Involvement of Interleukin-1 Receptor–Associated Kinase-1 in Vascular Smooth Muscle Cell Proliferation and Neointimal Formation After Rat Carotid Injury

Manish Jain,* Ankita Singh,* Vishal Singh, Manoj Kumar Barthwal

Objective—Reduced frequency of atherosclerotic plaques is observed in interleukin-1 receptor–associated kinase-1 (IRAK1)–deficient mice; however, the underlying mechanism is not clear. Therefore, this study investigate the role of IRAK1 in vascular smooth muscle cell proliferation and neointimal hyperplasia.

Approach and Results—Stimulation of rat primary vascular smooth muscle cells with fetal bovine serum (10%) or platelet-derived growth factor-BB (20 ng/mL) for 15 minutes to 24 hours induced a time-dependent increase in IRAK1 and extracellular signal–regulated kinase (ERK) activation, proliferating cell nuclear antigen upregulation and p27Kip1 downregulation as assessed by Western blotting. Inhibitors of ERK pathway (U0126, 10 μmol/L), IRAK (IRAK1/4, 3 μmol/L), protein kinase C (PKC; Ro-31-8220, 1 μmol/L), siRNA of toll-like receptor-4 (200 nmol/L), and PKC-ε (200 nmol/L) significantly attenuated these changes. Platelet-derived growth factor induced endogenous IRAK–ERK–PKC-ε association in a toll-like receptor-4 and PKC-ε–dependent manner. A time-dependent increase in IRAK1 and ERK activation was observed after 15 minutes, 30 minutes, 1 hour, 6 hours, 12 hours, and 24 hours of carotid balloon injury in rats. Balloon injury induced endogenous IRAK–ERK–PKC-ε interaction. Perivascular application of IRAK1/4 inhibitor (100 μmol/L), U0126 (100 μmol/L), and IRAK1 siRNA (220 and 360 nmol/L) in pluronic gel abrogated balloon injury–induced ERK phosphorylation, activation, and p27Kip1 downregulation. Hematoxylin and eosin staining and immunohistochemistry of proliferating cell nuclear antigen and smooth muscle actin demonstrated that balloon injury–induced intimal thickening and neointimal vascular smooth muscle cell proliferation were significantly abrogated in the presence of IRAK1/4 inhibitor, IRAK1 siRNA, and U0126.

Conclusions—IRAK1 mediates vascular smooth muscle cell proliferation and neointimal hyperplasia by regulating PKC-ε–IRAK1–ERK axis. (***Arterioscler Thromb Vasc Biol*. 2015;35:1445-1455. DOI: 10.1161/ATVBAHA.114.305028.)**

Key Words: cyclin-dependent kinase inhibitor p27 ■ extracellular signal–regulated map kinases ■ interleukin-1 receptor–associated kinases

Recent evidence suggests the involvement of innate and adaptive immune responses in the initiation and progression of atherosclerosis and restenosis.[1] Proliferation and migration of vascular smooth muscle cell (VSMC) play a pivotal role in such disorders.[2] Arterial injury during percutaneous coronary intervention induces multiple signaling pathways, which leads to the migration of VSMCs into the intimal layer of the arterial wall, where they proliferate and synthesize extracellular matrix components.[2] Drug-eluting stents releasing cytotoxic agents, such as paclitaxel and sirolimus, have been reported to show a remarkable reduction in angiographic restenosis. However, they have the potential drawback of delaying vascular healing and increasing the late thrombotic risk.[3]

Innate immune cells within the inflammatory microenvironment of the vessel wall secrete various proinflammatory cytokines, such as tumor necrosis factor-α, interleukins (IL)-1, IL-6, and IL-8.[4] These along with elevated expression of adhesion molecules, matrix-degrading metalloproteinases, and other proinflammatory mediators create an environment that promotes VSMC proliferation, migration,[5] and progression of atherosclerosis.[6] Toll-like receptors (TLRs) play an important role in innate immune response and pathogenesis of atherosclerosis.[7,8] Fetal bovine serum (FBS) and platelet-derived growth factor (PDGF) are potent VSMC mitogens[9] and also activate the TLR pathway.[10,11] However, the role of TLR pathway in FBS- and PDGF-induced VSMC proliferation is less understood. Signaling from these receptors is mediated by the adaptor protein MyD88 and further by IL-1 receptor–associated kinase (IRAK).[12] Inactivation of MyD88 reduces macrophage recruitment in the vessel wall

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Nonstandard Abbreviations and Acronyms	
ERK	extracellular signal–regulated kinase
FBS	fetal bovine serum
IRAK1	interleukin-1 receptor–associated kinase
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PKC	protein kinase C
TLRs	toll-like receptors
VSMC	vascular smooth muscle cell

and attenuates atherosclerosis progression.[13] Activation of IRAK pathway induces production of inflammatory cytokines IL-1β and IL-18,[14] which may also influence VSMC proliferation during atherosclerosis progression. Reduced frequency of atherosclerotic plaques is seen in IRAK1-deficient mice.[15] Moreover, TLR engagement by various ligands also activates protein kinase C (PKC),[16] p38 mitogen–activated protein kinase, c-Jun N-terminal kinases, and extracellular regulated kinase (ERK).[17] Several PKCs, including PKC-ε, may activate the ERK pathway[18] and play a role in VSMC proliferation.[19] ERK1 and ERK2 also play a pivotal role in VSMC proliferation.[20] In recent years, several studies have demonstrated that ERK–mitogen-activated protein kinase inhibitors reduce neointimal hyperplasia.[21] p27Kip1 is a critical determinant for cell cycle progression.[22] The role of p27Kip1 in hyperplasic response is well established, and target deletion of the p27 gene in mice leads to enhanced growth and hyperplasia in multiple organs.[23] p27Kip1 also plays an important role in controlling cell cycle progression during normal development and functions as a tumor suppressor.[22] p27Kip1 regulates VSMC proliferation through an ERK-dependent post-transcriptional mechanism.[24] It has been shown that direct activation of ERK leads to a reduction of p27 protein and mRNA, thus supporting the central role of ERK in regulation of p27 expression.[24] In this study, we hypothesized that IRAK1 may modulate VSMC proliferation and neointimal formation in balloon-injured carotid artery by modulating ERK and p27Kip1.

To test the hypothesis, we analyzed the expression and activation of ERK, IRAK1, and p27Kip1 in FBS- and PDGF-BB–stimulated rat aortic VSMCs and in a rat carotid balloon injury model.

We demonstrate for the first time that IRAK1 in a PKC-ε–dependent manner facilitates VSMC proliferation and intimal thickening after vascular injury by activating ERK1/2.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

IRAK1 and ERK Activation During VSMC Proliferation

Serum deprivation of rat aortic VSMCs for 24 hours resulted in ≈90% synchronization of the cell cycle in the G0 phase. Treatment of quiescent VSMCs with FBS induced a time-dependent increase in VSMC proliferation, cell cycle progression, proliferating cell nuclear antigen (PCNA) expression, cell count, BrdU incorporation, and decrease in p27Kip1 expression when compared with unstimulated quiescent VSMCs (Figure I in the online-only Data Supplement). A significant increase in IRAK1 phosphorylation was observed after 15 minutes of FBS stimulation (≈1.6-fold). This was further increased at 30 minutes (≈2.6-fold). Subsequently, a reduction in IRAK1 phosphorylation was observed (Figure 1A). Similarly, PDGF-BB also induced IRAK1 phosphorylation at 15 minutes (≈3.6-fold), and this was subsequently decreased at 30 minutes (≈2.0-fold), 60 minutes (≈1.8-fold), and 6 hours (≈1.9-fold); however, this was still significantly more than the control (Figure 1B). IRAK1 mRNA levels were unaltered after FBS stimulation (data not shown). FBS also induced a significant increase in ERK phosphorylation after 15 to 60 minutes (≈3-fold) of treatment (Figure 1C). No significant difference was observed at other time points. Likewise, PDGF-BB also induced a significant increase in ERK phosphorylation at 15 minutes (≈2.8-fold) and 30 minutes (≈2.1-fold), and subsequently, a decrease was observed (Figure 1D). At other time points, it was not significantly changed.

IRAK1 and ERK Mediate FBS- and PDGF-BB–Induced VSMC Proliferation

For ascertaining the role of IRAK1 and ERK in VSMC proliferation, MTT assay, cell cycle analysis, and PCNA expression were monitored in IRAK1/4 inhibitor and U0126-pretreated and FBS- or PDGF-BB–stimulated cells. FBS-induced proliferation (≈1.9-fold) was significantly prevented in VSMCs pretreated with IRAK1/4 inhibitor (≈1.5-fold) or U0126 (≈1.7-fold) as determined by MTT assay (Figure 2A). Likewise, PDGF-BB–induced VSMC proliferation (≈1.6-fold) was also significantly attenuated in the presence of IRAK1/4 inhibitor (≈1.4-fold) or U0126 (≈1.7-fold; Figure 2B). It was observed that FBS-induced increase in the S phase (≈6.9-fold) was significantly abrogated in the presence of IRAK1/4 inhibitor (≈1.8-fold) or U0126 (≈3.0-fold; Figure 2C). Likewise, PDGF-BB–induced increase in the S phase (≈3.8-fold) was also significantly reduced in IRAK1/4 inhibitor (≈1.6-fold) or U0126-pretreated VSMCs (≈2.0-fold; Figure 2D). FBS-induced increase (≈2.1-fold) in the PCNA protein expression was significantly prevented in VSMCs pretreated with IRAK1/4 inhibitor (≈1.8-fold) or U0126 (≈2.1-fold; Figure 2E). FBS-induced downregulation (≈2.4-fold) of p27Kip1 protein expression was significantly upregulated in the presence of IRAK1/4 inhibitor (≈2.0-fold) or U0126 (≈2.0-fold; Figure 2F). Similarly, PDGF-BB–induced increase (≈3.4-fold) in PCNA expression was also prevented in the presence of IRAK1/4 inhibitor (≈1.6-fold) or U0126 (≈1.8-fold; Figure 2G). Moreover, PDGF-BB–induced decrease (≈0.5-fold) in the p27Kip1 protein expression was also significantly increased in VSMCs pretreated with IRAK1/4 inhibitor (≈0.5-fold) or U0126 (≈0.5-fold; Figure 2H).

IRAK1 Induces ERK Activation During VSMC Proliferation

To ascertain the effect of IRAK1 on ERK activation during VSMC proliferation, ERK activity and phosphorylation were

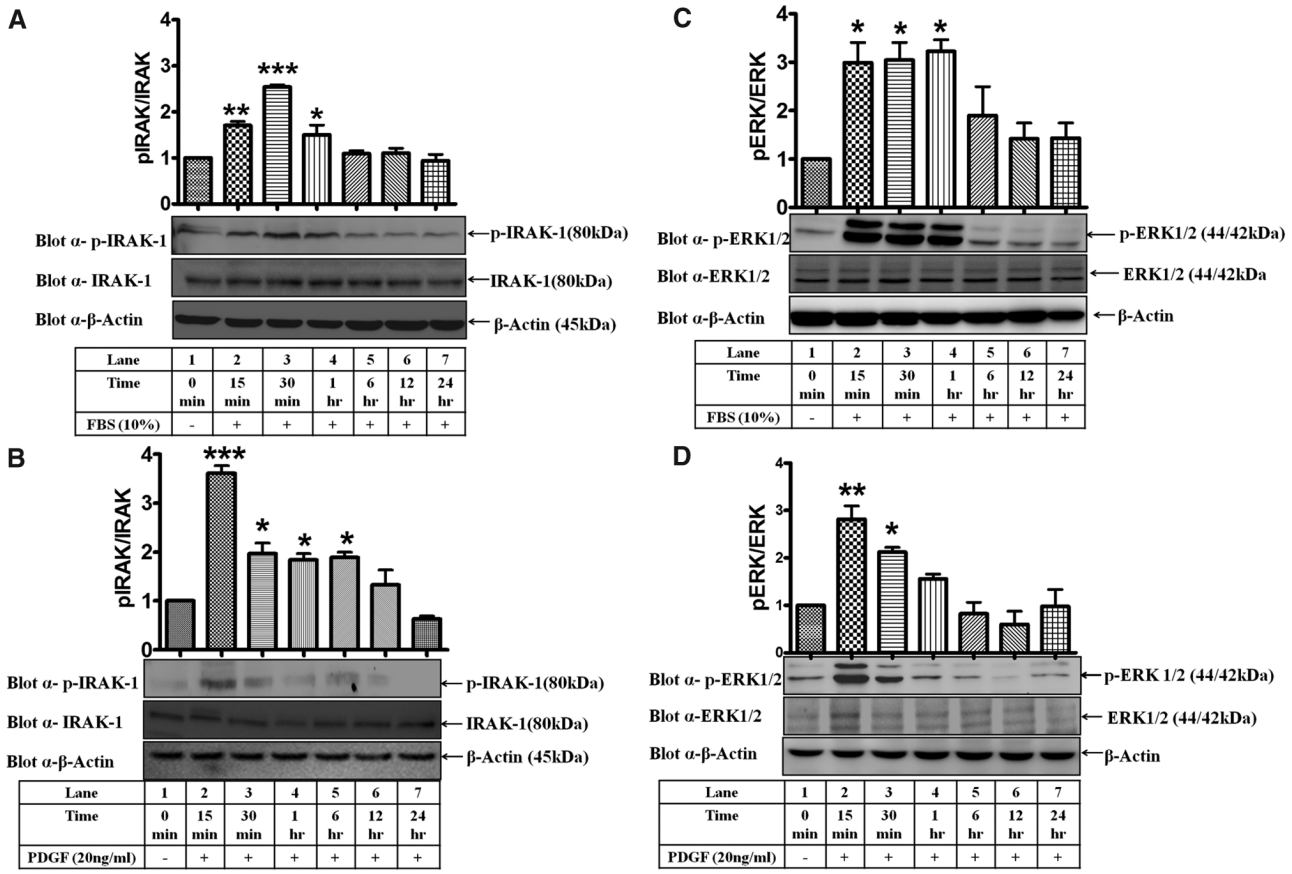

Figure 1. Fetal bovine serum (FBS) and platelet-derived growth factor (PDGF)-BB induces interleukin-1 receptor–associated kinase-1 (IRAK1) and extracellular signal–regulated kinase (ERK) activation in vascular smooth muscle cells (VSMCs). Quiescent VSMCs were stimulated with FBS (10%) or PDGF-BB (20 ng/mL) for 15 minutes, 30 minutes, 1 hour, 6 hours, 12 hours, and 24 hours. Cells were processed for p-IRAK1, IRAK1, p-ERK, ERK, and β-actin by Western blotting. Western blot analysis of IRAK1 phosphorylation (**A** and **B**) and ERK phosphorylation (**C** and **D**). Graph shows mean±SEM (n=3). **P*<0.05, ***P*<0.01, and ****P*<0.001 vs unstimulated quiescent VSMCs.

monitored in cells that were pretreated with IRAK1/4 inhibitor and U0126 and then stimulated with FBS or PDGF-BB. A significant increase in ERK activity was observed in VSMCs stimulated with FBS (≈1.9-fold; Figure 3A) or PDGF-BB (≈2.7-fold; Figure 3B). Concomitantly, VSMCs pretreated with IRAK1/4 inhibitor showed significant reduction in ERK activity after FBS (≈1.4-fold; Figure 3A) or PDGF-BB (≈1.5-fold) stimulation (Figure 3B). At the same time, a significant reduction in FBS (≈0.6-fold; Figure 3C) and PDGF-BB–induced (≈0.4-fold; Figure 3D) ERK phosphorylation was also observed in IRAK1/4 inhibitor–pretreated cells, suggesting that IRAK1 is upstream of ERK during VSMC proliferation. Transfection of IRAK1 siRNA in VSMCs led to a significant decrease in FBS-induced IRAK1 expression (≈0.5-fold) and ERK phosphorylation (≈0.6-fold; Figure 3E). However, no significant inhibition in IRAK phosphorylation was observed in cells pretreated with ERK pathway inhibitor U0126 (Figure 3F). Altogether, these results convincingly demonstrated that IRAK1 operates upstream of ERK during FBS- or PDGF-BB–induced VSMC proliferation.

TLR4- and PKC-ε–Dependent IRAK–ERK Activation During VSMC Proliferation

PDGF- and FBS-induced VSMC proliferation was significantly inhibited by pan PKC inhibitor (R0-31-8220) as shown by MTT, p27Kip1, and PCNA expression (Figure II in the online-only Data Supplement). Quiescent VSMCs were transfected scrambled, TLR4, PKC-ε, and PKC-δ siRNA with lipofectamine 2000 for 18 hours and then stimulated with PDGF for 24 hours. Significant (≈50%) knockdown of TLR4, PKC-δ, and PKC-ε was observed (Figure IIIA–IIIC in the online-only Data Supplement). TLR4 siRNA and PKC-ε siRNA–pretreated cells showed significant decrease in cell count and BrDU incorporation when compared with scrambled siRNA–treated cells (Figure IIID and IIIE in the online-only Data Supplement). However, there was no significant change in cell count and BrDU incorporation in PKC-δ siRNA–treated cells (Figure IIID and IIIE in the online-only Data Supplement). Moreover, PDGF-induced PCNA expression was significantly reduced in the presence of TLR4 and PKC-ε siRNA and not with PKC-δ siRNA (Figure IIIF in the online-only Data Supplement). A significant increase in p27Kip1 was observed in PDGF-stimulated and TLR4 and PKC-ε siRNA–pretreated (Figure IIIG in the online-only Data Supplement) cells when compared with PDGF and scrambled siRNA–treated cells. PDGF-induced IRAK (≈3-fold; Figure 4A) and ERK1/2 phosphorylation (≈2-fold; Figure 4B) was significantly reduced (*P*<0.05 and *P*<0.01, respectively) in the presence of Ro-31-8220. PDGF-stimulated IRAK (Figure 4C) and ERK1/2 (Figure 4D) phosphorylation was significantly

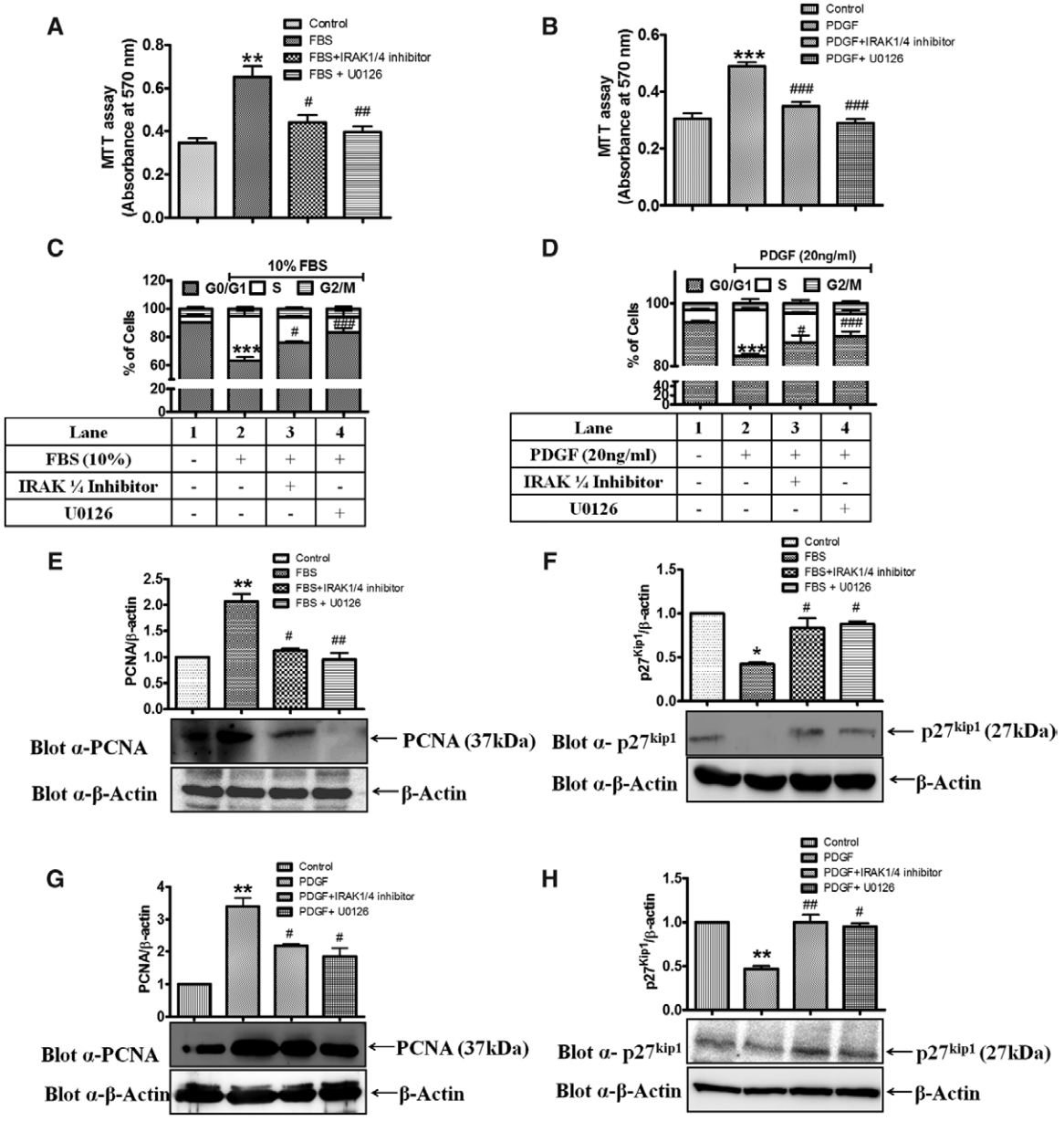

Figure 2. Role of interleukin-1 receptor–associated kinase-1 (IRAK1) and extracellular signal–regulated kinase (ERK) in vascular smooth muscle cell (VSMC) proliferation. Quiescent VSMCs were pretreated with IRAK1/4 inhibitor (10 μmol/L) and U0126 (3 μmol/L) for 1 hour and then stimulated with fetal bovine serum (FBS; 10%) or platelet-derived growth factor (PDGF)-BB (20 ng/mL) for 24 hours. Cells were processed for MTT assay (**A** and **B**) and cell cycle analysis (**C** and **D**). Bar diagrams represent MTT assay (**A** and **B**) and cell cycle analysis (**C** and **D**). Cells were also processed for proliferating cell nuclear antigen (PCNA), p27Kip1, and β-actin blotting. Western blot analysis of PCNA (**E** and **G**) and p27Kip1 (**F** and **H**) expression. The results are presented as mean±SEM (n=3). **P*<0.05, ***P*<0.01, and ****P*<0.001 vs unstimulated quiescent VSMCs. #*P*<0.05, ##*P*<0.01, and ###*P*<0.001 vs FBS or PDGF-BB–treated VSMCs.

inhibited (≈50%) when transfected with TLR4 siRNA and PKC-ε siRNA when compared with scrambled siRNA. However, there was no significant difference in IRAK phosphorylation when transfected with PKC-δ siRNA, suggesting TLR4- and PKC-ε–dependent and PKC-δ–independent IRAK and ERK phosphorylation (Figure 4C and 4D). To evaluate any physical association between PKC-ε, IRAK1, and ERK1/2, interaction studies were carried out. PKC-ε and ERK1/2 were detected in complex immunoprecipitated by anti-(α)-IRAK1 antibody or ERK1/2 antibody. A significant increase in PKC-ε–IRAK1–ERK1/2 interaction was observed in VSMCs on PDGF stimulation (2.0-fold; Figure 4E). There was significant reduction in PKC-ε and TLR4 expression after siRNA transfection. However, IRAK–ERK interaction was significantly reduced by PKC-ε siRNA, TLR4 siRNA, and R0-31-8220, and negligible interaction was observed with isotype IgG (Figure 4E). Significant knockdown of respective genes after siRNA treatment in IRAK–ERK phosphorylation experiments was confirmed by Western blotting (Figure IV in the online-only Data Supplement).

To ascertain whether agonism of IRAK by classical TLR ligands also activates ERK, studies were carried out by TLR4 agonist lipopolysaccharide. It was found that lipopolysaccharide treatment augmented TLR4 expression, suggesting that TLR4 operated

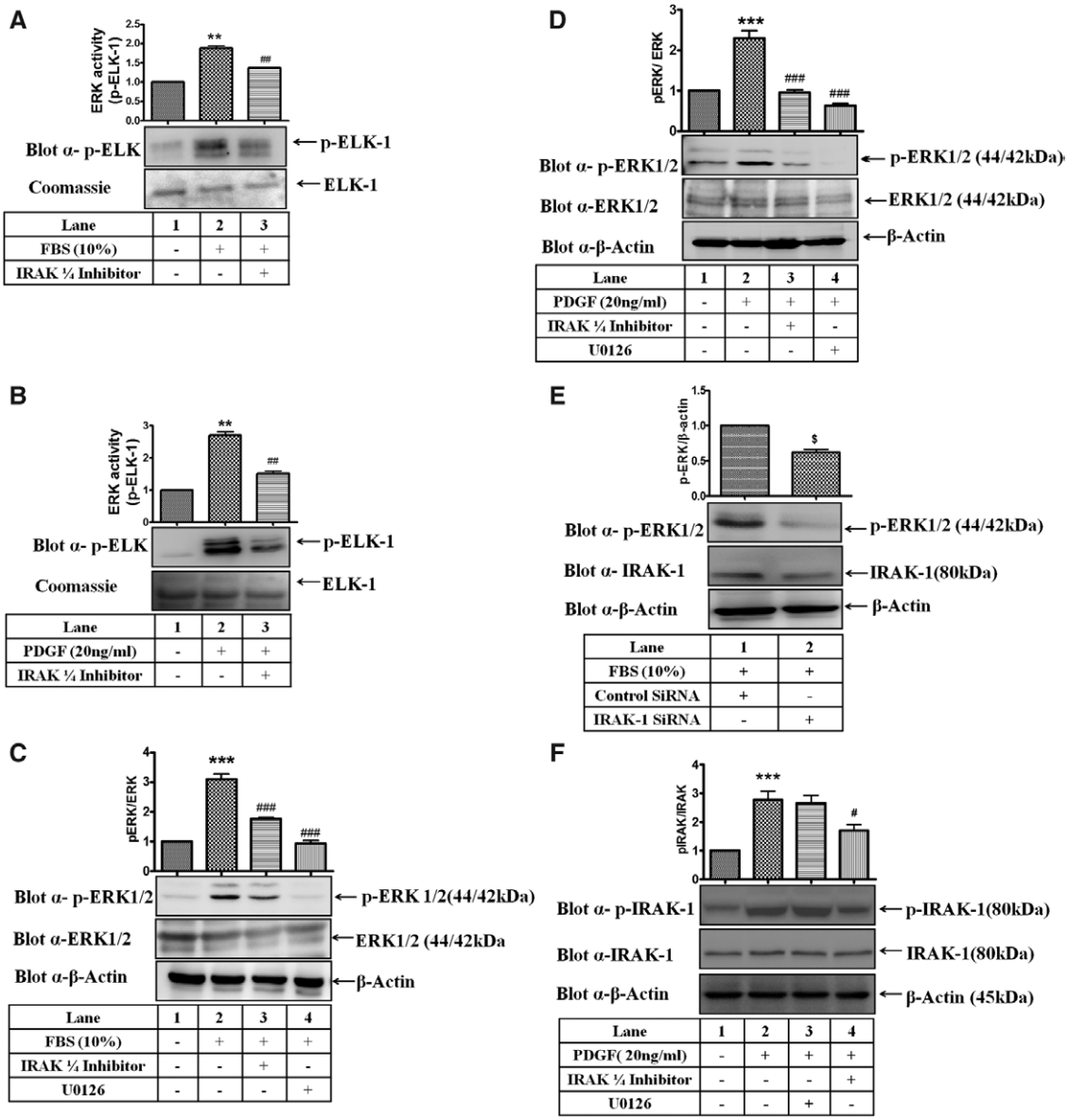

Figure 3. Interleukin-1 receptor–associated kinase-1 (IRAK1) induces extracellular signal–regulated kinase (ERK) activation during vascular smooth muscle cell (VSMC) proliferation. ERK activation was monitored by assessing ERK phosphorylation and ERK activity after fetal bovine serum (FBS; 10%) or platelet-derived growth factor (PDGF)-BB (20 ng/mL) stimulation of VSMCs for 30 minutes that were pretreated with IRAK1/4 inhibitor (10 μmol/L) and U0126 (3 μmol/L). Bar diagrams represent ERK activity (**A** and **B**) and ERK phosphorylation (**C** and **D**). **E**, IRAK1 expression and ERK activation in quiescent VSMCs transfected with control siRNA and IRAK1 siRNA (220 nmol/L) and stimulated with FBS (10%) for 30 minutes. **F**, IRAK phosphorylation was measured after pretreatment with IRAK1/4 inhibitor and U0126 followed by PDGF stimulation for 30 minutes (n=5). The results are presented as mean±SEM (n=3–5). ***P*<0.01 and ****P*<0.001 vs unstimulated quiescent VSMCs. #*P*<0.05, ##*P*<0.01, and ###*P*<0.001 vs FBS or PDGF-BB treated VSMCs. $*P*<0.05 vs control siRNA–transfected VSMCs.

upstream of IRAK–ERK–p27Kip1 axis. Lipopolysaccharide induced decrease in p27Kip1 expression, and it augmented PCNA expression and IRAK and ERK1/2 phosphorylation. This increased phosphorylation was significantly reduced in the presence of IRAK1/4 inhibitor again, establishing the IRAK–ERK axis (Figure V in the online-only Data Supplement).

IRAK1 Operates Upstream of ERK and p27Kip1 During Vascular Proliferation

Balloon injury induced a time-dependent increase in PCNA expression and decrease in p27Kip1 expression in the carotid arteries when compared with uninjured control (Figure VI in the online-only Data Supplement). Phosphorylation of IRAK1 and ERK was monitored to determine the activation of IRAK1 and ERK after injury to the rat carotid artery. A tendency of increase in arterial IRAK1 activation was observed at 15 minutes (≈1.7-fold) albeit not significant (Figure 5A). A significant increase in IRAK1 activation was observed after 30 minutes (≈2.5-fold) of balloon injury (Figure 5A). This was subsequently decreased at 1 hour (≈1.7-fold), 6 hours (≈1.2-fold), 12 hours (≈1.2-fold), and 24 hours (≈1.2-fold) after balloon injury (Figure 5A). IRAK1 mRNA levels were unaltered after balloon

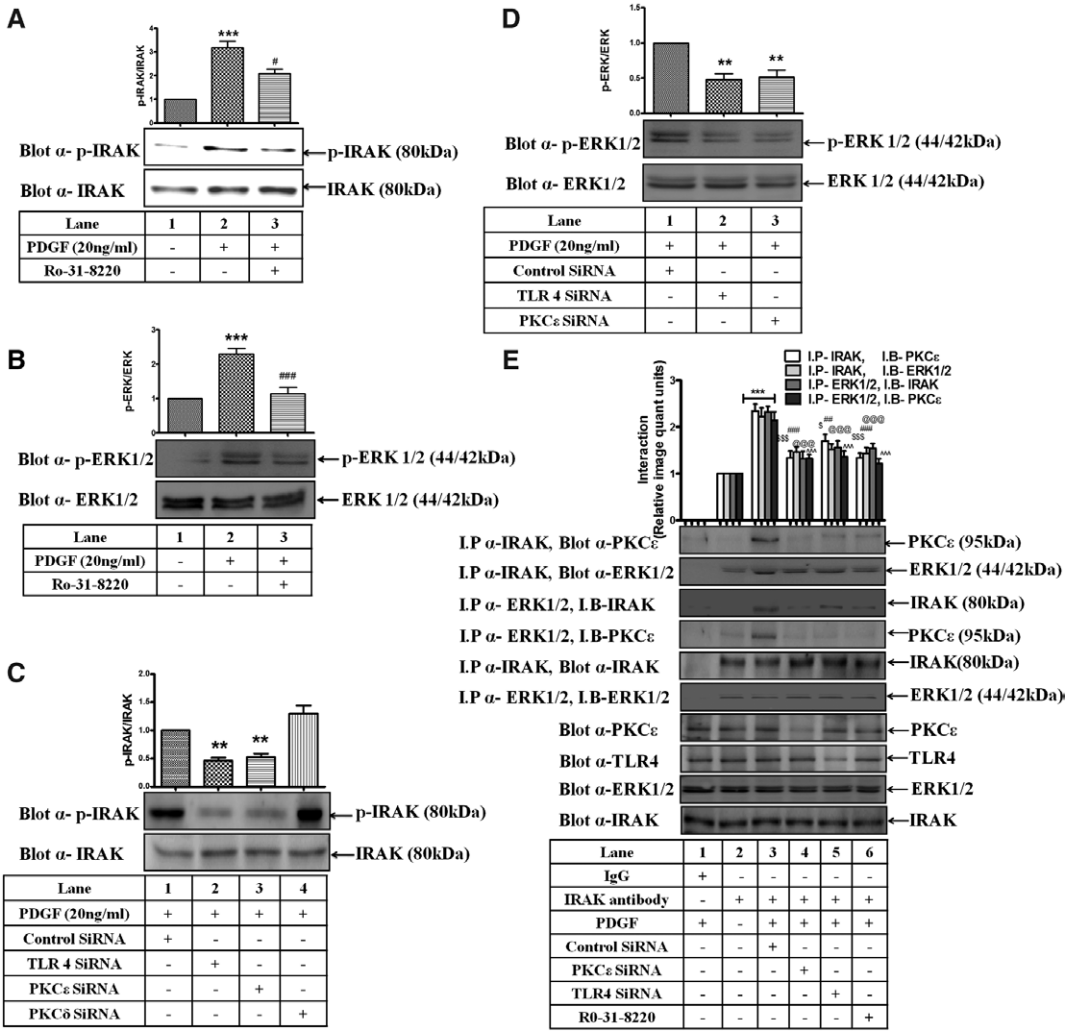

Figure 4. Platelet-derived growth factor (PDGF)–induced interleukin-1 receptor–associated kinase-1 (IRAK1)–extracellular signal–regulated kinase (ERK)-1/2 phosphorylation and association are toll-like receptor (TLR)-4 and protein kinase C (PKC)-ε dependent. Phosphorylation of IRAK (**A**) and ERK1/2 (**B**; n=5) was measured after Ro-31-8220 pretreatment and PDGF (20 ng/mL) stimulation for 30 minutes by immunoblotting. Quiescent vascular smooth muscle cells (VSMCs) were transfected with TLR4 siRNA, PKC-ε siRNA, and PKC-δ siRNA for 18 hours. Then stimulated with PDGF (20 ng/mL) for 30 minutes and phosphorylation of IRAK phosphorylation (n=5; **C**) and ERK1/2 (n=5; **D**) was analyzed by immunoblotting. **E**, VSMCs were pretreated with PKC-ε siRNA, TLR4 siRNA, and R0-31-8220 and stimulated with PDGF for 30 minutes (n=5). Then interaction studies were carried out by immunoprecipitation with IRAK and ERK and immunoblotting with PKC-ε, IRAK, and ERK1/2. The results are presented as mean±SEM (n=3–5). ***P*<0.01 and ****P*<0.001 vs unstimulated quiescent VSMCs or control siRNA–treated cells. $*P*<0.05 and $$$*P*<0.001 vs control siRNA–treated cells for immunoprecipitation (I.P)-IRAK and immunoblotting (I.B)-PKC-ε; #*P*<0.05, ##*P*<0.01, and ###*P*<0.001 vs PDGF-stimulated cells or control siRNA–treated cells for I.P-IRAK and I.B-ERK1/2; @@@*P*<0.01 vs control siRNA–treated cells for I.P-ERK1/2 and I.B-IRAK; ^^^*P*<0.01 vs control siRNA–treated cells for I.P-ERK1/2 and I.B-PKC-ε.

injury (data not shown). A significant increase in ERK 1/2 activation was observed after 30 minutes (≈2.4-fold), 1 hour (≈1.9-fold), 6 hours (≈2.0-fold), and 12 hours (≈1.9-fold) of balloon injury (Figure 5B). Subsequently, ERK activation declined to near control levels at 24 hours (≈1.2-fold) after injury (Figure 5B). To elucidate the mechanism by which IRAK1 and ERK induced vascular proliferation, p27Kip1 expression was monitored in uninjured artery and balloon-injured artery pretreated with or without IRAK1/4 inhibitor and U0126. Balloon injury–induced decrease in p27Kip1 expression (≈1.6-fold) was significantly increased in arteries pretreated with IRAK1/4 inhibitor (≈1.9-fold) or U0126 (≈2.2-fold; Figure 5C). At the same time, injury-induced enhanced PCNA expression (≈4.0-fold) was prevented in the presence of IRAK1/4 inhibitor (≈1.4-fold) or U0126 (≈1.9-fold; Figure 5D).

To ascertain the relative positioning of IRAK1 and ERK during vascular proliferation, IRAK1 and ERK phosphorylation was monitored in the presence of IRAK1/4 inhibitor, IRAK1 siRNA, and U0126. Balloon injury–induced ERK phosphorylation was significantly attenuated in the presence of IRAK1/4 inhibitor (≈1.8-fold; Figure 5E). However, IRAK1 phosphorylation was not significantly changed in the presence of U0126 (Figure 5F), suggesting that IRAK1 is upstream of ERK during neointimal lesion formation induced by balloon injury. siRNA-mediated gene silencing was done so as to confirm results with pharmacological inhibitors. IRAK1 siRNA dose dependently reduced the expression of IRAK1 by ≈42% and ≈62% at 220 nmol/L and 360 nmol/L concentrations, respectively (Figure 5G). IRAK1 siRNA also reduced ERK activation by ≈39% and ≈52% at 220 nmol/L and 360 nmol/L

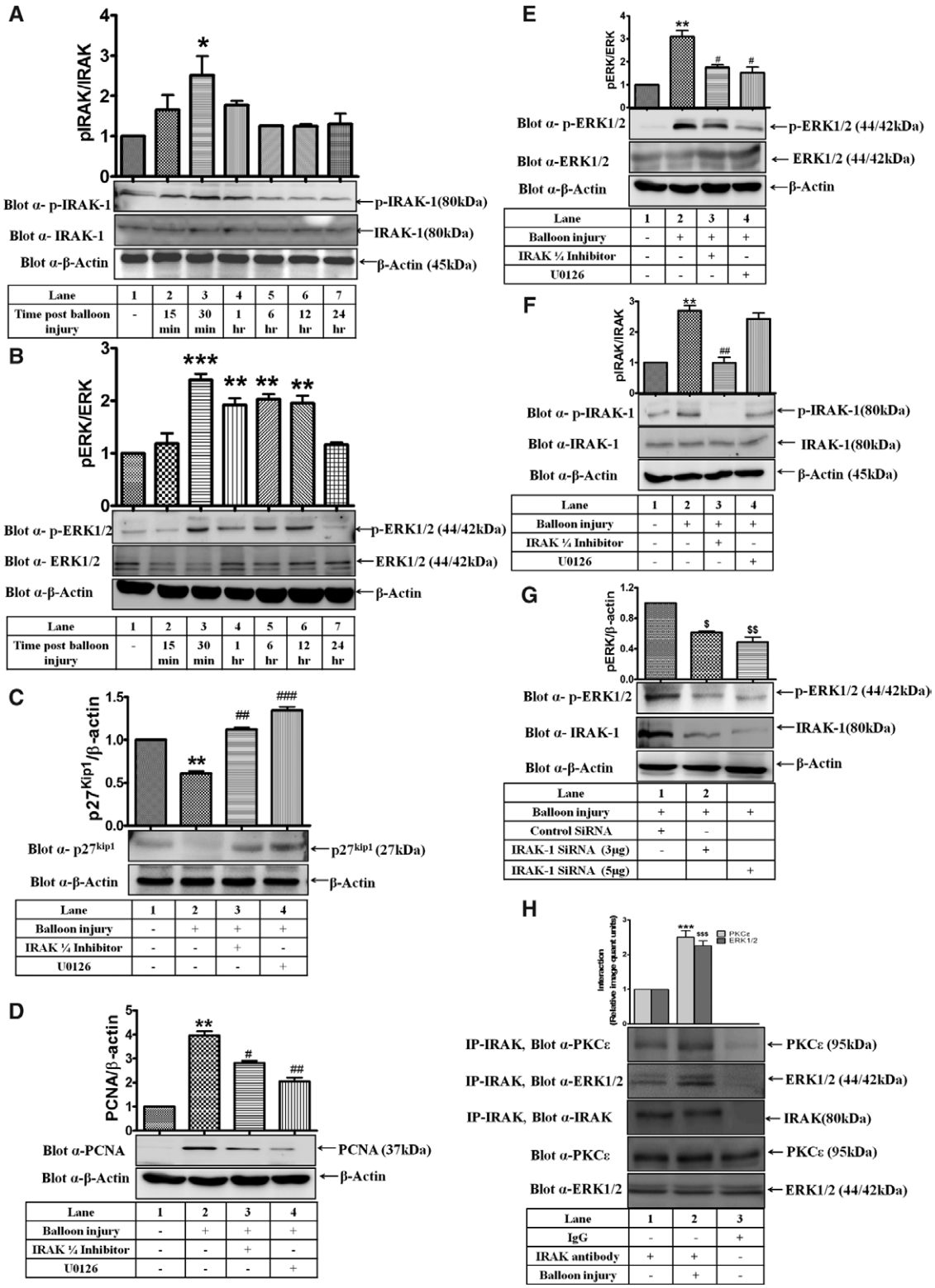

Figure 5. Interleukin-1 receptor–associated kinase-1 (IRAK1) mediates vascular proliferation by modulating extracellular signal–regulated kinase (ERK) and p27Kip1. Balloon injury was done in the carotid artery of rats. Phosphorylation of IRAK1 (**A**) and ERK (**B**) was determined after 15 minutes, 30 minutes, 1 hour, 6 hours, 12 hours, and 24 hours of balloon injury by Western blotting. Expression of p27Kip1 (**C**) and proliferating cell nuclear antigen (PCNA; **D**) was analyzed 24 hours after balloon injury, and phosphorylation of ERK (**E**) and IRAK1 (**F**) was monitored 30 minutes after balloon injury in carotid artery of rats pretreated for 1 hour with IRAK1/4 inhibitor or U0126. **G**, IRAK1 expression and ERK activation were analyzed 30 minutes after balloon injury in carotid arteries pretreated with IRAK1 siRNA (220 nmol/L [3 μg] and 360 nmol/L [5 μg]) for 24 hours. **H**, protein kinase C (PKC)-ε–IRAK–ERK interaction was studied in control and balloon-injured rats (n=5). Graph shows mean±SEM (n=3–5). **P*<0.05, ***P*<0.01, and ****P*<0.001 vs uninjured control artery; #*P*<0.05, ##*P*<0.01, and ###*P*<0.001 vs balloon-injured artery. $*P*<0.05, $$*P*<0.01, and $$$*P*<0.001 vs control siRNA–treated balloon-injured artery or control artery.

concentrations, respectively (Figure 5G). Moreover, there was significant increase in IRAK–ERK and PKC-ε interaction in the carotid artery after balloon injury when compared with uninjured control (Figure 5H). However, negligible interaction was observed with isotype IgG.

IRAK–ERK Axis Mediates Balloon Injury–Induced Neointimal Cell Proliferation and Hyperplasia

The role of IRAK1 and ERK on balloon injury–induced neointimal formation was determined by measuring the area of newly formed intima in carotid artery sections of rats that were pretreated with IRAK1/4 inhibitor, U0126, and IRAK1 siRNA. Hematoxylin and eosin staining in this study demonstrated blue nuclei and red-pink cytoplasm (Figure 6A–6E). There was no neointimal formation in the left carotid artery that was not subjected to vascular injury (Figure 6A). At 14 days post balloon injury, aggressive circumferential neointimal growth and luminal narrowing were evident in hematoxylin and eosin–stained cross-sections of carotid artery (Figure 6B) when compared with uninjured carotid artery, and this was prevented in IRAK1/4 inhibitor (Figure 6C), U0126 (Figure 6D), and IRAK1 siRNA-pretreated (Figure 6E) groups. The protection observed with U0126 was significantly more in comparison with IRAK1/4 inhibitor and IRAK1 siRNA. It was found that the percentage of PCNA-positive cells were rare in the uninjured control (Figure 6F), but a significant increase in the percent of PCNA-positive cells could be seen in the neointima of the balloon-injured artery (Figure 6G). Our results also showed prominent α-smooth muscle actin staining in the medial wall among all groups (Figure 6K–6O). Balloon injury–induced increase in neointimal area, intima/media area ratio, percent cross-sectional narrowing, and decrease in lumen area was significantly prevented in arteries pretreated with IRAK1/4 inhibitor, U0126, and IRAK1 siRNA (Figure 6 and Figure VII in the online-only Data Supplement, depicting quantification of the results shown in Figure 6). Altogether, these data show that IRAK and ERK inhibition leads to reduction in neointimal VSMC proliferation after balloon injury. At the same time, reduced phenylephrine–induced contractions in response to balloon injury were significantly increased in IRAK1/4 inhibitor, U0126, or IRAK1 siRNA–pretreated groups. Similarly, reduced acetylcholine–induced relaxation in phenylephrine precontracted rings from balloon-injured arteries was significantly increased in IRAK1/4 inhibitor, U0126, or IRAK1 siRNA–pretreated groups (Figure VIII in the online-only Data Supplement).

Discussion

Our in vitro and in vivo results demonstrate a novel role for IRAK1 in VSMC proliferation and neointimal hyperplasia in response to balloon injury. Besides this, we demonstrate existence and operation of PKC-ε–IRAK1–ERK axis during VSMC proliferation induced by FBS, PDGF-BB, and balloon injury.

FBS and PDGF are commonly used for inducing VSMC proliferation.[9] Because their stimulation is also associated with activation of TLR pathway,[10,11] it was considered worthwhile to study status of IRAK under such conditions. Therefore, the aim of this study was to decipher contribution of TLR pathway in the commonly used models of VSMC proliferation and neointimal hyperplasia. Because TLR4 is synonym with IRAK agonism[25] and is hypothesized to play a role in VSMC proliferation,[11] experiments were also performed with lipopolysaccharide. Stimulation with FBS often mimics the multiple factors environment faced in vivo; however, PDGF stimulation provides specificity. Therefore, we tested our hypothesis under 2 varied conditions that are known to activate the TLR pathway for understanding its role in VSMC proliferation.

During atherosclerotic lesion progression, VSMC proliferation is of particular pathophysiological importance.[26] Vascular response to mechanical injury involves complex cellular interactions that are coordinated and modulated by the elaboration of cytokines and growth factors.[27] Likewise, exposure of cells to growth factors or serum induces proliferation and activation of Ras/Raf/MEK pathway.[28] PCNA is considered to be a sign

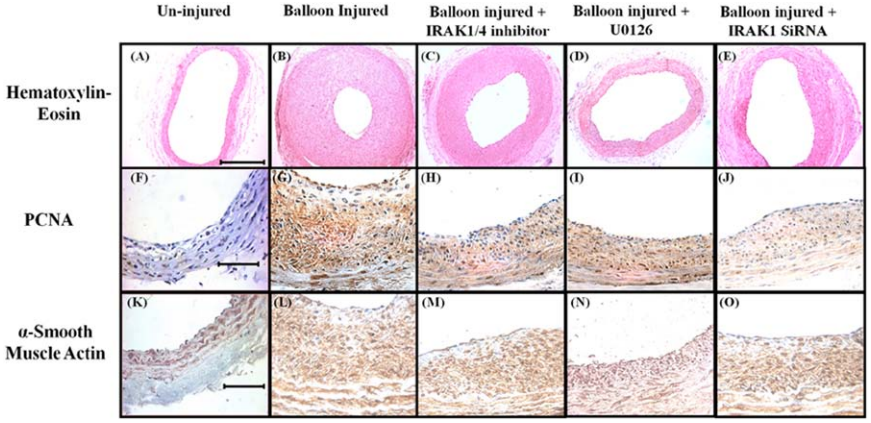

Figure 6. Interleukin-1 receptor–associated kinase-1 (IRAK1)–extracellular signal–regulated kinase (ERK) axis mediates balloon injury–induced neointimal cell proliferation and hyperplasia. Carotid arteries pretreated with IRAK1/4 inhibitor (100 μmol/L), U0126 (100 μmol/L), and IRAK1 siRNA (360 nmol/L) were analyzed 14 days after balloon injured. Representative images of hematoxylin and eosin (HE) staining (**A**–**E**) and immunohistochemical staining (**F**–**J**) showing proliferating cell nuclear antigen (PCNA) and α-smooth muscle actin (**K**–**O**). Results are representative arterial sections from at least 9 animals from each group. Magnification of photos is ×100 for HE and ×400 for α-smooth muscle actin and PCNA staining. Scale bar, 100 μm (**A**–**E**) and 20 μm (**F**–**O**).

of proliferation.[29] PCNA acts as a cell cycle cleavage protein and plays an important role in the process of DNA duplication and cleavage.[29] Higher PCNA expression is indicative of greater smooth muscle cell proliferation.[30] Our results showed that FBS and PDGF-BB stimulation leads to VSMC proliferation, cell cycle progression, and increased PCNA expression. Cellular proliferation is controlled by multiple holoenzymes comprising of a catalytic cyclin-dependent protein kinase and a cyclin regulatory subunit.[31] p27Kip1 is an inhibitor of cyclin E–cyclin-dependent protein kinase-2 complex and plays a critical role in cell cycle regulation by binding and inhibiting various cyclin-dependent protein kinase–cyclin complex activities.[22] p27Kip1 plays an important role in regulating entry into and exit from the mitotic cycle.[32] Overexpression of p27Kip1 in VSMCs results in G1 arrest and inhibition of cell growth.[33] A decrease in the level of p27Kip1 was observed in VSMCs stimulated with FBS and PDGF-BB, thus preventing G1 arrest and facilitating VSMC proliferation.

Previous reports suggest that TLRs are expressed constitutively on VSMCs from multiple vessel types, including human coronary artery and mouse aorta, and promote a proliferative VSMC phenotype.[34] Agonism of these receptors leads to the activation of IRAK pathway.[35] IRAK1 is also known to be expressed on human bronchial epithelial cells[36] and human airway smooth muscle.[37] In this study, both FBS and PDGF-BB induced IRAK1 phosphorylation. However, IRAK1 mRNA levels were unchanged indicating that IRAK1 activation was a consequence of an increase in its phosphorylation during VSMC proliferation. IRAK1/4 inhibitor used in the study does not rule out the role of IRAK4 in the FBS and PDGF-induced proliferation. Because TLR activation leads to IRAK4-dependent phosphorylation of IRAK1,[38] it is reasonable to think that the former will be involved. IRAK4 deficiency leads to reduced VSMC accumulation in vascular lesion indicating the role of IRAK4 in neointimal hyperplasia.[39]

PDGF is the potent regulator of VSMC migration and proliferation and exerts its effect, in part, through ERK1/2 activation.[40] PKC-δ mediated PDGF-BB–induced ERK1/2 activation in VSMCs.[41] In a previous study, it has been shown that stimulation of SMC migration by PDGF-BB involves the induction of PKC activity.[42] There are previous reports that also suggest that FBS, like phorbol esters, rapidly activates PKC in quiescent fibroblasts.[43] Recent work from the laboratory demonstrates the functional interaction between PKC-δ and IRAK1 in monocytic cells for IL-1 production.[44] Therefore, we tested this hypothesis in our present setup. Although PDGF and FBS-induced VSMC proliferation was PKC dependent, we did not observe any significant role of PKC-δ in PDGF-induced IRAK1 activation and VSMC proliferation. This may be because of varied cellular milieu in monocytic and smooth muscle cells required for regulating specific signaling pathway. Because PKC-ε was known to regulate VSMC proliferation,[19] we tested its role. PDGF induced VSMC proliferation and IRAK–ERK activation in PKC-ε–dependent manner. We did observe endogenous physical association between PKC-ε, IRAK, and ERK. Previous studies also indicate the role of PKC-ε in macrophage migration in an IRAK1-dependent manner.[45]

Our results clearly demonstrated that IRAK1/4 inhibitor and U0126 significantly prevented FBS or PDGF-BB–induced VSMC proliferation, PCNA expression, and cell cycle progression. These results agreed with those of previous studies that showed that ERK inhibition prevented VSMC proliferation.[46] Previous reports suggest that in rat aortic smooth muscle cells, PDGF-BB downregulated p27 protein and mRNA in an ERK-dependent post-transcriptional mechanism. The effect of PDGF on p27 protein and mRNA, as well as UTR-luciferase chimera expression, was blocked by ERK inhibition. However, overexpression studies showed that ERK activation leads to reduction of p27 protein and mRNA.[24] PDGF shortens the half-life of p27 mRNA and regulates p27 mRNA turnover through its 3′-UTR, whereas p27 promoter activity is not affected by PDGF.[24] These reasons may account for the increase in p27Kip1 protein expression observed after pretreating the cells with IRAK1/4 inhibitor or U0126.

In this study, both IRAK1 and ERK were activated on FBS and PDGF-BB stimulation, and in previous studies, IRAK1 seems to be essential for ERK activation.[47] We further tested the inter-regulation of these kinases during VSMC proliferation. IRAK1/4 inhibitor prevented FBS and PDFG-BB–induced ERK phosphorylation; however, U0126 did not affect IRAK1 phosphorylation. This indicated that IRAK1 operates upstream of ERK during VSMC proliferation. Furthermore, ERK1/2 activity was measured by in vitro kinase assay. The results demonstrated that both phosphorylation and activity of ERK1/2 are important. Because VSMC proliferation was inhibited by IRAK1/4 inhibitor and U0126 and also ERK activation was inhibited by IRAK1/4 inhibitor or IRAK1 siRNA, it can be said that IRAK–ERK axis is involved in FBS and PDGF-BB–induced VSMC proliferation.

To test the in vivo significance of IRAK–ERK–p27Kip1 regulation during VSMC proliferation, experiments were performed in a rat model of neointimal hyperplasia. Similar to in vitro results, balloon injury induced a time-dependent increase in PCNA protein expression, indicating early proliferative activity within the vessel wall. This proliferation was regulated by p27Kip1 because its expression was reduced in balloon-injured carotid arteries. A tendency of recovery in p27Kip1 was observed at later time points of balloon injury demonstrating a recovery response that has been shown earlier also.[48]

Early IRAK1 and ERK1/2 activation after balloon injury indicated the possible role of this pathway in neointimal hyperplasia. IRAK1 activation was at the phosphorylation level because its mRNA and protein levels were unchanged. In our study, IRAK1 and ERK inhibition was achieved by delivering their inhibitor through pluronic gel to the rat carotid artery. Recent studies show that perivascular application of mitogen-activated protein kinase inhibitors[21,49] or siRNA[50,51] using pluronic gel prevented intimal hyperplasia. IRAK1 and ERK regulated proliferation because pretreatment with their pharmacological inhibitors prevented the increase in balloon injury–induced PCNA expression. p27Kip1 played a role in proliferation because the inhibitors also prevented

its decrease. IRAK1 operates upstream of ERK and not the other way round because IRAK1/4 inhibitor and IRAK1 siRNA significantly inhibited ERK phosphorylation during neointimal lesion formation. However, U0126 had no effect on balloon injury–induced IRAK1 phosphorylation. We did observe endogenous association of PKC-ε, IRAK, and ERK after balloon injury. Because the TLR pathway feeds into the ERK pathway, inhibiting upstream of ERK may not completely abolish VSMC proliferation and neointimal hyperplasia. Therefore, targeting ERK directly may yield better results. However, if Ras–Raf–MEK–ERK pathway has a defining role in the activation of TLR pathway, it may be a better therapeutic target.

As shown earlier,[52] in this study also balloon injury showed endothelial denudation; however, other arterial structures, such the internal elastic lamina, media, and external elastic lamina, were found to be intact. Increased percentage of PCNA-positive cells and actin positive area in the neointima of balloon-injured carotid artery section indicated that neointima formation is substantially caused by excessive proliferation of VSMC leading to intimal thickening and luminal narrowing. This is further supported by previous reports where colocalization of PCNA and α-smooth muscle actin positive cells has been demonstrated within the neointima.[53] Interestingly, IRAK1 and ERK inhibition reduced the percentage of PCNA-positive cells in the neointima and the actin positive area, suggesting a potential role of these pathways in balloon injury–induced SMC proliferation. Our results revealed that neointimal hyperplasia was substantially retarded 14 days after balloon injury in arteries pretreated with IRAK1/4 inhibitor, IRAK1 siRNA, or U0126. However, U0126 offered better protection than IRAK1/4 inhibitor and IRAK1 siRNA, suggesting that other pathways may also feed into the ERK pathway during neointima formation. It can be hypothesized that PKC–IRAK–ERK axis regulates neointimal hyperplasia by regulating VSMC proliferation.

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Disclosures

None.

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Significance

The toll-like receptor pathway has significant presence in atherosclerosis. Interleukin-1 receptor–associated kinase (IRAK1) mediates the signaling downstream of toll-like receptors. Extracellular signal–regulated kinase (ERK) mediates cell proliferation, and in the vascular smooth muscle cell (VSMC), it regulates p27Kip1 during neointimal hyperplasia. In this study, we demonstrate the cross talk between IRAK1 and ERK during VSMC proliferation and neointimal hyperplasia. This study demonstrates activation of IRAK1, ERK, and p27Kip1 during fetal bovine serum and platelet-derived growth factor-BB–induced rat primary VSMC proliferation. Results demonstrate the importance of toll-like receptor-4 and protein kinase C-ε in IRAK–ERK–induced VSMC proliferation. Balloon injury in rat carotid artery also induces this pathway. More importantly, pharmacological or genetic ablation of IRAK1 by using specific inhibitor and siRNA in vitro and in vivo prevents fetal bovine serum, platelet-derived growth factor-BB, and balloon injury–induced ERK and p27Kip1 activation, VSMC proliferation, and neointimal hyperplasia. Our results propose a new protein kinase C–IRAK1–ERK axis for therapeutic intervention and also explain why targeting toll-like receptor pathway may be beneficial for atherosclerosis treatment.