Ceramide Triggers Weibel–Palade Body Exocytosis

Rinky Bhatia,* Kenji Matsushita,* Munekazu Yamakuchi, Craig N. Morrell, Wangsen Cao, Charles J. Lowenstein

Abstract—The sphingolipid ceramide mediates a variety of stress responses, including vascular inflammation and thrombosis. Activated endothelial cells release Weibel–Palade bodies, granules containing von Willebrand factor (vWF) and P-selectin, which induce leukocyte rolling and platelet adhesion and aggregation. We hypothesized that ceramide induces vascular inflammation and thrombosis in part by triggering Weibel–Palade body exocytosis. We added ceramide to human aortic endothelial cells and assayed Weibel–Palade body exocytosis by measuring the concentration of vWF released into the media. Exogenous ceramide induces vWF release from endothelial cells in a dose-dependent manner. Activators of endogenous ceramide production, neutral sphingomyelinase, or tumor necrosis factor-α also induce Weibel–Palade body exocytosis. We next studied NO effects on ceramide-induced Weibel–Palade body exocytosis because NO can inhibit vascular inflammation. The NO donor S-nitroso-N-acetylpenicillamine decreases ceramide-induced vWF release in a dose-dependent manner, whereas the NO synthase inhibitor Nω-nitro-L-arginine methyl ester increases ceramide-induced vWF release. In summary, our findings show that endogenous ceramide triggers Weibel–Palade body exocytosis, and that endogenous NO inhibits ceramide-induced exocytosis. These data suggest a novel mechanism by which ceramide induces vascular inflammation and thrombosis. (Circ. Res. 2004;95:319-324.)

Key Words: nitric oxide • sphingomyelin • granule • endothelial cells • N-ethylmaleimide sensitive factor

Inflammation and thrombosis play major roles in atherosclerosis pathogenesis. Activated endothelial cells release Weibel–Palade bodies, granules that contain procoagulant and proinflammatory substances including multimeric von Willebrand factor (vWF), P-selectin, and CD63.1–5 Interleukin-8 (IL-8), thromboplastinogen, calcitonin gene–related peptide, and endothelin have been reported in Weibel–Palade bodies as well.6–9 Exocytosis of Weibel–Palade bodies is triggered by a variety of agonists, including thrombin, histamine, complement, leukotrienes, superoxide anions, epinephrine, adenosine, and vasopressin.10–15 Weibel–Palade body exocytosis is mediated by calcium, cAMP, and G-proteins.10,11,15,16 After Weibel–Palade body exocytosis, P-selectin is translocated to the endothelial surface, where it facilitates leukocyte rolling, and vWF is released into the vessel lumen, where it mediates platelet adhesion and aggregation.17,18

NO is a messenger molecule that mediates vasodilation and inhibits vascular inflammation.19–24 NO inhibits atherogenesis in mice, and lack of NO synthesis is associated with atherosclerosis in humans.25–28 NO inhibits atherogenesis by multiple mechanisms such as blocking smooth muscle cell proliferation, decreasing leukocyte adhesion, and inhibiting platelet adhesion and aggregation.29–35 NO blocks nuclear factor κB (NF-κB)–directed transcription of proinflammatory molecules within endothelial cells.36 We showed recently that NO inhibits Weibel–Palade body exocytosis.37

Sphingolipids found in plasma membranes of all eukaryotic cells play an important signaling role in vascular inflammation and thrombosis.38–41 The sphingolipid ceramide mediates a variety of stress responses.41 Ceramide is produced de novo from serine condensation with palmitoyl-coenzyme A in the endoplasmic reticulum or from hydrolysis of sphingomyelin by sphingomyelinase in the plasma membrane, cytosol, lysosomes, or endoplasmic reticulum.42 A variety of factors induce ceramide production, including tumor necrosis factor-α (TNF-α), IL-1β, interferon-γ, Fas ligand, B7, CD40 ligand, oxidized low-density lipoprotein (LDL), ischemia, radiation, and chemotherapeutic agents.38–41 Ceramide and its metabolites serve as intracellular second messengers in pathways mediating inflammation, thrombosis, apoptosis, cell differentiation, proliferation, and vasomotor regulation by acting on a variety of phosphatases, proteases, kinases, phospholipases, and transcription factors (protein phosphatase [PP] 1, PP2A, cathepsin, protein kinases, endothelial differentiation gene receptors, NF-κB, mitogen-activated protein kinase, plasminogen activator inhibitor-1 (PAI-1), nicotinamide adenine dinucleotide phosphate oxidase, and endothelial NO synthase [NOS]).36–41,43–49

Ceramide can induce

---

Original received September 22, 2003; resubmission received May 3, 2004; revised resubmission received June 3, 2004; accepted June 11, 2004. From the Division of Cardiology, Department of Medicine (R.B., K.M., M.Y., C.N.M., W.C., C.J.L.) and the Departments of Comparative Medicine and Pathology (C.N.M.), The Johns Hopkins University School of Medicine, Baltimore, Md.

*These authors contributed equally to this work.

This manuscript was sent to Donald Heistad, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

Correspondence to Charles J. Lowenstein, 950 Ross Bldg, The Johns Hopkins University School of Medicine, 720 Rutland Ave, Baltimore, MD 21205.

E-mail clowenst@jhmi.edu

© 2004 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org

DOI: 10.1161/01.RES.0000136519.84279.7a
vascular inflammation by activating transcriptional pathways, but ceramide can also rapidly trigger vascular inflammation by nontranscriptional pathways, although the precise mechanism by which it does so is unclear.

We hypothesized that ceramide promotes rapid vascular inflammation in part by triggering Weibel-Palade body exocytosis. We furthermore proposed that NO will block ceramide-induced exocytosis.

Materials and Methods

Materials

Ceramide was purchased from Matreya; dihydroceramide and sphingosine 1-phosphate from Biomol; neutral sphingomyelinase from Bacillus cereus, thrombin, histamine, acetylpencillamine (AP), vascular endothelial growth factor (VEGF), N\(^2\)-nitro-arginine methyl ester (L-NAME), 1,2-bis(2-aminoxyloxy)ethane-\(N,N,N',N'\)-tetraacetic acid tetrakis (acetoxymethyl ester; BAPTA-AM), and TNF-\(\alpha\) from Sigma; DMEM, DMEM without calcium, PBS, and trypsin-EDTA from Gibco; vWF ELISA kit from American Diagnostica; dimethyl sulfoxide from J.T. Baker; 5-nitroso-N-acetylpencillamine (SNAP) from Cayman Chemical; and endothelium-based medium (EBM-2) with growth factors (FBS, hydrocortisone, VEGF, R3-insulin-like growth factor 1, ascorbic acid, and heparin) and EBM-2 without growth factors from Clonetics.

Cell Culture

Human aortic endothelial cells (HAECs) were obtained from Clonetics (Walkersville, Md) and grown in EGM-2 media with growth factors until 80% confluent. Cells were then washed with PBS, treated with trypsin-EDTA, and centrifuged at 1200 rpm for 5 minutes. The supernatant was removed, the cell pellet was resuspended in EBM-2 with growth factors, and 100 \(\mu\)L of the cell suspension was placed into each well of a 96-well plate and incubated for 24 hours at 37°C. The cells were then washed and reincubated in 100 \(\mu\)L of EBM-2 without growth factors.

Measurement of Ceramide-Induced Weibel-Palade Body Exocytosis

HAECs were pretreated or not with the NOS inhibitor L-NAME 1 mmol/L for 16 hours at 37°C, or with the NO donor SNAP or its control AP for 4 hours at 37°C, or with VEGF for 2 hours. Some HAECs were pretreated with BAPTA-AM or calcium-free media for 30 minutes, or with desipramine 5 \(\mu\)mol/L for 10 minutes. HAECs were then treated with various amounts of ceramide, neutral sphingomyelinase, or TNF-\(\alpha\) for 1 hour. Supernatants were harvested, stored at \(-20^\circ\)C, and later analyzed for vWF concentration using an ELISA (American Diagnostica). To confirm that the process of exocytosis was being studied, we also examined the ceramide-triggered release of IL-8. HAECs were stimulated with 10 \(\mu\)mol/L ceramide for 0 to 60 minutes, and the amount of IL-8 released into the media was measured by an ELISA (R&D Systems).

Statistical Analysis

Results were expressed as mean±SD. Significance between mean values was determined by the Student \(t\) test, with a value of \(P<0.05\) considered significant.

Figure 1. Exogenous ceramide activates Weibel-Palade body exocytosis. A, Dose-response. Increasing amounts of ceramide were added to HAECs for 1 hour, and the concentration of vWF released into the media was measured by an ELISA \((n=2±SD; \*P<0.01\) compared with \(0\) \(\mu\)mol/L; this experiment was repeated 3 \(\times\) with similar results). B, Time course; 1 U/mL thrombin or 10 \(\mu\)mol/L ceramide or its negative control 10 \(\mu\)mol/L dihydroceramide was added to HAECs for 1 hour, and the concentration of vWF released into the media was measured by an ELISA \((n=2±SD; \*P<0.01\) compared with \(0\) \(\mu\)mol/L). C, Ceramide activates IL-8 exocytosis over time; 1 U/mL thrombin or 10 \(\mu\)mol/L ceramide was added to HAECs for 1 hour, and the concentration of IL-8 released into the media was measured by an ELISA \((n=3±SD; \*P<0.01\) compared with \(0\) \(\mu\)mol/L). D, Comparison with other agonists of exocytosis; 10 \(\mu\)mol/L ceramide, 1 mmol/L histamine, 1 U/mL thrombin, or media alone was added to HAECs for 1 hour, and the concentration of vWF released into the media was measured by an ELISA \((n=2±SD; \*P<0.01\) compared with media). E, Ceramide has an insignificant effect on endothelial apoptosis. HAECs were treated with ceramide for 24 hours, and apoptosis levels were measured by 4',6-diamidino-2-phenylindole staining for fragmented chromatin (black bars; \(n=4±SD\)). As a positive control, HAECs were treated with 1 mmol/L hydrogen peroxide for 24 hours (gray bars; \(n=2±SD\); \*P<0.01 compared with media). F, Ceramide effects on total vWF in endothelial cells. HAECs were treated with 10 \(\mu\)mol/L ceramide, 1 U/mL thrombin, or 10 \(\mu\)mol/L cycloheximide (CHX) for 1 hour. Cells were washed, and vWF concentrations in cell lysates were measured by an ELISA \((n=3±SD; \*P<0.05\) vs Control).
Results

Ceramide Triggers Weibel-Palade Body Exocytosis

To explore the effect of ceramide on Weibel-Palade body exocytosis, we treated HAECS with ceramide for 1 hour and measured the concentration of vWF in the media by an ELISA. Ceramide activates vWF release from endothelial cells in a dose-dependent manner (Figure 1A). Ceramide induces vWF release over time, starting within 5 minutes of treatment and continuing through 60 minutes after treatment (Figure 1B). To confirm that ceramide activates exocytosis, we studied the effect of ceramide on the release of IL-8, another component of Weibel-Palade bodies. Ceramide activates endothelial release of IL-8 (Figure 1C). Ceramide is almost as effective in stimulating endothelial cell exocytosis as classical triggers of Weibel-Palade body release such as thrombin or histamine (Figure 1D). To confirm that ceramide does not damage endothelial cells, we treated HAECS with increasing amounts of ceramide for 24 hours and measured apoptosis by counting the number of cells with fragmented chromatin. Ceramide does not activate endothelial cell apoptosis (Figure 1E).

The effect of endogenous ceramide on Weibel-Palade body exocytosis was evaluated by adding to HAECS various concentrations of neutral sphingomyelinase, which hydrolyzes sphingomyelin to ceramide. The concentration of vWF in the media was measured 1 hour later using an ELISA. Sphingomyelinase activates vWF release from endothelial cells in a dose-dependent manner (Figure 2).

To examine the effect of endogenous sphingomyelinase on ceramide-induced exocytosis, we added various doses of TNF-α, an activator of sphingomyelinase, to HAECS for 1 hour. TNF-α stimulates vWF release in a dose-dependent manner (Figure 3A). This effect was partially blocked by desipramine, an inhibitor of acidic sphingomyelinase (Figure 3B). The addition of an inhibitor of neutral sphingomyelinase has no effect. Together, these data suggest that endogenous ceramide synthesis activates endothelial exocytosis.

Calcium plays a role in exocytosis of Weibel-Palade bodies. To determine whether ceramide induces exocytosis of Weibel-Palade bodies in a calcium-dependent manner, we pretreated HAECS with BAPTA or calcium-free media for 30 minutes and then incubated HAECS with 1 μmol/L ceramide for 1 hour. Pretreatment with BAPTA partially decreases ceramide-induced exocytosis, whereas pretreatment with calcium-free media does not affect Weibel-Palade body exocytosis (Figure 4). Pretreatment with BAPTA and calcium-free media also decreases Weibel-Palade body exocytosis (Figure 4). These data suggest that intracellular calcium pools mediate ceramide induction of endothelial cell exocytosis.

Discussion

The major finding of this study is that endogenous ceramide activates Weibel-Palade body exocytosis. Furthermore, NO inhibits ceramide-induced exocytosis.
Ceramide mediates a variety of stress responses including vascular inflammation.\textsuperscript{38,41} Multiple physical and biological stimuli activate ceramide synthesis, including ischemia and reperfusion, oxidized LDL, and inflammatory cytokines.\textsuperscript{38–41,50,51} Ceramide and its metabolites mediate vascular inflammation by increasing endothelial cell expression of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule, thereby inducing leukocyte and monocyte adhesion to the vascular wall.\textsuperscript{52–54} These activated leukocytes and macrophages secrete cytokines, which further induce inflammation and ceramide production. Ceramide also mediates lipoprotein accumulation in the vascular wall, and ceramide levels are increased in atherosclerotic plaques compared with the normal vessel wall.\textsuperscript{55–58} In addition, ceramide has been implicated in pathways leading to proliferation of smooth muscle cells.\textsuperscript{50,51,59} Ceramide is also involved in generation of reactive oxygen species, which leads to further inflammation and tissue injury.\textsuperscript{59} Our data show a novel mechanism by which ceramide might rapidly activate vascular inflammation. By triggering Weibel-Palade body exocytosis, ceramide increases endothelial P-selectin expression, which can induce leukocyte adhesion and aggregation to the vascular wall.

Ceramide also activates multiple pathways leading to vascular thrombosis. The ceramide metabolite sphingosine 1-phosphate induces platelet aggregation.\textsuperscript{60} In addition, ceramide induces endothelial cell release of prothrombotic tissue factor and PAI-1.\textsuperscript{61,62} Ceramide and its metabolites promote apoptosis of endothelial cells, which may play a role in plaque erosion and further thrombosis.\textsuperscript{63–65} Our data show a novel mechanism by which ceramide might induce thrombosis. By activating Weibel-Palade body exocytosis, ceramide induces vWF release, which can lead to platelet adhesion and aggregation.

Ceramide has 2 opposing effects on endothelial cells. In addition to triggering proinflammatory and prothrombotic pathways, ceramide and its metabolite sphingosine 1-phosphate also activate endothelial NOS (NOS3).\textsuperscript{63–69} NO has multiple effects on the vasculature, including inhibition of vascular inflammation and thrombosis.\textsuperscript{19–23} One mechanism by which NO decreases vascular inflammation is blocking Weibel-Palade body exocytosis.\textsuperscript{37} We found that ceramide activates a greater release of vWF when NOS is inhibited (Figure 5B). One possible explanation for this phenomenon is that ceramide normally activates exocytosis and NO, and NO then inhibits further exocytosis. Our results might partially explain why patients with endothelial dysfunction who cannot synthesize normal levels of NO have higher levels of vascular inflammation and a greater risk for atherosclerosis than patients with normal endothelial function and normal NO production.

In conclusion, our study shows that ceramide triggers Weibel-Palade body exocytosis and that NO blocks ceramide-induced exocytosis. These data suggest a novel mechanism by which ceramide induces vascular inflammation and thrombosis.

Acknowledgments
This research was supported in part by American Heart Association Established Investigator Grant EIG 0140210N (C.J.L.); National Institutes of Health grants P01 HL56091, P01 HL65608, NIH R01 HL53615, R01 HL63706 (C.J.L.); RR07002, and HL074945 (to C.M.); the Ciccarone Center for the Prevention of Heart Disease (C.J.L.); and the Cora and John H. Davis Foundation (C.J.L.).

References


Ceramide Triggers Weibel–Palade Body Exocytosis
Rinky Bhatia, Kenji Matsushita, Munekazu Yamakuchi, Craig N. Morrell, Wangsen Cao and Charles J. Lowenstein

Circ Res. 2004;95:319-324; originally published online June 24, 2004;
doi: 10.1161/01.RES.0000136519.84279.7a

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/95/3/319

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research 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 Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/