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 NH$_2$-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.$^1,^3$ This arterial repair process, characterized by vascular smooth muscle cell proliferation,$^1,^2$ apoptosis,$^4,^5$ or migration,$^3$ has been shown to be associated with significant changes in numerous gene expressions, such as immediate-early genes, growth factors, and extracellular matrix components.$^6,^7$ Generally, the gene expression is controlled by the intracellular signal transduction pathway (protein kinase cascade), indicating that the characterisation 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$^9,^10$ and JNKs$^{11-13}$ 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$^{14}$ and the regulation of various transcription factors such as AP-1$^{15-17}$ 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.$^{18}$

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.$^{19-21}$ 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
Balloon Injury
All procedures were in accordance with institutional guidelines for animal research. Ten- to 11-week-old male Sprague-Dawley rats (Clea Japan, Tokyo) weighing ~350 to 400 g were used in the present study. Rats were fed standard laboratory chow (MF, Oriental Kobo) and given tap water ad libitum. The present study was performed on 479 rats. The first set of experiments (n = 217 rats) were performed to examine the effects of balloon injury on arterial JNK and ERK activity and arterial AP-1 DNA binding activity. For balloon injury, rats were anesthetized with sodium pentobarbital (40 mg/kg IP), and endothelial denudation of the left common carotid artery was carried out by three passages of a Fogarty 2F balloon catheter (Baxter Healthcare), as previously described.6 For examination of ERK and JNK activities at 2, 5, 15, and 60 minutes and 3 and 24 hours after balloon injury, the carotid arteries were perfused via the left ventricle with PBS (pH 7.4) containing 2.5 mmol/L EDTA, 2 mmol/L β-glycerophosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate (Na3VO4), and 100 μg/mL PMSF at 4°C for a flow rate of 40 mL/min for 1 minute. After perfusion, injured left common carotid artery and noninjured right common carotid artery (control) were immediately excised, placed on PBS precooled at 4°C, dissected from adherent fat and connective tissues on ice, then frozen in liquid nitrogen and stored at −80°C until use. Extreme care was taken to be certain that the arteries were not stretched on dissection. For examination of AP-1 DNA binding activity at 1, 3, 6, and 24 hours after balloon injury, bilateral common carotid arteries were collected in the same manner as for examination of ERK and JNK activity and stored at −80°C. The second set of experiments (n = 262 rats) were undertaken to examine the effects of an ACE inhibitor and an angiotensin AT1 receptor antagonist on ERK and JNK activity and AP-1 DNA binding activity in balloon-injured artery. Rats were separated into three groups, including (1) vehicle-treated group (control group; 88 rats), (2) E4177-treated group (20 mg·kg−1·d−1; 87 rats), and (3) cilazapril-treated group (10 mg·kg−1·d−1; 87 rats). Preliminary experiments showed that E4177 (20 mg·kg−1·d−1) and cilazapril (10 mg·kg−1·d−1) prevented neointima formation after balloon injury to a similar extent. Therefore, in the present experiments, we used these drugs at the above-mentioned doses. Cilazapril and E4177, suspended with 5% gum arabic solution, were given to rats by gastric gavage once a day from 3 days before balloon injury until the end of the experiments. The control group of rats (vehicle-treated rats) were given an equal volume of gum arabic solution in the same manner. For balloon injury, rats were anesthetized with sodium pentobarbital (40 mg/kg IP), and balloon injury of the left common carotid artery was carried out as described above. The injured left carotid artery and noninjured right carotid artery were collected as described above at 2 and 5 minutes after balloon injury for the measurement of JNKs and ERKs and at 3 hours for the measurement of AP-1 DNA binding activity. Arterial tissues were stored at −80°C until use.

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 Na3VO4, 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 10,000g 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.24 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(1–7) specifically recognizing both p46JNK and p55JNK; polyclonal rabbit anti-p44ERK IgG(1–16); and polyclonal rabbit anti-p42ERK IgG(c14). 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 10,000g 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 A-agarose (50% vol/vol) was added, followed by incubation at 4°C for 12 hours. After centrifugation at 800g 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. Nonspecific 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 Na₂VO₄, 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. In brief, the samples of arterial protein extracts (10 μg protein) were incubated with 10 fmol of a [³²P]-labeled oligonucleotide probe containing the consensus AP-1 binding sequence (5'-CGCTTGAACGTCTGCGGGAA-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 (5'-CGCTTGAACGTCTGCGGGAA-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 (BAS-2000), as described previously. As a positive control sample of AP-1, we used nuclear extracts from raf-1–transformed rat fibroblasts stimulated with phorbol ester (3611-RP-phorboi), 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 247 to 263 portion of c-Fos and rabbit anti–c-Jun IgG raised against the amino acids 128 to 152 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 immuno-precipitates 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.

Figure 2. JNK (A) and ERK (B) activity at 0, 2, 5, 15, and 60 minutes and 3 and 24 hours after balloon injury of rat carotid artery. Upper panels indicate representative autoradiograms showing the activities of p46JNK and p55JNK (A) and p44ERK and p42ERK (B) from two different samples at 0, 2, 5, and 15 minutes after balloon injury, determined by in-gel kinase assay. The mean value of each JNK or ERK activity from noninjured artery. Upper panels indicate representative autoradiograms showing the activities of p46JNK and p55JNK (A) and p44ERK and p42ERK (B) from two different samples at 0, 2, 5, and 15 minutes after balloon injury, determined by in-gel kinase assay. The mean value of each JNK or ERK activity from noninjured artery. Upper panels indicate representative autoradiograms showing the activities of p46JNK and p55JNK (A) and p44ERK and p42ERK (B) from two different samples at 0, 2, 5, and 15 minutes after balloon injury, determined by in-gel kinase assay. The mean value of each JNK or ERK activity from noninjured artery.
peaked (10.3- and 14.0-fold, respectively; \( P \leq .01 \)) at 5 minutes. Thereafter, activity of both ERKs rapidly declined but remained higher \(( P \leq .01)\) 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; \( P \leq .01 \)), 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 \( \geq 86\% \) and \( \geq 80\% \) 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
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had the same mobility as specific AP-1 DNA complex in bands B and C). The major band (band A) in arterial extracts (lanes 1 to 3) showed one major band (band A) and two minor bands more, 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 DNA binding activity. 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 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.
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 inhibitor 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 collagen, endothelin-1, or transforming growth factor-β, by binding the AP-1 consensus sequence present in their promotor 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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