Angiotensin Blockade Inhibits Activation of Mitogen-Activated Protein Kinases in Rat Balloon-Injured Artery

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Background—The effect of balloon injury on the arterial signal transduction pathway has not been examined. In vitro studies show that extracellular signal-regulated kinases (ERKs) and c-Jun NH₂-terminal kinases (JNKs), belonging to the mitogen-activated protein kinase (MAPK) family, play a critical role in the activation of transcription factor activator protein-1 (AP-1) and cell proliferation or apoptosis. However, the activation and role of MAPKs in vascular diseases in vivo remain to be determined. Therefore, we examined the effect of balloon injury on arterial MAPKs and the possible role of angiotensin II.

Methods and Results—Arterial JNK and ERK activities were measured by in-gel kinase assay. AP-1 DNA binding activity was determined by gel mobility shift analysis. After balloon injury of rat carotid artery, JNK (p46JNK and p55JNK) and ERK (p44ERK and p42ERK) activities were increased as early as 2 minutes, reached their peak (6- to 18-fold) at 5 minutes, and thereafter rapidly declined to control levels. JNK and ERK activations were followed by a 3.9-fold increase in arterial AP-1 DNA binding activity, which contained c-Jun and c-Fos proteins. Arterial JNK activation at 2 or 5 minutes was remarkably suppressed by E4177 (an angiotensin AT1 receptor antagonist) and cilazapril (an ACE inhibitor). E4177 also prevented activation of ERKs by suppressing their tyrosine phosphorylation, whereas cilazapril failed to prevent such activation. The increased AP-1 DNA binding activity was significantly inhibited by both E4177 and cilazapril.

Conclusions—Arterial JNKs and ERKs are dramatically activated by balloon injury associated with the activation of the AP-1 complex. These MAPK activations, followed by AP-1 activation, are mediated at least in part by the AT1 receptor. Thus, activation of JNKs and ERKs may be responsible for balloon injury–induced neointima formation. (Circulation. 1998;97:1731-1737.)

Key Words: angiotensin • balloon • signal transduction • muscle, smooth • remodeling

Arterial balloon injury, which causes endothelial denudation and stretching of the medial smooth muscle cells, leads to progressive neointimal thickening. This arterial repair process, characterized by vascular smooth muscle cell proliferation, apoptosis, or migration, has been shown to be associated with significant changes in numerous gene expressions, such as immediate-early genes, growth factors, and extracellular matrix components. Generally, the gene expression is controlled by the intracellular signal transduction pathway (protein kinase cascade), indicating that the characterization of the cellular signal transduction pathway activated by balloon injury is essential to elucidate the molecular mechanism of neointima formation. However, in contrast to detailed studies on gene expression, the effects of balloon injury on cellular protein kinases have not been examined.

ERKs and JNKs are protein serine/threonine kinases and belong to the MAPK family. ERKs and JNKs are regulated by different upstream activators and play a central role in cell proliferation or apoptosis and the regulation of various transcription factors such as AP-1 and numerous gene expressions. However, previous reports on the regulation and function of MAPKs have largely come from in vitro studies using cultured cells, and the in vivo role of MAPKs remains unclear.

In rat balloon-injured artery, both an angiotensin AT1 receptor antagonist and an ACE inhibitor are well known to prevent neointima formation by suppressing the proliferation of vascular smooth muscle cells. In the present study, we examined MAPK activity in rat balloon-injured artery and obtained the first evidence that JNKs and ERKs are dramatically activated by balloon injury mediated by the AT1 receptor.

Methods

Drugs

Cilazapril, an ACE inhibitor, was donated by Nippon Roche, Ltd (Tokyo, Japan). E4177, a specific and potent nonpeptide AT1
Preparation of Arterial Protein Extracts for Protein Kinase Assay

Arterial protein extracts were prepared from the pooled tissue from five to six rat carotid arteries in each group to minimize animal-to-animal and procedural variability. Arteries, pooled from five to six rats, were homogenized on ice with polytron homogenizer (PCU-11, Kinematica AG) in lysis buffer (20 mmol/L HEPES) (pH 7.2), 25 mmol/L NaCl, 2 mmol/L EGTA, 50 mmol/L NaF, 1 mmol/L Na_{3}VO_{4}, 25 mmol/L β-glycerophosphate, 0.2 mmol/L DTT, 1 mmol/L PMSF, 60 µg/mL aprotinin, 2 µg/mL leupeptin, and 0.1% Triton X-100). After incubation at 4°C for 30 minutes, the homogenates were sonicated (Sonifier 250, Branson Ultrasonics Co) on ice for 1 minute and centrifuged at 10,000 g at 4°C for 30 minutes. The protein concentrations of the supernatants were measured with a protein assay kit (Pierce) and stored at −80°C until protein kinase assay.

Measurement of Arterial JNK and ERK Activity

JNK and ERK activities were measured by use of the in-gel kinase method, as previously described in detail.23 GST-c-Jun(1–79) and MBP were used as the substrate of JNKs and ERKs, respectively. In brief, samples of arterial protein extracts (10 µg), denatured in Laemmli sample buffer, were electrophoresed on SDS-polyacrylamide (12%) gel containing 0.1 mg/mL of GST-c-Jun(1–79) for JNK assay or 0.5 mg/mL of MBP for ERK assay. After electrophoresis, protein kinases in the gels were denatured by guanidine-HCl and renatured in Tris-HCl (pH 8.0), and the gels were incubated with (γ-32P)ATP, washed extensively, dried, and subjected to autoradiography, as described in detail elsewhere.24 The densities of autoradiograms were measured by use of a bioimaging analyzer (BAS-2000, Fuji Photo Film Co).

Identification of JNKs and ERKs by Immunoprecipitation With Specific Antibodies

To confirm that arterial JNK and ERK activities can be specifically measured by in-gel kinase assay, we performed in-gel kinase assay of arterial extracts immunoprecipitated with specific antibodies. Specific antibodies used were as follows: polyclonal rabbit IgG(Gc-17) specifically recognizing both p46JNK and p53INK; polyclonal rabbit anti-p44ERK IgG(c-16); and polyclonal rabbit anti-p42ERK IgG(c-14). All antibodies were purchased from Santa Cruz Biotechnology, Inc. Normal rabbit IgG (control) was purchased from Vector Laboratories, Inc. Arterial protein extract (50 µg of protein) was preabsorbed with 10 µL of recombinant protein A-agarose (50% vol/vol) (Upstate Biotechnology) at 4°C for 2 hours. After centrifugation at 10,000 g at 4°C for 15 minutes, the supernatants were incubated with each specific antibody (1 µg each) or normal rabbit IgG (1 µg) at 4°C for 2 hours, and 20 µL of recombinant protein A-agarose (50% vol/vol) was added, followed by incubation at 4°C for 12 hours. After centrifugation at 800 g for 10 minutes, the pellets were washed four times with lysis buffer containing 0.5 mol/L NaCl. Finally, the pellets were suspended with 25 µL of lysis buffer, boiled for 5 minutes in Laemmli sample buffer, and centrifuged, and the resulting supernatants were electrophoresed on 12% SDS–polyacrylamide gel containing 0.2 mg/mL of GST-c-Jun or 0.5 mg/mL of MBP and subjected to in-gel kinase assay for JNKs or ERKs, as described above.

Western Blot Analysis

By using rabbit polyclonal phospho-specific ERK antibody (New England Biolabs, Inc) recognizing tyrosine-phosphorylated forms (active forms) of p44ERK and p42ERK, we measured arterial phosphorylated ERK proteins with Western blot analysis. Arterial protein extracts (10 µg protein), prepared as described above, were boiled for 5 minutes in Laemmli sample buffer, then electrophoresed on an SDS–polyacrylamide gel (12%), and the separated proteins were electrophoretically transferred to Hybond-PVDF membranes (Amersham Life Sciences). Complete protein transfer to the membrane was ensured by staining the gels with Coomassie blue. Non-specific background was blocked by incubating the membrane.
with 5% bovine serum albumin in TBS-T at 4°C overnight. The membrane was then incubated with phospho-specific ERK antibody (1:1000 dilution) for 1 hour at room temperature, washed 4 times with TBS-T, and then incubated with horseradish peroxidase–conjugated donkey anti-rabbit immunoglobulin (Amersham) at a dilution of 1:5000 in TBS-T. After a further washing with TBS-T, the membrane was treated with ECL reagent (Amersham), and chemiluminescence was detected by exposure to Hyperfilm-ECL. The intensity of the bands was measured by use of a Macintosh LC-III computer with an optical scanner (EPSON GT-8000, Seoko), using the public domain NIH Image program.

Gel Mobility Shift Assay

For gel mobility shift assay, seven to eight rat carotid arteries were pooled to obtain one sample. Arteries were homogenized in 0.4 mL of 20 mmol/L HEPES (pH 7.9) containing 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1.5 mmol/L MgCl2, 20% glycerol, 10 mmol/L NaF, 1 mmol/L Na3VO4, 0.2 mmol/L DTT, 20 mmol/L β-glycerophosphate, 0.5 mmol/L PMSF, 60 μg/mL aprotinin, and 2 μg/mL leupeptin, incubated on ice for 15 minutes, and centrifuged at 15 000 rpm at 4°C for 10 minutes. The resulting supernatant was assayed for protein concentrations and stored at −80°C until use. The detailed procedure of the gel mobility shift assay has been described previously.24 In brief, the samples of arterial protein extracts (10 μg protein) were incubated with 1 fmol of a 32P-labeled oligonucleotide probe containing the consensus AP-1 binding sequence (5'-CGCTTGAGTACCTGCGGGGA-3') at room temperature for 20 minutes in 20 μL of the binding buffer, consisting of 20 mmol/L HEPES (pH 7.9), 0.2 mmol/L EDTA, 0.2 mmol/L EGTA, 80 mmol/L NaCl, 0.3 mmol/L MgCl2, 1 mmol/L DTT, 0.2 mmol/L PMSF, 6% glycerol, and 2 μg of polydeoxyinosinic-deoxyctydilic acid (poly[dI-dC]; Pharmacia) as a nonspecific competitor. For competition experiments, a mutant AP-1 oligonucleotide competitor (5'-CGCTTGAGTACCTGCGGGGA-3') was also used. The DNA-protein complexes were electrophoresed on 4% nondenaturing polyacrylamide gels, and the gels were then dried, subjected to autoradiography, and analyzed with the use of a bioimaging analyzer (LAS-2000), as described previously.24 As a positive control sample of AP-1, we used nuclear extracts from raf-1–transformed rat fibroblasts stimulated with phorbol ester (3611-RF-phorbol), known to be rich in AP-1 (Santa Cruz Biotechnology, Inc).

Supershift assays were performed with rabbit polyclonal anti–c-Fos IgG raised against the amino acids 128 to 152 portion of c-Fos and rabbit anti–c-Jun IgG raised against the amino acids 247 to 263 portion of c-Jun (Santa Cruz Biotechnology, Inc). Each antibody (1 μg each) was added to the samples after the initial binding reaction between the arterial protein extracts and 32P-labeled consensus AP-1 oligonucleotide; the reaction was allowed to occur at room temperature for 1 hour and subjected to electrophoresis, as described above.

Statistical Analysis

Data are expressed as mean±SEM. Statistical significance was determined with one-way ANOVA, followed by Duncan multiple-range test (SuperANOVA, Abacus Concepts). Differences were considered statistically significant at a value of P<.05.

Results

JNK and ERK Activity in Arterial Extracts

As shown by in-gel assay with GST-c-Jun (Figure 1A) used as a substrate, it was apparent that the 46-kD and 55-kD kinase bands in crude arterial extracts were due to p46JNK and p55JNK, respectively. In-gel assay with MBP (Figure 1B) showed that the 42-kD and 44-kD kinase bands corresponded to p42ERK and p44ERK, respectively. Thus, in this study, JNK and ERK activities in crude arterial extracts were successfully measured by using an in-gel kinase assay.

Figure 1. Identification of JNK and ERK activity in crude arterial extracts. A, In-gel kinase assay with GST-c-Jun as a substrate was carried out on crude arterial extracts (lane 1), the immunoprecipitates with anti-JNK IgG recognizing both p46JNK and p55JNK (lane 2), and the precipitates with normal rabbit IgG (lane 3). B, In-gel kinase assay with MBP as a substrate was performed on crude arterial extracts (lane 1), the immunoprecipitates with both anti-p44ERK IgG and anti-p42ERK IgG (lane 2), and the precipitates with normal rabbit IgG (lane 3). In both A and B, there was no band of protein kinase activity in the sample treated with normal rabbit IgG (lane 3). The positions of molecular mass markers are indicated by 97.4 kD, 66 kD, 46 kD, and 30 kD. K indicates kilodalton.

Time Course of Arterial JNK and ERK Activities After Balloon Injury

As shown by autoradiograms in Figure 2A, arterial JNKs consisted of two isoforms, p46JNK and p55JNK, and the majority of arterial JNK activity was due to p46JNK. Compared with noninjured artery, arterial p46JNK and p55JNK activities were increased by 3.6- and 2.2-fold, respectively, as early as 2 minutes after balloon injury and reached peak levels (17.9- and 6.0-fold, respectively; P<.01) at 5 minutes. Although p46JNK and p55JNK activities remained increased by 3.1-fold (P<.01) and 2.4-fold (P<.05), respectively, at 15 minutes, their activities rapidly decreased and returned almost to control levels at 60 minutes. At 24 hours after balloon injury, conversely, arterial p46JNK activity was decreased to 49.1% of control value (P<.05).

As indicated by autoradiograms in Figure 2B, arterial ERKs were composed of two isoforms, p44ERK and p42ERK. Like the time course of JNK activity, p44ERK and p42ERK activities were increased by 8.4- and 10.7-fold (P<.01), respectively, at 2 minutes after balloon injury and...
peaked (10.3- and 14.0-fold, respectively; \( P < .01 \)) at 5 minutes. Thereafter, activity of both ERKs rapidly declined but remained higher \(( P < .01)\) than control values at 3 hours after injury. At 24 hours after injury, conversely, p44ERK activity was significantly lower than control (48% of control value; \( P < .01 \)), and p42ERK also tended to be lower than control (although not statistically significant).

**Effects of E4177 and Cilazapril on Arterial JNK Activity**

Figure 3 illustrates the effects of E4177 (AT1 receptor antagonist) and cilazapril (ACE inhibitor) on arterial JNK activity at 2 and 5 minutes after balloon injury. Both drugs prevented the increase in p46JNK and p55JNK activity by 86% and 80% at 2 and 5 minutes \(( P < .01)\), respectively, after injury. There was no significant difference between E4177 and cilazapril in the inhibitory effects of JNK activation at either time point.

**Effects of E4177 and Cilazapril on Arterial ERK Activity and Tyrosine Phosphorylation of ERKs**

Figure 4 indicates the effects of E4177 and cilazapril on arterial ERK activity at 2 and 5 minutes after balloon injury. At 2 minutes, E4177 tended to suppress p44ERK or p42ERK activation (although not statistically significant). At 5 minutes (peak point), E4177 significantly prevented the activation of p44ERK and p42ERK by 42% and 47% \(( P < .01)\), respectively. On the other hand, cilazapril did not significantly inhibit the activation of p44ERK or p42ERK at 2 or 5 minutes after balloon injury.

To examine whether the inhibition of arterial ERK activation by E4177 was due to the inhibition of tyrosine phosphorylation of ERK, we specifically determined tyrosine-phosphorylated p44ERK and p42ERK contents in arterial extracts at 5 minutes after balloon injury with Western blot analysis using specific antibody recognizing only tyrosine-phosphorylated p44ERK and p42ERK. Figure 5 shows that tyrosine phosphorylation of p44ERK and p42ERK was significantly increased in injured artery and that E4177 but not cilazapril significantly prevented tyrosine phosphorylation of both ERK isoforms.

**Time Course of Arterial AP-1 DNA Binding Activity After Balloon Injury and Effects of E4177 and Cilazapril**

As shown in Figure 6, the results obtained with the gel mobility shift assay of 3611-RF, used as a positive control for AP-1, confirmed that specific AP-1 DNA binding complex could be successfully detected by our present method. Furthermore, the major band (band A) in rat carotid arterial extracts (lanes 1 to 3 in Figure 6) had nearly the same mobility as the AP-1 DNA complex in the positive control (3611-RF). In both carotid arterial extracts (lanes 1 to 3) and 3611-RF (lanes 4 to 6), the use of 10 fmol of a labeled AP-1 oligonucleotide probe gave less nonspecific binding (bands B and C) than the use of 90 or 40 fmol of an AP-1 probe. Therefore, all gel mobility shift assays examining carotid arterial AP-1 DNA binding were performed with the use of 10 fmol of a labeled AP-1 probe.

We further characterized band A in arterial extracts in Figure 6. As shown in Figure 7A, the band designated with a half bracket (corresponding to band A in Figure 6) was
Carotid 3611-RF

Figure 6. Validity of gel mobility shift assay using a labeled AP-1 oligonucleotide probe. In lanes 1 to 3, carotid arterial extracts (10 μg protein), collected at 3 hours after balloon injury, were subjected to gel mobility shift assay using different concentrations of a labeled AP-1 probe (90, 40, and 10 fmol in lanes 1, 2, and 3, respectively). In lanes 4 to 14, nuclear extracts (10 μg protein) from rat-1-transformed rat fibroblasts (3611-RF), which are known to be rich in AP-1, were subjected to gel mobility shift assay. The detailed method is described in “Methods.” The major band (corresponding to position A) in 3611-RF was effectively competed for by addition of 10-, 40-, 100-, and 200-fold excess of a cold AP-1 oligonucleotide (lanes 7, 8, 9, and 10, respectively) but not by addition of 200-fold excess of a cold mutant AP-1 oligonucleotide (lane 11). Furthermore, as shown by arrows, this major band in 3611-RF super-shifted with the addition of anti–c-Fos IgG (lane 12) or anti–c-Jun IgG (lane 13) but not with control IgG (lane 14). These results showed that the major band (corresponding to position A) in 3611-RF indeed represented specific AP-1 complex containing both c-Fos and c-Jun, confirming the validity of our assay. Gel mobility shift assay of carotid arterial extracts (lanes 1 to 3) showed one major band (band A) and two minor bands (bands B and C). The major band (band A) in arterial extracts had the same mobility as specific AP-1 DNA complex in 3611-RF (positive control).

efficiently competed for by increasing concentrations of a cold AP-1 oligonucleotide but not by a mutant AP-1. Furthermore, the addition of anti–c-Fos or anti–c-Jun antibody to the binding reaction produced supershifted complexes. Thus, the band designated with a half bracket (Figure 7A) had nearly the same characteristics as the specific AP-1 band in 3611-RF (a positive control) with regard to electrophoretic mobility, competition with cold competitors, and supershift with specific antibodies, thereby confirming that this band indeed represented specific AP-1 DNA binding activity.

As shown in Figure 7B, arterial AP-1 DNA binding activity was increased by 3.9-fold (P < .01) at 3 hours after injury and gradually decreased thereafter.

As shown in Figure 8, treatment with E4177 and cilazapril prevented the increase in arterial AP-1 DNA binding activity at 3 hours after balloon injury by 47% (P < .01) and 36% (P < .01), respectively.

Discussion
Cells respond to extracellular stress or stimuli by activating intracellular signal transduction pathways, which cause the changes in various gene expressions. These molecular changes finally lead to the modification of cell function, including cellular phenotypic changes, or cell growth or apoptosis. Previous studies demonstrate that numerous growth-associated genes responsible for vascular remodeling, such as proto-oncogenes, growth factors, or extracellular matrices, are activated in balloon-injured rat artery, indicating the contribution of the altered gene expressions to neointima formation after balloon injury. However, the signal

Figure 7. Specificity of AP-1 DNA binding activity from carotid artery (A) and time course of arterial AP-1 DNA binding activity after balloon injury (B). A, Arterial protein extracts, collected at 3 hours after balloon injury, were incubated with a 32P-labeled AP-1 consensus oligonucleotide probe in the absence of unlabeled AP-1 oligonucleotide probe (-), and in the presence of 10-, 40-, 100-, and 200-fold molar excess of unlabeled AP-1 probe (X 10, X 40, X 100, and X 200, respectively) and 200-fold molar excess of unlabeled mutant AP-1 probe (X 200 mutant AP-1). Furthermore, supershift assays were performed with anti–c-Fos IgG (Anti-c-Fos), anti–c-Jun IgG (Anti-c-Jun), or nonimmunized rabbit IgG (Normal IgG). The half bracket indicates specific AP-1 DNA binding complexes. NS indicates nonspecific binding; F, free probe; and unlabeled AP-1 oligonucleotide competitor. B, Representative autoradiogram of gel mobility shift assay of arterial AP-1 binding activity (top) and the value of arterial AP-1 DNA binding activity (bottom) at 0, 1, 3, 6, and 24 hours after balloon injury. Each bar represents mean ± SEM (each, n = 3). The mean value of AP-1 binding activity in noninjured artery (time 0) is expressed as 1. †P < .05, ‡P < .01 vs time 0.

Figure 8. Effect of cilazapril and E4177 on arterial AP-1 DNA binding activity at 3 hours after balloon injury. Arterial AP-1 DNA binding activity at 3 hours after balloon injury was compared among rats treated with vehicle (Veh), cilazapril (Cilaza), and E4177. Left, Representative autoradiogram of gel mobility shift assay of two different samples from each group. For gel mobility shift assay, seven to eight rat carotid arteries were pooled and extracted to obtain one sample. The half bracket indicates specific AP-1 binding complexes. NS indicates nonspecific binding; F, free probe. Right, Each bar represents mean ± SEM (each, n = 4). The mean value in vehicle is defined as 100.
transduction pathway underlying the changes in gene expression in balloon-injured artery has not been examined, which encouraged us to examine the activation of MAPKs in vivo. In the present study, using in-gel kinase assay, we successfully measured arterial MAPK activity and obtained the first evidence that both JNKs and ERKs are rapidly and dramatically activated in balloon-injured artery.

Accumulating evidence on the in vivo effects of AT1 receptor antagonists and ACE inhibitors indicates that angiotensin II, via the AT1 receptor, plays a critical role in the development of various vascular diseases induced by balloon injury, hypertension, or diabetes. However, the signal transduction pathway associated with the vascular protective effects of angiotensin II blockade in vivo remains to be determined. In vitro studies of cultured vascular smooth muscle cells show that ERKs are activated by angiotensin II. Furthermore, very recently, angiotensin II has been shown to activate JNKs in cultured hepatocytes and neonatal rat cardiac myocytes, although the effect of angiotensin II on JNKs in cultured vascular smooth muscle cells has not been reported. Therefore, to examine the possible contribution of the AT1 receptor to the activation of ERKs and JNKs in balloon-injured artery, we examined the effects of an AT1 receptor antagonist and an ACE inhibitor. In the present study, both the AT1 receptor antagonist and the ACE inhibitor significantly inhibited the activation of JNKs in injured artery, demonstrating that angiotensin II, via the AT1 receptor, is responsible for balloon injury–induced arterial JNK activation. Furthermore, in-gel kinase assay and Western blot analysis showed that the AT1 receptor antagonist also significantly inhibited arterial ERK activation at its peak time point (5 minutes) after balloon injury by suppressing the tyrosine phosphorylation of ERKs. Thus, our present work provided the first evidence that balloon injury–induced activation of JNKs and ERKs is at least in part mediated by the AT1 receptor. Furthermore, in light of the fact that JNKs and ERKs have different upstream signaling cascades and substrate specificities, the AT1 receptor seems to be responsible for the activation of multiple signaling cascades in the balloon-injured artery.

It has been believed that JNKs or ERKs play a central role in the formation of transcription factor AP-1 complex. JNKs, which are the only potent activator of c-Jun protein, increase c-Jun transactivational activity by their phosphorylation or induce c-jun mRNA expression. Furthermore, JNKs can also induce c-fos mRNA expression. On the other hand, unlike JNKs, ERKs cannot activate c-Jun protein or induce c-jun mRNA expression, although they can induce c-fos mRNA expression by phosphorylating Elk-1/TCF transcription factors. Previously, we and other groups of investigators reported that arterial mRNA expression of c-jun and c-fos is significantly enhanced 30 to 60 minutes after balloon injury. However, it remains to be determined whether or not the increased arterial mRNAs for c-jun and c-fos induced by balloon injury can lead to the increase in DNA binding activity of the AP-1 complex. Our present study, using gel shift analysis, provided the first evidence that AP-1 DNA binding activity, which contained c-Jun and c-Fos proteins, is significantly increased in balloon-injured artery. AP-1 is involved in the expression of numerous genes responsible for cell proliferation and tissue remodeling, such as collagenase, endothelin-1, or transforming growth factor-β, by binding the AP-1 consensus sequence present in their promoter region. Therefore, it is likely that the activation of AP-1 is implicated in intimal thickening after balloon injury.

Our present data on gel shift analysis of AP-1 activity, taken together with our previous findings that the AT1 receptor antagonist prevents the induction of c-fos and c-jun mRNAs in balloon-injured artery, demonstrate that the inhibition of c-fos and c-jun gene expression by the AT1 receptor antagonist is associated with suppression of the activation of the AP-1 complex. Of note are the observations that the inhibitory effect of the ACE inhibitor on arterial AP-1 DNA binding activity was comparable to that of the AT1 receptor antagonist, although the ACE inhibitor suppressed JNKs but not ERKs. Homodimers of c-Jun proteins or heterodimers of c-Jun and c-Fos proteins can form a stable AP-1 complex. On the other hand, unlike c-Jun, c-Fos cannot form homodimers and therefore needs c-Jun to form the AP-1 complex. Furthermore, the expression and activation of c-Jun protein are significantly induced by JNKs but not by ERKs. These findings suggest that the suppression of arterial AP-1 activity by the AT1 receptor antagonist and ACE inhibitor in balloon injury might be mediated, at least in part, by the suppression of JNK activation and that JNKs might be responsible for AP-1 activation, although our present work provided no direct evidence of this.

The present study did not enable us to elucidate the reason for the differential effects of the AT1 receptor antagonist and ACE inhibitor on ERKs. A previous report clearly showed that the inhibition of neointima formation in balloon-injured artery by an ACE inhibitor was at least partly mediated by increased bradykinin accumulation. Furthermore, in vitro investigations indicate that bradykinin causes the activation of ERKs. Therefore, our present work suggests that the lack of a decrease in arterial ERK activity by an ACE inhibitor might be partly due to the increased bradykinin accumulation and that both the ACE inhibitor and the AT1 receptor antagonist prevent neointima formation with different effects on the intracellular signal transduction cascades. However, further study is needed to elucidate our proposal.

In conclusion, we obtained the first evidence that JNKs and ERKs, the two main subgroups of the MAPK family, are rapidly and dramatically activated in balloon-injured artery, and that this is associated with activation of the transcription factor AP-1 complex. The activation of JNKs and ERKs in balloon-injured artery is at least partially mediated by the angiotensin AT1 receptor. We propose that the activation of MAPKs may be involved in vascular remodeling after balloon injury. However, further work is needed to determine whether our present observations apply to human atherosclerosis, because the balloon-injury model has limited relevance for human atherosclerosis.

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References
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