ANG II-induced neointimal growth is mediated via cPLA$_2$- and PLD$_2$-activated Akt in balloon-injured rat carotid artery

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Angiotensin II (ANG II) promotes neointimal growth in the balloon-injured rat carotid artery. However, the mechanism by which ANG II stimulates neointimal growth during vascular injury is not known. In cultured vascular smooth muscle cells, ANG II activates Akt through cytosolic phospholipase A$_2$ (cPLA$_2$)-dependent phospholipase D$_2$ (PLD$_2$). This study was conducted to determine whether ANG II-induced neointimal thickening is mediated via cPLA$_2$- and PLD$_2$-activated Akt in balloon-injured rat carotid arteries. ANG II-stimulated neointimal growth was inhibited by exposure of the injured carotid arteries to an adenovirus containing a dominant negative Akt mutant (intima-to-media ratio from 3.01 ± 0.31 to 1.44 ± 0.14, P < 0.01) or a retrovirus containing cPLA$_2$ small interfering RNA (siRNA; intima-to-media ratio from 3.01 ± 0.31 to 1.16 ± 0.36, P < 0.001) or PLD$_2$ siRNA (intima-to-media ratio from 3.01 ± 0.31 to 1.33 ± 0.11, P < 0.001). The effect of cPLA$_2$ and PLD$_2$ siRNA to reduce the ANG II-induced increase in neointimal thickening was associated with reduced expression of cPLA$_2$ and PLD$_2$ as determined by immunohistochemical analysis in injured carotid arteries. Western blot analysis showed that Akt phosphorylation that was increased by ANG II was inhibited in injured carotid arteries 2 days after exposure to cPLA$_2$ or PLD$_2$ siRNA or in injured arteries isolated after exposure to these agents for 30 min and then placed in tissue culture media for 24 h in the presence of these agents. These data suggest that the ANG II-induced neointimal growth is mediated by the activation of Akt through a mechanism dependent on cPLA$_2$ and PLD$_2$ activation in balloon-injured rat carotid arteries.

The major pathophysiological process that is common to carotid artery stenosis and other vascular diseases involves injury of the endothelium and proliferation, migration, and accumulation of smooth muscle cells in the intima (37). Acute arterial injury promotes vascular smooth muscle cell (VSMC) proliferation and migration across the internal elastic lamina where replication of VSMCs results in neointimal formation (38). The rat carotid artery model of transluminal balloon catheter angioplasty has provided much insight into the pathogenesis of intimal thickening after injury and is a good model for the study of restenosis after injury associated with angioplasty.

Several growth factors including angiotensin II (ANG II) have been implicated in neointimal growth and restenosis in injured arteries (38, 46). The infusion of ANG II in rats has been shown to augment intimal thickening in balloon-injured carotid arteries (5, 16). The intimal thickening caused by injury in the rat carotid artery is inhibited by ANG II type 1 receptor (AT$_1$R) antagonists, suggesting an AT$_1$R-mediated effect of ANG II during this process (16, 46, 48), although the AT$_2$R antagonist also decreased neointimal growth after balloon injury (46). ANG-converting enzyme inhibitors also reduce neointimal formation in the rat carotid model of injury (16, 32). However, the mechanism by which ANG II promotes intimal thickening remains elusive, because ANG II is a poor mitogen for VSMCs in vitro (11).

ANG II activates both cytosolic phospholipase A$_2$ (cPLA$_2$) and phospholipase D (PLD) in VSMCs (9, 34), and the increase in PLD activation is dependent on cPLA$_2$ activity (30). PLA$_2$ hydrolyzes phospholipids into free fatty acids and lysophospholipids. Arachidonic acid (AA) is the main fatty acid generated by PLA$_2$ activation, and it is metabolized by cyclooxygenase (COX), lipoxigenase (LO), and cytochrome P-450 (CYP) enzymes into various biologically active products (3). The inhibition of 85-kDa cPLA$_2$ is associated with reductions in DNA synthesis in VSMCs, suggesting a possible relationship between cPLA$_2$ and proliferation (25, 45), whereas the inhibition of a 14-kDa secretory PLA$_2$ with SB-203347 [(2-{2-[3,5-bis(trifluoromethyl)sulfonamido]-4-trifluoromethylphenoxyl} benzoic acid)] and a calcium-independent PLA$_2$ with the haloenol lactone suicide substrate had no effect on VSMC growth (2). Whether cPLA$_2$ activation is involved in ANG II-induced neointimal growth or VSMC proliferation after vascular injury is not known.

PLD hydrolyzes phospholipids, mainly phosphatidylcholine, into phosphatidic acid (PA) and choline. PA is an important...
second messenger, which is involved in membrane trafficking (41), cell proliferation, cell migration, and cell transformation through activation of mitogen-activated protein kinases (35). ANG II and exogenous PLD (Streptomyces chromofuscus) stimulate [3H]thymidine incorporation (8). PA also increases [3H]thymidine incorporation (8). However, it is not known whether PLD or PA contributes to ANG II-induced neointimal growth after vascular injury.

ANG II also increases Akt activity, which, in turn, causes activation of various downstream targets such as B-cell leukemia/lymphoma-2 (Bcl-xL/Bcl-2)-associated death promoter, mammalian target of rapamycin, glycogen synthase kinase-3β (GSK-3β), and forkhead family members (40). Activated GSK-3β phosphorylates downstream transcription factors and causes their ubiquitination, nuclear exit, or decrease in DNA binding (13). Phosphorylation and inactivation of GSK-3β by Akt suppress the antihypertrophic effect of GSK-3β in the heart and increases protein synthesis (13). Furthermore, ectopic expression of constitutively active (dephosphorylated) GSK-3β inhibits the proliferation of VSMCs and reduces neointimal hyperplasia after balloon injury in rat carotid arteries (50). Akt has been implicated in neointimal growth and VSMC proliferation after balloon injury (42). High levels of total phospho-Akt and phospho-ribosomal S6 kinase (p70S6K) have been shown in rat embryonic aorta and adult balloon-injured carotid arteries compared with quiescent adult rat aorta and uninjured carotid arteries (24). Akt is phosphorylated at 30 and 60 min after injury and to a lesser degree at 6 h after injury (39). Whether Akt mediates ANG II-induced neointimal growth in injured vessels is not known. Because cPLA2 and PLD2 regulate Akt phosphorylation stimulated by ANG II in cultured VSMCs (21, 22), it is possible that ANG II promotes neointimal growth by increasing Akt activity through activation of cPLA2 and PLD2. To test this hypothesis, we have investigated the effect of Akt dominant negative, Akt wild-type (WT), cPLA2 small interfering RNA (siRNA), and PLD2 siRNA on ANG II-stimulated neointimal growth and on ANG II-induced Akt activation in balloon-injured rat carotid arteries.

METHODS

Materials. Adenoviral Akt WT (Ad-Akt WT), Ad-Akt T308/A, S473/A, K179/A, and Ad-β-galactosidase (Ad-β-Gal) were generously provided by Dr. Kenneth Walsh (Molecular Cardiology/CVI, Boston University School of Medicine). The titers of Akt WT, Akt triple A (AAA) mutant, and cytomegalovirus-β-Gal were 3 × 10^9, 1 × 10^9, and 1 × 10^8 plaque-forming units (PFU)/ml, respectively. Retroviral (retro) β-Gal reporter gene LacZ (1 × 10^9 PFU/ml), retro-cPLA2 siRNA (1 × 10^9 PFU/ml), and retro-PLD2 siRNA (5 × 10^8 PFU/ml) were prepared with the use of the GeneSuppressor system from Imgenex (San Diego, CA) as follows.

Preparation of retro-siRNA and amplification. Retro-siRNA was prepared as described (22). Briefly, the primers with forward sequences 5'-TGG AGC ACA TGA AGC CAT GAG CGG AGT ACT GGG CAC GTA GAA CCA CTA CTG TTT TTT-3' for cPLA2; 5'-TCG AGC ACA TGA AGC CAT GAG CGG AGT ACT GGG CAC GTA GAA CCA CTA CTG TTT TTT-3' for PLD2; and reverse sequences 5'-CTA GGA AAA ACA GTA GTG GTC GTA CGT GGC CAC TAG TGC GCC TCG TAC TCG GCA CGT AGA ACC ACT ACT GTC-3' for cPLA2; and 5'-CTA GGA AAA ACA GTA GTG GTC GTA CGT GGC CAC TAG TGC GCC TCG TAC TCG GCA CGT AGA ACC ACT ACT GTC-3' for PLD2 were synthesized by Integrated DNA Technologies. The annealed forward and reverse oligonucleotides were inserted into the linearized pSuppressorRetro viral vector with the use of a ready-to-go T4 DNA ligase kit (Amersham, Piscataway, NJ). Competent DH5α cells (GIBCO-BRL, Carlsbad, CA) were transformed with ligated plasmid DNA. After transformation, the colonies were amplified and purified with the use of a Miniprep purification kit (Qiagen), and plasmid DNAs were sequenced with the use of primers complementary to pSuppressorRetro viral vector. Plasmids with correct sequences were then amplified and purified with Qiagen Maxi plasmid DNA kit. Plasmids (pEco Packaging vector and pSuppressorRetro vector containing the cPLA2 or PLD2 siRNA insert) were used to transfect human embryonic kidney-293 cells with the calcium phosphate precipitation method. The virus was harvested by filtering the virus containing supernatant.

* Rat carotid artery balloon injury. Balloon injury in the rat carotid artery was performed as described (44, 52). Briefly, male Sprague-Dawley rats (350 ± 10 g; Harlan, Indianapolis, IN) were anesthetized by intraperitoneal administration of ketamine (60 mg/kg) and xylazine (5 mg/kg) (VetMed Drugs). With the use of a dissecting microscope, the right common carotid artery was exposed through a midline cervical incision, and blood flow to the site of surgical manipulation was temporarily interrupted by ligation of the right common, internal, and external carotid arteries with vessel clips. Heparin (100 IU/kg) was administered intraperitoneally to prevent coagulation. A Fogarty 2-Fr embolectomy catheter (Baxter Healthcare) was introduced into the common carotid artery through the external carotid branch, advanced, the balloon inflated, and withdrawn thrice. The catheter was removed, and a polyethylene catheter was introduced through the external carotid branch for administration of various agents. Immediately after the injury, 100 μl of a saline solution containing Ad-β-Gal, Ad-Akt WT, or Ad-Akt AAA mutant (at a final concentration of 1 × 10^10 PFU/ml) or 100 μl of a saline solution containing 8 μg/ml polybrene and retroviral cPLA2 or PLD2 siRNA (at a concentration of 1 × 10^10 PFU/ml) with or without 200 nM ANG II were infused and remained in contact with the carotid artery for 1 h. The adenovirus, retrovirus, or saline solution was then removed, the carotid artery section was flushed, and the blood flow was restored. The incision was closed with sutures, and the animals were given buprenorphine (0.5 mg/kg subcutaneously; Geenpark Pharmacy) for analgesia. After full recovery, the animals were returned to the animal care facility and were provided standard rat chow and water ad libitum. At specific times (14 days after injury), rats were euthanized with pentobarbital sodium (50 mg/kg) and perfused with 10% saline-10% formalin, and the tissues were harvested for specific protocols.

In a second series of experiments, injured carotid arteries were exposed to vehicle or ANG II (200 nM) with or without retroviral cPLA2 siRNA, PLD2 siRNA, or LacZ for 1 h. The animals were euthanized 2 days after surgery, and the common carotid arteries from these groups of animals were removed for the detection of Akt phosphorylation by Western blot analysis (described in Western blot analysis).

In a third series of experiments, the common carotid arteries after injury were exposed to ANG II with or without retroviral cPLA2 siRNA, PLD2 siRNA, or LacZ for 30 min and removed immediately. The carotid arteries were then incubated overnight with ANG II alone or combined with their respective retrovirus containing cPLA2 or PLD2 siRNA in medium 199 (M199) tissue culture medium and placed in the incubator under compressed gas (95% O2-5% CO2). Akt phosphorylation was determined by Western blot analysis by using antibody that recognizes phospho-Ser473-Akt as described in Western blot analysis. All experimental protocols used were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center.

Morphometric and immunohistochemical analysis. Carotid artery segments embedded in paraffin were cut into serial sections (5-μm thick) at equally spaced intervals, deparaffinized, and stained with hematoxylin and eosin for morphometric analysis as described (51, 52). The medial area was calculated by subtracting the area defined by
the internal elastic lamina from the area defined by the external elastic lamina, and the intimal area was determined by subtracting the lumen area from the area defined by the internal elastic lamina. Finally, the intimal-to-medial area ratio (I/M) was calculated, and the values from at least three to four sections from each animal were averaged.

For immunohistochemical analysis, antibodies against CD45 (BD Biosciences Pharmingen, San Diego, CA), cPLA2 (Santa Cruz Biotechnology, Santa Cruz, CA), and PLD2 (generously supplied by Dr. Sylvain Bourgoin) were applied to the sections of carotid arteries for 2 h. Before being incubated with indicated dilutions of the antibodies, tissue sections were treated with hydrogen peroxide to quench endogenous peroxidase activity. After treatment with primary antibodies, the arterial sections were exposed to biotinylated anti-rabbit (for detection of PLD2) or anti-mouse (for detection of CD45 and cPLA2) IgG secondary antibody for 1 h. After being incubated with a biotinylated secondary antibody, immunostains were detected with the use of the avidin-biotinylated enzyme complex (ABC) kit (Vector Laboratories, Burlingame, CA).

Western blot analysis. Carotid arteries from four to six rats of each treatment group in the second and third series of experiments described above were pooled and ground to a fine powder in liquid nitrogen and incubated in ice-cold 0.1% Triton lysis solution [10 mM HEPES (pH 7.4), 50 mM sodium pyrophosphate, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 100 μM Na3VO4, 50 mM NaCl, 0.1% Triton X-100, 500 μM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin] for 1 h. Insoluble matter was removed by centrifugation. Equal amounts of protein were loaded on a 8% SDS-polyacrylamide gel and transferred to nitrocellulose. Nonspecific binding was blocked with 5% nonfat dry milk for 1 h. The blots were incubated overnight with antibodies against cPLA2 (Santa Cruz Biotechnology), Akt (Santa Cruz Biotechnology), PLD2 and phospho-Ser473-Akt (Cell Signal, Beverly, MA). Horseradish peroxidase-labeled IgG secondary anti-

![Fig. 1. Retroviral (retro-) cytosolic phospholipase A2 (cPLA2) small interfering RNA (siRNA) and phospholipase D2 (PLD2) siRNA decreased angiotensin II (ANG II)-stimulated neointimal growth. Balloon-injured carotid arteries were exposed to vehicle, retro-β-galactosidase (β-Gal) reporter gene LacZ, retro-cPLA2 siRNA, or retro-PLD2 siRNA with or without ANG II (200 nM) for 1 h. Rats were euthanized after 14 days, and arterial sections were stained with hematoxylin and eosin. Neointimal growth was determined by ratio of intima area to media area (I/M; n = 4–7). I/M ratio calculated as (area defined by internal elastic lamina – lumen area)/(area defined by external elastic lamina – area defined by internal elastic lamina). A: photomicrographs of control and retrovirus-treated groups. B: I/M ratio of control and retrovirus-treated groups. Values are means ± SE. *Value significantly different from the corresponding value obtained with vehicle in absence of ANG II and virus (P < 0.05); †value significantly different from the corresponding value obtained with ANG II in absence of virus (P < 0.01).]
bodies were used in conjunction with ECL Plus (Amersham, Arlington Heights, IL) to visualize the bands. The density of bands was measured by using ImageJ software version 1.32. PLD$_2$ and cPLA$_2$ expression among different treatments was compared with the band density obtained with ANG II treatment alone (taken as 1.00). Each sample was run several times, and the Akt activity was expressed as the ratio of phosphorylated Akt to total Akt. The values of the density of bands from four to five blots were expressed as means ± SE.

**RESULTS**

cPLA$_2$ siRNA and PLD$_2$ siRNA decrease neointimal growth induced by ANG II in balloon-injured rat carotid arteries. Because the rat carotid balloon-injury model has numerous features that are identical to those in clinical restenosis and arterial injury diseases, it is used extensively to study the mechanism underlying restenosis and its prevention (56). VSMCs start proliferating in the media 24 h after injury, migrate into the intima, and proliferate in the intima 4 days after injury (56). The I/M ratio is an index for VSMC growth after injury. In this study, ANG II significantly increased neointimal growth in balloon-injured carotid arteries. Balloon injury increased the I/M ratio from none to 1.02 ± 0.19 (n = 5 rats; injury alone), which was further increased by ANG II to 3.01 ± 0.31 (P < 0.001, n = 7 rats). Exposure of injured arteries to retrovirus containing cPLA$_2$ siRNA or PLD$_2$ siRNA minimized ANG II-stimulated neointimal growth (Fig. 1, A and B) without affecting the neointimal growth induced by injury alone. The control retro-LacZ virus did not alter intimal thickening caused by injury alone or ANG II-induced neointimal growth in balloon-injured vessels. Arterial sections after 14 days of injury were used for immunohistochemical analysis. Although the injured arteries were exposed to retro-cPLA$_2$ siRNA or retro-PLD$_2$ siRNA for 1 h, 14 days after injury, immunostaining showed that cPLA$_2$ siRNA or PLD$_2$ siRNA decreased the expression of cPLA$_2$ and PLD$_2$, respectively (Fig. 2, A and B). The fact that cPLA$_2$ siRNA did not alter PLD$_2$ expression and PLD$_2$ siRNA did not affect cPLA$_2$...
expression demonstrates the selectivity of the retro-siRNA of cPLA2 and PLD2 (Fig. 2, A and B). β-Gal-positive staining in vessels infected with retro-LacZ further confirmed the efficiency of transfection, indicating that 1 h exposure to retro-siRNAs was sufficient to decrease the expression of target proteins in neointima 14 days after injury (Fig. 3).

Akt AAA mutant decreases neointimal growth induced by ANG II in balloon-injured rat carotid artery. Akt has been implicated in neointimal growth caused by balloon injury and in VSMC proliferation through altering the activity or expression of GSK-3β (50, 53), P70S6K (24), forkhead transcription factor (29), p21Cip1 (42), and p27 Kip1 (29). Whether or not Akt mediates ANG II-induced neointimal growth is not known. Therefore, injured carotid arteries were infected with Ad-Akt kinase-dead mutant in which Ser473, Thr308, and Lys179 have been mutated to alanine, Ad-β-Gal, or Ad-Akt WT with or without ANG II. Infection with Ad-Akt WT significantly increased the neointimal thickness caused by balloon injury in the absence of ANG II compared with vehicle alone, and it was not further increased by exposure to ANG II (Fig. 4). However, Ad-Akt AAA mutant inhibited neointimal growth caused by injury alone as well as that stimulated by ANG II in balloon-injured arteries (Fig. 4), suggesting that Akt regulates the neointimal growth induced by balloon injury as well as that stimulated by ANG II during injury.

Akt phosphorylation stimulated by ANG II in balloon-injured carotid artery is inhibited by cPLA2 and PLD2 siRNA. Previous studies in our laboratory (21, 22) have shown that ANG II-induced Akt activation and phosphorylation is mediated by activated PLD2 that depends on cPLA2 stimulation in cultured rat aortic VSMCs. To determine the contribution of cPLA2 and PLD2 to ANG II-induced neointimal growth through Akt activation caused by ANG II, we examined the phosphorylation of Akt at Ser473 as an index for Akt activation in balloon-injured carotid arteries. In the balloon-injured rat carotid artery, Akt is phosphorylated at 30 and 60 min after injury and to a lesser degree after 6 h and 1 and 2 days (39). In our study, Akt phosphorylation was also increased by injury (Fig. 5, A and B). However, ANG II, which stimulates Akt phosphorylation in cultured rat VSMCs (21), also increased Akt phosphorylation in injured arteries 2 days after 60 min of exposure to ANG II (Fig. 5, A and B). Retro-PLD2 siRNA treatment decreased the expression of PLD2 but not cPLA2 in ANG II-treated groups (Fig. 5A). On the other hand, retro-cPLA2 siRNA decreased cPLA2 but not PLD2 expression, although ANG II alone increased cPLA2 expression in injured vessels (Fig. 5A). Retro-cPLA2 and PLD2 siRNA decreased the ANG II-induced

Fig. 3. Infection with retro-LacZ or Ad-β-Gal of injured arteries increased LacZ expression in neointima after 14 days. Injured carotid arteries were exposed to vehicle, retro-LacZ, or Ad-β-Gal with or without ANG II (200 nM) for 1 h, and rats were euthanized after 14 days. Immunohistochemical staining of LacZ was performed on sections of carotid arteries with use of ABC kit (Vector Laboratory) with DAB peroxidase substrate. LacZ antibodies were incubated with tissue sections for 2 h. Anti-mouse IgG was applied to sections for 1 h before addition of ABC and DAB peroxidase substrate.
increase in the ratio of phosphorylated Akt to total Akt (an index of Akt activity) in injured arteries; there was no significant change in this ratio in vessels treated with retro-LacZ (Fig. 5, A and B).

**Akt phosphorylation decreased by cPLA2 or PLD2 siRNA is due to their direct effect in vascular cells.** Neointimal growth is known to be associated with initial immune cell attachment, cytokine release, and VSMC growth. The immunostaining of α-smooth muscle actin in neointima suggested that VSMC migration and proliferation are involved in neointimal growth (see online data supplement at http://ajpheart.physiology.org/cgi/content/full/00450.2005/DC1). To exclude the effects of immune cells, the carotid arteries that were injured and exposed to ANG II with retro-LacZ, cPLA2 siRNA, or PLD2 siRNA for 30 min in vivo were removed from the animals, washed with M199 tissue culture medium to eliminate blood cells from the injured vessels, and then incubated in M199 with ANG II with or without retro-cPLA2 siRNA, PLD2 siRNA, or LacZ for 24 h. Compared with treatment with vehicle alone, ANG II increased Akt phosphorylation in injured vessels. The in vitro treatment of injured vessels with retro-cPLA2 and PLD2 siRNA also decreased the expression of their respective proteins (Fig. 6). These data indicate that both cPLA2 siRNA and PLD2 siRNA decrease Akt phosphorylation caused by ANG II by their direct action on the vessel wall rather than on monocytes, leukocytes, or other blood elements.

**Neointimal growth inhibited by cPLA2 siRNA, PLD2 siRNA, or Akt AAA mutant is also related to inhibition of immune cell infiltration.** It is well known that vascular injury causes inflammation and infiltration of immune cells, which are involved in

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**Fig. 4.** Ad-Akt kinase-dead triple A (AAA) mutant decreased neointimal growth caused by injury and that stimulated by ANG II in balloon-injured carotid arteries. Balloon-injured carotid arteries were exposed to vehicle, Ad-β-Gal, Ad-Akt wild type (WT), or Ad-Akt AAA mutant with or without ANG II (200 nM) for 1 h. Rats were euthanized after 14 days, and arterial sections were stained with hematoxylin and eosin to determine neointimal growth by I/M ratio (n = 3–6). A: photomicrographs of control and adenovirus-treated groups. B: I/M ratio of control and adenovirus-treated groups. Values are means ± SE. *Value significantly different from the corresponding value obtained with vehicle in absence of ANG II and virus (P < 0.01); †value significantly different from the corresponding value obtained with ANG II in absence of virus (P < 0.001).

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**Table 1.**

<table>
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<tr>
<th>Condition</th>
<th>Ratio of I/M</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>1</td>
<td>5</td>
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<tr>
<td>Ad-β-gal</td>
<td>1.5 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>Ad-Akt Wt</td>
<td>2 ± 0.3</td>
<td>3</td>
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<tr>
<td>Ad-Akt AAA mutant</td>
<td>1 ± 0.1</td>
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*Values are means ± SE. *Value significantly different from the corresponding value obtained with vehicle in absence of ANG II and virus (P < 0.01); †value significantly different from the corresponding value obtained with ANG II in absence of virus (P < 0.001).*
Fig. 5. Retro-cPLA2 siRNA and PLD2 siRNA decreased ANG II-stimulated Akt phosphorylation in injured vessels in vivo. Injured carotid arteries were incubated with saline or ANG II (200 nM) in absence or presence of retro-LacZ, retro-cPLA2 siRNA, or retro-PLD2 siRNA for 1 h, and rats were euthanized after 2 days. Injured common carotid arteries were homogenized and lysed to perform Western blot analysis with use of phospho-Akt, Akt, cPLA2, and PLD2 antibodies (each sample contains tissue lysate from 4 to 6 rats). Equal amounts of protein from each tissue sample were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Total Akt, cPLA2, PLD2, and phospho-Akt were measured by Western blot analysis as described in METHODS. A: representative Western blot analysis. Blots are representative of 2–5 different Western blots from same samples prepared from vessels pooled from 4 to 6 rats for each treatment. Density of bands was measured by using ImageJ software version 1.32. Quantification of cPLA2 or PLD2 expression was normalized to density of bands obtained with ANG II alone, which was expressed as 1.00. Representative blots with density ratio of bands for PLD2 and cPLA2 are shown. Average ratio of PLD2 and cPLA2 protein from various groups of vessels [uninjured:injured (vehicle:ANG II:LacZ + ANG II:cPLA2 siRNA + ANG II:PLD2 siRNA + ANG II)] is as follows: cPLA2 = 0.96:(1.00:1.55:0.97:0.55) from 5 runs; PLD2 = 1.06:(1.00:0.94:0.48:0.81) from 3 runs, respectively. B: densitometric analysis of ratio of phosphorylated Akt/total Akt. Quantification of ANG II-induced Akt phosphorylation was normalized to corresponding density of Akt protein, and value was expressed as percentage of that obtained with vehicle. Values are expressed as means ± SE of the density of bands from 5 blots of the same samples obtained from vessels pooled from 4 to 6 rats with various treatments.

Fig. 6. Retro-cPLA2 siRNA and PLD2 siRNA decreased ANG II-stimulated Akt phosphorylation in cultured injured vessels in vitro. Carotid arteries were injured and exposed to vehicle, retro-LacZ, retro-cPLA2 siRNA, or retro-PLD2 siRNA together with ANG II (200 nM) for 30 min in vivo. Carotid arteries were then removed and placed in tissue culture plates containing medium 199 tissue culture medium and incubated with the above agents for 24 h. Injured common carotid arteries were homogenized and lysed to perform Western blot analysis with use of phospho-Akt, Akt, cPLA2, and PLD2 antibodies (each sample contains tissue lysate from 4 rats). Equal amounts of protein from each tissue sample were resolved by SDS-PAGE and transferred to nitrocellulose membranes, and total Akt, cPLA2, PLD2, and phospho-Akt were measured by Western blot analysis as described in METHODS. A: representative Western blot analysis. Blots are representative of 2–4 different Western blots from same samples of vessels pooled from 4 rats for various treatments. Density of bands was measured by using ImageJ software version 1.32. Quantification of cPLA2 or PLD2 expression was normalized to density of bands obtained with ANG II alone, which is expressed as 1.00. Representative blots with density ratio of bands for PLD2 and cPLA2 are shown in A. Average ratio of PLD2 and cPLA2 protein from various groups of vessels [uninjured:injured (vehicle:ANG II:LacZ + ANG II:cPLA2 siRNA + ANG II:PLD2 siRNA + ANG II)] is as follows: cPLA2 = 0.96:(1.00:1.55:0.97:0.55) from 2 runs; PLD2 = 1.06:(1.00:0.94:0.48:0.81) from 3 runs, respectively. B: densitometric analysis of ratio of phosphorylated Akt/total Akt. Quantification of ANG II-induced Akt phosphorylation was normalized to corresponding density of Akt protein, and value was expressed as percentage of that obtained with vehicle. Values are expressed as means ± SE of bands from 4 different Western blots of the same sample obtained from vessels pooled from 4 rats with various treatments.
neointimal growth. To determine whether cPLA$_2$, PLD$_2$, and Akt also contribute to the infiltration of immune cells in neointima, we examined the immunostaining of CD45, a marker for leukocytes, in neointima. ANG II increased the density of CD45-positive cells in neointima compared with injury alone. Infection of injured arteries with Ad-Akt kinase-dead AAA mutant compared with Ad-Akt WT and retro-cPLA$_2$ siRNA or PLD$_2$ siRNA compared with retro-LacZ decreased the density of CD45-positive cells in neointima (Fig. 7).

DISCUSSION

ANG II is known to stimulate neointimal growth caused by balloon injury in the rat carotid artery (16). This study, which was conducted to elucidate the mechanism of ANG II-induced increase in neointimal growth, demonstrates that this effect of the peptide is mediated through cPLA$_2$- and PLD$_2$-dependent activation of Akt. It has been reported that during vascular injury, Akt activation promotes VSMC proliferation and migration, which contribute to neointimal growth, whereas in endothelial cells, activated Akt phosphorylates endothelial nitric oxide synthase and reduces endothelial cell apoptosis (20). Moreover, immunohistochemical and Western blot analysis of balloon-injured rat carotid artery showed that a transient increase in phospho-phosphatase and tensin homolog (inactive), a negative regulator of the PI3K/Akt pathway, is correlated to neointimal growth (10). These divergent actions of Akt in endothelial cells and VSMCs synergistically promote neointimal thickening after injury (4). In the present study, exposure of balloon-injured carotid arteries to Ad-Akt WT increased neointimal growth, whereas exposure to Akt kinase-dead AAA mutant (dominant negative) decreased neointimal growth, suggesting the involvement of Akt in neointimal growth. However, the mechanism by which Akt is activated during vascular injury is not known. It is possible that exposure of VSMCs to growth factors including ANG II during injury promotes activation of Akt, which, in turn, leads to neointimal growth. The finding that the AT$_1$R antagonists TCV-116 (1, 16 –18, 43) and losartan (14, 28, 33, 46, 47, 54) inhibit intimal thickening of rat balloon-injured carotid arteries links ANG II to neointimal growth during injury. The AT$_1$R is overexpressed in injured vessels (7). This observation, together with our demonstration that the exposure of balloon-injured rat carotid arteries to ANG II increased neointimal growth, which was inhibited by treatment with the adenovirus containing the kinase-dead Akt but not the WT Akt mutant, suggests that Akt activation mediates the neointimal thickening caused by ANG II in injured carotid arteries.

Our previous studies (21, 22) have shown that in cultured VSMCs, activation of Akt by ANG II is mediated by cPLA$_2$-dependent PLD$_2$ activation. Inhibition of PLD by 1-butanol has been shown to inhibit norepinephrine-stimulated VSMC proliferation (31). Our finding that the exposure of injured carotid arteries to retroviral vectors containing cPLA$_2$ or PLD$_2$ siRNA diminished the increase in neointimal growth elicited by ANG II, which was also reduced by exposure of Akt kinase-dead AAA mutant, suggests that ANG II-stimulated neointimal growth mediated by Akt activation in injured vessels is dependent on cPLA$_2$ and PLD$_2$ activity. In support of this was our observation that exposure of injured vessels to ANG II increased Akt phosphorylation after 2 days of injury, which was inhibited in carotid arteries exposed to retroviral cPLA$_2$ or PLD$_2$ siRNA. It has been reported that during injury, there is increased migration of lymphocytes to the injured vessel and cytokine release that contributes to neointimal growth (19). Although we cannot exclude an effect of cPLA$_2$ and PLD$_2$ siRNA on Akt in immune cells to reduce ANG II-induced increase in neointimal thickening, it is most likely due to their direct effect in the injured vessel wall for the following reason. Akt phosphorylation induced by ANG II was inhibited in the

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Fig. 7. Ad-Akt kinase-dead AAA mutant, retro-cPLA$_2$ siRNA, and retro-PLD$_2$ siRNA, but not Ad-Akt WT and retro-LacZ, decreased ANG II-stimulated CD45-positive cell density in injured vessels. Injured carotid arteries were exposed to vehicle, retro-LacZ, retro-cPLA$_2$ siRNA, retro-PLD$_2$ siRNA, Ad-Akt WT, or Ad-Akt AAA mutant with ANG II (200 nM) for 1 h, and rats were euthanized after 14 days. Immunohistochemical staining of CD45 was performed on sections of carotid arteries with use of ABC kit (Vector Laboratory) with DAB peroxidase substrate. CD45 antibodies were incubated with tissue sections for 2 h. Anti-mouse IgG was applied to sections for 1 h before addition of ABC and DAB peroxidase substrate.

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injured carotid arteries that were isolated from the animals and placed in M199 tissue culture medium for 24 h in the presence of ANG II and cPLA2 or PLD2 siRNA.

Although the exposure of injured carotid arteries to cPLA2 or PLD2 siRNA inhibited the expression of cPLA2 and PLD2, respectively, as well as the increase in neointimal growth caused by ANG II, these agents did not alter the neointimal growth stimulated by injury alone. However, Akt kinase-dead mutant decreased neointimal growth caused by injury alone as well as that stimulated by ANG II. Therefore, it appears that ANG II-induced neointimal growth during injury is dependent on one or more phospholipid products generated by the activation of cPLA2 and PLD2 in the vessel wall but not on the basal activity of these enzymes. However, Akt kinase-dead AAA mutant decreased not only Akt activity induced by ANG II but also the basal Akt activity and consequently intimal thickness caused by injury alone, suggesting that cPLA2 and PLD2 regulate only Akt activity caused by ANG II but not the basal Akt activity. Activation of cPLA2 releases AA from tissue phospholipids, which is metabolized by COX, LO, and CYP (23, 27, 36). Although aspirin, a COX-1 inhibitor, does not reduce the neointimal growth after balloon injury (50), adenoviral gene transfer of COX-1 to balloon-injured arteries of atherosclerotic rabbits prevents thrombosis and reduces intimal thickening as a result of increased prostaglandin I2 synthesis (55). On the other hand, the COX-2 inhibitor celecoxib significantly reduced the VSMC proliferation and neointimal growth (50), although the COX-2 inhibitor increased the thrombotic process (6, 55). Celecoxib also suppressed the phosphorylation of Akt and GSK in cultured VSMCs (50). Moreover, constitutively active Myr-Akt reversed the inhibition of intimal hyperplasia caused by celecoxib (50). Expression of 12-LO has also been reported to increase neointimal formation after balloon injury (26). The 12-LO antisense oligonucleotide decreases VSMC fibronectin expression and migration as well as neointimal growth (26). These results imply that AA metabolites derived through COX-2 and LO are involved in neointimal growth. Moreover, in neointimal smooth muscle cells, CYP1A1 expression level is high (12), and CYP1A1 antisense inhibits ANG II- and AA-induced increase in neointimal growth in injured rat carotid arteries (49), suggesting that CYP4A metabolites of AA are also involved in neointimal growth (49). An AA metabolite of CYP4A1, 20-HETE, has been shown to promote VSMC proliferation (45), increase Akt phosphorylation (21), and stimulate neointimal growth in injured carotid arteries (49).

It has been reported that AA metabolites derived via LO and CYP subsequent to activation of cPLA2 increase PLD2 activity in VSMCs (30). Previous studies from our laboratory (22) have shown that activation of cPLA2-dependent PLD2, through generation of PA, promotes transactivation of the epidermal growth factor receptor and, consequently, activation of Akt. Therefore, it is possible that Akt activated by PA mediates the effect of ANG II to increase neointimal formation. It has been reported that the AT1R blocker TCV-116 inhibits both platelet-derived growth factor (PDGF) receptor tyrosyl phosphorylation and intimal thickening, linking ANG II to neointimal thickening through transactivation of the PDGF receptor (1). Whether PDGF transactivation by ANG II is also dependent on PLD/PA and whether Akt activation is responsible for neointimal thickening remain to be determined.

In conclusion, this study demonstrates that the ANG II-induced increase in neointimal thickening in balloon-injured rat carotid artery is mediated by Akt phosphorylation by a mechanism dependent on cPLA2 and PLD2 activation. This could provide a novel target for the development of therapeutic agents for the prevention and treatment of restenosis after angioplasty in carotid arteries and other vascular diseases, including coronary artery disease and atherosclerosis.

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