

Activation of MMP-2 in response to vascular injury is mediated by phosphatidylinositol 3-kinase-dependent expression of MT1-MMP

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Zahradka, Peter, Greg Harding, Brenda Litchie, Shawn Thomas, Jeffrey P. Werner, David P. Wilson, and Natalia Yurkova. Activation of MMP-2 in response to vascular injury is mediated by phosphatidylinositol 3-kinase-dependent expression of MT1-MMP. *Am J Physiol Heart Circ Physiol* 287: H2861–H2870, 2004. First published August 5, 2004; doi:10.1152/ajpheart.00230.2004.—Phosphatidylinositol 3-kinase (PI3K) is required for smooth muscle cell (SMC) proliferation. This study reports that inhibitors of PI3K also prevent SMC migration and block neointimal hyperplasia in an organ culture model of restenosis. Inhibition of neointimal formation by LY-294002 was concentration and time dependent, with 10 μ M yielding the maximal effect. Continuous exposure for at least the first 4–7 days of culture was essential for significant inhibition. To assess the role of matrix metalloproteinases (MMPs) in this process, we monitored MMP secretion by injured vessels in culture. Treatment with LY-294002 selectively reduced active MMP-2 in media samples according to zymography and Western blot analysis without concomitant changes in latent MMP-2. Parallel results with wortmannin indicate that MMP-2 activation is PI3K dependent. Previous research has shown a role for both furin and membrane-type 1 (MT1)-MMP (MMP-14) in the activation of MMP-2. The furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone did not prevent MMP-2 activation after balloon angioplasty. In contrast, balloon angioplasty induced a significant increase in the levels of MT1-MMP, which was suppressed by LY-294002. No change in MT1-MMP mRNA was observed with LY-294002, because equivalent amounts of this mRNA were present in both injured and noninjured vessels. These results implicate PI3K-dependent regulation of MT1-MMP protein synthesis and subsequent activation of latent MMP-2 as critical events in neointimal hyperplasia after vascular injury.

matrix metalloproteinase; LY-294002; wortmannin; furin; restenosis

PHOSPHATIDYLIOSITOL 3-KINASE (PI3K) is a heteromeric protein consisting of an 85-kDa (p85) regulatory subunit and a 110-kDa (p110) catalytic subunit. PI3K functions as a lipid kinase and phosphorylates phosphoinositides on the 3' position of the inositol ring. The biological functions of PI3K can be grouped into four distinct categories: mitogenic signaling, inhibition of apoptosis, cell adherence and motility, and intracellular vesicle trafficking (5). A role in cell motility and cell adherence was indicated by evidence showing PDGF-dependent membrane ruffling and chemotaxis requires an interaction between PI3K and the PDGF receptor (25, 55). In addition, PI3K is involved in microtubule reassembly in response to both insulin and PDGF (21) and actin rearrangement by PDGF (59). The involvement of PI3K in growth factor regulation of integrins

and cell adherence has also been established (16, 23). In particular, PI3K has been shown to associate with focal adhesion kinase (FAK) as well as participate in PDGF-mediated phosphorylation of both FAK and paxillin (40). A recent study (41) has also demonstrated that PI3K promotes cell migration on fibronectin by facilitating the binding of FAK to Src and p130^{Cas}. In contrast, integrin-mediated migration of macrophages involves a PI3K-dependent, but FAK-independent, mechanism (6).

PI3K is activated in vascular smooth muscle cells (SMCs) in response to angiotensin II (ANG II) (44). Furthermore, inhibition of PI3K activity with LY-294002 or wortmannin prevents the stimulation of DNA synthesis by ANG II, thus implicating PI3K in the proliferation of SMCs. Interestingly, a similar relationship between SMC migration and PI3K activation has also been reported (56). However, it is recognized that cell proliferation and migration are independent processes that are controlled by distinct mechanisms (4). In fact, it is now evident that matrix metalloproteinases (MMPs) have a major role in SMC migration (22) and that their contribution is likely independent of cell proliferation (38, 67).

The ability to regulate MMP activity is essential for the maintenance of normal vascular architecture, and three levels of control have been identified. MMP expression is transcriptionally regulated by NF- κ B (3, 10). MMPs are also produced as zymogens, and activation requires proteolytic cleavage of the proenzyme form. As well, secreted MMPs are found complexed with tissue inhibitors of MMPs (TIMPs), which suppress proteolytic activity. The mechanisms responsible for MMP activation have not been identified in all cases; however, membrane-type 1 (MT1)-MMP is one factor that has been shown to cleave pro-MMP-2 to its active form (46). Interestingly, expression of MT1-MMP, which is anchored on the cell surface through a transmembrane domain, is also modulated by tissue damage (39, 48). Indeed, a correlation between MT1-MMP expression and neointimal hyperplasia suggests this protein may be involved in the vascular response to injury (34).

Although no connection between MMPs and PI3K has been reported in SMCs, recent studies with tumour and endothelial cells support the existence of such a link (9, 20). Furthermore, these reports suggest that PI3K contributes to both MMP activation and expression. We therefore investigated whether PI3K modulates MMP-dependent migration by vascular SMCs. A coronary artery organ culture model was also employed to examine the correlation between PI3K-dependent

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MMP activation and the development of a neointimal lesion in response to balloon angioplasty. Our study provides insight into the role of PI3K in SMC migration and identifies a mechanism by which PI3K may control MMP activation.

MATERIALS AND METHODS

SMC culture. Primary cultures of porcine coronary artery SMCs were generated from the left anterior descending coronary artery by an explant organ culture method (45). To obtain a quiescent cell population, SMCs were grown to 70% confluence and then placed into serum-free DMEM supplemented with 11 $\mu\text{g/ml}$ pyruvate, 5 $\mu\text{g/ml}$ transferrin, 10^{-9} M selenium, 2×10^{-4} M ascorbate, and 10^{-8} M insulin for 5 days. To maintain consistency between cultures, only second passage cells with confirmed expression of smooth muscle markers (>95% of cells staining for myosin) were used for all experiments.

Coronary artery organ culture. The left anterior descending coronary artery of pig hearts obtained from the local abattoir was exposed and flushed with PBS, and an angioplasty catheter (3.5×20 mm) was inserted into the vessel distal to the first major bifurcation. The catheter was inflated to 5 atmospheres for 1 min. The vessel was dissected free, and the damaged region of the vessel was cut into four equal 5-mm segments. Control vessels were treated in an identical manner with the exception of catheter insertion and inflation. One vessel segment was randomly placed per well of a 24-well culture dish containing 20% FBS in DMEM. Media, including treatments, were changed every second day. Vessels harvested from culture were placed into OCT embedding media and stored at -70°C . A detailed characterization of this model has been published elsewhere (58).

Boyden chamber migration. Proliferating SMCs were incubated in DMEM (no FBS) for 48 h in a Boyden chamber, with chemoattractant (10^{-6} M ANG II) added to the lower compartment and antagonists added to the upper compartment. Cells migrating to the underside of the membrane separating these compartments were stained with Giemsa and counted as described previously (63).

Immunohistochemistry. SMCs grown on Superfrost Plus glass slides (Fisher Scientific) were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 before antibody treatment. The slides were blocked for 60 min at room temperature, rinsed with Tris-buffered saline (TBS), and then incubated with MT1-MMP antibody (MAb 3317, Chemicon, diluted 1:100 in 1% BSA-TBS-0.1% Triton X-100) for 60 min at room temperature (44). The primary antibody was detected with Cy3-coupled secondary antibody (diluted 1:200 with 1% BSA-TBS-Triton X-100). Nuclei were visualized with Hoechst 33342 (0.5 mg/ml diluted 1:4,000 in TBS). Digital images were captured with a DAGE-MTI charge-coupled device camera.

Western blot analysis. Western blotting of cellular proteins separated by SDS-PAGE in a 7.5% gel and transferred to polyvinylidene difluoride membrane was conducted as previously described (62). Horseradish peroxidase-conjugated secondary antibody (1:10,000 diluted) was detected using the ECL chemiluminescent system (Amersham). Band intensity was quantified by scanning densitometry. Antibodies employed include MMP-2 (latent and active, Oncogene, diluted 1:1,000), MMP-9 (latent and active, NeoMarkers, diluted 1:2,000), and MAb 3317 (diluted 1:1,000).

Gelatin zymography. Culture medium was diluted 1:1 in SDS sample buffer [0.5 M Tris·HCl (pH 6.8), 2.0% SDS, 10% glycerol, and 0.001% bromophenol blue] without reducing agent, and 7 μl were loaded per well of a 7.5% polyacrylamide gel containing 0.1% gelatin. Gels were washed in glycine-Triton buffer [0.025 M glycine (pH 8.3) and 2.5% Triton X-100] twice for 10 min at 4°C to remove the SDS and permit partial renaturation of the protein. Gels were incubated in buffer [0.05 M Tris·HCl (pH 8.0), 5 mM CaCl_2 , and 0.1 M PMSF] at 37°C for 12 h and then stained with Coomassie blue R-250. Protein loading and incubation time were adjusted to ensure lytic activity was in the linear range (10–200 pg). Gels were subsequently

scanned with an imaging densitometer, with transmittance correlating to activity (57).

RT-PCR amplification. Coronary artery segments were flash frozen in liquid nitrogen and pulverized with a mortar and pestle. Total RNA was isolated from the powder with TRIzol. RNA was resuspended in RNase-free water, and concentration was determined by spectrophotometric absorbance at 260 nm. Reverse transcription of 1 μg RNA was conducted (after removal of possible genomic DNA contamination with DNase I) according to the protocol (25 cycles of amplification and 62°C annealing temperature) recommended for the Access RT-PCR System (Promega). The specific forward and reverse oligodeoxynucleotide primers employed were as follows: MT1-MMP (sense) 5'-AAGGCCAATGTTCTCGAAGGAA-3' and (antisense) 5'-AAGAAGATCATGATGTCCT-3'; and L32 ribosomal protein (rP-L32) (sense) 5'-TAAGCGAAACTGGCGGAAAC-3' and (antisense) 5'-GCTGCTCTTTCTACGATGGCTT-3'. Oligonucleotide primers for MT1-MMP were designed with Primer3 (42) using a sequence reported for *Sus scrofa* (GI:5051631). Amplification products were analyzed by electrophoresis in 2% agarose gels, and ethidium bromide-visualized bands were captured on Polaroid 667 black and white instant film. Control reactions (without RNA, without RT, and without primers) were used to demonstrate the specificity of the PCR.

Data analysis. Morphometric data, cell number, and band intensity for both autoradiographic and zymography experiments were quantified and graphically represented as means \pm SE. In most experiments, the sample size was three to six; however, six to eight vessel segments were used per treatment group in organ culture. All experiments were replicated at least three times, with each replicate employing independent cell or vessel isolations. Treatment means were compared using ANOVA, whereas all other data were analyzed using the unpaired Student's *t*-test. Significance was set to $P < 0.05$ in all cases. Quantification of data obtained on film or autoradiograms was accomplished with a Bio-Rad model 670 Imaging Densitometer under nonsaturating conditions. Background subtraction was achieved by reading the absorbance of an equal-sized region directly adjacent (above, below, or beside) to the band. Although multiple exposures were acquired to ensure the absence of film saturation, the longest exposures were typically selected for visual presentation and not used for data analysis.

RESULTS

PI3K is required for SMC migration. ANG II is both a mitogen and a chemoattractant for SMCs (44, 66). Although it was previously established that PI3K is required for SMC proliferation in response to ANG II (44), its role in SMC migration was not examined. Migration of cells was measured with a Boyden chamber. Basal migration over 48 h in the absence of ANG II was set to 100% (Fig. 1A). ANG II increased cell migration by 142%, a statistically significant change that was somewhat less than the 205% increase obtained with serum. The addition of LY-294002 (10 μM) reduced migration to basal levels (Fig. 1A). A requirement for PI3K activation was confirmed by the concentration-dependent decrease in migration obtained with both LY-294002 (Fig. 1B) and wortmannin (Fig. 1C). EC_{50} values of 3.0 and 0.25 μM were calculated for LY-294002 and wortmannin, respectively.

Inhibition of PI3K prevents neointimal formation. The ability to block both SMC migration and proliferation suggests that LY-294002 may be able to prevent neointimal formation (also termed neointimal hyperplasia or restenosis) in response to a vascular injury such as balloon angioplasty. We employed an organ culture model of neointimal hyperplasia (58) in which the left descending coronary artery of an isolated porcine heart is subjected to balloon angioplasty in situ, and segments of the

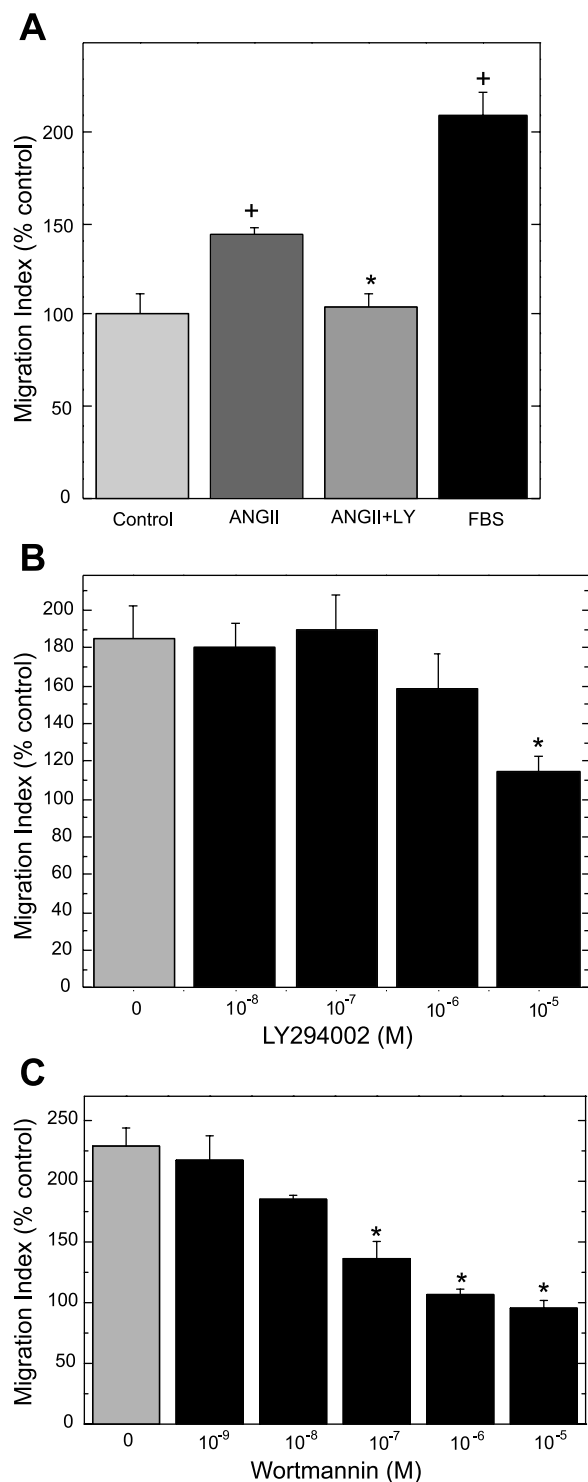


Fig. 1. Smooth muscle cell (SMC) migration is reduced in the presence of phosphatidylinositol 3-kinase (PI3K) inhibitors. Directional movement of growing SMCs was measured with a Boyden chamber as described in MATERIALS AND METHODS. Bars show means \pm SE; $n = 6$. Values represent the ratio of cells migrated for each treatment relative to untreated control (migration index). Statistically significant differences ($P < 0.05$) from control (+) and from stimulated cells (*) are indicated. *A*: cell migration in the presence of either ANG II (1 μ M) or FBS (2%) and with LY-294002 (LY; 10 μ M) in the presence of ANG II. *B* and *C*: concentration effects of LY-294002 and wortmannin (Wt).

injured artery are then placed into culture for 14 days. A 2-wk period was selected to ensure the presence of sufficient neointimal formation for quantitative purposes. Visual examination of vessel cross sections stained with Lee's methylene blue revealed the presence of a neointima in balloon-injured vessels (Fig. 2*B*) compared with noninjured controls (Fig. 2*A*). In the presence of 1 μ M LY-294002 (Fig. 2*C*), there was a decrease in the amount of neointima present, whereas 10 μ M LY-294002 (Fig. 2*D*) completely suppressed neointimal formation. Quantitative analysis of the neointimal index (intimal area/medial area) revealed a concentration-dependent effect of LY-294002 (Fig. 3*A*) that exhibited an EC_{50} of 1.9 μ M.

LY-294002 affects an early event postangioplasty. To evaluate the mode of action of LY-294002 on neointimal formation, LY-294002 was added to the culture medium for varying periods of time. The inhibitor was subsequently removed, and incubation of the vessel segments was allowed to continue in the absence of LY-294002 for the remainder of the 14-day culture period. In agreement with the previous experiment (Fig. 3*A*), balloon injury in the absence of LY-294002 resulted in a marked increase in the neointimal index. No significant change in the neointimal index was observed when 10 μ M LY-294002 was included in the culture medium for either 1 or 2 days (Fig. 3*B*). After 4 days with 10 μ M LY-294002, however, a small but nonsignificant decrease in the neointimal index was observed. Exposure to 10 μ M LY-294002 for 7 days completely inhibited neointimal formation (Fig. 3*B*), as did periods of 10 and 14 days (data not shown). These results suggest that LY-294002 disrupts a process that is completed within the first 7 days and is likely activated within 4 days after injury.

Inhibition of migration by LY-294002 prevents neointimal hyperplasia. Neointimal formation involves both cell migration and cell proliferation, and both processes are sensitive to LY-294002. Our data indicate that LY-294002 operates through a process that is activated early in response to injury, which would favour SMC migration as the more likely target. To determine whether inhibition of migration is sufficient to prevent neointimal formation, LY-294002 was compared with GM-6001, a nonselective MMP inhibitor that does not block cell proliferation (29), and aphidicolin, a classical inhibitor of DNA synthesis. We initially confirmed that SMC migration in a Boyden chamber was blocked by GM-6001 (Fig. 4*A*) to the same extent as LY-294002. Aphidicolin had no effect on this process, in agreement with the report of Geimer and Bade (12), who employed epithelial cells in their study. When examined in the organ culture system (Fig. 4*B*), GM6001 was highly effective in reducing neointimal formation (by 85%), whereas aphidicolin was much less potent (43% decrease).

The results obtained with GM-6001 and aphidicolin suggest that migration is essential during the early stages of neointimal formation, although it has been reported that SMC proliferation also occurs during the 4- to 7-day period postangioplasty (15). To confirm the role of migration, cell movement to the luminal side of the internal elastic lamina (IEL) was quantified (Fig. 4*C*). This analysis indicated that cell migration is detectable as early as 2 days postinjury. Furthermore, the number of intimal cells increased consistently over time, whereas the cell number remained constant in the uninjured vessel. It was also evident that both GM-6001 and LY-294002 effectively blocked cell

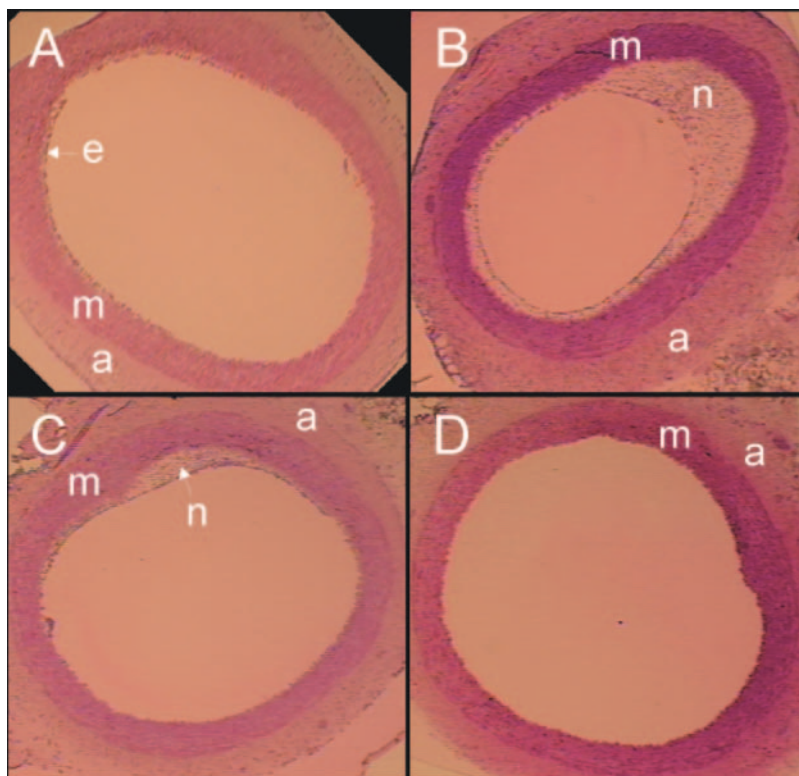


Fig. 2. Treatment with LY-294002 suppresses neointimal formation after balloon angioplasty. Porcine coronary arteries subjected to balloon angioplasty were placed into culture for 14 days. Vessel segments were subsequently sectioned, stained with Lee's methylene blue, and visualized by microscopy. A–D: representative sections that were uninjured (A), balloon injured (B), and balloon injured but incubated with either 1 (C) or 10 μ M LY-294002 (D). The location of the neointimal (n), medial (m), adventitial (a), and endothelial (e) layers are indicated.

movement. In contrast, aphidicolin did not reduce the intimal cell number below that of the untreated control vessel before 7 days, although there was a marked decline relative to control on days 10 and 14. Thus the degree of inhibition of neointimal formation obtained with LY-294002 corresponds more closely with cell migration than with cell proliferation.

Vascular injury stimulates PI3K-dependent MMP-2 activation. MMPs have a role in SMC migration and neointimal formation (22, 67). Consequently, MMPs may be a target for inhibition by LY-294002. To investigate the possible connection between MMPs and PI3K, injured vessel segments were cultured for 14 days, and samples of media from the final 2 days of the culture period were processed for analysis by gelatin zymography. This method detected the primary gelatinases, including MMP-2 and MMP-9, secreted by the vessel segments (Fig. 5A). No difference in lytic band activity between the injured untreated (*lane 0*) and noninjured (*lane NI*) samples was observed. One interpretation of this result is that vessels not subjected to balloon angioplasty still exude MMPs from the cut site generated during preparation of the artery segments. The inclusion of LY-294002 in the medium for the first 4 days of the 14-day culture period produced no change in gelatinase levels (Fig. 5A). In contrast, a decrease in the amount of active MMP-2 (68-kDa band A) was evident when LY-294002 was present for either 7 or 14 days. Because no apparent change in latent MMP-2 (72 kDa band L) could be seen under these conditions, it was concluded that neither MMP-2 expression nor secretion were affected by LY-294002. Therefore, to examine the relationship between LY-294002 and MMP-2 activation, media samples were collected at each media change (i.e., every second day), and gelatinase levels were monitored by zymography. This experiment revealed that

active MMP-2 present in the media samples, which was quantified by densitometry, is greatly increased after balloon angioplasty, reaching a peak after 8 days (Fig. 5B). In contrast, the amount of active MMP-2 remained at control values when LY-294002 was present for the same time period, indicating that activation of latent MMP-2 was prevented.

Quantification of MMP-2 in the media samples by Western blot analysis confirmed that injury to the vessels resulted in an increase in both latent and active MMP-2 over 8 days (Fig. 5C). Whereas the increase in latent MMP-2 was unaffected by the presence of LY-294002, formation of active MMP-2 was significantly reduced in the presence of 10 μ M LY-294002. No comparable change was observed for either active or latent MMP-9 (Fig. 5D). The effectiveness of 1 μ M wortmannin in reducing active MMP-2 levels (Fig. 5E) confirmed that PI3K activity was required for modulating MMP-2 activation. Furthermore, the effect of LY-294002 on MMP-2 was restricted to its activation, because no significant change was observed in the intensity of the latent MMP-2 band from LY-294002-treated samples relative to control. A second band that migrated slightly slower than latent MMP-2 in the zymogram (\sim 78 kDa, marked with *) was reduced in parallel with active MMP-2 (Fig. 5A), indicating that it is also sensitive to LY-294002. This band was not recognized by antibodies specific for either MMP-2 or MMP-9 (latent or active), thus revealing a second gelatinase of unknown identity is regulated by the same mechanism.

MMP-2 activation correlates with MT-1 MMP expression. Activation of MMP-2 requires proteolytic cleavage of the latent enzyme after its secretion from the cell. Two distinct enzymes have been proposed as catalysts for this event, furin and MT1-MMP (also designated MMP-14) (8, 32). To distin-

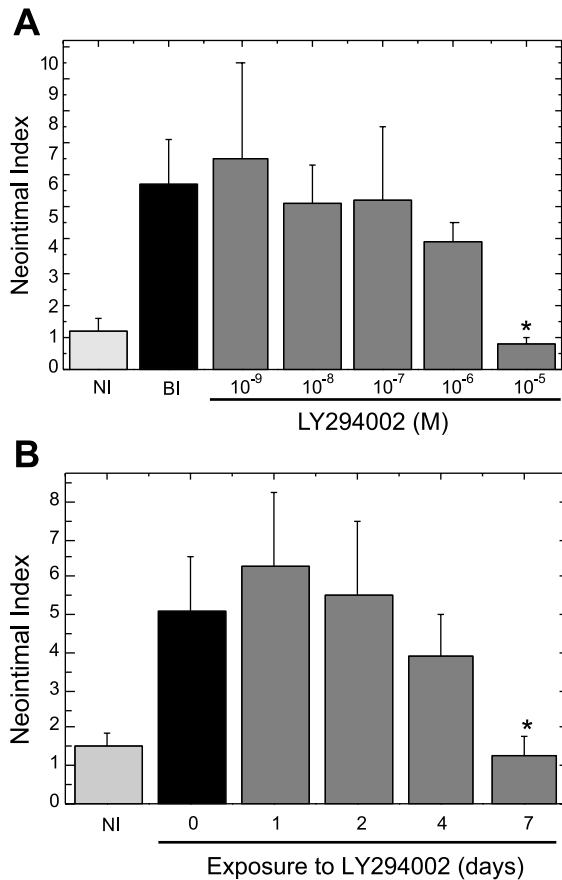


Fig. 3. Concentration- and time-dependent effects of LY-294002 on neointimal formation. *A*: vessel sections were incubated for 14 days with varying concentrations of LY-294002. Neointimal index was quantified as described in MATERIALS AND METHODS and is plotted as means \pm SE; $n = 8$. Statistically significant differences ($P < 0.05$) from balloon-injured control (BI) are indicated (*). *B*: LY-294002 ($10 \mu\text{M}$) was included in the culture medium for varying lengths of time, beginning with the first day of incubation. The inhibitor was then removed from the medium after the indicated number of days had passed, and incubation of the vessel segments was continued for a total of 14 days. Neointimal formation was quantified as described for *A* and plotted as means \pm SE; $n = 8$. Statistically significant differences ($P < 0.05$) from balloon-injured control (no LY-294002 exposure) are indicated (*). NI, noninjured.

guish between these candidates, two approaches were employed. A potent furin inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (DCMK), was added to the organ culture media for the entire 14-day incubation period, and MMP-2 activation was monitored by Western blot analysis. Quantification of these results (Fig. 6) revealed that DCMK had no effect, because only LY-294002 reduced the level of active MMP-2 accumulating in the culture media over time. These data suggest that furin does not mediate the conversion of latent MMP-2 to its active form by SMCs.

Because selective inhibitors of MT1-MMP have not been identified [e.g., GM-6001 inhibits MMP-9 $<$ MMP-2 $<$ MT1-MMP (60)], expression of MT1-MMP was monitored in cultured vessel segments after balloon angioplasty. In uninjured vessels, MT1-MMP was not detectable by immunological staining of the tissue (Fig. 7A). After angioplasty, however, elevated levels of MT1-MMP were observed after 4 and 10 days of culture (Fig. 7, A-C). The increase in MT1-MMP was

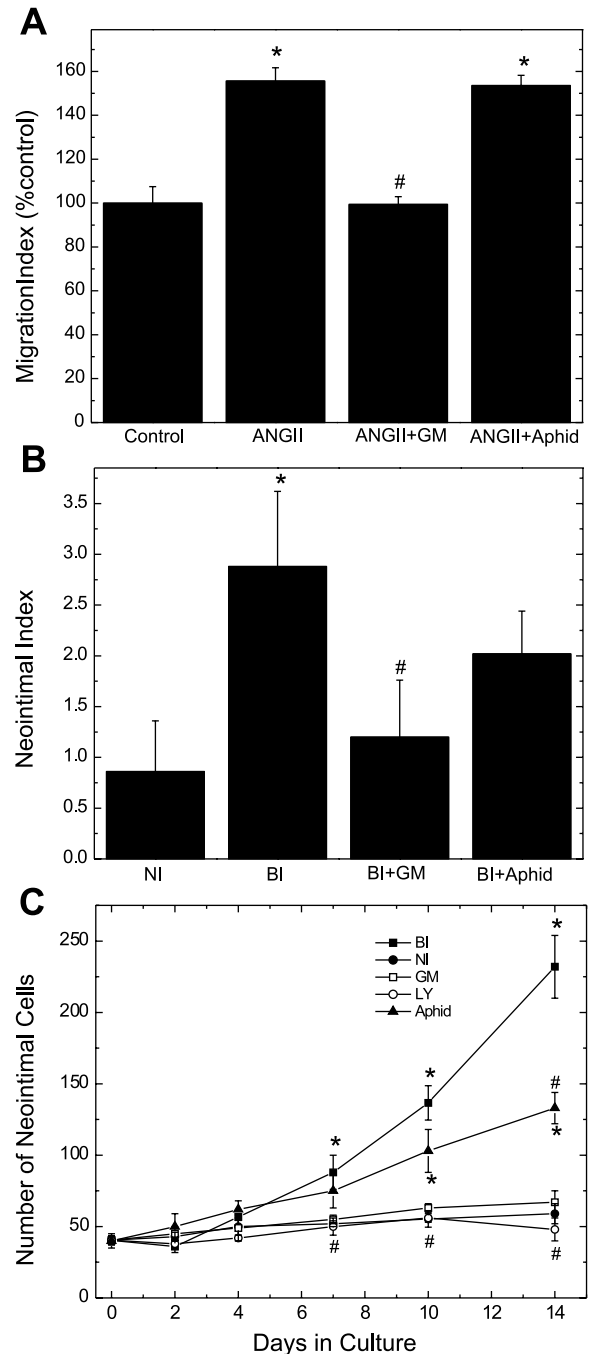


Fig. 4. Role of migration in neointimal formation. *A*: SMC migration was measured in a Boyden chamber with chemoattractant ($1 \mu\text{M}$ ANG II) in the lower reservoir and either GM-6001 (GM; 10^{-4} M) or aphidicolin (Aphid; $5 \mu\text{g/ml}$) in the upper reservoir. The migration index for each condition is presented as means \pm SE ($n = 6$) and is set relative to cell migration in the absence of ANG II (control). Statistically significant differences ($P < 0.05$) from control (*) and from ANG II-stimulated cells (#) are indicated. *B*: organ culture was conducted as described in Fig. 3A in the presence of either GM-6001 (10^{-4} M) or aphidicolin ($5 \mu\text{g/ml}$). The neointimal index is plotted as means \pm SE; $n = 8$. Statistically significant differences ($P < 0.05$) from noninjured control (*) and from untreated balloon-injured vessels (#) are indicated. *C*: neointimal cell number was quantified by counting cells in stained sections after 14 days in culture postangioplasty under the described conditions. Values represent means \pm SE per $2\text{-}\mu\text{m}$ -thick sections; $n = 6$ with 3 random fields counted per vessel. Statistically significant differences ($P < 0.05$) from uninjured control (*) and from untreated balloon-injured vessels (#) are indicated.

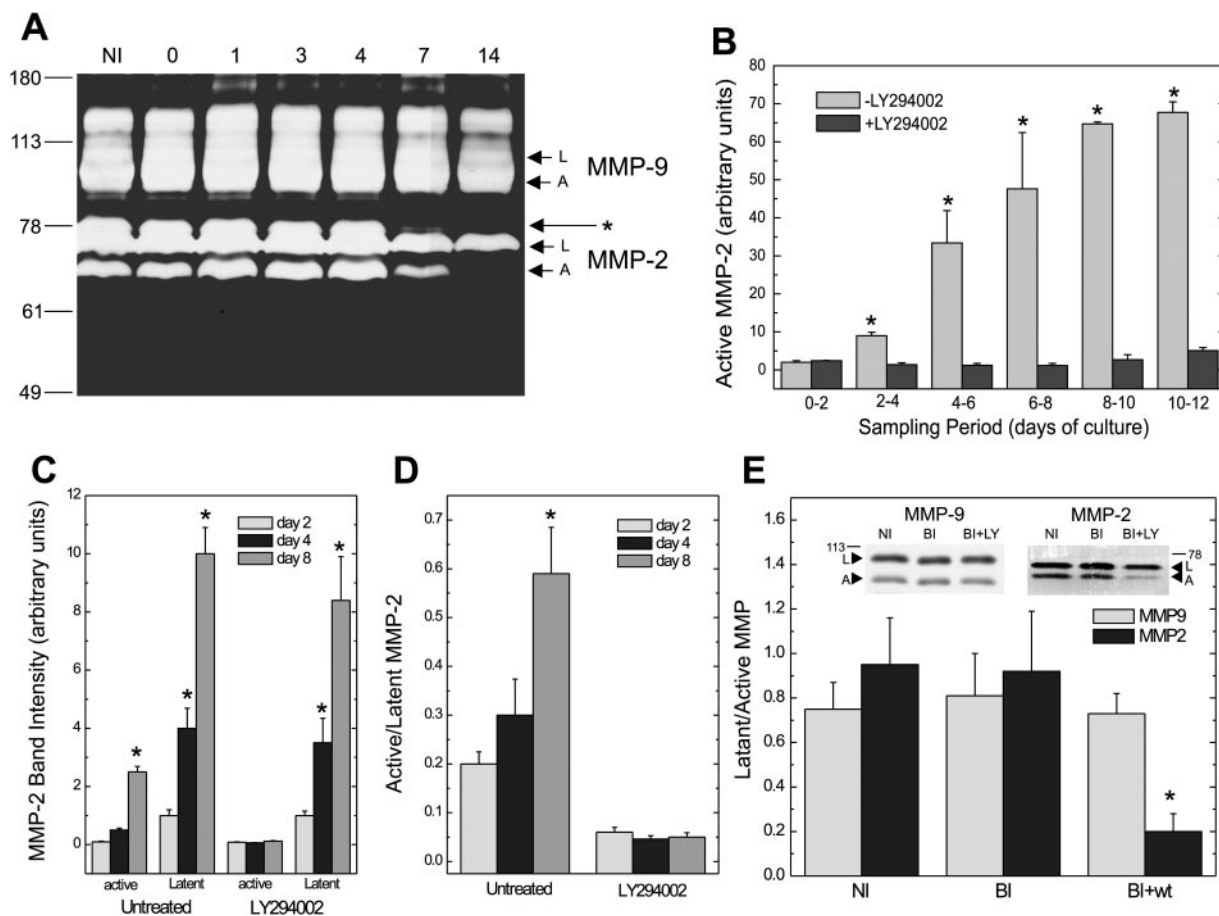


Fig. 5. PI3K inhibitors prevent activation of matrix metalloproteinase (MMP)-2 after vascular injury. *A*: organ culture media was obtained from balloon-injured vessel segments cultured for 14 days and exposed for varying lengths of time (0, 1, 2, 4, 7, and 14 days) to 10 μ M LY-294002 during this period, as described in Fig. 3*B*. The latent (L) and active (A) forms of MMP-9 and MMP-2 were detected by zymography. A noninjured control has been included for comparison. *B*: vessel segments were balloon injured and placed into culture in the absence or presence of 10 μ M LY-294002. Media were harvested every 2 days over a 12-day period and analyzed for MMP-2 activation by zymography. Lytic bands were quantified by scanning densitometry, and the data are presented as means \pm SE; $n = 8$. *C* and *D*: media from vessel segments incubated for 2, 4, or 8 days in the absence or presence of 10 μ M LY-294002 were analyzed by Western blot analysis for MMP-2. Relative intensity of the latent and active bands was determined by scanning densitometry, and the data are plotted as means \pm SE ($n = 4$) for each condition (*C*). The ratio of active MMP-2 to latent MMP-2 is presented separately (*D*). Statistically significant differences ($P < 0.05$) from day 2 samples (*) are indicated. *E*: MMP-9 and MMP-2 activation was monitored by Western blot analysis of media obtained after vessel segments were cultured for 14 days in the presence and absence of PI3K inhibitors. *Insets*, raw data with 10 μ M LY-294002 (the positions of the latent and active bands are indicated), whereas the graph shows data obtained with wortmannin (1 μ M). Treatment conditions included noninjured, balloon-injured, and balloon injured with either LY-294002 or wortmannin incubation. The data presented in the graph represent 3 independent experiments with $n = 4$ segments/condition. Statistically significant differences ($P < 0.05$) from the noninjured control (*) are indicated.

visible primarily within the forming neointima in the vicinity of the IEL, as previously reported by Wang and Keiser (53). Nevertheless, staining was also apparent at the boundary of the medial and adventitial layers, with weaker staining seen within the media. Treatment with 10 μ M LY-294002 was sufficient to prevent the increase in MT1-MMP, and staining was comparable to the control segment (Fig. 7, *A*, *D*, and *E*). Interestingly, breaks in the IEL was visible in sections prepared from both 4- and 10-day incubation periods (yellow arrows, Fig. 7*C*), whereas no breaks were detected in the LY-294002-treated sections. Western blot analysis of vessel extracts confirmed that there was a marked increase in MT1-MMP (both 63-kDa latent and 60-kDa mature forms) after 10 and 14 days of culture, whereas the presence of LY-294002 suppressed MT1-MMP expression (Fig. 7*B*).

Total RNA was extracted from uninjured vessels as well as balloon injured and injured LY294002-treated vessels that had been incubated for 6 days in organ culture and analyzed by semiquantitative RT-PCR amplification. MT1-MMP mRNA levels in these samples were compared by concurrently measuring mRNA for rP-L32, a gene known to be translationally regulated (28). No differences in MT1-MMP mRNA levels (relative to rP-L32 mRNA) were observed between control, injured, and LY-294002-treated vessels (Fig. 7*C*). These data indicate that MT1-MMP is constitutively expressed in these vessels, which therefore suggests that translational control of MT1-MMP expression may be the primary mode of regulation in response to injury. These data concur with those of Shofuda et al. (48), who reported high constitutive expression of MT1-MMP in rat carotid arteries.

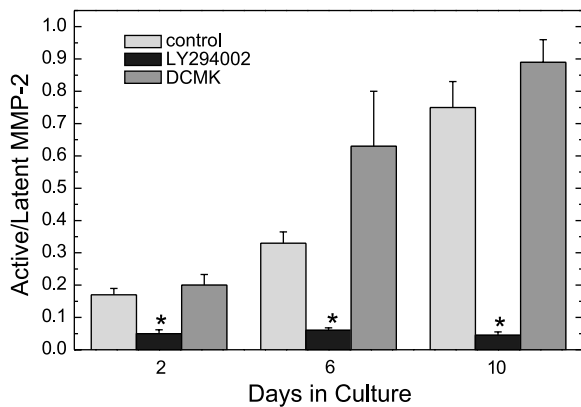


Fig. 6. Effect of furin inhibition on MMP-2 activation. Balloon-injured vessel segments were cultured in the absence or presence of either 10 μ M LY-294002 or 100 μ M decaanoyl-Arg-Val-Lys-Arg-chloromethylketone (DCMK) for 2, 6 or 10 days. Media samples taken at those times were analyzed by Western blot analysis for MMP-2, and the relative intensity of latent and active MMP-2 bands was quantified by scanning densitometry. The ratio of active to latent MMP-2 is plotted as means \pm SE; $n = 4$. Statistically significant differences ($P < 0.05$) from the respective controls (*) are indicated.

DISCUSSION

Modulation of the SMC phenotype is a critical event in the vascular response to injury. The consequent ability to migrate and proliferate enables SMCs to participate in remodelling of the damaged vessel. We have previously reported that PI3K is an important mediator of SMC proliferation (44). In this study, we present evidence that PI3K is also an important factor for SMC migration. Furthermore, our data suggest that PI3K contributes to this process by controlling the activation of a critical subset of MMPs, particularly MT1-MMP and MMP-2. The apparent function of PI3K is to regulate the expression of MT1-MMP after injury, which subsequently promotes the proteolytic conversion of latent MMP-2 to its active form. The presence of active MMP-2 then enables migration of SMCs into the lumen space, leading eventually to the formation of a neointimal lesion.

MMP expression is increased in response to vascular injury (49, 54), and their activation is required for SMC migration (2). Participation of MMPs in vascular repair was established by experiments showing both chemical and molecular inhibitors of MMP activity suppress neointimal formation (11, 67). The presence of both active and latent forms of these MMPs, particularly MMP-2 and MMP-9 (Fig. 5A), also suggests that the mechanisms required to activate the zymogens must be operational. On the other hand, the ability to selectively prevent MMP-2 activation with inhibitors of PI3K (Fig. 5) indicates that MMP-2 is differentially regulated in relation to other MMPs. On the basis of these differences, it may be argued that the various MMPs have distinct roles in vascular repair and their functions are linked to a specific route of activation. This view is supported by evidence that showed inhibition of a single MMP is sufficient to prevent neointimal hyperplasia (13, 30). However, identifying the precise physiological contribution of individual MMPs has been difficult, because there is a considerable overlap in substrate specificity of the many ECM degrading enzymes.

Recently, several research groups have attempted to define MMP function in terms of cell motility and cell invasiveness.

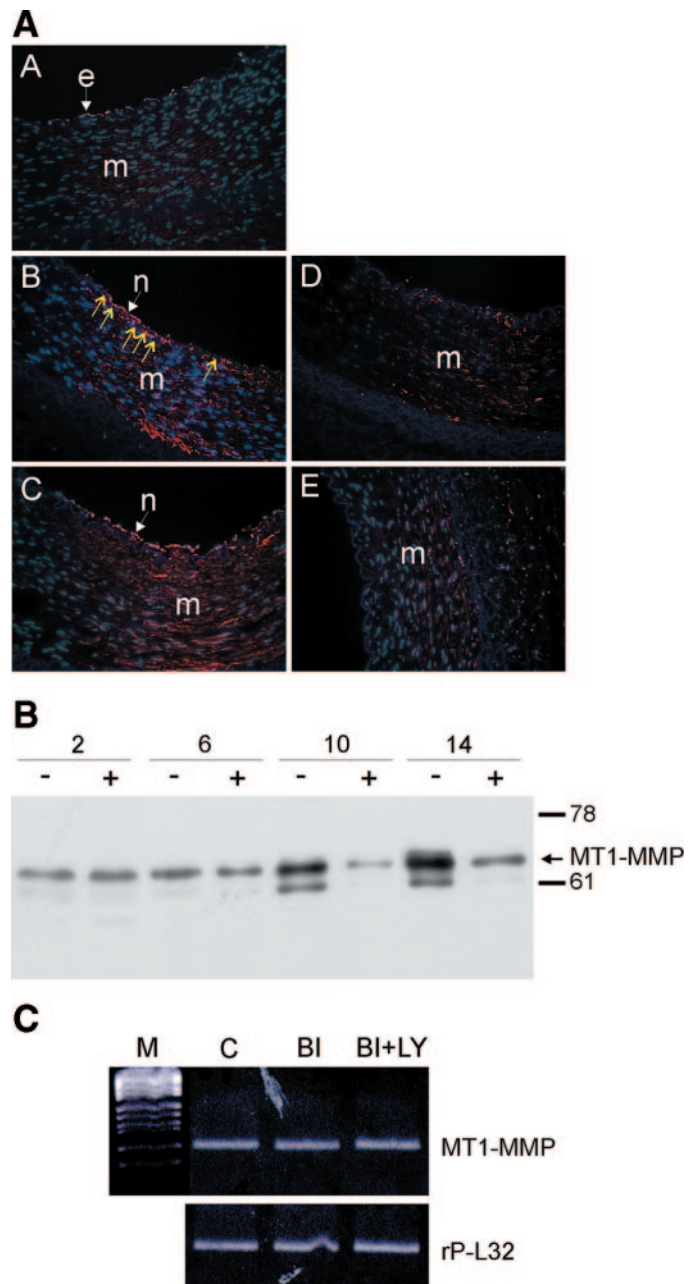


Fig. 7. PI3K inhibitors prevent injury-induced membrane-type 1 (MT1)-MMP expression. A: coronary artery vessels were balloon injured and placed into culture for 4 or 10 days in the absence or presence of 10 μ M LY-294002. MT1-MMP expression was assessed by immunohistological staining. Culture conditions include noninjured control (A,A), 4 days postballoon injury (A,B), 10 days postballoon injury (A,C), 4 days with LY-294002 (A,D), and 10 days with LY-294002 (A,E). MT1-MMP is visible as red staining, whereas nuclear staining with Hoescht 33342 is seen in blue. The locations of the medial layer (M), endothelial layer (e), and forming neointima (n) are indicated. B: vessel segments placed into culture after balloon injury for 2, 6, 10, and 14 days were extracted and analyzed by Western blot analysis for MT1-MMP. The vessels exposed to 10 μ M LY-294002 (+) and those not subjected to LY-294002 (-) are indicated. C: total RNA was extracted from noninjured control vessels and balloon-injured vessels incubated for 6 days in the absence or presence of 10 μ M LY-294002. RNA for MT1-MMP and ribosomal protein L32 (rP-L32) was amplified concurrently by RT-PCR, and the amplification products were analyzed on 2% agarose gels. Lane assignments are molecular markers (M), noninjured control (C), balloon injured, and balloon injured with LY-294002. Product lengths were 233 nucleotides for MT1-MMP and 255 nucleotides for rP-L32.

This approach has merit, because active MMP-2 is a prerequisite for migration through the IEL (27, 37) and subsequent development of a neointimal lesion (27). The data presented in Fig. 7A support the idea that MMP-2 may be essential for egress of SMCs from the media to the intima, as indicated by the decreased number of breaks in the IEL in the absence of active MMP-2. Furthermore, the absence of active MMP-2 as a result of inhibition with LY-294002 (which appears to be a more selective inhibitor than GM-6001) prevents movement of SMCs to the intima (Fig. 2D), because degradation of the IEL likely precedes migration into the luminal space. Thus the ability to regulate MMP-2 activation independent of other MMPs may be an important control point for modulating lesion formation given that inhibition of MMP-2 activity is sufficient to prevent neointimal hyperplasia in the presence of other active proteinases.

In addition to demonstrating the importance of MMP-2 activation in neointimal formation, this study has also established that activation of MMP-2 correlates with expression of MT1-MMP (MMP-14). Sato et al. (43) were the first to show activation of MMP-2 was coupled to transient expression of MT1-MMP, a member of the membrane-anchored metalloproteinase family (35). Subsequent investigations have established that MT1-MMP is essential for MMP-2 activation in many tissues, and MT1-MMP is likely the primary route for processing of latent MMP-2 *in vivo* (68). For this reason, modulation of MT1-MMP must be a critical event in the regulation of SMC motility after vascular injury (32, 36, 48). The data presented in this report (Fig. 7) agree with published evidence showing that MT1-MMP expression is induced in vascular tissues by injury (39, 48). A recent study (34) from this laboratory has confirmed that MT1-MMP becomes detectable after balloon angioplasty and extended these observations further by showing that expression of MT1-MMP is coupled to activation of the AT₁ receptor. This relationship provides an obvious link between vascular injury and PI3K, because PI3K activation is controlled via the AT₁ receptor (44). However, the mechanism by which MT1-MMP expression is regulated in vascular tissues has not been previously explored. Our data also indicate that translation of MT1-MMP mRNA, which is already present at high levels in noninjured vessels (Fig. 7C), is likely the critical point for exerting control of MMP-2 activation. But is this the only mechanism for controlling MT1-MMP? As is seen with other MMPs, processing of proMT1-MMP (63 kDa) is required to produce the mature and active form of MT1-MMP (61 kDa). Furin, a proprotein convertase known to be expressed by proliferating SMCs (51), has been shown to mediate MT1-MMP maturation (32). As a result, inhibition of furin with DCMK (1) can prevent MMP-2 activation in certain cell types (32). Our results, in contrast, show that DCMK does not inhibit MMP-2 processing in damaged coronary artery (Fig. 7), which likely suggests that furin does not participate in vascular tissue repair. Nevertheless, the presence of mature MT1-MMP (Fig. 7) implies that a mechanism for MT1-MMP activation must exist. Identification of the convertase responsible for MT1-MMP maturation and determination of whether its activity is constitutive or regulated will be necessary before it can finally be established whether PI3K contributes to the post-translational processing of MT1-MMP in addition to regulating expression of this gene. One possible candidate for this role is

PC5, a proprotein convertase whose expression by proliferating SMCs is PI3K dependent (50).

Our identification of PI3K as a critical factor in the control of MMP-2 activation and neointimal hyperplasia is based on data obtained with the inhibitors LY-294002 and wortmannin. Although individually these compounds are not sufficiently specific to clearly establish a role for PI3K [LY-294002 also inhibits casein kinase 2, whereas wortmannin inhibits myosin light chain kinase (7)], when employed together they have proven adequate for confirming the involvement of PI3K in various experimental systems (7, 19, 24, 26). Even in the absence of our data, there is considerable published evidence, based on both biochemical and molecular interventions, to link PI3K to MMP activation and cell migration. For instance, PI3K-dependent SMC migration has been reported in response to IGF-1, urokinase, PDGF, and VEGF (14, 19, 26, 61). Furthermore, these data are supported by the recent report of Huang and Kontos (18), who established that depletion of PIP₃ by overexpressing PTEN blocked SMC migration. On the other hand, the connection between PI3K and invasiveness is more tenuous. The most compelling evidence has been presented by Kubiawski et al. (24), who showed the invasive properties of glioma cells were inhibited by LY-294002 and wortmannin. Additionally, these researchers identified a link between PI3K and MMP activity, which had previously been shown necessary for the invasive properties of gliomas and other tumour cells (33, 52). Recently, Yoon et al. (64) have shown that PI3K is one element in the signaling pathway that is responsible for the activation of pro-MMP-2 in response to hydrogen peroxide treatment of fibrosarcoma and gliosarcoma cells. Not only was the elevation in active MMP-2 levels correlated with increased capacity to penetrate a collagen I coating but there was also a requirement for MT1-MMP in this process. Similarly, Hess et al. (17) have shown that PI3K regulates the MMP-2 and MT1-MMP activity of invasion-competent melanoma cells. In both latter cases, however, the mechanism by which PI3K regulated MT1-MMP activity was not identified.

What is the contribution of PI3K to other processes essential for neointimal formation? Our data suggest that PI3K operates primarily by stimulating SMC migration. PI3K has also been linked to SMC proliferation (44), which is essential for elaboration of the lesion after penetration of SMCs to the intima. Shigematsu et al. (47) reported that wortmannin blocked replication of medial SMCs *in vivo* if administered within 11 h after arterial injury yet had no effect on intimal SMC replication if administration was 7 days after injury. It may be argued that medial replication did not occur because the cells remained in contact with an undegraded extracellular matrix, whereas intimal cells were not similarly constrained. Also, the lack of effect of wortmannin on intimal cell replication supports Lynn et al. (31), who reported that SMC proliferation was inhibited by LY-294002 but not by wortmannin. The differential inhibition seen with these compounds suggests that SMC replication does not require PI3K but likely involves a wortmannin-independent, LY-294002-dependent mediator such as casein kinase 2 (7). As a result, there may exist the potential for selectively interfering with PI3K-dependent SMC migration after arterial injury, thus making it reasonable to propose PI3K as a target for developing novel therapeutic interventions designed to prevent the formation of vascular lesions (6, 65).

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