Nitric Oxide and N-Acetylcysteine Inhibit the Activation of Mitogen-activated Protein Kinases by Angiotensin II in Rat Cardiac Fibroblasts*

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Angiotensin II acts on the cardiac fibroblast to produce a mitogenic response. Nitric oxide and N-acetylcysteine have been used to determine if oxidative stress influenced the effects of angiotensin II on the cardiac fibroblast. Angiotensin II activated the mitogen-activated protein kinases designated extracellular signal-regulated kinases within 5 min by interacting with the AT₁ receptor. This activation was completely independent of protein kinase C and was inhibited when farnesyltransferase was blocked, implicating Ras involvement. Pretreatment of cardiac fibroblasts with either N-acetylcysteine for 8 h or nitric oxide for 10 min suppressed this activation by angiotensin II in a dose-dependent manner. However, when both agents were added, inhibition was essentially complete. This combined effect of N-acetylcysteine and nitric oxide to block ERKs activation also was found if the activity was stimulated by either another growth factor (platelet-derived growth factor) or by the addition of phorbol ester, suggesting the effect was not limited to the receptor site alone. The results are consistent with the hypothesis that hormonal activation of mitogenic steps such as ERKs is influenced by increased oxidative stress, which is reduced by the combined effects of N-acetylcysteine and nitric oxide.

Angiotensin II (Ang II) has pleiotrophic effects on several cell types, leading to diverse responses including the regulation of cell growth, programmed cell death, cell migration, and modification of the extracellular matrix (1–3). Ang II has been shown to activate many signaling pathways through its Gq-modification of the extracellular matrix (1–3). Ang II has been shown to regulate cell growth, programmed cell death, cell migration, and cell types, leading to diverse responses including the regulation of transcription factors such as Elk1 and c-Fos, whereas JNKs and p38 MAPK exhibit substantial sequence homology and respond primarily to various cellular stress conditions such as proinflammatory cytokines and UV irradiation, although it is not clear whether this occurs via common or parallel upstream kinases (8–10).

The signaling system leading to the activation of MAP kinases is subject to diverse and complex regulation. Several recent studies have suggested that the balance of the oxidative and reductive potentials within the cell (cellular redox state) may substantially influence this pathway (11, 12). In the presence of reactive oxygen species, such as superoxide anion or hydroxyl radical, or by the addition of N-acetylcysteine (NAC), an agent that is thought to influence intracellular glutathione and the redox state of the cell, the response of cells to mitogens or cytokines can be impaired, presumably by changing the conditions for thiol oxidation or reduction (13–15). Nitric oxide (NO), a redox-active molecule, has been identified as an important potential regulator of certain signaling events. NO acts by stimulating soluble guanylate cyclase, leading to enhanced production of intracellular cyclic GMP, an intracellular second messenger that can activate cyclic GMP-dependent protein kinases (16). NO also is capable of reacting with oxygen radicals such as superoxide anion (17) as well as directly modulating the activity of signaling molecules (18, 19). The interaction with superoxide anion was suggested to be important in mechanisms where NO was implicated in modulating cytotoxic mechanisms, presumably by influencing oxidative stress (19).

Oxidative stress, the term used to encompass changes in the cellular redox state, has been implicated in inflammatory processes such as fibrosis. The cardiac fibroblast, which is the cell type known to proliferate during cardiac fibrosis and produce the excess matrix proteins characteristic of that condition, is a target cell for Ang II. Ang II has been shown to cause proliferation of cardiac fibroblasts in culture (20). We have recently shown that nitric oxide can modulate this proliferative effect (21), consistent with the known ability of NO to antagonize the actions of Ang II in many cell types. To better understand the role that NO might have in influencing Ang II action in cardiac fibroblasts, we have characterized the activation of ERKs in these cells by Ang II and have examined the effects of N-acetylcysteine and NO on this pathway. We have found novel effects of both NAC and NO on Ang II-induced ERKs activation, suggesting that both NO and oxidative stress, which ac-
company the development of cardiac fibrosis, could modulate the effects of Ang II on the cardiac fibroblasts.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium/F-12, fetal calf serum, PDGF-BB, and tissue culture reagents were from Life Technologies, Inc. S-Nitroso-N-acetylpenicillamine (SNAP) was from Alexis Corp. (San Diego, CA); PDI23319, bisindolylmaleimide I (GF109203X), GI69853, ODQ, genistein, and farnesyltransferase inhibitor-3 were from Calbiochem. [γ-32P]ATP (10 mCi/ml), p42/p44 MAP kinase enzyme assay kit, and the ECL detection system were from Amersham Pharmacia Biotech. MEK1 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PDI90659 and phosphospecific ERK antibodies were from New England Biolabs (Beverly, MA). Losartan was generously provided by DuPont. Ang II, FMA, NAC, 5,5’-dithiobis(nitrobenzoic acid), t-buthionine-(S,R)-sulfoximine (BSO), n-cysteine, glutathione monothi- ylester, and all other chemicals were purchased from Sigma.

Cell Culture—Cardiac fibroblasts were obtained from 8-day-old rats following an isolation procedure described previously by us (22). Cells in the 3rd to 4th passage were grown to 80% confluence in either 60- or 100-mm culture dishes and then maintained for 24 h in 0.4% fetal calf serum/Dulbecco’s modified Eagle’s medium/F-12. Fresh medium was routinely changed in the 2nd passage before the experiment.

Cell Treatments—Ang II was routinely added to the cells for 5 min at a concentration of 0.1 μM. SNAP was routinely added 10 min prior to the addition of agonists. NAC pretreatment was typically for 8 h, and the medium containing NAC was replaced with Dulbecco’s modified Eagle’s medium/F-12 lacking NAC for 2 h prior to adding other agonists. Care was taken to adjust the pH of medium containing NAC prior to adding it to the cells. Cell viability was monitored routinely using either trypan blue exclusion or by a measurement of lactate dehydrogenase activity into the culture medium using a commercially available kit.

Radioassay for ERKs—This radioassay was essentially that provided in a kit purchased from Amersham Pharmacia Biotech (catalog no. RPNI84) in which a cell lysate is used to generate a radiolabeled product using a specific substrate and an antibody (1:1000) specific for a polyclonal antibody against MEK-1 (Santa Cruz Biotechnology, catalog no. sc-436). Protein A-Sepharose beads were added, and the immunoprecipitated enzyme was incubated in 30 μl of kinase buffer (25 mM Tris, pH 7.5, 2 mM dithiothreitol, 0.1 mM orthovanadate, 10 mM MgCl2, 5 μM ATP, 10 μM of [γ-32P]ATP) with a recombinant GST- [K71A]Erk44 lacking enzymatic activity as a substrate (Upstate Biotechnology, Inc., catalog no. 14-135). The labeled substrate was detected by autoradiography following 10% SDS-PAGE.

Glutathione Assay—Intracellular glutathione was measured spectrophotometrically following a minor modification of the method of Tietze (23). Cells were lysed with 0.2 ml of 0.35% perchloric acid. Following centrifugation at 10,000 × g for 10 min at 4 °C, different aliquots of the lysate were incubated with 0.1 mM sodium phosphate, pH 7.5, 5 mM EDTA, 1 mM NADPH, 1 unit of glutathione reductase (Boehringer Mannheim), and 0.6 mM 5,5’-dithiobis(nitrobenzoic acid) in a total volume of 1 ml. The rate of change in absorbancy at 412 nm was measured over a 5-min period to reflect the formation of reaction product and correlated with a standard curve using reduced glutathione. Data are expressed as nmol of glutathione/mg of protein in the lysate.

Statistics—Data are presented as the mean ± 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

Characteristics of ERK Activation by Ang II—Fig. 1A shows the rapid and transient increase in the activity of ERKs following the addition of 0.1 μM Ang II in quiescent rat cardiac fibroblasts, where maximal activation occurred 2–5 min after hormone addition and then gradually decreased to basal levels. Fig. 1B shows that the 5-min response to Ang II was dose-de- pendent with peak activity occurring at concentrations between 0.01–0.1 μM Ang II and a clear increase even at concentrations less than 1 nM. In 35 separate experiments using five different preparations of cardiac fibroblasts in the 3rd or 4th passage, 0.1 μM Ang II increased ERK activity an average of 8-fold over control levels, with a range of 5–15-fold. Fig. 1C shows that the response to Ang II was mediated through AT1B receptors, based on the almost complete blockade of activation if cells were pretreated with losartan (an AT1 receptor antagonist), whereas pretreatment with PD123319 (an AT2 receptor antagonist) was without effect. The experiments shown in Fig. 1, A and C, were performed using tied to radio- active donor ERK. To further establish that Ang II and AT1B experiments using an immunoprecipitation assay for ERKs were performed (Fig. 2, A–C). This assay detects immunoreac- tive phosphorylated Elk1, a specific substrate for the immunoprecipitated ERKs. In Fig. 2, A–C, the effects of genistein, farnesyltransferase inhibitor-3, and PD98059, respectively, were studied. In each case, pretreatment with the respective

33028 NO and NAC Inhibit MAP Kinase Activation by Ang II

Centrifugation at 10,000 × g for 10 min and allowed to thaw at 4 °C, and then the cell lysates were solubilized, subjected to phosphocellulose and then cell lysis was accomplished by adding a lysis buffer containing 0.1% Triton X-100, 10 mM Tris, pH 7.4, 50 mM NaCl, 2 mM EGTA, 1 mM diithiothreitol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. 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 a 1.5-ml Eppendorf tube for subsequent assay, 200 μl against phosphorylated Elk1 as described in the kit. In the p38 MAPK assay, 200 μg of protein was immunoprecipitated with 1 μg of a polyclonal antibody against phosphorylated p38 MAPK. The phosphorylated proteins were determined by Western blot analysis using an antibody against phosphorylated p38 MAPK as described in the kit. In the JNKs assay, 300 μg of protein was incubated with 2 μg of GST-c-Jun-(1–89) fusion protein bound to glutathione-Sepharose beads to selectively precipitate JNKs. The kinase reaction was performed following the addition of 100 μM ATP for 30 min at 30 °C. c-Jun phosphorylation was selectively measured using a phosphospecific c-Jun antibody as described in the kit. Immune complexes on nitrocellulose membrane were treated with an appropriate secondary antibody conjugated with horseradish peroxidase and visualized with ECL (Amersham Pharmacia Biotech). Densitometric analysis of immunoblots from these assays and all other immunoassays used in this study were performed using a PDI scanner (model 420oe), and the data were reported as a ρ-fold increase over unstimulated cells (control), which arbitrarily were set at 1.

Direct Immunooassay for ERKs Using Whole Cell Lysates—Following treatment with Ang II for 5 min, cells were washed twice with ice-cold PBS and then cell lysis was accomplished by adding a lysis buffer containing 250 mM Tris, pH 6.8, 8% SDS, 40% glycerol, 200 mM dithiothreitol, and 0.04% bromphenol blue. Following boiling for 5 min, the suspension was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was used for direct application onto 10% SDS-PAGE. Following transfer to nitrocellulose membrane and blockage with 5% nonfat milk in a Tris-buffered saline solution containing 0.1% Tween 20, the blot was incubated with antibody (1:1000) specific for phospho-ERK44 and -42 (New England Biolabs). After extensive washing, the blot was incubated with a second antibody conjugated with horseradish peroxidase and visualized with ECL (Amersham Pharmacia Biotech).

Radioimmunoassay for MEK1—500 μg of protein in cell lysates obtained as described above for the ERKs radioimmunoassay were incubated at 4 °C with 1 μg of a polyclonal antibody against MEK-1 (Santa Cruz Biotechnology, catalog no. sc-436). Protein A-Sepharose beads were added, and the immunoprecipitated enzyme was incubated in 30 μl of kinase buffer (25 mM Tris, pH 7.5, 2 mM dithiothreitol, 0.1 mM orthovanadate, 10 mM MgCl2, 5 μM ATP, 10 μM of [γ-32P]ATP) with a recombinant GST- [K71A]Erk44 lacking enzymatic activity as a substrate (Upstate Biotechnology, Inc., catalog no. 14-135). The labeled substrate was detected by autoradiography following 10% SDS-PAGE.

The procedures for these assays were essentially those provided in the protocol (11). The instructions provided with the kit were followed except for the initial procedures used to obtain a cell lysate. Following treatment of the cells with hormone or drugs, the cells were washed twice with ice-cold PBS, and then cell lysis was accomplished by adding a lysis buffer containing 0.1% Triton X-100, 10 mM Tris, pH 7.4, 50 mM NaCl, 2 mM EGTA, 1 mM diithiothreitol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. 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 a 1.5-ml Eppendorf tube for subsequent centrifugation at 10,000 × g for 10 min. Fifteen-μl aliquots were then used for the radioassay, following the instructions provided in the kit. Following incubation of the extract with labeled ATP and the synthetic substrate, the radiolabeled products were solubilized, separated on a polyacrylamide gel and electrophoresed to what extent the kinases were inhibited by a particular compound. The instructions from these assays and all other immunoassays used in this study were performed using a PDI scanner (model 420oe), and the data were reported as a ρ-fold increase over unstimulated cells (control), which arbitrarily were set at 1.
bated with 0.1 μM Ang II receptor antagonists on activation of ERKs. Cells were incubated with either 10 μM losartan for 5 min following a 30-min preincubation with 50 μM PD123319 (PD). All data are shown as the means ± S.E. for three separate experiments and expressed as fold increase relative to the unstimulated cells (Con), arbitrarily defined as 1 unit. *, p < 0.05 versus control.

Role of Protein Kinase C in ERK Activation—Fig. 3 shows experiments examining the role of protein kinase C in the Ang II-induced activation of ERKs. In Fig. 3A, using the radioassay for ERKs, cells were pretreated for only 15 min with protein kinase C inhibitors GF109203X (1 μM) or G6983 (1 μM), and the subsequent activation by PMA was abolished, whereas no effect on Ang II activation was found. Similar results were shown in Fig. 3B by using the radioassay for ERKs. Cells were preincubated with GF109203X. Responses to PMA were blocked with no effect on Ang II activation. In Fig. 3C, cells were pretreated for 24 h with 1 μM PMA to down-regulate protein kinase C, and then either Ang II (0.1 μM) or PMA (1 μM) was added. After 5 min, the cells were assayed for ERK activity. Again, pretreatment with PMA for 24 h completely prevented subsequent activation of ERKs by additional PMA but had almost no effect on the increased activity in response to Ang II. Thus, it appears that Ang II activates ERKs through a mechanism that is predominantly independent of protein kinase C.

Role of Glutathione in ERK Activation—To assess the role of intracellular GSH, a major determinant of the redox state of the cell, on the activation of ERKs by Ang II, cells were pretreated with BSO, an inhibitor of glutathione biosynthesis that decreases intracellular GSH levels. Cells also were treated with NAC, an agent known to increase GSH levels, and additionally with a combination of both drugs. Fig. 4A shows the effects of these drugs on GSH levels in the cardiac fibroblasts. BSO (50 μM) dramatically decreased cellular GSH levels when pretreatment of the cells was performed for 24 h. NAC (8 mM) increased GSH approximately 3-fold if pretreatment was for at least 8 h. Higher concentrations of NAC (20–30 mM) caused cell damage within 30 min of treatment as measured by the release of lactate dehydrogenase into the culture medium. The combination of BSO and NAC pretreatment blocked the increase in GSH levels generated by exposure to NAC, indicating that the effects of BSO override those of NAC. However, as shown in Fig. 4B, activation of ERKs by Ang II was essentially unaffected by BSO treatment, whereas NAC pretreatment caused a decrease in ERK activity. This effect of NAC was clearly independent of GSH levels, since ERK activity was lowered even when BSO was added in the presence of NAC. Fig. 4C shows that the inhibition of ERK activation by NAC was dose-dependent, but NAC did not completely inhibit the response. Since a low GSH concentration presumably makes the cell more sensitive to oxidative stress, and Ang II-mediated effects may be mediated by reactive oxygen species (24, 25), we performed a series of experiments with BSO-pretreated cells to determine if the response to suboptimal levels of hormone would be influ-

![Figure 1](http://www.jbc.org/)

**Fig. 1. Characteristics of the ERK activation by Ang II in cardiac fibroblasts.** A, effect of incubation time on activation of ERKs. Cells were incubated with 0.1 μM Ang II for the designated time and then assayed for ERK activity using the radioassay as described in “Experimental Procedures.” B, effect of Ang II concentration on the activation of ERKs. Cells were incubated with the designated concentration of Ang II for 5 min and then assayed for ERK activity. C, effect of Ang II receptor antagonists on activation of ERKs. Cells were incubated with 0.1 μM Ang II for 5 min following a 30-min preincubation with either 10 μM losartan (Los) or 10 μM PD123319 (PD). All data are shown as the means ± S.E. for three separate experiments and expressed as fold increase relative to the unstimulated cells (Con), arbitrarily defined as 1 unit. *, p < 0.05 versus control.

![Figure 2](http://www.jbc.org/)

**Fig. 2. Effect of inhibitors of tyrosine kinase (A), protein farnesyltransferase inhibitor-3 (FT-3), a commercially available farnesyltransferase inhibitor-3 (FT-3), a commercially available enzyme inhibitor (Calbiochem) for 1 h, the cells were incubated with either 0.1 μM Ang II or 20 ng/ml PDGF-BB for 5 min, and then extracts were prepared for immunoprecipitation. C, following pretreatment with 50 μM PD98059 for 45 min, the cells were incubated for 5 min with 0.1 μM Ang II, 1 μM PMA, or 20 ng/ml PDGF-BB, and then extracts were prepared for immunoprecipitation. Each immunoblot is representative of at least two separate experiments. pElk1, phosphorylated Elk1.

NO and NAC Inhibit MAP Kinase Activation by Ang II

33029
throughout a concentration range of 10−10 to 10−6 M. In separate experiments, not shown, we also found that BSO dose-response curves to Ang II activation were similar in the presence of cysteine, 50 μM GST (0.5 mM), which increased intracellular GSH levels about 2-fold, did not affect Ang II-activated ERK activity, whereas relatively brief pretreatment (60 min) with either β-mercaptoethanol (1 mM) or dithiothreitol (0.5 mM) reduced Ang II activation of ERKs by 50−75%. These latter findings suggested that it was the efficacy of NAC as a reducing agent that might account for its inhibitory effect. In all experiments with BSO and NAC pretreatments, cell viability was monitored visually by trypan blue exclusion and in selected experiments by measuring the release of lactate dehydrogenase into the culture medium. No evidence of cell damage to the cardiac fibroblasts was found during the pretreatment protocols.

The Effect of NO on ERK Activation—To assess the role of NO, we pretreated the cells with varying concentrations of the NO donor SNAP. Fig. 5A shows that pretreatment for 10 min with varying doses of SNAP resulted in a dose-dependent inhibition of ERK activation, but complete inhibition was not obtained at SNAP concentrations that maintained cell viability. In the above experiments, equimolar amounts of cysteine were added with SNAP to increase the efficacy of NO release. Fig. 5B shows that in the absence of cysteine, 50 μM SNAP was only slightly effective in inhibiting ERK activation, whereas a statistically significant reduction in activity was found when cysteine was included. Using another NO donor, S-nitrosothiolamine, a similar effect of cysteine addition was observed. We routinely checked to see if the SNAP addition, either in the presence or absence of cysteine, influenced the basal levels of ERK activity and found no change whatsoever in the control groups with or without BSO pretreatments (two-way analysis of variance).
NAC and SNAP treatment on ERK activation using Ang II, PDGF-BB, or PMA as the agonist. The most striking finding with all agonists used was that in combination, SNAP and NAC produced greater inhibition than when either drug was added alone.

Direct Measurement of ERK Phosphorylation—Since both a radioassay and an immunoassay have been used in experiments described above to document the effects of NAC and NO either alone or in combination, and some quantitative discrepancies sometimes were found, we performed an additional assay for ERK activation, which directly measures the phosphorylated ERK44 and ERK42 in a whole cell extract. Fig. 7 shows representative results and densitometric analysis from such assays using either SNAP or NAC, alone or in combination, prior to the Ang II addition. Consistent with the other assays, there was a greater inhibition of activity when both agents were added together. In this procedure, we found it necessary to obtain concentrated extracts of the cells prior to SDS-PAGE in order to have enough activated enzyme to detect by Western blot analysis.

Measurement of MEK1 Activity—To obtain insight into the possible upstream sites involved in the above effects of NAC and NO, we measured the effects of these agents on the activation of MEK1, an upstream kinase of ERKs, by Ang II. Fig. 8 shows that MEK1 activation by Ang II is marked and that pretreatment with either NAC or SNAP caused significant inhibition. However, combined treatment with both NAC and SNAP inhibited MEK1 activation by Ang II completely. In an additional set of experiments, we directly added SNAP to the assay tubes containing the immunoprecipitated, activated MEK1. There was no effect on MEK1 activity even when SNAP was added at a final concentration of 100 μM, suggesting that there was no in vitro nitrosation and subsequent inactivation of MEK1.
Cells were preincubated with 50 \( \mu \)M SNAP/cysteine for 10 min, 8 mM NAC for 8 h, or the combination and then incubated with 0.1 \( \mu \)M Ang II for 5 min. MEK1 activity was determined by using a recombinant GST-[K71A]Erk44 as a substrate in an radioimmunoassay as described under “Experimental Procedures.” The upper part is a representative autoradiograph of Ang II-induced MEK1 activation. pErk44, phosphorylated Erk44. The lower part shows the densitometric result, expressed as the mean ± S.E. of three separate experiments.

**DISCUSSION**

This study shows that NO inhibited the activation of ERKs by Ang II in rat cardiac fibroblasts, providing a molecular model for the known counterregulatory roles that Ang II and NO have in diverse tissues. The inhibitory effect of NO was enhanced when cells were pretreated with NAC, which by itself also inhibited the response, albeit to a lesser extent than when combined with NO treatment. The combination of NAC pretreatment and the addition of SNAP almost completely inhibited ERK activation by pathways both dependent on and independent of protein kinase C and also inhibited ERK activation initiated by PDGF and PMA, suggesting the effect was not localized to the hormone receptor alone.

We characterized the response to Ang II using agents that implicated the AT1 receptor (losartan), tyrosine kinase activity (genistein), Ras activation (farnesyltransferase inhibitor-3), and the requirement for MEK1 activation (PD98059). The activation of ERKs through such a signaling pathway has been implicated in other studies using cardiac fibroblasts and is consistent with recent studies showing that divergent pathways were involved in Ang II action, which conventionally had been thought to work solely through G protein-mediated activation of phospholipase C (4). The absence of a role for protein kinase C in our study is in agreement with recent findings showing that protein kinase C was not involved in Ang II activation of ERKs using cultured neonatal cardiac fibroblasts and vascular smooth muscle cells (26, 27), but differs from other studies where protein kinase C was involved in Ang II action both in cardiac myocytes and aortic smooth muscle cells (28, 29). We inactivated protein kinase C using both down-regulation by PMA pretreatment for 24 h and by using specific inhibitors (GF109203X and Go6983) that only required brief pretreatment. Since it is known that many isoforms of protein kinase C exist, some being independent of PMA down-regulation, it is difficult to absolutely rule out any involvement of protein kinase C, although the inhibitors we used are thought to also act on the PMA-independent forms of protein kinase C (30). A recent study (31) has shown that Ang II activates ERKs in vascular smooth muscle cells through a mechanism involving protein kinase C-\( \gamma \). This kinase is calcium-independent and not activated by diacylglycerol or phorbol esters.

Studies with the agent that inhibited farnesyltransferase implicated Ras activation in the signaling pathway. Ras is known to be involved in mediating the effects of Ang II in several cell types, although a recent study has reported that Ras was not activated by Ang II in cultured cardiac myocytes (28). Of particular interest to our studies is the report showing that Ras can be directly nitrosated and activated by NO (19) and studies showing that basal activity of ERKs and other MAP kinases were increased following the addition of NO (32). Those studies, conducted in the Jurkat human T-cell line, clearly differ from our own, since we repeatedly found no effect of SNAP addition on basal activity, either under our standard assay conditions or if assays were performed between 30 s and 30 min following the SNAP addition (data not shown).

Cysteine increased the ability of the NO donor SNAP to inhibit ERK activity. Cysteine was shown to enhance the effect of the S-nitrosothiol SNAP, which commonly is used as an NO donor in *in vitro* experiments with intact cultured cells. It was suggested (33) that cysteine could act by promoting transnitrosation from SNAP to S-nitrosocysteine, which would be more susceptible to intracellular decomposition, leading to more effective storage or transport of NO within the cell. Reduced GSH...
would presumably be the major intracellular mediator of NO storage and transport, implicating a transnitrosation from the original NO donor (SNAP) to a labile intermediate carrier S-nitrosocysteine and ultimately to intracellular GSH.

The reported effects of NAC pretreatment on several cell types include increased levels of reduced glutathione (34) and action as a reducing agent (35), a potential contributory factor in the intracellular storage and transport of NO (36), and a free radical scavenger (37). NAC has been shown to both increase survival and inhibit the proliferation of PC12 cells by mechanisms that are independent of intracellular glutathione levels and do not appear to be dependent on its antioxidant/radical scavenging properties (15, 38). In one study (38), the effect of NAC was suggested to be dependent on its ability to act as a reducing agent or on the transcription activation of as yet unidentified genes. Our findings with NAC required prolonged pretreatment (about 8 h) of the cardiac fibroblasts to partially inhibit the acute activation of ERKs in response to Ang II or to further enhance the inhibitory effect of SNAP. We also found that glutathione monoethylster, an agent that also increases intracellular level of glutathione, does not share with NAC the capability to inhibit Ang II-induced ERK activation. Although we have shown that changes in the absolute amount of glutathione do not account solely for the inhibitory effects of NAC on the activation of ERKs, it is possible that changes in the concentration of reduced glutathione combined with an increased ratio of reduced/oxidized glutathione (GSH/GSSG) might account for the effect of NAC. It has been proposed that the GSH/GSSG ratio, by controlling the reduction state of critical sulfhydryl groups, may reflect the redox state of the cell and regulate enzyme activity (39). Recently, the effect of NAC on cell redox environment has been studied in endothelial cells (40), where NAC caused a dramatic increase in GSH/GSSG ratio from 200 to more than 400, which has been suggested as one possible mechanism through which NAC exerts its inhibitory effect.

A role for reactive oxygen species influencing the MAP kinase pathway has been suggested, with the data available indicating a link between NO and the superoxide anion (41). Ang II, by virtue of activating the NADPH oxidase system, produces the superoxide anion, which may not be rapid at physiological conditions. Nonetheless, the inhibition of ERK activation by Ang II, although the reaction may not be rapid at physiological conditions, has been presented (19), where NO binds to a cysteine residue on the surface of the activated Ras molecule, leading to activation of the downstream MAP kinase cascade. This apparently does not happen in our system, since no activation of ERKs in the basal state was observed when SNAP was added to the cardiac fibroblasts. However, NO could react with superoxide anion, giving rise to the formation of peroxynitrite, a reactive substance that could lead to modification of cellular thiol (17).

We found that MEK1 was activated by Ang II and inhibited by both NAC and SNAP, suggesting that it could be a potential site of action for these drugs, although any site upstream of MEK1 also could be implicated. Evidence suggesting that MEK1 is not the sole site of action for these drugs comes from our observations that both JNKs and p38 MAPK activation also were inhibited by NAC and SNAP, and these pathways are generally believed to be independent of MEK1.

In our studies, NAC facilitated the effects of NO on the inhibition of an essential signaling system. This is a novel finding that contrasts with recent studies in other cell types where NAC pretreatment reduced heme oxygenase activity induced by NO (44) and in another study where the inhibitory effect of NO on the respiration of digitonin-treated ascites hepatoma cells was suppressed by NAC (45). The relationship between NO and the superoxide anion has been intensely studied in recent years, and an emerging hypothesis is that such interactions have an important physiological role in modulating the biological activities of both NO and the superoxide anion (46). It is plausible that the Ang II signaling pathway consists of redox-sensitive steps and that NAC pretreatment could modulate the redox state of the cell in an as yet undefined manner that would lead to NO being either stabilized or transported intracellularly in a more effective manner so that it could either interact more readily with superoxide anion or could act directly on redox-sensitive target proteins by nitrosylation reactions.

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Nitric Oxide and N-Acetylcysteine Inhibit the Activation of Mitogen-activated Protein Kinases by Angiotensin II in Rat Cardiac Fibroblasts
Deming Wang, Xin Yu and Peter Brecher

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Page 33031, Fig. 5A: The plus (+) designation for AII in the second bar from the left is incorrect and should be a minus sign, so that the bar represents an experiment lacking AII(−), containing Cys(+), and lacking SNAP(−). A corrected version of Fig. 5 is shown below:

Hypoxia-inducible factor 1α (HIF-1α) is a non-heme iron protein. Implications for oxygen sensing.

V. Srinivas, X. Zhu, Susana Salceda, R. Nakamura, and Jaime Caro

We have been unable to reproduce the studies on the iron content of the recombinantly expressed HIF-1α protein, as represented in Table I of the above cited article. It appears that the iron was due to a contaminated reagent. Consequently, we wish to retract those data. The results concerning the effect of CO and heme synthesis inhibitors on the hypoxia response of B-1 cells are not in doubt. We apologize for the inconvenience caused by our mistake.