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c-Jun N-terminal kinase 2 phosphorylates endothelial nitric oxide synthase at serine 116 and regulates nitric oxide production

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ABSTRACT

The c-Jun N-terminal kinases (JNKs) belonging to the mitogen-activated protein kinase (MAPK) superfamily play important roles in foam-cell formation, hypercholesterolemia-mediated endothelial dysfunction, and the development of obesity. Although decreased nitric oxide (NO) production via decreased phosphorylation of endothelial NO synthase at serine 1179 (eNOS-Ser^{1179}) was reported to be partly involved in JNK2-derived endothelial dysfunction, JNK2 seems likely to be indirectly involved in this signaling pathway. Here, using bovine aortic endothelial cells, we examined whether JNK2 directly phosphorylated eNOS-Ser^{116}, a putative substrate site for the MAPK superfamily, and this phosphorylation resulted in decreased NO release. JNK inhibitor SP60012 increased NO release in a time- and dose-dependent manner, which was accompanied by increased eNOS-Ser^{116} phosphorylation. Purified JNK2 directly phosphorylated eNOS-Ser^{116} *in vitro*. Ectopic expression of dominant negative JNK2 repressed eNOS-Ser^{116} phosphorylation and increased NO production. Coimmunoprecipitation and confocal microscopy studies revealed a colocalization of eNOS and JNK2. However, all these observed effects were not manifested when JNK1 probes were used. Overall, this study indicates that JNK2 is a physiological kinase responsible for eNOS-Ser^{116} phosphorylation and regulates NO production.

1. Introduction

The c-Jun N-terminal kinases (JNKs) are members of a large group of serine/threonine protein kinases known as the mitogen-activated protein kinase (MAPK) superfamily. The MAPK superfamily is one subgroup of the CMGC class of protein kinases; CMGC represents cyclin-dependent kinases (CDKs), MAPKs, glycogen synthase kinases (GSKs), and CDK-like kinases. This CMGC class is distinctive in favoring their substrates from the AGC kinase superfamily; AGC represents protein kinases A, G and C. The mammalian JNKs, JNK1, JNK2, and JNK3, are encoded by three distinct genes, jnk1, jnk2, and jnk3. Although JNK3 is restricted primarily to the brain, heart, and testes, JNK1 and JNK2 are expressed in a variety of tissues including vessels, indicating tissue-specific roles for JNKs. Studies using gene targeting and JNK inhibitors demonstrated that JNK1 and JNK2 play important roles in cardiovascular and metabolic diseases. For example, deficiency in JNK1 prevented the development of obesity and improved insulin sensitivity in diabetic db/db mice [1]. Use of a cell permeable peptide inhibitor of JNK1 also demonstrated an attenuation of vasoconstriction derived by norepinephrine and angiotensin II [2]. Furthermore, activation of JNK2 stimulated oxidized low-density lipoprotein (oxLDL)-mediated foam-cell formation in atherosclerotic plaques [3]. In contrast, JNK2 knock-out mice were shown to be protected from hypercholesterolemia-induced endothelial dysfunction [4].

Endothelial nitric oxide synthase (eNOS) is an essential enzyme responsible for the production of endothelium-derived nitric oxide (NO), which is a key molecule with multiple functions including vascular homeostasis [5]. Thus, eNOS dysregulation contributes to the pathogenesis of certain diseases, such as atherosclerosis and hypertension [6,7]. It is well known that eNOS is regulated at the level of its phosphorylation [8]. Several specific phosphorylation sites have been identified, among which the phosphorylation of eNOS at serine 1179 (in bovine sequence) (eNOS-Ser^{1179}) increases NO production [9–11], while phosphorylation at eNOS-Thr^{497} or eNOS-Ser^{116} decreases eNOS activity [12,13]. *In silico* motif scan analysis reveals that both eNOS-Ser^{1179} and eNOS-Thr^{497} sites represent putative substrates for the AGC kinase superfamily. Use of specific inhibitors and/or gene knock-out methods showed that several protein kinases phosphorylate eNOS-Ser^{1179}, which

Abbreviations: NO, nitric oxide; eNOS, endothelial NO synthase; BAEC, bovine aortic endothelial cell(s); JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; AMPK, AMP-activated protein kinase; PKA, protein kinase A; CDK, cyclin-dependent kinase; dn, dominant negative.

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include protein kinase B, AMP-activated protein kinase (AMPK), calmodulin-dependent kinase II, PKA, and [9–12,14]. Similarly, the phosphorylation of eNOS-Thr^{497} is also reported to be mediated by AMPK [15] and PKC [16]. Unlike eNOS-Ser^{1179} and eNOS-Thr^{497}, the eNOS-Ser^{116} site is a putative substrate for the CMGC family. Recently, we showed that eNOS-Ser^{116} phosphorylation is mediated by CDK5 in basal endothelial cells, which is the only kinase to date to directly phosphorylate eNOS-Ser^{116} [17].

Although several studies have reported that JNK1 and JNK2 play important roles in mediating cardiovascular problems [18], only one study reported an association between eNOS phosphorylation and JNK-mediated endothelial dysfunction; a significant increase in eNOS-Ser^{1179} phosphorylation was observed in JNK2-deficient mice [4]. However, it seemed unlikely that JNK2 directly phosphorylated eNOS-Ser^{1179} in those experimental conditions. It is rather likely that the level of eNOS-Ser^{1179} phosphorylation must be reduced in JNK2-deficient mice if JNK2 directly phosphorylates eNOS. Furthermore, eNOS-Ser^{1179} does not represent a putative substrate for the JNK family. Here, we examined whether and which eNOS sites are phosphorylated by JNK and found that JNK2 phosphorylated eNOS-Ser^{116} and decreased NO release in the basal state.

2. Materials and methods

2.1. Reagents and antibodies

JNK inhibitor (SP600125) and recombinant bovine eNOS were purchased from Calbiochem (Darmstadt, Germany). Antibodies against eNOS and p-eNOS-Ser^{116} were purchased from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology Inc. (Lake Placid, NY), respectively. Antibodies against JNK1, JNK2, and glutathione-S-transferase (GST) were purchased from Santa Cruz Biotechnology (La Jolla, CA). The antibody against hemagglutinin (HA) was obtained from Covance (Berkeley, CA). Minimal essential medium (MEM), Dulbecco's phosphate-buffered saline (DPBS), newborn calf serum (NCS), penicillin and streptomycin, L-glutamine, and trypsin–EDTA solution used for cell culture were purchased from Gibco-BRL (Gaithersberg, MD). All other chemicals were of the purest analytical grade.

2.2. Cell culture, drug treatment, and transfection

Bovine aortic endothelial cells (BAEC) were isolated and maintained in MEM supplemented with 5% NCS at 37 °C under 5% CO_2 as described [19]. Cells between passages 5 and 9 were used. After the BAEC reached confluence, they were further maintained for the indicated times in MEM supplemented with 0.5% NCS containing the indicated drugs or chemicals. For transfection, BAEC were transferred to 60-mm plates and allowed to grow until they were approximately 60% confluent, at which time they were transfected for 48 h with 3 μg of cDNAs encoding the dominant negative (dn) genes using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.3. Measurement of NO release

NO production was measured as nitrite (a stable metabolite of NO) concentration in cell culture supernatants, as described in our previous study [9]. Briefly, after cells were treated with SP600125, the culture medium was changed to Kreb's solution (pH 7.4) and equilibrated for 1 h at 37 °C. In some experiments, cells were transfected with empty vector, dn-JNK1, or dn-JNK2. At the end of the incubation, 150 μl of each supernatant was carefully transferred into a 96-well plate, with the subsequent addition of 100 μl of Griess reagent (50 μl of 1% sulfanilamide containing 5% phosphoric acid and 50 μl of 0.1% N-(1-naphthyl) ethylenediamine). After color development at room temperature for 10 min, absorbance was measured on a microplate reader at a wavelength of 520 nm.

2.4. Western blot analysis

For Western blot analysis, cells were treated with various chemicals, washed with ice-cold DPBS and then lysed in lysis buffer as previously described [9] [20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM Na_3VO_4, and 1 × Protease Inhibitor Cocktail™ (Roche Molecular Biochemicals, Indianapolis, IN)]. Protein concentrations were then determined using a BCA protein assay kit (Sigma, St. Louis, MO). Equal quantities of protein (20 μg) were separated on sodium dodecyl sulfate–polyacrylamide gel under reducing conditions, after which they were electrophoretically transferred onto nitrocellulose membranes. The blots were then probed with the appropriate antibodies followed by the corresponding secondary antibody, and finally developed using enhanced chemiluminescence reagents (Amersham, Buckinghamshire, UK).

2.5. In silico analysis of the eNOS protein sequence

The eNOS protein sequence was obtained from SWISS-PROT (locus NOS3_BOVIN, accession P29473), and the sequence motifs most likely to be phosphorylated by specific protein kinases were identified using the motif scan program (Scansite) available at http://scansite.mit.edu.

2.6. In vitro phosphorylation assay

In vitro phosphorylation assay was accomplished as described [17] with modifications. Briefly, GST-tagged-JNK1 or JNK2 (200 ng each) was separately incubated with purified bovine eNOS (200 ng) as a substrate in the presence or absence of 100 μM SP600125 in kinase buffer (25 mM HEPES pH7.5, 1 mM DTT, 0.2 mM EGTA, 1 mM PMSF, protease inhibitor cocktail, 1 mM Na_3VO_4, 1 mM β-glycerophosphate) containing 20 μM ATP for 1 h at 30 °C. After the reactions, mixtures were boiled in SDS sample buffer at 95 °C.

2.7. Coimmunoprecipitation assay

BAEC were harvested with lysis buffer (0.5% NP-40, 10 mM HEPES pH7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail and phosphatase inhibitor cocktail). Lysate (500 μg) was collected and incubated overnight with specific antibody against JNK1, JNK2, or preimmune IgG as a negative control at 4 °C. Protein G–Sepharose beads were added to the lysates containing antibody for a 2 h incubation at 4 °C and washed three times with immunoprecipitation buffer (0.5% CHAPS, 20 mM Tris pH7.4, 125 mM NaCl, and 1 mM EDTA). Immunoprecipitated proteins were boiled in SDS loading buffer at 95 °C.

2.8. Immunofluorescence assay

BAEC grown on coverslips were fixed with 4% (wt/vol) paraformaldehyde in DPBS, followed by 5 min-permeabilization in 0.2% (vol/vol) Triton X-100 in DPBS at room temperature. After permeabilization, cells were blocked in 2% bovine serum albumin in DPBS for 10 min. The presence of eNOS and JNKs were detected by appropriate dilutions of the primary antibodies (anti-eNOS, 1:100; anti-JNK1, 1:100; and JNK2, 1:100), and with 1:100 dilution of Alexa Fluro 488- or 594-conjugated secondary antibody (Invitrogen).

Nuclei were examined with DAPI (Invitrogen). Colocalized images were photographed using a confocal microscope (LSM5 Pascall, Carl ZEISS).

2.9. Statistical analysis

All results are expressed as the mean ± standard deviation (S.D.), with n indicating the number of experiments. Statistical significance was determined by a Student's *t*-test for two points. All differences were considered significant at a *p* value of <0.05.

3. Results

3.1. JNK inhibitor SP600125 increases NO release with a concomitant decrease in eNOS-Ser^{116}phosphorylation

Although a previous study showed that JNK2 deficiency in *JNK2*$^{-/-}$mice with hypercholesterolemia preserved NO bioavailability compared with control mice in part by increased eNOS-Ser^{1179} phosphorylation as well as increased eNOS expression [4], JNK2 is likely to indirectly phosphorylate eNOS-Ser^{1179} for this mechanism. Here, using BAEC we examined whether the phophorylation of eNOS at other site(s) is directly mediated by JNK2, resulting in altered NO release. *In silico* analysis using the motif scan program revealed that a (P)-S/T-P motif around the eNOS-Ser^{116} sequence (see the amino acid sequences of eNOS from 113 to 120: ^{113}TRPSPGPP^{120}) represents a putative substrate sequence for the CMGC kinase superfamily which includes JNK1 and JNK2. Therefore, we tested whether eNOS-Ser^{116} phosphorylation is involved in a direct effect of JNK2-mediated signaling pathways on NO release. As shown in Fig. 1A, treatment with the specific JNK inhibitor SP600125 significantly increased NO release in a dose-dependent manner. This increase was accompanied by dose-dependent decreases in the phosphorylation of eNOS-Ser^{116} (Fig. 1B). Furthermore, we also found that these alterations were time-dependent (Figs. 1C and D).

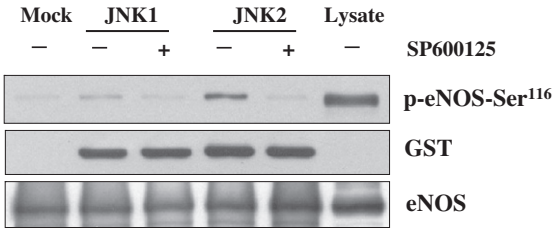

Fig. 2. JNK2 directly phosphorylates eNOS-Ser^{116} *in vitro*. For the *in vitro* phosphorylation assay, 200 ng of GST-tagged JNK1 or JNK2 was separately incubated with 200 ng of bovine eNOS as a substrate in the presence (+) or absence (−) of 100 μM SP600125 in kinase assay buffer as described in Section 2. Mock represents an incubation without JNKs. After 1 h incubation at 30 °C, the reaction was terminated by adding SDS sample buffer. Each kinase was confirmed by detecting the tagged-GST. After the reaction, levels of p-eNOS-Ser^{116}, eNOS, and GST were analyzed using corresponding antibodies as described in Fig. 1. The blot shown is representative of at least three experiments.

3.2. JNK2 directly phosphorylates eNOS-Ser^{116} in vitro

To confirm whether JNK2 directly phosphorylates eNOS-Ser^{116}, we performed an *in vitro* phosphorylation assay. When compared with the control, purified JNK2 significantly phosphorylated

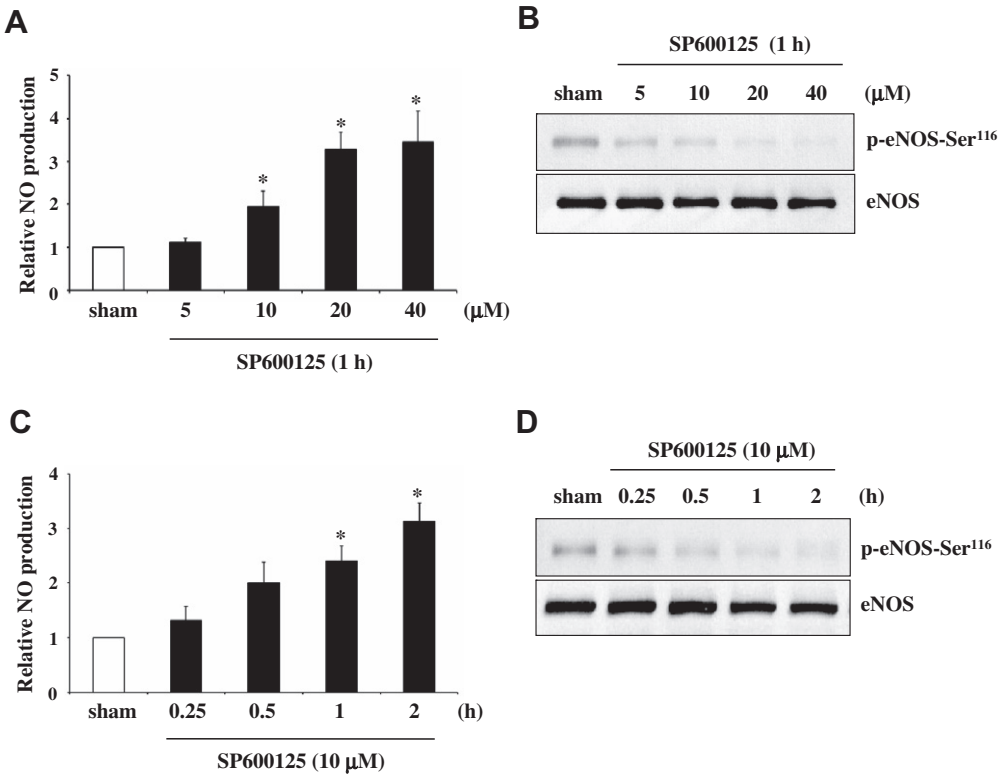

Fig. 1. JNK inhibitor SP600125 increases NO release and decreases phosphorylation of eNOS-Ser^{116} in BAEC in a dose and time-dependent manner. BAEC were treated with SP600125 at various doses (5, 10, 20 or 40 μM) for 1 h. Control cells (sham) were not treated. NO release was measured by the Griess method (A). Bars show mean NO production (after normalization to total cellular protein as fold increases above controls ± SD) and represent at least four experiments. Differences were statistically significant at $^{*}P < 0.05$. After treatment with SP600125, cells were harvested with lysis buffer as mentioned in Section 2. For Western blot analysis, cell proteins separated on SDS–polyacrylamide gel were electrophoretically transferred onto nitrocellulose membranes. Proteins transferred on membranes were immunoblotted with antibodies specific for eNOS phosphorylated at Ser116 (p-eNOS-Ser^{116}) and total eNOS (B). BAEC were treated with 10 μM SP600125 for various times (0.25, 0.5, 1 or 2 h). NO release (C) and Western blot analysis (D) were done as described in Fig. 1A and B, respectively.

eNOS-Ser^{116}, although JNK1 had a small effect (Fig. 2). Furthermore, this phosphorylation was almost completely blocked by SP600125, which suggests that eNOS-Ser^{116} phosphorylation is specifically mediated by JNK2. As JNK3 is expressed primarily in the brain, heart, and testes, we did not use a JNK3 probe in this study.

3.3. Ectopic expression of dominant negative JNK2 inhibits eNOS-Ser^{116} phosphorylation and increases NO production in BAEC

We next determined whether JNK2 was a kinase responsible for phosphorylation of eNOS-Ser^{116} in BAEC. As shown in Fig. 3A, ectopic expression of dn-JNK2, but not dn-JNK1, repressed eNOS-Ser^{116} phosphorylation. Similarly, ectopic expression of dn-JNK2 significantly increased basal NO production in BAEC (Fig. 3B). An increase in NO production was also seen in cells transfected with dn-JNK1, but this increase was not statistically significant.

3.4. JNK2 interacts with eNOS in the cytosol of BAEC

JNK2 but not JNK1 coimmunoprecipitated with eNOS, suggesting a physical interaction between JNK2 and eNOS in cells (Fig. 4A). Confocal microscopy also showed colocalization of JNK2 and eNOS in the cytosol (Fig. 4B), although JNK1 also appeared to be colocalized with eNOS. Taken together, these results showed

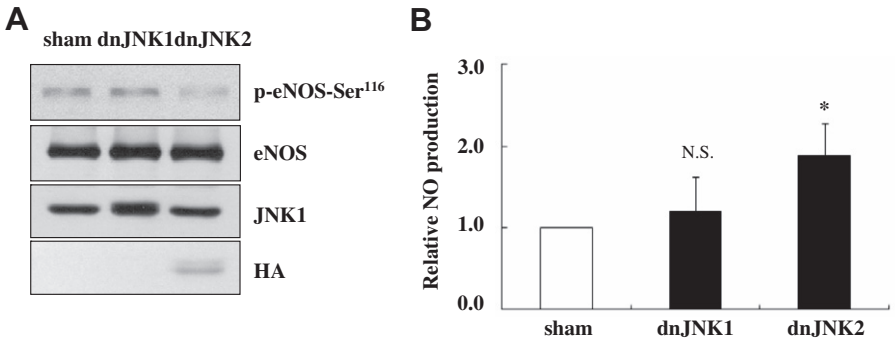

Fig. 3. JNK2 phosphorylates eNOS-Ser^{116} and decreases NO release in cells. BAEC were transfected with domin negative (dn-) JNK1 or dn-JNK2. Control cells (sham) were transfected with empty plasmid (pcDNA3.1). After transfection for 48 h, cells were harvested. Proteins from cell lysates were detected by Western blotting assay with specific antibodies for p-eNOS-Ser^{116}, total eNOS, JNK1 or HA (A), as described in Fig. 1B. After transfection with each dn-JNK, NO release was measured (n = 6) and statistical analysis was performed (B) as described in Fig. 1A. N.S., not significant.

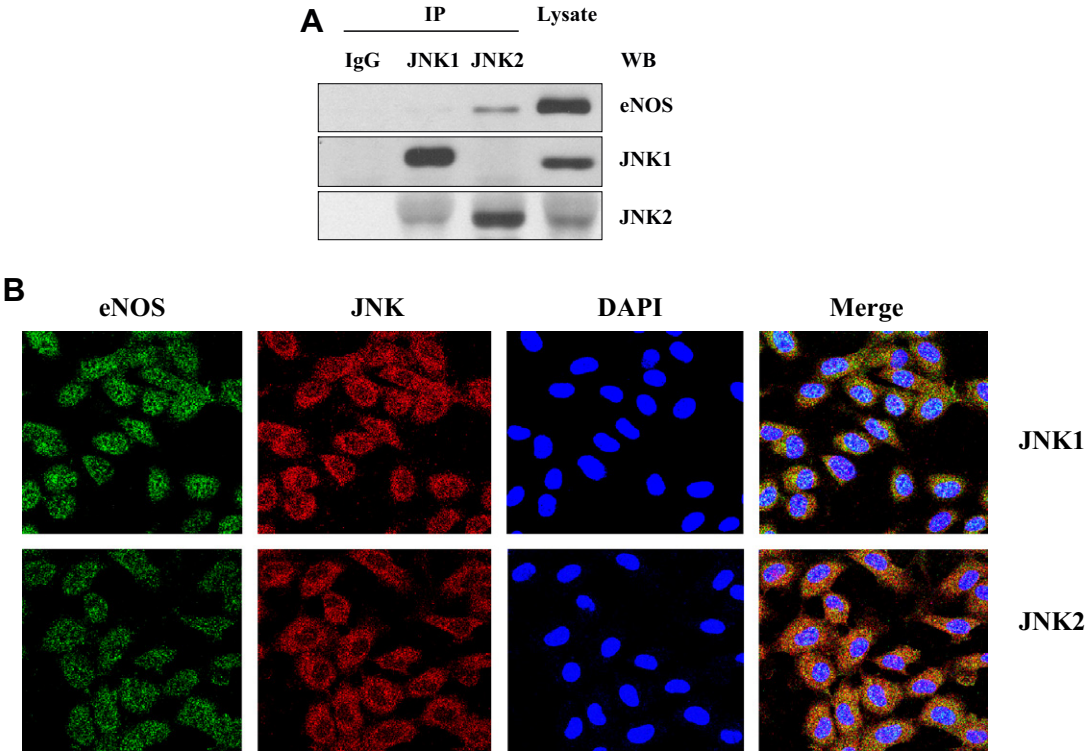

Fig. 4. JNK2 physically interacts with eNOS in intact cells. BAEC were lysed in lysis buffer and lysates were immunoprecipitated using antibody against JNK1 or JNK2, as described in Section 2. A control (IgG) using non-immune IgG was also performed. Immunoprecipitates were dissolved with SDS sample buffer and separated by SDS-gel electrophoresis. Bound proteins in the immunoprecipitates were subjected to Western blot (WB) analysis using antibody specific for eNOS, JNK1, or JNK2. The blot shown is representative of at least three experiments (A). Cells were grown on coverslips and subcellular locations of JNK1, JNK2 and eNOS were detected by confocal microscopy as described in Section 2. DAPI was used for nuclear staining. Confocal images shown are representative of at least three experiments (B).

that JNK2 is a physiological kinase that mediates eNOS-Ser^{116} phosphorylation within the cell.

4. Discussion

Although considerable data shows that JNK1 or JNK2 plays an important role in the development of metabolic and cardiovascular diseases, the underlying mechanism is not fully understood. To date, only one report showed that decreases in eNOS-Ser^{1179} phosphorylation and NO release were attributable to JNK2-mediated hypercholesterolemia-mediated endothelial dysfunction [4]. However, the effect of JNK2 on eNOS-Ser^{1179} phosphorylation seems to be indirect. In this study, we report that JNK2 directly phosphorylated eNOS-Ser^{116}, resulting in decreased NO release, which may provide the molecular mechanism underlying JNK2-mediated endothelial dysfunction.

Several lines of evidence suggest that JNK2 phosphoryates eNOS-Ser^{116} directly. First, JNK-specific inhibitor SP600125 repressed eNOS-Ser^{116} phosphorylation in a dose- and time-dependent manner. Second, purified JNK2 phosphorylated eNOS-Ser^{116} *in vitro*. Third, transfection with dn-JNK2 significantly attenuated eNOS-Ser^{116} phosphorylation. Fourth, JNK2 interacted physically and colocalized with eNOS in cells. Finally, *in silico* analysis using the motif scan program revealed a P-S/T-P motif around the eNOS-Ser^{116} sequence, representing a putative substrate sequence for the CMGC kinase superfamily which includes JNK2. Furthermore, we also found a R/K$_{2-3}$-X$_{1-6}$-L/I-X-L/I motif around eNOS-Ser^{116} (see the amino acid sequences of eNOS from 98 to 108: ^{98}PRRCLGSLVLP^{108}), representing a consensus sequence for a JNK2 binding domain [20].

Although the consensus substrate motifs are closely similar for these two JNK isoforms [20], it seems likely that JNK1 and JNK2 have isoform-specific roles in eNOS-Ser^{116} phosphorylation and NO regulation in the basal state. In earlier gene targeting studies, these two JNK isoforms were suggested to play redundant roles. However, using isoform-specific deficient animals, it has now become clear that they play different roles in physiological and pathological states. For example, the animals lacking both ApoE and JNK2 (*ApoE*$^{-/-}$ *JNK2*$^{-/-}$ mice) developed fewer atherosclerotic lesions than did *ApoE*$^{-/-}$ mice. However, the absence of JNK1 (*ApoE*$^{-/-}$ *JNK1*$^{-/-}$ mice) had no effect [3], suggesting an existence of isoform-specific roles in the same tissues. In this study, JNK2 is predominant over JNK1 as the kinase for eNOS-Ser^{116}. Nonetheless, JNK1 also phosphorylated eNOS-Ser^{116} *in vitro* but to a lesser extent compared with JNK2 (Fig. 2), suggesting that some overlapping substrate specificity is present for these kinases. However, it is unlikely that JNK1 is responsible for eNOS-Ser^{116} phosphorylation in cells because dn-JNK1 transfection studies and coimmunoprecipitation with JNK1 did not show significant effects (Figs. 3 and 4A). It is also noted that under immunofluorescence microscopy, JNK1 appeared to colocalize with eNOS in the cytosol as JNK2 did (Fig. 4B). At present time, however, we are unable to verify that JNK1 in fact interacts with eNOS due to the limitations in immunofluorescence microscopy and a lack of coimmunoprecipitation. In this regard, in a PC12 model of dopaminergic cell death, JNK2 but not JNK1 was reported to translocate to the mitochondria [21], suggesting that isoform-specific subcellular distribution exists in the cells. Further studies using highly sensitive methods such as immunoelectronmicroscopy are needed to know whether these JNK isoforms are localized in the same compartment in the cytosol of BAEC.

It was previously reported that eNOS-Ser^{116} phosphorylation was repressed by pretreatment with the PKC inhibitor calphostin C [22], implying that it is regulated by PKC. However, calphostin C has rather broad ability to inhibit several kinases in addition to PKC, such as myosin light chain kinase, PKA, and protein kinase C; therefore this evidence cannot be regarded as definitive. Furthermore, significant increases in eNOS-Ser^{116} phosporylation were also found in response to both high density lipoprotein and apolipoprotein AI, which are associated with AMPK activation [23], suggesting a role for AMPK in eNOS-Ser^{116} phosphorylation. However, it is also not clear whether AMPK phosphorylates eNOS-Ser^{116} directly because this previous study did not present direct evidence evaluating the effects of wide-type AMPK or dn-AMPK constructs on eNOS-Ser^{116} phosporylation. Recently, we reported that CDK5 is a physiological kinase that mediates eNOS-Ser^{116} phosphorylation in BAEC that are in the basal state [17]. In this previous study, we assumed that MAPK superfamily was not involved in eNOS-Ser^{116} phosphorylation because we found that treatment with MAPKK inhibitor U0126 did not alter eNOS-Ser^{116} phosphorylation. However, in this study, we clearly found that JNK2, another MAPK superfamily member, is a physiological kinase that phosphorylates the same eNOS site. It is interesting to know how different kinases can phosphorylate the same site of eNOS in the cells. At this time, it is likely that different extracellular stimuli activate distinct kinase pathways, leading to phosphorylation of eNOS-Ser^{116} with different time course in cells. For example, CDK5 was reported to mediate biphasic activation of its substrate c-Jun in HT22 immortalized mouse hippocampus cells; it directly phosphorylated c-Jun at the first phase independent of reactive oxygen species (ROS) and JNK, but at the second phase it activated c-Jun via ROS-mediated JNK phosphorylation [24]. Whether CDK5 is still upstream of JNK2 in eNOS-Ser^{116} phosphorylation in BAEC like c-Jun phosphorylation in HT22 cells warrants further investigation. Furthermore, it is also interesting to find whether CDK5 is colocalized with JNK2 in the cytosol in basal cells and whether these kinases play a role in eNOS-Ser^{116} phosphorylation differently in agonist-stimulated states.

In summary, this study is the first to show that JNK2 directly phosphorylates eNOS-Ser^{116} and thus decreases NO release. Because JNKs play a pivotal role in cardiovascular and metabolic diseases such as foam-cell formation, endothelial dysfunction and insulin insensitivity, these results may extend our understanding of the molecular mechanism involved in other JNK-mediated and NO-related chronic diseases such as cancer. Like CDK5, JNK2 is also likely to be registered as an eNOS-Ser^{116} kinase in EC, leading to decrease in NO release. However, care should be exercised when defining the molecular mechanism of JNK2 or CDK5 involved, as it may be context-dependent.

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References

[1] G. Tuncman, J. Hirosumi, G. Solinas, L. Chang, M. Karin, G.S. Hotamisligil, Functional in vivo interactions between JNK1 and JNK2 isoforms in obesity and insulin resistance, Proc. Natl. Acad. Sci. USA 103 (2006) 10741–10746.

[2] M.S. Zhou, I.H. Schulman, K. Chadipiralla, L. Raij, Role of c-Jun N-terminal Kinase in the Regulation of Vascular Tone, J. Cardiovasc. Pharmacol. Ther. 15 (2010) 78–83.

[3] R. Ricci, G. Sumara, I. Sumara, I. Rozenberg, M. Kurrer, A. Akhmedov, M. Hersberger, U. Eriksson, F.R. Eberli, B. Becher, J. Borén, M. Chen, M.I. Cybulsky, K.J. Moore, M.W. Freeman, E.F. Wagner, C.M. Matter, T.F. Lüscher, Requirement of JNK2 for Scavenger Receptor A-Mediated Foam Cell Formation in Atherogenesis, Science 306 (2004) 1558–1561.

[4] E. Osto, C.M. Matter, A. Kouroedov, T. Malinski, M. Bachschmid, G.G. Camici, U. Kilic, T. Stallmach, J. Boren, S. Iliceto, T.F. Lüscher, F. Cosentino, c-Jun N-terminal kinase 2 deficiency protects against hypercholesterolemia-induced endothelial dysfunction and oxidative stress, Circulation 118 (2008) 2073–2080.

[5] A. Chatterjee, J.D. Catravas, Endothelial nitric oxide (NO) and its pathophysiologic regulation, Vascul. Pharmacol. 49 (2008) 134–140.

[6] B.S. Oemar, M.R. Tschudi, N. Godoy, V. Brovkovich, T. Malinski, T.F. Luscher, Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis, Circulation 97 (1998) 2494–2498.
[7] H. Duplain, R. Burcelin, C. Sartori, S. Cook, M. Egli, M. Lepori, P. Vollenweider, T. Pedrazzini, P. Nicod, B. Thorens, U. Scherrer, Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase, Circulation 104 (2001) 42–345.
[8] P.F. Mount, B.E. Kemp, D.A. Power, Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation, J. Mol. Cell Cardiol. 42 (2007) 271–279.
[9] D.H. Cho, Y.J. Choi, S.A. Jo, I. Jo, Nitric oxide production and regulation of endothelial nitric-oxide synthase phosphorylation by prolonged treatment with troglitazone, J. Biol. Chem. 279 (2004) 2499–2506.
[10] D. Fulton, J.P. Gratton, T.J. McCabe, J. Fontana, Y. Fujio, K. Walsh, T.F. Franke, A. Papapetropoulos, W.C. Sessa, Regulation of endothelium-derived nitric oxide production by the protein kinase Akt, Nature 399 (1999) 597–601.
[11] Y. Zhang, T.S. Lee, E.M. Kolb, K. Sun, X. Lu, F.M. Sladek, G.S. Kassab, T. Garland Jr., J.Y. Shyy, AMP-activated protein kinase is involved in endothelial NO synthase activation in response to shear stress, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 1281–1287.
[12] I. Fleming, B. Fisslthaler, S. Dimmeler, B.E. Kemp, R. Busse, Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity, Circ. Res. 88 (2001) 68–75.
[13] P.M. Bauer, D. Fulton, Y.C. Boo, G.P. Sorescu, B.E. Kemp, H. Jo, W.C. Sessa, Compensatory phosphorylation and protein-protein interactions revealed by loss of function and gain of function mutants of multiple serine phosphorylation sites in endothelial nitric-oxide synthase, J. Biol. Chem. 278 (2003) 14841–14849.
[14] S.W. Bae, H.S. Kim, Y.N. Cha, Y.S. Park, S.A. Jo, I. Jo, Rapid increase in endothelial nitric oxide production by bradykinin is mediated by protein kinase A signaling pathway, Biochem. Biophys. Res. Commun. 306 (2003) 981–987.
[15] Z.P. Chen, K.I. Mitchelhill, B.J. Michell, D. Stapleton, I. Rodriguez-Crespo, L.A. Witters, D.A. Power, P.R. Ortiz de Montellano, B.E. Kemp, AMP-activated protein kinase phosphorylation of endothelial NO synthase, FEBS Lett. 443 (1999) 285–289.
[16] M. Oubaha, J.P. Gratton, Phosphorylation of endothelial nitric oxide synthase by atypical PKC zeta contributes to angiopoietin-1-dependent inhibition of VEGF-induced endothelial permeability in vitro, Blood 114 (2009) 3343–3351.
[17] D.H. Cho, J. Seo, J.H. Park, C. Jo, Y.J. Choi, J.W. Soh, I. Jo, Cyclin-dependent kinase 5 phosphorylates endothelial nitric oxide synthase at serine 116, Hypertension 55 (2010) 345–352.
[18] G. Sumara, M. Belwal, R. Ricci, ''Jnking'' atherosclerosis, Cell Mol. Life Sci. 62 (2005) 2487–2494.
[19] H.P. Kim, J.Y. Lee, J.K. Jeong, S.W. Bas, H.K. Lee, I. Jo, Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor localized in caveolae, Biochem. Biophys. Res. Commun. 263 (1999) 257–262.
[20] M.A. Bogoyevitch, B. Kobe, Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases, Microbiol. Mol. Biol. Rev. 70 (2006) 1061–1095.
[21] S. Eminel, A. Klettner, L. Roemer, T. Herdegen, V. Waetzig, JNK2 translocates to the mitochondria and mediates cytochrome c release in PC12 cells in response to 6-hydroxydopamine, J. Biol. Chem. 279 (2004) 55385–55392.
[22] R. Kou, D. Greif, T. Michel, Dephosphorylation of endothelial nitric-oxide synthase by vascular endothelial growth factor. Implications for the vascular responses to cyclosporin A, J. Biol. Chem. 277 (2002) 29669–29673.
[23] B.G. Drew, N.H. Fidge, G. Gallon-Beaumier, B.E. Kemp, B.A. Kingwell, High-density lipoprotein and apolipoprotein AI increase endothelial NO synthase activity by protein association and multisite phosphorylation, Proc. Natl. Acad. Sci. USA 101 (2004) 6999–7004.
[24] K.H. Sun, H.G. Lee, M.A. Smith, K. Shah, Direct and indirect roles of cyclin-dependent kinase 5 as an upstream regulator in the c-Jun NH2-terminal kinase cascade: relevance to neurotoxic insults in Alzheimer's disease, Mol. Biol. Cell 20 (2009) 4611–4619.