transcriptional activity. Because it appears that there is a decrease in NF-E2 expression after exposure of Meg-01 cells to NO (data not shown), we hypothesize that NO acts downstream from NF-E2. Thus, the presence of decreased NF-E2 protein expression and decreased DNA binding on exposure to NO may be caused by increased degradation of NF-E2 such that megakaryocyte maturation and proplatelet formation are terminated to initiate platelet elaboration. We therefore speculate that NF-E2 and TPO act at early stages of platelet formation such that megakaryocytic cytoplasmic maturation essential for proplatelet development can occur before the involvement of NO in the platelet release phase. This concept of NF-E2 as an early effector of platelet formation is supported by the fact that the basal level of active NF-E2 is high in the Meg-01 cell line before treatment with NO (data not shown).

We have previously shown that NO plays a role in megakaryocyte turnover through induction of apoptosis (10). We now hypothesize that NO-induced apoptosis is related to the process by which megakaryocytes produce platelets (Fig. 8). By using annexin V labeling, we have shown that megakaryocytes producing platelets are simultaneously undergoing apoptosis. After platelets have been formed from the megakaryocyte cytoplasm, the megakaryocyte remains as a naked nucleus that is removed by macrophages in a process suggestive of apoptosis (19). Previously, it has been reported that megakaryocytes die by a paraapoptotic process (20). We therefore suggest that one explanation for the apoptosis seen in megakaryocytes producing platelets is the result of removal of "spent" senescent megakaryocytes. Yet another possibility is that apoptosis and platelet production are simultaneous events. Morphological observations of megakaryocytic maturation and thrombopoiesis are suggestive of an apoptotic response. As the megakaryocyte matures, its nucleus becomes eccentric in location and the nuclear-to-cytoplasmic ratio increases; this, along with platelet blebbing, is suggestive of an apoptotic process. In the experiments presented here, the cells producing platelets also showed signs of apoptosis.

Because a primary mechanism of NO's action involves activation of guanylyl cyclase, thereby increasing cellular cGMP, analysis of cGMP responses was performed in association with both platelet release and apoptosis. Treatment with NO led to a time-dependent increase in cGMP activity in Meg-01 cells. Surprisingly, we found that TPO completely suppressed the NO-mediated increase in cGMP formed in Meg-01 cells treated with NO. Pretreatment of Meg-01 cells with TPO led to no detectable increase in cGMP levels over basal levels in comparison to cells that were treated with NO alone. Because our previous experiments have shown that TPO suppresses NO-induced apoptosis yet enhances NO-induced platelet formation, it appears that NO-mediated apoptosis may be cGMP-dependent, whereas NO's ability to induce platelet formation does *not* depend on cGMP (10). To address the role of guanylyl cyclase in platelet formation and apoptosis in Meg-01 cells, we treated the cells with the cGMP analog 8-Br-cGMP. Treatment with 8-Br-cGMP was found to produce apoptosis, but no platelet formation was observed (data not shown). Because TPO enhances platelet-sized particle production yet attenuates NO-induced increases in cGMP, the importance of this signal in NO-induced apoptosis is clear, whereas its role in platelet production is not immediately discernible. One potential explanation is that TPO may function to enhance platelet production and simultaneously suppress cGMP responses in an attempt to produce functional platelets in an environment rich in NO, a known cGMP-dependent inhibitor of platelet function.

The data presented here show that a megakaryocytoid cell line can be stimulated by NO alone or in combination with TPO to produce functional platelet-sized particles. These experiments provide a model with which to study the process of platelet formation and offer a system in which to develop potential treatments for disorders of platelet formation.

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