Role of the JAK/STAT Pathway in Rat Carotid Artery Remodeling After Vascular Injury

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Abstract—In cultured vascular smooth muscle cells (VSMCs), Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) are expressed constitutively and play a role in angiotensin II (Ang II)–induced intracellular signaling and proliferation. However, little is known regarding the relevance of these proteins to the process of vascular remodeling. The role of JAK and STAT proteins in vascular remodeling and their functional coupling with Ang II were examined in balloon-injured rat carotid artery. Immunoreactive Jak2, Tyk2, Stat1, and Stat3 were not detected in the intact artery. Immunohistostaining showed transient expressions of these JAKs and STATs in medial and neointimal VSMCs at days 2 and 5, respectively, with a peak at day 7 in both layers. The expressions declined to insignificant levels by day 14. Ang II type 1 receptors (AT1s) were coexpressed in the medial and neointimal VSMCs expressing Jak2 and Stat3. The Jak2 and Stat3 inductions in the injured artery were accompanied by constitutive Jak2 and Stat3 phosphorylations, which were enhanced by ex vivo Ang II stimulation via AT1. Additionally, a Jak2 inhibitor, AG490, blocked the Ang II–induced Stat3 phosphorylation. Furthermore, local treatment with AG490 inhibited constitutive Stat3 phosphorylation and neointimal VSMC replication and subsequently reduced neointima formation in the injured artery. In conclusion, JAK and STAT proteins were inducible in medial and neointimal VSMCs after vascular injury and were functionally coupled to AT1. The inductions of JAKs and STATs would be involved in the mechanisms of neointima formation after vascular injury. (Circ Res. 2000;87:12-18.)

Key Words: signal transduction ▪ remodeling ▪ arteries ▪ muscle, smooth ▪ angiotensin
inhibitors or AT1 antagonists prevented the injury-induced neointima formation.10,11 Furthermore, Ang II stimulates VSMC proliferation in the injured artery.8,12 Moreover, gene expressions of most components of the renin-angiotensin system, including renin, angiotensinogen, angiotensin-converting enzyme, and AT1, are transiently induced in the neointima after vascular injury.13-16 These findings underscore the likelihood that Ang II is one of the potent contributors to neointima formation in this model.

Accordingly, we investigated not only the expression of JAKs and STATs in quiescent VSMCs in the rat intact carotid artery but also the temporal and spatial changes in JAK and STAT expressions in medial and neointimal VSMCs after balloon injury. Furthermore, functional coupling of JAK and STAT proteins in response to ex vivo Ang II stimulation was examined in the injured artery. We also investigated the effects of local AG490 treatment on the injury-induced neointima formation.

Materials and Methods

Vascular Injury Model

All procedures were approved by the institutional animal use and care committee and conducted in conformity with institutional guidelines. Male Sprague-Dawley rats were anesthetized by sodium pentobarbital (50 mg/kg), and then balloon injury of the left carotid artery was performed as described previously.17

Morphometry and Immunohistostaining Study

After rats (n=5) were killed with an overdose of pentobarbital, the carotid artery was perfusion-fixed at 100 mm Hg, excised, and embedded in paraffin. Serial sections of the transverse arterial rings were subjected to morphometry for assessing the intima/media area ratio (I/M ratio) and to immunohistostaining using a denoted primary antibody and a commercially available detection system (DAKO). An anti-AT1 polyclonal antibody18,19 was a gift of Dr H. Rakugi (Osaka University).

Immunoblotting Study

Protein sample was extracted from the homogenates of the carotid artery (n=5), separated by 10% SDS-PAGE, and blotted onto polyvinylidene difluoride membrane, as described elsewhere.20 Blots were probed with a denoted primary antibody, and the signals were analyzed by the chemiluminescence detection system and the laser digital analyzer (FluorImager, Amersham Pharmacia Biotech).

Jak2 and Stat3 Phosphorylation in the Balloon-Injured Artery

At day 7, the injured artery was excised and cut into strips. The arterial strips were equilibrated in HBSS for 60 minutes (37°C) in the presence of 100 nmol/L candesartan (Takeda Chemical Industries) or vehicle. Ang II (1 μmol/L, Sigma) or vehicle was applied to the strips for 20 minutes (37°C), and the strips were immediately frozen in dry ice/acetone (n=5). In some experiments, the arterial strips were incubated in HBSS for 16 hours (37°C) with 10 μmol/L AG490 (Calbiochem) or vehicle, and then Ang II was applied to the strips (n=5). Phosphorylated Stat3 was detected by immunoblotting using phosphospecific Stat3 antibody. Jak2 phosphorylation was assessed on the basis of anti-Jak2 immunoprecipitation followed by anti-phosphotyrosine immunoblotting, as described previously.15 Immunoreactive bands were quantified by FluorImager, and the signal intensity of phosphorylated Jak2 and Stat3 was normalized by that of total Jak2 and Stat3, respectively.

![Figure 1. Representative immunoblots for Jak2, Tyk2, Stat1, and Stat3 in intact (left) and balloon-injured carotid arteries at days 7 and 14 (n=3).](image)

Local Treatment With AG490 of the Balloon-Injured Artery

AG490 was dissolved in 25% pluronic F127 gel (Sigma) with the final concentrations of 0.5, 1, or 2 mg/mL. At the time of balloon injury (n=5), gel containing AG490 or vehicle was applied around the middle segment (2 cm in length) of the injured carotid artery (1 mL per 1-cm length of the artery segment), as previously described.21 As a control experiment, 2 mg/mL AG490 was applied around the sham-operated carotid artery.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

After balloon injury, the neointima was first seen in the denuded left carotid artery at day 5, and then a transient burst phase of VSMC proliferation was observed during days 5 to 14. At day 14, the neointimal area peaked, with an I/M ratio of 1.87±0.24 (0.03±0.01 in the sham-operated rats, P<0.01).

Transient Induction of JAK and STAT Expression

Immunoblotting demonstrated that Jak2, Tyk2, Stat1, and Stat3 proteins were not expressed in the intact rat carotid

<table>
<thead>
<tr>
<th>Time Courses of Expressions of Jak2, Stat3, AT1, SM2, and SMemb in VSMCs of Balloon-Injured Carotid Artery (n=5)</th>
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Intensity of the signal of the protein of interest was graded to negative (−), weak (+), moderate (++), and strong (+++). NA, no neointima was observed.
artery (Figure 1). In balloon-injured artery, expression of these JAKs and STATs was transiently observed at day 7 and decreased to insignificant levels at day 14.

Immunohistostaining was performed to investigate the temporal and spatial changes in the expression of JAK and STAT proteins in intact and balloon-injured carotid arteries (Table). Immunoreactive Jak2 and Stat3 were not detectable in medial VSMCs in the intact carotid artery (Figure 2). Transient expression of Jak2 and Stat3 was observed after vascular injury. In medial VSMCs, immunoreactive Jak2 and Stat3 were observed beginning at day 2 and peaked at days 5 to 7. Proliferating neointimal VSMCs showed immunoreactivities of Jak2 and Stat3 at day 5, and the Jak2 and Stat3 expressions peaked at day 7. Jak2 and Stat3 were scarcely found in both layers at day 14. Transient Tyk2 and Stat1 expressions were detected in a time course and distribution similar to those of Jak2 and Stat3 (data not shown). The transient induction of JAKs and STATs was not observed in either sham-operated carotid artery or right carotid artery of the injured rats. The cells expressing Jak2 and Stat3 in the media and neointima were identified as VSMCs on the basis of coimmunostaining with a VSMC marker, α-smooth muscle actin (data not shown).

Coexpression of AT1 with Jak2 and Stat3
Temporal and spatial changes of AT1 expression were examined in balloon-injured artery (Figure 3A) because AT1 is the upstream molecule that activates the JAK/STAT pathway in cultured VSMCs.5 The baseline AT1 expression was observed constitutively in medial VSMCs in the intact artery. Vascular injury triggered a transient increase in AT1 expression in medial and neointimal VSMCs at days 2 and 5, respectively, with a peak at day 7 in both layers. The AT1 expression returned to control level in both layers by days 14 to 28. These temporal changes of AT1 expression were in accord with earlier studies.13,16 Immunohistostaining for AT1, Jak2, or Stat3 was performed using serial tissue sections at day 7 when all of these proteins showed the maximum expression. AT1 was coexpressed in the VSMCs, which showed strong immunoreactivities against both Jak2 and Stat3 (Figure 3B).

Phenotype Transition of VSMCs After Balloon Injury
To determine the relation of the phenotype transition of VSMCs to the transient expression of JAK and STAT
proteins after vascular injury, immunohistostaining against the phenotype-specific smooth muscle myosin heavy chain (MHC) isoforms SM2 and SMemb was performed using serial sections (Table). The media of the intact carotid artery consisted of SM2 VSMCs, suggesting that these VSMCs were of the differentiated and contractile phenotype. After balloon injury, the most medial VSMCs were SM2+ throughout the observation period, whereas SMemb+ cells, indicative of the dedifferentiated and proliferative phenotype, were transiently found at days 2 to 7, but were not observed at day 14. In the neointima, SMemb+ VSMCs were exclusively observed from days 5 to 14, although SM2+ cells were scarcely found.

Jak2 and Stat3 Phosphorylations

Immunoblotting studies showed that a transient Stat3 induction was accompanied with constitutive phosphorylation in balloon-injured artery (Figure 4A). The constitutive Stat3 phosphorylation was detected in the injured artery after day 5, peaked at day 7, and declined to insignificant levels at day 14. To determine functional coupling of the inducible Jak2, Stat3, and AT1, balloon-injured arterial strips were stimulated by Ang II ex vivo, and then Jak2 and Stat3 phosphorylation levels were investigated on the basis of immunoblotting. Signal of phosphorylated Jak2 or Stat3 was not detected in the strips of the intact artery, irrespective of ex vivo Ang II stimulation (data not shown). Constitutive Jak2 and Stat3 phosphorylations were found in balloon-injured artery at day 7. The Jak2 and Stat3 phosphorylations were markedly enhanced by ex vivo Ang II stimulation (Figure 4B). The Ang II–induced Jak2 and Stat3 phosphorylations were blocked by an AT1–specific inhibitor, candesartan, although candesartan did not have significant effects on the constitutive Jak2 and Stat3 phosphorylations. Furthermore, the Ang II–induced Stat3 phosphorylation was significantly reduced by ex vivo pretreatment with AG490, a specific Jak2 inhibitor, whereas the constitutive Stat3 phosphorylation was not affected by the ex vivo AG490 pretreatment (Figure 4C).

**Effects of Local Treatment With AG490 on Neointima Formation**

To determine the role of JAK/STAT proteins in VSMC regulation in vivo, we studied the effects of periadventitial treatment with AG490 dissolved in pluronic F127 gel, on neointima formation. There were no apparent differences in body weight or other systemic conditions between controls and AG490-treated rats. Inflammation or necrosis was not found in the AG490-treated artery, irrespectively of vascular injury, during the observation period. Additionally, locally applied AG490 showed neither macroscopic nor microscopic changes in the sham-operated artery over the course of this study. At day 14, neointima formation after balloon injury was reduced by the AG490 treatment, and the inhibitory effect of AG490 was dose dependent (Figure 5). The I/M ratio of the injured artery was reduced by 65% in the AG490-treated rats versus the vehicle-treated rats (P<0.01, Figure 5B). The vehicle treatment had no effect on the I/M ratio at day 14 (1.75±0.14).

Effects of AG490 on neointimal VSMC replication were evaluated at day 7 on the basis of immunohistostaining for proliferating cell nuclear antigen (PCNA), a marker of cell replication in vivo (Figure 6A), because preliminary experiments showed that balloon injury induced a transient increase in the PCNA+ neointimal VSMCs after day 5 with a peak at day 7, declining to lower levels at day 14. Local treatment with AG490 reduced the number of PCNA+ neointimal VSMCs at day 7 by 60% and the I/M ratio at day 7 by 57%, as compared with vehicle treatment (Figure 6B, P<0.05). Furthermore, at day 7, the constitutive Stat3 phosphorylation in the injured artery was remarkably inhibited by the local AG490 treatment (Figure 6C, P<0.01).

**Discussion**

The present study demonstrated for the first time that JAK (Jak2, Tyk2) and STAT (Stat1, Stat3) proteins were below
detection levels in in vivo VSMCs of the rat intact carotid artery and were transiently expressed in medial and neointimal VSMCs during the process of neointima formation after balloon injury. The Jak2 and Stat3 inductions in the injured artery were accompanied by the constitutive Jak2 and Stat3 phosphorylations, which were enhanced by ex vivo Ang II stimulation via AT1. AG490 inhibited the ex vivo Ang II–induced Stat3 phosphorylation. Furthermore, local treatment with AG490 not only inhibited the constitutive Stat3 phosphorylation and VSMC replication but also reduced neointima formation after vascular injury.

The processes of vascular remodeling after balloon injury in rats can be divided into the following 4 phases. The first phase is the burst of medial VSMC proliferation that peaks within a few days after injury. The second phase involves VSMC migration into the intima, beginning at days 4 to 5. The third phase is characterized by extracellular matrix deposition. Nevertheless, very little is known about intracellular signal events after vascular injury. Recently, involvement of the extracellular signal–regulated kinase (ERK)-1/ERK2 pathway, which is commonly related to cell growth in response to growth factors, has been suggested in vascular remodeling of rat balloon-injured artery. However, despite the constant ERK1/ERK2 expression levels, an ERK kinase inhibitor reduces medial VSMC replication but does not...
affect neointimal VSMC proliferation.21 Thus, although ERK1/ERK2 activation may induce medial VSMC replication in the first phase in response to vascular injury, an alternative signaling pathway must be involved in neointima formation in the second and third phases. As shown in Figure 2 and the Table, JAKs and STATs were inducible simultaneously in medial and neointimal VSMCs, beginning in the first phase, reaching the maximum in the second and third phases. Inducible Jak2 and Stat3 were accompanied by constitutive phosphorylation in the injured artery (Figure 4). Moreover, local treatment with AG490 inhibited constitutive Stat3 phosphorylation and neointimal VSMC replication in the injured artery (Figure 6). Because the size of the neointimal lesion is mainly dependent on VSMC accumulation, inhibition of VSMC replication by AG490 was considered to reduce neointima formation. As expected, AG490 reduced neointima formation (Figures 5 and 6). Taken together, constitutive Stat3 phosphorylation at day 7 is dependent on Jak2 activation in the injured artery, and it is suggested that induction and activation of the JAK/STAT pathway play a role in the mechanisms underlying neointima formation after balloon injury via VSMC replication. However, we do not deny the possibility that additional signaling pathways are involved in the mechanism of neointima formation, because the inhibitory effects of AG490 on VSMC replication and neointima formation were partial.

The present study suggests that Jak2 and Stat3, as well as AT1, are not only coexpressed but also coupled functionally in the vascular remodeling process, given that candesartan blocked the Jak2 and Stat3 phosphorylations induced by ex vivo Ang II stimulation and the Ang II–induced Stat3 phosphorylation was dramatically reversed by AG490 (Figure 4). The rationale for testing the functional coupling of Jak2 and Stat3 by applying Ang II to the injured artery derives from the evidence that activation of Jak2 and Stat3 is involved in the Ang II–mediated proliferation in cultured VSMCs24 and that AT1 was coexpressed with Jak2 and Stat3 in VSMCs of the injured artery (Figure 3). Moreover, it is well known that components of the tissue renin-angiotensin system as well as AT1 are upregulated in response to vascular injury.13–16 Taken together, it seems likely that Ang II is one of the in vivo ligands that activate the inducible JAK/STAT pathway and induce the constitutive Jak2 and Stat3 phosphorylation in the injured artery. However, we do not necessarily consider Ang II to be the only essential agonist coupling to inducible JAKs and STATs in balloon-injured artery. Several cytokines and growth factors, including basic fibroblast growth factor and platelet-derived growth factor, are also considered to be candidates responsible for VSMC migration and/or proliferation after vascular injury.7–9 These agonists also can activate the intracellular signaling pathways utilizing JAK and STAT proteins.3 An expected finding was that Jak2 and Stat3 were already somewhat phosphorylated in the injured artery. Thus, the constitutive Jak2 and Stat3 phosphorylation in the injured artery may reflect the phosphorylation induced by these agonists or hitherto unidentified factors produced in the vessel wall in response to vascular injury, which is possibly related to neointima formation.

Molecular mechanisms of transient inductions of JAKs and STATs are currently unknown. Phenotypic modulation of VSMCs from the differentiated state to the dedifferentiated state is suggested to be involved in the pathogenesis of neointima formation after vascular injury. The induction of expressions of several proteins was indeed observed during the phenotype transition.22 Thus, transient JAK and STAT expressions were possibly related to the VSMC phenotype transition. To address this issue, we did immunostaining for the phenotype-specific MHC isoforms, SM2 and SMemb, for assessing VSMC phenotypes; expression of SM2, a marker of differentiated VSMCs, and SMemb, a marker of undifferentiated VSMCs, have been reported to be downregulated and upregulated in neointimal VSMCs after injury, respectively.23 As shown in the Table, time course of the VSMC phenotypic transition, however, was not in accord with those of JAK and STAT expressions. Thus, it is suggested that transient expressions of JAKs and STATs are not necessarily associated with the phenotype transition. Recent studies documented transient inductions of JAKs and STATs in the nerve system after neural damages.24,25 Additionally, the JAK/STAT pathway, especially the Stat3-mediated pathway, induces the expression of a variety of genes that dramatically increase with tissue injury and inflammation.2 Taken together, it is possible that induction of JAKs and STATs could be a ubiquitous response to tissue injury. It is important to clarify the molecular mechanisms triggering and modulating JAK and STAT expression in vascular remodeling in future studies.

Limitations
Recent studies have demonstrated that a single periadventitial administration of various drugs from pluronic F127 gel, a sustained-release polymer, is an effective means of delivering drug locally and limiting neointima formation in rat models, but little is known regarding the pharmacodynamics of delivered drugs.26–28 Currently, neither labeled compound nor quantitative assay technique is available for direct measurements of tissue AG490 concentrations. Moreover, no data are available regarding the in vivo degradation rate of AG490. Therefore, the kinetics of AG490 released from pluronic gel matrix and the intramura Ag490 concentrations remain to be elucidated. However, locally applied AG490 showed neither macroscopic nor microscopic changes in sham-operated artery during the observation period. Thus, it seemed unlikely that the observed effects of local AG490 treatment on neointima formation were attributable to the toxic effects of the drug.

In conclusion, it is suggested that inductions of JAKs and STATs may be involved in the mechanisms of neointimal formation after balloon injury, especially in the second and third phases of the tissue response. Modulation of JAK and STAT expressions may be a regulatory mechanism of cellular responses to the agonists that activate the pathway, given that the enrichment of the components of the signaling cascade could be of crucial importance in effective signaling through the pathway. Finally, the present study may provide insight into a possible treatment strategy for the prevention of the progression of atherosclerosis and restenosis after angioplasty, especially after stent implantation.
Acknowledgments
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