Nitric Oxide Inhibits Angiotensin II-induced Activation of the Calcium-sensitive Tyrosine Kinase Proline-rich Tyrosine Kinase 2 without Affecting Epidermal Growth Factor Receptor Transactivation*

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In a previous study, we showed that nitric oxide donors and N-acetylcysteine, either alone or in combination, inhibited the activation of several mitogen-activated protein kinases by angiotensin II in rat cardiac fibroblasts (Wang, D., Yu, X., and Brecher, P. (1998) J. Biol. Chem. 273, 33027–33034). In the present study, we have focused on the mechanism by which nitric oxide exerts this effect on the activation of extracellular signal-regulated kinase (ERK). We contrasted the effects of nitric oxide on ERK activation by angiotensin II and epidermal growth factor (EGF), since the transactivation of the EGF receptor has been implicated as a response to angiotensin II. We found that nitric oxide inhibited ERK activation by angiotensin II but did not inhibit the relatively slight but significant transactivation of the EGF receptor by angiotensin II. The tyrosinostin AG1478, known to inhibit EGF receptor phosphorylation, also inhibited the angiotensin II and EGF-induced activation of ERK, the phosphorylation of the EGF receptor, and the subsequent association of Shc and Grb2. Nitric oxide did not affect either EGF receptor phosphorylation or Shc-Grb2 activation induced by either Ang II or EGF. However, the activation of the calcium-sensitive tyrosine kinase PYK2, which occurred in response to angiotensin II, but not EGF, was inhibited by nitric oxide. The data suggested that PYK2 activation may be an important inhibitory site in signaling pathways affected by nitric oxide.

Angiotensin II (Ang II) is thought to have an important role in the cardiovascular remodeling processes associated with hypertension, heart failure, and atherosclerosis. The mitogenic response to Ang II in target cells such as the cardiac fibroblast or cardiac myocyte has been well documented (1). This mitogenic response requires the rapid activation of one or several mitogen-activated protein kinases including extracellular signal-regulated kinase (ERK), stress-activated c-Jun N-terminal kinases, and p38 mitogen-activated protein kinase, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase typically are activated in response to various cellular stresses, whereas the ERK cascade has a major role in signal transduction from both G protein-coupled receptors and receptors with intrinsic tyrosine kinase activity (2).

The coupling mechanisms between G protein-coupled receptors, including the AT1 receptor, and the ERK cascade are still incompletely characterized. However, there is some indication that the signaling of G protein-coupled receptors involves Shc-Grb2 and Sos complex formation prior to activation of a member of the Ras family (3), and it appears to be mediated by one or several tyrosine kinases, including the proline-rich tyrosine kinase 2 (PYK2), Src family tyrosine kinases, platelet-derived growth factor (PDGF) receptor, and epidermal growth factor (EGF) receptor (4–6). Of particular interest, both PYK2 and the EGF receptor were suggested to be central to ERK activation by G protein-coupled receptors (4, 6). The EGF receptor can serve as a scaffolding structure to which other signaling proteins are recruited, and signaling events induced by Ang II that involve tyrosine phosphorylation may be initiated by the transactivation of EGF receptor, since the addition of the tyrosinostin AG1478, a specific inhibitor of EGF receptor tyrosine kinase, blocked the ERK activity induced by Ang II as well as by EGF (7, 8). Furthermore, stimulation of cells by several G-protein-coupled receptors, including the AT1 receptor, results in the tyrosine phosphorylation of PYK2, a Cα2+-dependent tyrosine kinase also described as RAFTK, CAKβ, or CADTK, that has been implicated as an upstream component in ERK, c-Jun N-terminal kinase and p38 signaling cascades (4, 9, 10). Recently, vascular smooth muscle cells and cardiac fibroblasts were used to demonstrate that Ang II could activate PYK2 (11, 12).

Nitric oxide (NO) is an important free radical that has been suggested to contribute to the regulation of several hormone-mediated responses (13). NO acts principally through the stimulation of soluble guanylyl cyclase, leading to enhanced production of intracellular cGMP, which then activates cGMP-dependent protein kinases, although NO also has been shown to influence cellular events other than cGMP production (14). NO is capable of reacting with oxygen radicals such as superoxide anion to form the reactive peroxynitrite radical (15) as well as to directly nitrosate cysteine residues in different proteins (16). Because many of the physiological and pathophysiological effects of Ang II are known to be opposed by NO, it seems plausible that NO might influence Ang II action by inhibiting one or several of the tyrosine kinases implicated during the early signaling events leading to mitogen-activated
protein kinase activation.

In a previous study, we have shown that NO inhibits Ang II-induced activation of three major mitogen-activated protein kinases in rat cardiac fibroblast (17). Our study focused on ERK activation, but we also showed that there was increased activity for stress-activated c-Jun N-terminal kinase and p38 mitogen-activated protein kinase in response to Ang II and that these increases were attenuated by the addition of the NO donor S-nitroso-N-acetylpenicillamine (SNAP). This effect of SNAP was not limited to ERK activation by Ang II but was also found when ERK was activated by other agonists such as PDGF or phorbol esters. To gain more detailed insight into the possible mechanism by which NO might influence Ang II-sensitive signaling pathways in the cardiac fibroblast and to test the possibility that transactivation of the EGF receptor may have a role in these interactions, we have delineated several steps involved in the activation of ERK and found that NO selectively inhibits PYK2 tyrosine phosphorylation in response to Ang II but does not influence any of the steps involved in ERK activation in response to EGF.

**EXPERIMENTAL PROCEDURES**

**Reagents—** Dulbecco’s modified Eagle’s medium/F-12, Ca2+-free Dulbecco’s modified Eagle’s medium, fetal calf serum, and tissue culture reagents were from Life Technologies, Inc.; SNAP, ionophore A23187, and 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ) were from Alexis Corp. (San Diego, CA); tyrphostin AG1478, AG1295, calmidazolium, BAPTA-AM, and GF109203x were from Calbiochem. PDGF-BB and EGF were from Upstate Biotechnology, Inc. (Lake Placid, NY). The ECL detection system was from Amersham Pharmacia Biotech; Ang II, 1H-cysteine, and all other chemicals were purchased from Sigma.

**Antibodies—** Anti-Shc and anti-Grb2 antibodies were from Upstate Biotechnology; anti-phosphotyrosine (PY20) and anti-PYK2 antibodies were obtained from Transduction Laboratories (Lexington, KY); anti-EGFR antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho ERK antibody was from New England Biolabs (Beverly, MA).

**Cell Culture—** Rat cardiac fibroblasts were obtained from 8-day-old rats following an isolation procedure described previously by us (18). Cells in the fifth to seventh passage were grown to 85% confluence in 100-mm culture dishes and then maintained for 24 h in 0.1% fetal calf serum/Dulbecco’s modified Eagle’s medium/F-12. Fresh medium without fetal calf serum was routinely added 5 h before the experiment.

**Cell Treatments—** Ang II was routinely added to the cells at a final concentration of 0.1 µM. SNAP was routinely added with equimolar amounts of 1H-cysteine 15 min prior to the addition of agonists. 1H-Cysteine was then added without SNAP, had no effect on EGF receptor phosphorylation, ERK activation, or PYK2 phosphorylation. Cell viability was monitored either using trypan blue exclusion or by a measurement of lactate dehydrogenase activity into the culture medium using a commercially available kit. All experiments shown are representative of multiple experiments using separate cell preparations.

**Direct Immunoassay for ERK Using Whole Cell Lysates—** Following treatment with Ang II, EGF, or PDGF-BB for 5 min, cells were washed twice with ice-cold phosphate-buffered saline and then lysed with a concentrated buffer solution containing 250 mM Tris, pH 6.8, 8% SDS, 40% glycerol, 200 mM dithiotreitol, and 0.04% bromphenol blue. Cells were scraped into an Eppendorf tube and incubated on ice for 10 min. Following boiling for 5 min, the suspension was centrifuged at 10,000 × g for 10 min at 4 °C, and an aliquot of the supernatant was separated by 10% SDS-polyacrylamide gel electrophoresis. Following transfer to a nitrocellulose membrane and blockage with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), the blot was incubated with antibody (1:2000) specific for phospho-ERK1/2. After extensive washing, the immunoblot was then incubated with a second antibody conjugated with horseradish peroxidase and visualized with ECL (Amersham Pharmacia Biotech).

**Immunoprecipitation and Immunoblotting—** Following treatment of the cells with hormone or drugs, the cells were washed twice with ice-cold phosphate-buffered saline, and then cell lysis was accomplished by adding an ice-cold modified radioimmune precipitation buffer (50 mM Tris, pH 7.4, 1% Nonidet P-40, 1 mM NaF, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM orthovanadate, 1 mM PMSF, 10 µg/ml aprotenin, and 1 µg/ml leupeptin). The cells were briefly frozen at −70 °C for 10 min and allowed to thaw at 4 °C, and then the lysed cells were transferred to an Eppendorf tube for subsequent centrifugation at 10,000 × g for 10 min. Protein concentration was determined using the Bio-Rad protein assay system with bovine serum albumin as a standard. To immunoprecipitate phosphotyrosine-containing proteins from the cell lysates, we added 4 µg of anti-phosphotyrosine monoclonal antibody (PY20) to a volume of lysate containing 400 µg of protein. Antibodies were allowed to equilibrate with the lysate overnight at 4 °C. The immunocomplex was recovered by the addition of protein A-Sepharose for an additional 2 h at 4 °C. The immunoprecipitates were then washed once with ice-cold lysis buffer and twice with wash buffer (50 mM Tris, 150 mM NaCl, pH 7.4). The immunoprecipitated proteins were then resuspended in a sample buffer containing 62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromphenol blue. The sample was boiled for 5 min and then separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane at 100 V for 2.5 h, and the membrane was then incubated with the designated antibodies. Proteins were detected by using a horseradish peroxidase conjugated to goat anti-mouse or goat anti-rabbit IgG and visualized with an ECL kit. In selected experiments, cell lysates were initially immunoprecipitated with the designated specific antibody, and the nitrocellulose membranes obtained following Western blotting were incubated with the anti-phosphotyrosine antibody (PY20) and then visualized with ECL as described above. Images were obtained using a PDI scanner (model 420oe).

**Statistics—** Data are presented as means ± S.E. of at least three experiments unless designated otherwise. Statistical analysis was performed using analysis of variance and Student’s t test as appropriate. A value of p < 0.05 was considered to be statistically significant.

**RESULTS**

**AG1478 and SNAP Inhibit ERK Activation by Ang II—** Fig. 1A shows the activation of ERK by 0.1 µM Ang II, 20 ng/ml PDGF, or 50 ng/ml EGF after 5 min of treatment. All agonists induced a strong and comparable response. If cells were pretreated for 30 min with 1 µM tyrphostin AG1478, reported to be a selective inhibitor of the tyrosine kinase activity of the EGF receptor (19), both the EGF and Ang II-induced
activation of ERK were completely blocked, whereas the PDGF-induced response was essentially unaffected. In contrast, when cells were pretreated for 30 min with 10 μM tyrphostin AG1295, a selective inhibitor of PDGF receptor tyrosine kinase (19), the response to both Ang II and EGF was unaffected, whereas PDGF-induced activation of ERK was completely blocked (Fig. 1B). These results were consistent with other studies suggesting that Ang II activates ERK through a transactivation of the EGF receptor (7, 8). Interestingly, when cells were pretreated for 15 min with the NO donor SNAP, Ang II activation of ERK was markedly inhibited, consistent with our previous studies (17), whereas activation of ERK by EGF was essentially unaffected (Fig. 1C), implying that components of the EGF receptor signaling cascade may not be important sites for the inhibitory effect of SNAP. This latter observation is not completely consistent with transactivation of the EGF receptor being an essential mediator of Ang II action.

Tyrosine Phosphorylation of the EGF Receptor Occurs Relatively Weakly in Response to Ang II and Is Not Affected by SNAP Pretreatment—Fig. 2A shows data where EGF receptor phosphorylation was measured directly following incubation with either 0.1 μM Ang II for varying times or with 50 ng/ml EGF for 2 min. EGF receptor phosphorylation was relatively weak when incubated with Ang II throughout a 10-min time period but was markedly and rapidly increased in response to EGF. The lower panel of Fig. 2A indicates total EGF receptor to show equal loading of samples. Fig. 2B compares EGF receptor phosphorylation with ERK activation at different concentrations of EGF. EGF levels from 1 to 50 ng/ml produced progressive increases in EGF receptor phosphorylation, whereas ERK activation reached the peak at submaximal dose of EGF (10 ng/ml), indicating that there may be a relatively low threshold phosphorylation level for the EGF receptor that is sufficient for ERK activation. The extent of EGF receptor phosphorylation produced by 0.1 μM Ang II was actually less than the small response seen with the low dose of EGF (1 ng/ml). A more detailed examination of the effect of SNAP on EGF receptor phosphorylation was shown in Fig. 2C, indicating that SNAP did not influence EGF-induced EGF receptor phosphorylation using 1–50 ng/ml of EGF. Importantly, SNAP was ineffective in reducing the small degree of EGF receptor transactivation induced by 0.1 μM Ang II.

Ang II and EGF Cause Shc Phosphorylation and Association with Grb2, but SNAP Does Not Inhibit This Process—Fig. 3A shows that the addition of either 0.1 μM Ang II or 50 ng/ml EGF causes Shc phosphorylation as evidenced by increased intensity of three bands, consistent with previous studies showing three isoforms of phosphorylated Shc in rat cardiac fibroblasts (20). The tyrphostin AG1478 inhibited the action of both agonists, whereas SNAP addition has no effect on Shc phosphorylation. Complex formation between Shc and Grb2, determined by immunoprecipitating Shc and then detecting Grb2 on the resulting immunoblot, also rapidly occurred in response to both Ang II and EGF (Fig. 3B), and this process also was completely inhibited by AG1478 but was unaffected by SNAP pretreatment.

Intracellular Calcium Is Required for ERK Activation by Ang II—To validate the role of intracellular Ca2+ in ERK activation, cells were pretreated with the intracellular Ca2+ chelator BAPTA-AM using a calcium-containing or calcium-depleted medium and subsequently treated with 0.1 μM Ang II (Fig. 4A). Although pretreatment of the cells in Ca2+-depleted medium did not prevent Ang II activation of ERK, ERK activation was markedly inhibited when BAPTA-AM was added. Further information on the Ca2+-dependence of Ang II induced ERK activation was provided using 2.5 μM calmidazolium and 25 μM W7, agents used as selective inhibitors of calmodulin-dependent enzymes (Fig. 4B). Neither drug affected the Ang II-induced activation of ERK. It was noted that a higher concentration of calmidazolium (>5 μM) could cause cell damage during a 15-min pretreatment in our experimental system, so higher concentrations could not be used. The addition of the ionophore A23187 (5 μM) also activated ERK rapidly, independent of Ang II addition, but in this case the activation was prolonged (Fig. 4C) rather than transient as when Ang II was added (17). Of interest, the addition of SNAP attenuated the ionophore-induced activation of ERK (Fig. 4C), analogous to the inhibition found with Ang II-induced ERK activation. In contrast to Ang II, EGF caused rapid activation of ERK independent of BAPTA-AM pretreatment (Fig. 4D) and was unaffected by SNAP pretreatment (Fig. 1C).
SNAP Inhibits PYK2 Activation in Response to Ang II—Fig. 5A shows that Ang II treatment resulted in marked activation of PYK2, a calcium-sensitive tyrosine kinase recently shown to be activated by Ang II in vascular smooth muscle cells, cardiac fibroblasts, and liver epithelium cells (11, 12, 21). Of particular interest, the activation of PYK2 by Ang II was effectively blocked by SNAP pretreatment but not by tyrphostin AG1478 (Fig. 5A). In contrast to Ang II, EGF addition (50 ng/ml) did not activate PYK2 either rapidly or throughout a 30-min period (Fig. 5B).

The temporal and concentration-dependent aspects of the relationship between PYK2 and ERK were investigated. Fig. 6A shows the time-dependent changes in both PYK2 and ERK activation in response to 0.1 μM Ang II. PYK2 activation could be detected clearly after just 0.5 min of Ang II treatment, whereas ERK activation was only seen to occur after 1 min. This set of experiments was performed three times, and statistical analysis of the combined data did show a significantly greater increase in PYK2 than in ERK at both 0.5 and 1 min. Interestingly, both PYK2 and ERK remained active for a comparable time period before eventually declining in activity by 30 min. Fig. 6B shows the response of both PYK2 and ERK activation to a broad concentration range of Ang II. Phosphorylated PYK2 and ERK were determined after cells were stimulated with Ang II for 2 and 5 min, respectively, as the exposure time for PYK2 and ERK. Comparable increases were seen for both PYK2 and ERK in response to varying concentrations of Ang II.

To further validate the effect of SNAP on PYK2 and ERK activation, cells were pretreated with SNAP for times ranging between 10 and 30 min and then incubated with Ang II for 2 min (PYK2) or 5 min (ERK). Fig. 7A shows no appreciable difference due to SNAP pretreatment times with respect to the subsequent inhibition of either PYK2 or ERK. In addition, we performed experiments (not shown) where pretreatment periods ranging from 5 min to 24 h were employed. The results indicated that 5-min pretreatment was about as effective as 10–30 min, but if pretreatment was for 3 h or longer, there was no subsequent suppression of either PYK2 or ERK activation by Ang II. The data are consistent with complete dissociation of NO from SNAP and subsequent oxidation to nitrite and nitrate during the longer preincubation times. In Fig. 7B, we performed a series of experiments comparing SNAP effects at concentrations ranging from 1 to 500 μM using a 15-min pretreatment period. There was a comparable dose-dependent suppression of Ang II activation for both PYK2 and ERK. Again, these experiments were repeated three times, and the results were analyzed densitometrically in the lower panel.

Effects of ODQ and GF109203x on SNAP inhibition of PYK2 and ERK in Response to Ang II—Fig. 8A shows the dose-dependent effects of ODQ, a selective inhibitor of soluble guanylyl cyclase, on both PYK2 and ERK activation. In both cases, increasing amounts of ODQ between 0.1 and 10 μM reversed the inhibitory effects of SNAP on the activation of each kinase by Ang II, suggesting that cGMP may be a possible mediator in the action of SNAP. In separate experiments (not shown), we found that 10-, 30-, or 60-min pretreatment with 10 μM ODQ produced a similar reversible effect when cells were exposed to
SNAP and subsequently to Ang II.

To determine if protein kinase C has a role in Ang II activation of PYK2 and ERK, we pretreated cells for 30 min with 1 μM GF109203x, a drug we have previously shown to prevent PMA-induced ERK activation in our cells (17). As shown in Fig. 8B, the addition of GF109203x had no effect on Ang II activation of ERK, whereas it effectively blocked the activation of ERK by phorbol 12-myristate 13-acetate (PMA). Despite the effectiveness of the drug in blocking protein kinase C isoforms, the inhibitory effect of SNAP on ERK was essentially unchanged when GF109203x was added, although SNAP did appear to be more effective as an inhibitor in the presence of GF109203x (Fig. 8C). The data suggest that protein kinase C inhibition is not a major determinant to the mechanism by which SNAP inhibits Ang II-induced activation of ERK.

DISCUSSION

In the present study, we have examined the effects of NO on several of the enzymatic steps necessary for the rapid activation of ERK by Ang II in rat cardiac fibroblasts. Our data suggest that the Ca2+-sensitive phosphorylation of PYK2 is a likely site for the previously described inhibition of Ang II action by NO (17). We also have excluded an inhibitory effect of NO on the steps leading to the transactivation of the EGF receptor by Ang II.

Transactivation of the EGF receptor by Ang II has recently been documented in vascular smooth muscle cells (8) and cardiac fibroblasts (20). This process of transactivation has been invoked as a potential pathway to explain how agonists known to interact with G protein-linked receptors can produce responses usually attributed to tyrosine kinase-linked receptors (22). In our studies, we showed that Ang II did cause a relatively slight phosphorylation of the EGF receptor, but this process was not affected by SNAP. In fact, the signaling events leading to EGF receptor activation, Shc phosphorylation, and association with Grb2 and downstream ERK activation initiated by EGF were not inhibited by SNAP, in marked contrast to other agonists, including Ang II, PDGF, and phorbol esters, where SNAP inhibited ERK activation (17). Our findings contrast with those reported by Yu et al. (23), who indicated that NO could inhibit EGF-induced ERK activation, with Estrada et al. (24) showing that EGF receptor tyrosine kinase was inhibited by NO, and with Peranovich et al. (25) showing that NO potentiates EGF-evoked tyrosine kinase activity.

Although the Ang II-induced transactivation of the EGF receptor was slight, it is possible that this is a necessary event to permit activation of ERK by Ang II, since our data suggest that submaximal activation of the EGF receptor by EGF, nevertheless, can induce maximal downstream activation of ERK. Furthermore, tyrphostin AG1478, reportedly a specific inhibitor for EGF receptor tyrosine kinase activity, did prevent Ang II-induced ERK activation. However, other agonists, such as PDGF, also can activate ERK, but through mechanisms that do not involve transactivation of the EGF receptor.

Phosphorylation of Shc, its association with Grb2, and Sos have been considered as downstream components of EGF receptor activation (26). We found that Shc phosphorylation was induced by both Ang II and EGF and was inhibited completely by pretreatment with AG1478. However, SNAP addition had no effect on either Shc phosphorylation or its association with Grb2 when cells were treated with either Ang II or EGF. These findings are consistent with Ang II acting via transactivation of

Fig. 5. PYK2 phosphorylation occurs in response to Ang II, but not EGF. A, cells were treated directly with 0.1 μg/ml Ang II for 2 min or following a pretreatment with either 1 μM tyrphostin AG1478 for 30 min or 100 μM SNAP for 15 min. In this protocol, phosphorylated PYK2 was determined by first immunoprecipitating the extract with antibody against PYK2 (α PYK2) and then immunoblotting with an anti-phosphotyrosine antibody (α pTyr). The lower panel shows the total amount of PYK2 in each lane detected by using anti-PYK2 antibody for both immunoprecipitation and immunoblotting procedures. B, cells were incubated with EGF (50 ng/ml) for the designated time or with Ang II (0.1 μg/ml) for 2 min. Phosphorylated PYK2 was determined with an antibody against phosphotyrosine (α pTyr) and analyzed by immunoblotting with an antibody against PYK2 (α PYK2).

Fig. 6. The parallel phosphorylation of PYK2 and ERK following Ang II stimulation. In all experiments, ERK was determined by the direct assay for ERK using whole cell lysates and a specific antibody directed against the phosphorylated forms of ERK, and PYK2 was immunoprecipitated with an antibody against phosphotyrosine (α pTyr) and analyzed by immunoblotting with an antibody against PYK2 (α PYK2). A, cells were incubated with Ang II (0.1 μg/ml) for the designated times, and then tyrosine-phosphorylated ERK and PYK2 were determined. The lower panel shows the mean ± S.E. from densitometric analysis of three independent experiments. *, p < 0.05, indicating the significant differences between PYK2 and ERK activation at designated times. B, cells were incubated with the designated concentrations of Ang II for 5 min (PYK2) or 5 min (ERK). Cell extracts were analyzed for tyrosine-phosphorylated ERK (α pERK) or PYK2 (α PYK2) as indicated above. The lower panel indicates the densitometric analysis of three separate sets of experiments.
the EGF receptor, although it is possible that Shc phosphorylation could occur through a pathway independent of the EGF receptor as well (27).

Perhaps the most novel and important finding in this study is the inhibition of PYK2 phosphorylation by NO. This is the first example of NO inhibiting a Ca
\(^{2+}\)-sensitive tyrosine kinase and could represent an important mechanism by which NO regulates signaling by other agonists. Although the precise role of PYK2 in ERK activation has not been fully established, it could act as an intermediate that links various calcium signals to cellular response, since PYK2 can be activated by various agonists that change intracellular levels (28). In fact, PYK2 has recently been shown to be essential for the activation of ERK by Ang II in both vascular smooth muscle cells and cardiac fibroblasts (11, 12). Our data clearly showed the temporal and concentration-dependent aspects of the relationship between PYK2 and ERK. Furthermore, comparable suppression of Ang II action could occur through a pathway independent of the EGF receptor as well (27).

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Our studies with BAPTA using both Ca
\(^{2+}\)-containing and Ca
\(^{2+}\)-free medium show that ERK activation by Ang II is dependent upon intracellular Ca
\(^{2+}\). Activation of PYK2 by Ang II also is clearly Ca
\(^{2+}\)-dependent. The activation of PYK2 by Ang II occurred rapidly and was inhibited by SNAP but not by AG1478. PYK2 phosphorylation did not occur when cells were treated with EGF, and unlike Ang II, EGF could activate ERK independently of intracellular Ca
\(^{2+}\). Furthermore, ionophore-induced activation of ERK was inhibited by SNAP. These data are consistent with a mechanism where SNAP inhibits a Ca
\(^{2+}\)-dependent step or steps and emphasize the potential importance of Ca
\(^{2+}\)-dependent tyrosine kinases.

There are many paths that can be used to activate ERK. EGF action in cardiac fibroblasts does not activate PYK2, whereas Ang II action does involve PYK2 activation. It is possible that weak activation of the EGF receptor causes partial phosphorylation of its many potential sites, leading to a degree of recruitment and activation of a downstream effector such as Shc, which by itself may be insufficient to activate ERK. However, when PYK2 is activated by Ang II, it can interact with other signaling molecules associated with the relatively weakly phosphorylated EGF receptor, and that combination would be sufficient for ERK activation. This may explain why tyrphostin AG1478 would block Ang II action, since it prevents Shc recruitment. Alternatively, if the tyrphostin inhibited an as yet unelucidated intermediate in a pathway independent of the EGF receptor phosphorylation, that also would explain our data.

In summary, we have used two distinct signaling pathways leading to the activation of ERK in rat cardiac fibroblasts to show specificity for the inhibitory effects of NO. We have presented data suggesting that an important site of NO inhibition of ERK activation by Ang II is the Ca
\(^{2+}\)-sensitive tyrosine kinase PYK2. NO did not affect ERK activation mediated by EGF, a signaling pathway that appeared independent of...
changes in intracellular Ca^{2+}. This effect of NO on the inhibition of specific tyrosine kinases may offer a partial explanation for the antagonistic effects of NO on Ang II action in other cell types as well. The ability of NO to interfere with diverse signaling pathways in various cell types strongly suggests that a single site of action probably is not sufficient to explain all of the effects of this substance; indeed, there is a large body of literature suggesting that the pleiotropic effects of NO may be mediated by diverse mechanisms involving changes in cyclic nucleotide levels, covalent modification of regulatory proteins by nitrosation, or effects mediated through interactions with other free radicals (29).

REFERENCES
1. Sadoshima, J., and Izumo, S. (1993) Circ. Res. 73, 413–423
2. Force, T., and Bonventre, J. V. (1998) Hypertension 31, 152–161
3. Sadoshima, J., and Izumo, S. (1996) EMBO J. 15, 775–87
4. Dikic, I., Tokiwa, G., Lev, S., Courtnidge, S. A., Schlessinger, J. (1996) Nature 383, 547–550
5. Linseman, D. A., Benjamin, C. W., Jones, D. A. (1995) J. Biol. Chem. 270, 12563–12568
6. Dusb, H., Weiss, F. U., Wallasch, C., Ulrich, A. (1996) Nature 379, 557–560
7. Li, X., Lee, J. W., Graves, L. M., and Earp, H. S. (1996) EMBO J. 15, 2574–2583
8. Eguchi, S., Numaguchi, K., Iwasaki, H., Matsumoto, T., Yamakawa, T., Utsumi, K., Iwasaka, T., Inada, M., and Matsubara, H. (1998) J. Biol. Chem. 273, 8890–8896
9. Tokiwa, G., Dikic, I., Lev, S., and Schlessinger, J. (1996) Science 273, 792–794
10. Pandey, P., Avraham, S., Kumar, S., Nakazawa, A., Place, A., Ghanem, L., Rana, A., Kumar, V., Majumder, P. K., Avraham, H., Davis, R. J., and Kharbanda, S. (1999) J. Biol. Chem. 274, 10140–10144
11. Sabri, A., Govindarajan, G., Griffin, T. M., Byron, K. L., Samarel, A. M., and Luchesi, P. A. (1998) Circ. Res. 83, 841–851
12. Murasawa, S., Mori, Y., Nozawa, Y., Masaki, H., Maruyama, K., Tsutsui, Y., Moriguchi, Y., Shibasaki, Y., Tanaka, Y., Iwasaka, T., Inada, M., and Matsubara, H. (1998) Hypertension 32, 668–675
13. Schmidt, H. H. W., and Walser, U. (1994) Cell 78, 919–925
14. Mayer, B., Pfeiffer, S., Schrammel, A., Koesling, D., Schmidt, K., and Brunner, F. (1998) J. Biol. Chem. 273, 3264–3270
15. Kelm, M., Dahnmann, H., Wink, D., and Peedem, M. (1997) J. Biol. Chem. 272, 9922–9932
16. Wink, D. A., Cook, J. A., Kim, S. Y., Vodovotz, Y., Pacelli, R., Krishna, M. C., Russo, A., Mitchell, J. B., Jourd’heuil, D., Miles, A. M., and Grisham, M. B. (1997) J. Biol. Chem. 272, 11147–11151
17. Wang D., Yu X., and Brecher P. (1998) J. Biol. Chem. 273, 33027–33034
18. Farrier, R. S., Chobanian, A. V., and Brecher, P. (1996) Circ. Res. 78, 759–768
19. Levitzki, A. and Gazit A. (1995) Science 267, 17921787
20. Murasawa, S., Mori, Y., Nozawa, Y., Gotoh, N., Shibuya, M., Masaki, H., Maruyama, K., Tsutsui, Y., Moriguchi, Y., Shibasaki, Y., Tanaka, Y., Iwasaka, T., Inada, M., and Matsubara, H. (1998) Circ. Res. 82, 1338–1348
21. Li, X., and Earp, H. S. (1997) J. Biol. Chem. 272, 14341–14348
22. Luttrell, L. M., Della, Rocca, G. J., van Biesen, T., Luttrell, D. K., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 4637–4644
23. Yu, S. M., Hung, L. M., and Lin, C. C. (1997) Circulation 95, 1269–1277
24. Estrada, C., Gomez, C., Martin-Nieto, J., De Frutos, T., Jimenez, A., and Villalobo, A. (1997) Biochem. J. 326, 369–376
25. Ponsanovich, T. M. S., De Silva, A. M. Pries, D. M., Stern, A., and Monteiro H. P. (1995) Biochem. J. 303, 613–619
26. Pomereau, M., Multon, M. C., Parker, F., Venot, C., Blondeau, J. P., Toque, B., and Schweighoffer, F. (1998) J. Biol. Chem. 273, 24301–24304
27. Jiao, H., Cui, X. L., Tori, M., Chang, C. H., Alexander, L. D., Lapetina, E. G., and Douglas, G. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7417–7421
28. Lev, S., Moreno, H., Martinez, R., Canosi, P., Peles, E., Musacchio, J., M., Plowman, G. D., Ruby, B., and Schlessinger, J. (1996) Nature 376, 737–745
29. Loscalzo, J. and Welch, G. (1995) Prog. Cardiovasc. Dis. 38, 87–104