Abstract—Neointimal hyperplasia at the site of surgical intervention is a common and deleterious complication of surgery for cardiovascular diseases. We hypothesized that direct delivery of a cell-permeable growth-arresting lipid via the balloon tip of an embolectomy catheter would limit neointimal hyperplasia after stretch injury. We have previously demonstrated that sphingolipid-derived ceramide arrested the growth of smooth muscle cell pericytes in vitro. Here, we show that ceramide-coated balloon catheters significantly reduced neointimal hyperplasia induced by balloon angioplasty in rabbit carotid arteries in vivo. This ceramide treatment decreased the number of vascular smooth muscle cells entering the cell cycle without inducing apoptosis. In situ autoradiographic studies demonstrated that inflating the balloon catheter forced cell-permeable ceramide into the intimal and medial layers of the artery. Intercalation of ceramide into vascular smooth muscle cells correlated with rapid inhibition of trauma-associated phosphorylation of extracellular signal–regulated kinase and protein kinase B. These studies demonstrate the utility of cell-permeable ceramide as a novel therapy for reducing neointimal hyperplasia after balloon angioplasty. (Circ Res. 2000;87:282-288.)

Key Words: hyperplasia ■ angioplasty ■ ceramide ■ smooth muscle ■ MAP kinase

Restenosis still persists as a major complication in the maintenance of vessel patency after percutaneous transluminal coronary angioplasty (PTCA). Restenosis is a consequence of multiple factors, including vessel recoil, negative vascular remodeling, residual plaque burden, and neointimal hyperplasia.1,2 Neointimal hyperplasia reflects the migration and proliferation of vascular smooth muscle (VSM) cells with subsequent deposition of extracellular matrix components at the site of injury.1,3 Considerable evidence indicates that, in restenosis, growth factors stimulate the VSM cells to proliferate, resulting in a thickening of the tunica intima.4 Nearly 40% of all patients develop significant luminal narrowing within 6 months after angioplasty procedures.4 Consequently, despite the initial therapeutic benefits of angioplasty, within a few months after surgery, blood flow through the affected vessels can again become compromised. Conventional therapies, which include angiotensin-converting enzyme inhibitors, anticoagulants, and statins, are ineffective in preventing or reducing neointimal hyperplasia after stretch injury.1,5 Endovascular radiation therapy has shown some success in both animal and human trials, yet the long-term deleterious effects of this therapy on the artery have not been adequately evaluated.1,6 We propose that direct delivery, to the site of vascular injury, of a cell-permeable lipid that blocks growth factor–mediated signaling cascades has the potential to reduce neointimal hyperplasia without systemic complications.

Sphingolipids are ubiquitous membrane lipids that serve as substrates for the formation of second messengers.7 Ceramide, a second messenger derived from cytokine receptor–activated sphingomyelin catabolism, stimulates differentiation, inhibits proliferation, and has been associated with apoptosis.7 We previously demonstrated that increasing endogenous ceramide concentration by inhibition of ceramide catabolism induces growth arrest in smooth muscle pericytes.8 Moreover, cell-permeable ceramide (C₆-ceramide) mimics the effect of interleukin-1 to inhibit both tyrosine kinase receptor–linked and G protein receptor–linked mitogenesis in A7r5 aortic smooth muscle cells and rat glomerular mesangial cells.8–10 In vitro, ceramide inhibits VSM cell proliferation by differentially regulating members of the mitogen-activated protein kinase (MAPK) cascade. Ceramide stimulates c-Jun N-terminal kinases (JNKs), whereas it suppresses extracellular signal–regulated kinases (ERKs).10,11 In addition, ceramide could regulate mitogenesis by inhibiting cell survival kinases, such as protein kinase B (PKB).12 The experiments described here were designed to determine whether a cell-permeable ceramide could diminish VSM cell proliferation in vivo and, if so, to characterize the mechanisms responsible for this effect.

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Ceramide-Coated Balloon Catheters Limit Neointimal Hyperplasia After Stretch Injury in Carotid Arteries

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Materials and Methods

Animal Model

We chose the carotid artery of the New Zealand white rabbit as a model system for neointimal hyperplasia after stretch injury. The rabbit carotid artery responds to stretch injury with marked, rapid, and reproducible neointimal hyperplasia.13–15 In addition, this model serves as an excellent source for explanted and cultured smooth muscle cells so that in vivo studies can be confirmed with in vitro experiments.13

The details of the balloon angioplasty procedure are described in the online Materials and Methods (available at http://www.circresaha.org). Briefly, the left carotid artery was exposed and a small incision was made in the vessel 20 mm above the bifurcation of the internal and external carotid. A 3F Intimax arterial embolectomy catheter from Applied Medical Vascular Division was inserted retrograde into the common carotid artery 70 mm below the incision. The balloon was inflated to 4 mm, which distended the vessel 3-fold. The inflated balloon was withdrawn 50 mm, deflated, rotated 120°, and inserted back to the original position in the common carotid. This procedure was repeated 3 times.

Lipid Therapeutics

The lipid gels were applied to the catheters by dipping the balloons 10 times into a DMSO/ethanol, 1:1 vol/vol solution containing 5 mmol/L C6-ceramide (d-erythro-N-hexanoylsphingosine) or dihydro-C6-ceramide (d-erythro-N-hexanoylsphinganine) (Biomol), interspersed with drying under nitrogen. The coated latex balloon catheter, inserted into 50 mmol/L ceramide solution, remained intact after 50 inflations as evidenced by enveloping the balloon with a loading dye. In situ autoradiography with radiolabeled C6-ceramide was used to document the pharmacokinetics of ceramide transfer to carotid arteries, and details of these methods can be found in the online Materials and Methods.16–19 (available at http://www.circresaha.org).

Immunohistochemistry

An adapted “ABC” (avidin-biotin-peroxidase complex) procedure was used to stain for α-smooth muscle cell actin and proliferating cell nuclear antigen (PCNA) 2 weeks after angioplasty.16,20

Apoptosis Measurement

We initially assessed apoptosis of primary VSM cells isolated from rabbit carotid arteries by fluorescence-activated cell sorting after propidium iodide staining.15 To confirm these measurements, we also assessed apoptosis in situ by quantifying the percentage of pyknotic propidium iodide– or hematoxylin-stained nuclei per arte-}

Results

Experiments were designed to determine whether ceramide-coated embolectomy catheters would diminish neointimal hyperplasia after balloon-induced stretch injury. Initial studies assessed the extent of neointimal hyperplasia in rabbit carotid arteries after balloon angioplasty as a function of time. Animals were euthanized at 15 and 60 minutes as well as at 1, 2, 4, and 6 weeks after balloon injury. Marked neointimal hyperplasia was observed as early as 1 week and peaked at 4 weeks (Figure 1A). Neointimal hyperplasia was not observed in damaged arteries 15 and 60 minutes after angioplasty. Sham-treated carotid arteries showed no signs of neointimal hyperplasia at any time point. On the basis of these results, we chose to investigate the effects of ceramide on dynamic VSM growth 2 weeks after balloon injury.

Figures 1B through 1E show hematoxylin and eosin (H&E)–stained cryostat sections of rabbit carotid arteries 2 weeks after balloon injury. In addition to the sham-treated control artery (B), the 3 treatment groups included a vehicle-treated balloon (C), a C6-ceramide–coated balloon (D), and a dihydro-C6-ceramide–coated balloon (E). Quite strikingly, C6-ceramide treatment significantly reduced the neointimal hyperplasia induced by balloon angioplasty. A quantitative analysis revealed that balloon catheters coated with C6-ceramide diminished the number of neointimal concentric cell layers by \(~50\%\) (Figure 2A). This corresponds to a reduction of neointimal thickness from 0.21±0.06 to 0.12±0.09 mm. As a control for the lipid vehicle, noncoated balloon embolectomy catheters always induced the same degree of neointimal hyperplasia as vehicle-coated balloons. In accordance with Komukai et al15 and Negoro et al,22 we also quantified neointimal stenosis as a ratio of neointimal/medial cross-sectional areas and showed a 92% reduction of stenosis with ceramide treatment (Figure 2B). Stretch injury induced a slight but significant increase in medial hypertrophy that was not reduced by ceramide treatment (Figure 2C). Dihydro-C6-ceramide, an inactive analogue of C6-ceramide, did not significantly reduce neointimal hyperplasia, nor did it reduce medial hypertrophy after balloon injury (Figures 2A through 2C). Thus, the selective reduction in neointimal hyperplasia after stretch injury requires bioactive ceramide, and this effect cannot be mimicked using structurally similar

![Figure 1. C6-ceramide but not dihydro-C6-ceramide limited neointimal hyperplasia after balloon angioplasty in rabbit carotid arteries. A, Time course of neointimal hyperplasia after angioplasty. Each time point depicts 2 to 6 arteries. B through E, Representative H&E-stained sections from excised arteries 2 weeks after angioplasty. These photomicrographs are representative of 5 to 8 arteries. B, Sham-treated control artery. C, Artery treated with a DMSO/ethanol (1:1, vol/vol)–coated balloon. D, Artery treated with a C6-ceramide–coated balloon. E, Artery treated with dihydro-C6-ceramide, a biologically inactive form of ceramide. Bar=200 μm.](image-url)
but inactive lipids. It can be inferred that the effects of ceramide are due to biochemical actions and not to lipophilic properties.

We next assessed the pharmacokinetics of ceramide transfer and delivery from the balloon catheter to the damaged artery. Using \(^{[3]H}\)C\(_6\)-ceramide as a tracer, we calculated that 70 ± 10 nmol of C\(_6\)-ceramide was applied to the balloon as a gel from a solution of 5 μmol of C\(_6\)-ceramide. Figure 3A shows that, after insertion and inflation, ∼12 ± 2 nmol remained on the balloon. This translates to roughly 58 nmol of C\(_6\)-ceramide being transferred from the balloon catheter during the angioplasty procedure. To test whether inflation of the balloon within the carotid artery was essential for optimal transfer of the ceramide, we repeated the surgical procedure using noninflated balloons. The recovered ceramide mass on the inserted but noninflated balloon was 14 ± 3 nmol. We next asked whether the difference in ceramide mass between the inflated and noninflated balloons (∼2 nmol) corresponded to the calculated mass of \(^{[3]H}\)C\(_6\)-ceramide isolated from damaged carotid arteries. Rabbit carotid arteries treated with radiolabeled lipid were homogenized, and lipid products were separated by thin-layer chromatography (TLC) (Figure 3A, inset). The mass of intact ceramide isolated 15 minutes after angioplasty was 2.7 ± 0.4 nmol for inflated balloon treatments and 0.7 ± 0.2 nmol for noninflated balloon treatments. The amount of ceramide recovered from excised tissues did not differ significantly from the amount of ceramide transferred to the tissue as a consequence of balloon inflation. As the transferred ceramide was initially delivered to 0.0365 cm\(^3\) of carotid artery luminal volume, the effective concentration of ceramide at the site of balloon injury was estimated to be 1.5 mmol/L. Thus, we suggest that an effective and reproducible dose of ceramide can be delivered to the damaged artery as a consequence of the balloon inflation.

We next used in situ autoradiography to document arterial penetrance for \(^{[3]H}\)C\(_6\)-ceramide transferred from the balloon catheter after angioplasty (Figures 3B through 3D). Compared with unlabeled arteries (panel B), \(^{[3]H}\)C\(_6\)-ceramide was observed throughout the medial layers of the artery 15 minutes after angioplasty (panel C). This increase in pixel intensity reflects an increase in intact ceramide, as at this time point ∼89 ± 4% of the radiolabel comigrates with authentic C\(_6\)-ceramide standards. Pixel intensity was more intense in inflated (panel C) versus noninflated (panel D) arteries. Expressed as pixel density per square millimeter for 10 randomly selected blocks with background values subtracted, medial staining was increased 4.7 ± 0.2-fold for ceramide-coated inflated versus noninflated balloons. Again, this supports the finding that balloon inflation leads to maximal delivery and penetrance. Thus, a lipid-coated balloon delivers a therapeutic dose of ceramide to tissues underlying the site of vascular stretch injury. These studies also suggest that a short-term application of cell-permeable ceramide is sufficient to completely penetrate injured arteries and to reduce intimal proliferation despite an inflammatory milieu.

We next assessed degradation of the rapidly intercalated radiolabeled ceramide by TLC. For the 15-minute postangioplasty time point, 89 ± 4% of the TLC-separated lipid comigrated with authentic C\(_6\)-ceramide standards. This corresponded to a recovered mass of 2.7 ± 0.4 nmol of ceramide. At 60 minutes after angioplasty, 1.3 ± 0.6 nmol of ceramide was recovered. Thus, ∼50% radiolabel can still be recovered as intact ceramide in 1 hour. This decrease in ceramide mass corresponds to an increase in TLC-separated gangliosides and cerebrosides but not sphingosines (data not shown).
To prevent thrombus formation, patients routinely receive anticoagulants before PTCA. Thus, the consequences of anticoagulation therapy on the effectiveness of ceramide therapy were investigated. Neither ceramide- nor vehicle-treated balloon angioplasty induced thrombus formation. Lovenox, a low molecular weight heparin, administered subcutaneously (2.5 mg/kg) for 7 days after surgery, did not by itself diminish neointimal hyperplasia. Nor did it augment ceramide-induced inhibition of neointimal hyperplasia (data not shown). These findings suggest that ceramide treatment is equally effective in both anticoagulated and untreated rabbits.

We next examined the effects of ceramide treatment on VSM cell growth in vivo 2 weeks after angioplasty. Immunohistochemical techniques were used to identify VSM cells using smooth muscle cell-specific α-actin antibody (Figures 4A and 4B) and cell growth using PCNA antibody (Figures 4C through 4F). The positive staining with the actin antibody indicates that VSM cells or myofibroblasts were a major component of balloon injury–induced neointimal formation (panel B). Also, this photomicrograph shows dramatic balloon angioplasty–induced ruffling and dispersion of VSM cells in the medial layer. PCNA is synthesized in early G1 and S phases of the cell cycle and thus can be used as a marker for...
cell proliferation. In Figures 4C through 4F, representative photomicrographs depicting PCNA-positive staining are shown for control, balloon-injured, ceramide-treated, and dihydro-ceramide–treated carotid arteries, respectively. The percentage of PCNA-positive cells in balloon-injured arteries (2.8% ± 0.1%) was dramatically increased compared with control vessels (0.2 ± 0.1%). C6-ceramide (0.6 ± 0.2%) but not dihydro-C6-ceramide (1.9 ± 0.3%) diminished the number of PCNA-positive cells in the neointimal layer but not in the medial layer of the carotid artery (n = 4 to 8 experimental arteries, P < 0.05, 1-way repeated-measures ANOVA, 6 arteries. Data are mean ± SEM; P > 0.05).

Figure 5. C6-ceramide did not induce appreciable cellular apoptosis in carotid artery explants or in VSM cells in situ. A, Apoptosis by fluorescence-activated cell sorting after propidium iodide staining in explanted and cultured cells from untreated carotid arteries. n = 4 cultures, P < 0.05, Student t test. B, Apoptosis in situ by quantifying the number of pyknotic nuclei in H&E-stained arterial sections at 15 to 60 minutes after angioplasty. These experiments were analyzed by a double-blind method; 1-way repeated-measures ANOVA, 6 arteries. Data are mean ± SEM; P > 0.05.

Minimal morphological injury was observed after acute stretch injury. Representative H&E-stained sections from control and stretch-injured carotid arteries 15 and 60 minutes after angioplasty. A through C, Control, vehicle-treated balloon, and C6-ceramide-coated balloon arteries 15 minutes after angioplasty, respectively. The arrow reflects endothelial cells present in panel A but not in panels B or C. D, Carotid artery 60 minutes after angioplasty. The small arrow marks neutrophils and the large arrow macrophages; the phenotypes of which were confirmed by immunohistochemistry. These photomicrographs are representative of 3 arteries for each condition and time point. Bar=50 μm.

We next investigated both early morphological and biochemical determinants for an inflammatory or proliferative phenotype in stretch-injured VSM cells. Figures 6A through 6D show H&E staining of control (Figure 6A), vehicle-coated (Figure 6B), or ceramide-coated (Figures 6C and 6D) arteries at 15 or 60 minutes after angioplasty. There was minimal evidence of macrophage or neutrophil invasion (panel D), which was confirmed by immunohistochemistry with antibodies to either induced significant apoptosis in primary VSM cells isolated from rabbit carotid arteries. Primary cultured rabbit VSM cells treated with 5 μmol/L C6-ceramide or dihydro-C6-ceramide for either 24 or 40 hours showed <1% apoptotic cell death. As a control, okadaic acid treatment (100 nmol/L) significantly induced apoptosis after 24 hours (52 ± 3%) and 40 hours (69 ± 2%) (Figure 5A). To confirm these studies, apoptosis was assessed in situ at time points when apoptotic medial cells were identified after balloon angioplasty injury.14 Minimal pyknotic nuclei were evident in either vehicle-treated or ceramide-treated arteries at 15 to 60 minutes after angioplasty (Figure 5B). In data not shown, pyknotic nuclei were not observed in sections from ceramide-treated arteries 2 weeks after angioplasty. In addition, we were unable to observe any evidence of apoptotic cells in stretch-injured arteries at any time point by in situ end labeling of nicked DNA (data not shown). Taken together, it is suggested that cell-permeable ceramide limits stenosis by arresting VSM cell growth without inducing significant apoptosis.
arteries. A, Representative Western blot for ERK2 and PKB after ceramide-coated balloon angioplasty in rabbit carotid arteries. A, Representative Western blot for ERK2 and PKB probed using phosphorylation-specific antibodies. Lysates from NIH3T3 cells treated with or without platelet-derived growth factor were used as positive and negative controls, respectively. B and C, Immunoblot data. n=4 carotid preparations for each time point and condition; mean±SEM. Stars indicate P<0.05, Student t test.

macrophages (RAMIII, DAKO) or neutrophils (LY6G, Pharmingen) (data not shown). Positive controls for these antibodies included thrombolytic arteries.

Even though there was little evidence of severe clinical damage, there were significant elevations in the phosphorylation states of critical kinases involved in the proliferative response to stretch injury. Evidence from in vitro studies suggests that ceramide arrests cell growth by inhibiting the growth factor–induced ERK cascade and possibly by inhibiting the PKB cascade. Thus, to elucidate mechanisms by which ceramide limits neointimal hyperplasia, the phosphorylation states of critical kinases involved in the proliferative and inflammatory responses of VSM cells to stretch injury.

Discussion
Rapid advances in the field of sphingolipid-based signal transduction have identified several metabolic products as potential targets for pharmacological manipulation. Receptor-generated ceramide has been implicated in growth regulation, apoptosis, and cellular differentiation in vitro. The present study extends these observations to an in vivo model of arterial stretch injury as a consequence of balloon angioplasty. We demonstrate that a cell-permeable ceramide selectively limits neointimal hyperplasia without inducing significant apoptosis. In addition, we further demonstrate the utility of delivering cell-permeable ceramide directly to the site of vascular injury by applying the bioactive lipid as a gel on the balloon catheter itself. Our studies indicate that the efficacy of therapy might be a consequence of physical force, which transfers ceramide from the inflated balloon to the site of vascular lesions. Pharmacokinetic studies indicate that the transferred ceramide rapidly penetrates the medial layer of the VSM at a therapeutic dose. Thus, intra-arterial delivery of cell-permeable ceramide has a high likelihood of clinical success, as the failure of experimentally effective therapies to succeed in clinical trials is often the consequence of suboptimal dosing being delivered to the site of injury for the appropriate duration.

It is noteworthy that stretch injury resulted in rapid changes in ERK and PKB activities that preceded marked signs of inflammation. The sustained phosphorylation of these kinases most likely reflects continuous remodeling of damaged arteries. The downregulation of both ERK and PKB activities within 15 minutes of ceramide treatment argues very strongly for the seminal roles played by these mitogenic and cell survival pathways in the pathology of neointimal hyperplasia. The rapid inhibition of kinase activity precedes any substantive morphological changes as assessed by H&E staining. We are intrigued by the observation that ceramide treatment inhibits PKB activity leading to growth arrest without apoptosis. This might reflect the fact that the cell cycle transcription factor E2F is downstream of PKB. Regardless of mechanism, direct administration of ceramide to the site of vascular injury results in a chronic inhibition of kinase signaling cascades linked to mitogenesis.

Even though altered ceramide metabolism has been implicated in atherosclerosis, diabetes mellitus, and cancer, ceramide analogues have not yet been considered as therapeutics for proliferative vascular diseases. Increased concentrations of lactosyl- and glyceroceramide conjugates at the expense of endogenous ceramide were noted in models of atherosclerosis and diabetes mellitus, and this diminished level of ceramide correlated with VSM cell proliferation and vasoconstriction. Thus, it is logical to consider the use of exogenous ceramide analogues as antimitogenic agents.

We have used an animal model that responds to stretch injury with significant and reproducible neointimal hyperplasia. However, restenosis in humans reflects other mechanisms, such as vessel recoil and negative vascular remodeling, in addition to neointimal hyperplasia. The interactions between these complications are only now being identified. Growth factors that induce neointimal hyperplasia also contribute to vessel narrowing caused by recoil through inflammatory and myofibroproliferative mechanisms. In addition, adventitial proliferation and fibrosis may also contribute to negative vascular remodeling. Therefore, it is possible that delivery of antiproliferative, cell-permeable lipid therapeutics that block growth factor signaling cascades can contribute to a decrease in restenosis after PTCA through multiple mechanisms.
Documentation that cell-permeable ceramide can be used as an efficacious treatment for neointimal hyperplasia after stretch injury has important ramifications for control of dysregulated smooth muscle proliferation not only after angioplasty but also after stent placement, hemodialysis access failure, and diabetic retinopathy. In fact, neointimal formation is more significant after stenting than after balloon angioplasty. Our studies demonstrating that ceramide delivery is an effective treatment in a model of neointimal hyperplasia after stretch injury argue for the applicability and efficacy of ceramide-coated stents. The ability to deliver the bioactive lipid directly at the site of injury has strong clinical potential. In addition to delivering this drug on the tip of balloon catheters or through infusion ports, antimitogenic ceramide analogues can be delivered as components of conventional or cationic liposomal vectors, potentially augmenting the efficacy of gene transfer and targeting strategies.

In this report, we have demonstrated that intra-arterial delivery of ceramide analogues via the balloon tip of embolectomy catheters is technically feasible and targets the drug precisely where it is needed. Use of endogenous lipid-derived metabolites as well as lipomimetic drugs promises high efficacy with low toxicity. This study establishes ceramide analogue–coated balloon catheters as an efficacious therapy to reduce neointimal hyperplasia after stretch injury. Moreover, this study documents a signal transduction mechanism responsible, in part, for ceramide-induced VSM growth arrest in vivo.

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