Distinct Effects of N-Acetylcysteine and Nitric Oxide on Angiotensin II-induced Epidermal Growth Factor Receptor Phosphorylation and Intracellular Ca\textsuperscript{2+} Levels*

Deming Wang, Xin Yu, Richard A. Cohen, and Peter Brecher‡

From the Department of Biochemistry and Medicine and Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Massachusetts 02118

These studies describe inhibitory effects of N-acetylcysteine on several biochemical events associated with the activation of extracellular signal-regulated kinases (ERK) by angiotensin II in the cardiac fibroblast and compare these effects with those of the nitric oxide donor, S-nitroso-N-acetylpenicillamine, an agent we showed previously to inhibit angiotensin II-induced ERK activation and the concomitant phosphorylation of proline-rich tyrosine kinase 2 (Wang, D., Yu, X., and Brecher, P. (1999) J. Biol. Chem. 274, 24342-24348). The transactivation of the epidermal growth factor receptor by angiotensin II, a process required for the activation of ERK, was inhibited by N-acetylcysteine but not by nitric oxide. The transactivation of the epidermal growth factor receptor by angiotensin II was shown to be independent of intracellular calcium increases. Nitric oxide, but not N-acetylcysteine, inhibited the angiotensin II-induced increase in intracellular Ca\textsuperscript{2+}. Neither nitric oxide nor N-acetylcysteine inhibited either phospholipase C activation or inositol triphosphate generation in response to angiotensin II. N-Acetylcysteine did inhibit the phosphorylation of the calcium sensitive tyrosine kinases PYK2 and Src, effects that also occurred using nitric oxide. These studies describe a novel effect of N-acetylcysteine on cross-talk between a G protein-linked receptor and a tyrosine kinase receptor and offer additional molecular insight to explain how N-acetylcysteine and nitric oxide act at different sites and might have an additive effect on specific hormonal responses.

The diverse response of target cells to angiotensin II (Ang II)\textsuperscript{1} with respect to activation of signaling pathways has been well documented. Most studies have focused on either the vascular smooth muscle cell or the cardiac fibroblast to delineate the intracellular steps involved in the activation of the MAP kinase system, a characteristic response now associated with hypertrophy, proliferation, or apoptosis (1–3). This kinase cascade has been shown to be under complex regulation, and its activation by Ang II has been used as a model for the study of how G protein-linked receptors can influence systems normally activated by tyrosine kinase-linked receptors (4–6). One suggested mechanism for this response was proposed to be the transactivation of the EGF receptor by Ang II and the requirement for that receptor tyrosine kinase in the ultimate activation of MAP kinases (7, 8).

In a preceding study (9), we have characterized the acute activation of MAP kinases in quiescent cardiac fibroblasts by Ang II, and shown that nitric oxide (NO), a substance known to functionally oppose the physiological effects of Ang II in several organ systems, including the heart (10), can attenuate the activation of ERK by Ang II. This effect of NO on ERK activation also occurred in the same cell type when either of the other major MAP kinases (JNK or p38) were activated by Ang II, suggesting regulation at a site, or sites, upstream of Ras or proteins within the Ras superfamily (9). A subsequent study (11) showed that NO inhibits the activation by Ang II of the proline-rich tyrosine kinase 2 (PYK2), a nonreceptor tyrosine kinase that has been implicated in signaling cascades involving both receptor tyrosine kinases and G protein-linked receptors (12). Interestingly, NO had no effect on either the transactivation of the EGF receptor by Ang II, nor did NO influence the autophosphorylation of the EGF receptor. Thus these studies suggested that NO affects PYK2 and subsequently ERK activation, although the specific mechanism by which PYK2 phosphorylation was inhibited by NO remains to be defined.

We also showed previously (9) that N-acetylcysteine (NAC), a reducing agent known to alter the redox state of the cell (13), also inhibited ERK activation by Ang II and other agonists, and when cells were pretreated with both NO and NAC, inhibition of ERK was greater than when either agent was added alone. Superoxide anion or other free radicals have also been implicated as potential mediators of signaling systems initiated by Ang II (14); thus the possibility that NAC could affect ERK activation by altering the redox state in a manner that would selectively change steady state levels of free radicals offered a possible explanation for the effect. In the current manuscript, we have evaluated the role of NAC on the transactivation of the EGF receptor by Ang II and have shown that it acts at sites distinct from that of NO. We also have further defined the site of action for NO in the signaling cascade by assessing phospholipase C activation and inositol triphosphate generation, measuring changes in intracellular Ca\textsuperscript{2+} and for the activation of both PYK2 and another Ca\textsuperscript{2+}-dependent, nonreceptor tyrosine kinase, Src. Our findings offer a possible explanation for how both NAC and NO, acting in a concerted manner but at different sites, might have an additive effect on specific hormonal responses.

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‡ To whom correspondence should be addressed; Boston University School of Medicine, 715 Albany St., Boston, MA 02118. Tel.: 617-638-4022; Fax: 617-638-4066; E-mail: pbrecher@bu.edu.

1 The abbreviations used are: Ang II, angiotensin II; MAP kinases, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases; EGF, epidermal growth factor; NAC, N-acetylcysteine; PYK2, proline-rich tyrosine kinase 2; SNAP, S-nitroso-N-acetylpenicillamine; BAPTA, 2,4-bis(aminophenoxyl)-ethane-N, N, N', N'-tetraacetic acid; BSO, l-buthionine-(S,R)-sulfoximine; IP\textsubscript{3}, inositol triphosphate; PLC, phospholipase C; PDGF, platelet-derived growth factor.
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EXPERIMENTAL PROCEDURES

Reagents—Dulbecco’s modified Eagle’s medium/Ham’s F-12, Ca\textsuperscript{2+}+-free Dulbecco’s modified Eagle’s medium, fetal calf serum, and tissue culture reagents were from Life Technologies, Inc. S-Nitroso-n-acetylpenicillamine (SNAP) and ionophore A23187 were from Alexis Corp (San Diego, CA); tyrophostin AG1476 and BAPTA-acetoxyethyl ester were from Calbiochem (La Jolla, CA). Fura-2-acetoxyethyl methyl ester was purchased from Molecular Probes (Eugene, OR). Platelet-derived growth factor-BB (PDGF-BB) and epidermal growth factor (EGF) were from Sigma. The ECL detection system were from Amersham Pharmacia Biotech; Ang II, \textit{dL}-cysteine, N-acetylcysteine, \textit{iL}-buthionine-(S,R)-sulfoximine (BSO), and all other chemicals were purchased from Sigma.

Antibodies—Anti-Grb2 antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-Shc, anti-phosphotyrosine (PY20), and anti-PYK2 antibodies were obtained from Transduction Laboratories (Lexington, KY); anti-EGF receptor antibody was from Santa Cruz Biotechnology; and anti-phospho ERK antibody was purchased from Cell Signaling Technology. Anti-Grb2 antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-Shc, anti-phosphotyrosine (PY20), and anti-PYK2 antibodies were obtained from Transduction Laboratories (Lexington, KY); anti-EGF receptor antibody was from Santa Cruz Biotechnology; and anti-phospho ERK antibody was purchased from Cell Signaling Technology. Anti-Grb2 antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-Shc, anti-phosphotyrosine (PY20), and anti-PYK2 antibodies were obtained from Transduction Laboratories (Lexington, KY); anti-EGF receptor antibody was from Santa Cruz Biotechnology; and anti-phospho ERK antibody was purchased from Cell Signaling Technology.

Cell Culture—Rat cardiac fibroblasts were obtained and prepared for experiments exactly as described previously by us (15). Fresh medium without fetal calf serum was routinely added 3–5 h before the experiment unless designated otherwise.

Cell Treatments—Ang II was routinely added to the cells at a final concentration of 0.1 μM. NAC (10 mM) was routinely added 12 h prior to treatment of cells with Ang II or other agonists. 1 h prior to addition of agonist, the medium was changed to fresh Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium lacking NAC, and cells were equilibrated with that medium prior to Ang II addition. SNAP was routinely added with equimolar amounts of \textit{dL}-cysteine 15 min prior to addition of agonists. \textit{dl}-Cysteine, when added without SNAP, had no effect on any of the parameters measured. All experiments shown are representative of multiple experiments using separate cell preparations.

Immunoprecipitation and Immunoblotting—Following treatment of the cells with hormones or drugs, the cells were washed twice with ice-cold phosphate-buffered saline, and then cell lysis was performed. ERK phosphorylation was measured directly by immunoblotting of the cell extract with the anti-phospho ERK antibody. Immunoprecipitation of phosphotyrosine-containing proteins from the cell lysates and subsequent SDS-polyacrylamide gel electrophoresis and immunoblotting procedures were exactly as described previously (11). Images were obtained using a PDI scanner (model 420oe).

Measurement of Inositol 1,4,5-Trisphosphate—Following exposure to agonists, the incubation was terminated by the addition of 1.5 ml of ice-cold 1% trichloroacetic acid. After incubation on ice for 10 min, the cells were harvested by scraping and transferred to a polystyrene tube. The cell extract was sonicated briefly on ice and then centrifuged for 10 min at 5,000 × g. The supernatant was removed and warmed to room temperature for 15 min. IP3 was extracted with a solution of trichloroacetic acid-triocytlamine (3:1) and measured using a radioisotope assay kit from NEN Life Science Products following the instructions supplied by the manufacturer. Results were calculated from a standard curve prepared by using the purified IP3 included in the kit. Data are normalized to total cell number, and each experiment typically included duplicate or triplicate determinations for each parameter measured.

Measurement of Intracellular Ca\textsuperscript{2+}—Intracellular free Ca\textsuperscript{2+} levels were determined with the Ca\textsuperscript{2+}+-dye fura-2 essentially as described (16). Briefly, cells were grown to subconfluency on coverslips in Dulbecco’s modified Eagle’s medium/Ham’s F-12 with 10% fetal calf serum and 5 mM NAC were incubated with the designated concentration of EGF for 3 min and then phosphorlated EGFR receptor measured. The bar graph summarizes three separate experiments performed as described for C. D, cells that were either untreated with NAC or pretreated for 12 h with 10 mM NAC were incubated with 20 ng/ml EGF or 0.1 μM Ang II for 3 min. The cell extracts were then immunoprecipitated with antibody against Shc (α Shc) and subsequently immunoblotting procedures were performed with antibody against Grb2 (α Grb2). The bar graph summarizes three separate experiments performed as described above with the asterisk indicating a significant difference between the Ang II-treated cells pretreated with or without NAC.

Fig. 1. Effects of NAC on the Ang II-induced EGFR receptor phosphorylation. For measurement of EGFR receptor phosphorylation, cell extracts were immunoprecipitated (IP) with antibody against the EGFR receptor (α EGFR) and then immunoblotting (IB) was used with a phosphotyrosine antibody (α pTyr) as described under “Experimental Procedures.”

Total EGFR receptor was measured using the anti-EGFR receptor antibody for both immunoprecipitation and immunoblotting. A, cells were treated with Ang II (0.1 μM) for 3 min following either preincubation for 15 min with 100 μM SNAP or preincubation for 12 h with 10 mM NAC followed by a change of medium to one lacking NAC for 1 h prior to adding the Ang II. B, cells were pretreated for 12 h with the designated concentration of NAC and then treated with Ang II as described above. The bar graph is a summary of three separate experiments performed as described for B. The asterisk indicates a significant difference (p < 0.05) from the Ang II-treated cells not given NAC. C, cells that were either untreated with NAC or pretreated for 12 h with 10 mM NAC were incubated with the designated concentration of EGF for 3 min and then phosphorlated EGFR receptor measured. The bar graph summarizes three separate experiments performed as described for C. D, cells that were either untreated with NAC or pretreated for 12 h with 10 mM NAC were incubated with 20 ng/ml EGF or 0.1 μM Ang II for 3 min. The cell extracts were then immunoprecipitated with antibody against Shc (α Shc) and subsequently immunoblotting procedures were performed with antibody against Grb2 (α Grb2). The bar graph summarizes three separate experiments performed as described above with the asterisk indicating a significant difference between the Ang II-treated cells pretreated with or without NAC.
Subsequently, the cells were gently washed three times and then suspended in physiological salt solution. The glass coverslip was placed in a cuvette containing the same buffer with continuous stirring. Intracellular Ca^{2+} levels were measured in a Hitachi F-4500 spectrophotometer and calculated from the ratio of the 340/380-nm fluorescence values as described (16).

**Statistics**—Data are presented as the 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

**NAC Inhibits the Ang II-induced EGF Receptor Transactivation but Does Not Influence EGF-induced EGF Receptor Autophosphorylation**—The tyrosine phosphorylation of the EGF receptor following addition of Ang II is shown in Fig. 1A. As we showed previously (11) this phenomenon, referred to as transactivation, was not influenced by the nitric oxide donor SNAP using experimental conditions that attenuate Ang II activation of MAP kinases. However, preincubation with 10 mM NAC, which by itself had no effect on receptor phosphorylation, markedly suppressed the Ang II-activated EGF receptor phosphorylation. The ability of NAC to inhibit EGF receptor phosphorylation by Ang II was dose-dependent with respect to NAC, with almost complete inhibition occurring between 5 and 10 mM NAC (Fig. 1B). This was confirmed statistically in triplicate experiments where significant differences between NAC-treated or untreated cells were found at concentrations of 5 and 10 mM NAC. In all cases, the total content of EGFR receptor was also measured and remained unchanged. In contrast to the inhibitory effect of NAC on Ang II-induced activation of the EGF receptor, NAC had no inhibitory effect on EGF-induced activation of the same receptor over a broad range of ligand concentrations (Fig. 1C). As we had indicated previously (11), the extent of EGFR receptor phosphorylation in response to about 1 ng/ml EGF was equivalent to that found with the maximal transactivation in response to Ang II. Fig. 1D shows that the formation of the Shc-Grb2 complex, an event occurring downstream of the activated EGF receptor, was initiated by the addition of either Ang II or EGF, but NAC pretreatment only inhibited the Ang II-induced effect and was without effect on the EGF-induced formation of the Shc-Grb2 complex. Statistical evaluation of triplicate experiments confirmed the selective inhibition by NAC of the effect of Ang II on the Shc-Grb2 complex. Thus, NAC, but not SNAP, inhibited Ang II-induced transactivation of the EGF receptor and the subsequent association of Grb2 and Shc. The lack of effect of SNAP was reported by us previously (11).

**Neither NAC nor SNAP Has an Effect on the Activation of Phospholipase C or Inositol Triphosphate Generation**—Another protein associated with tyrosine kinase receptors following activation is phospholipase C-γ, which becomes phosphorylated when activated by certain agonists. In the cardiac fibroblast, PLC-γ was not significantly phosphorylated by either Ang II or EGF but was strongly phosphorylated by 20 ng/ml PDGF within 2 min (Fig. 2A). Ang II did not phosphorylate the PLC-γ isozyme throughout a 10-min time period, ruling out a delayed activation of PLC-γ by Ang II that had been reported previously in vascular smooth muscle cells (17). Both SNAP and NAC were without effect on the activation of phospholipase C-γ by PDGF over a wide concentration range (Fig. 2B and C). Trplicate determinations of the experiments in Fig. 2 (B and C) showed no significant effect of either NAC or SNAP on PLC-γ phosphorylation by PDGF (Fig. 2D).

The inability of Ang II to activate phospholipase C-γ suggested that the β isozyme was activated in response to Ang II. To determine whether Ang II did indeed activate phospholipase C in the cardiac fibroblast, IP₃ levels were determined. Prelimi-
affected basal levels of IP₃, and addition of EGF (20 ng/ml) did not increase IP₃ over basal levels.

SNAP but Not NAC Inhibits the Increase in Intracellular Ca²⁺ Induced by Ang II—To determine whether NAC might influence changes in intracellular free Ca²⁺ induced by Ang II, we measured such changes by using the fluorescent indicator fura-2 (Fig. 3). The data show that Ang II clearly increased intracellular Ca²⁺ with a characteristic pattern (Fig. 3A). This increase was unaffected by NAC pretreatment (Fig. 3B) but clearly was significantly reduced with SNAP/cysteine pretreatment (Fig. 3C). Combined treatment with both SNAP and NAC (Fig. 3D) caused a slightly greater inhibition than that seen with SNAP addition alone. The addition of cysteine alone had no effect on Ang II-induced Ca²⁺ changes (Fig. 3E). Statistical analysis of four experiments performed separately showed that the Ang II-induced Ca²⁺ increase was significantly reduced by either SNAP alone or SNAP + NAC, whereas there was no statistical difference between Ang II treatment versus Ang II + NAC or Ang II + cysteine (Fig. 3F).

SNAP and NAC Inhibit Ca²⁺-dependent Activation of Both Src and PYK2—Our previous study showed that Ang II activated and SNAP inhibited the Ang II-induced phosphorylation of PYK2, a Ca²⁺-dependent, nonreceptor tyrosine kinase (11). To determine the effect of SNAP and NAC on another important signaling intermediate, we characterized the phosphorylation of Src in response to Ang II. Fig. 4A shows that Src is rapidly phosphorylated in response to 0.1 μM Ang II, and this occurred in a dose-dependent manner (Fig. 4B). The phosphorylation of Src by Ang II in the cardiac fibroblast was a Ca²⁺-dependent process that was progressively eliminated when cells were pretreated with 1–50 μM BAPTA (Fig. 4C), a characteristic shared with PYK2, as we described previously (11). A relatively high concentration of EGF (20 ng/ml) did not phosphorylate Src within a 30-min period, in contrast to the marked effect of Ang II (Fig. 4D). This relative difference between the response to Ang II and EGF also was reported with regard to PYK2, which was readily phosphorylated by Ang II but not by EGF (11).

The data in Fig. 5 characterize in more detail the response of the cells to Ang II with regard to PYK2 and Src. In Fig. 5A data are presented showing that a selective inhibitor of Src activation, PP2, blocks the Ang II-induced phosphorylation of Src in a dose-dependent manner, with almost complete inhibition observed at 10 μM PP2. That dose-dependent inhibition of Src by PP2 also was found when the phosphorylation of either PYK2 or ERK was determined. The effects of SNAP pretreatment on Src activation are shown in Fig. 5B where SNAP (1–500 μM) was added 15 min prior to Ang II addition, and phosphorylated
Src was measured 3 min following Ang II addition. Inhibition of Src phosphorylation by SNAP was dose-dependent, an effect comparable with the inhibition of Ang II-induced phosphorylation of PYK2 by SNAP (11). NAC inhibited Src phosphorylation by Ang II in a dose-dependent manner and also reduced PYK2 phosphorylation in a manner comparable with that observed for Src (Fig. 5C). The activation of Src and PYK2 by Ang II led to complex formation between Src and PYK2, and both NAC and SNAP pretreatment significantly attenuated the Src-PYK2 complex formation as indicated both in the representative immunoblot and the bar graph summarizing several separate experiments below (Fig. 5D).

NAC, but Not Intracellular Ca\(^{2+}\), Influences Ang II-induced EGF Receptor Phosphorylation—Fig. 6A shows the effect of BAPTA pretreatment on phosphorylated EGF receptor in response to Ang II and indicates the transactivation of the EGF receptor was not markedly influenced by BAPTA preincubation between 1–20 \(\mu\)M, whereas BAPTA had a clear inhibitory effect on the phosphorylation of Src using the identical cell extracts for analysis. The bar graph summarizes four separate experiments showing the selective effect of BAPTA pretreatment on both the EGF receptor and Src phosphorylation in a manner comparable with that observed for Src (Fig. 5C). The activation of Src and PYK2 by Ang II led to complex formation between Src and PYK2, and both NAC and SNAP pretreatment significantly attenuated the Src-PYK2 complex formation as indicated both in the representative immunoblot and the bar graph summarizing several separate experiments below (Fig. 5D).

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![Fig. 5. Effects of Src inhibition, NAC, or SNAP treatment on Ang II-induced phosphorylation of Src and PYK2.](image)

![Fig. 6. Increases in intracellular Ca\(^{2+}\) are not required for the Ang II-induced transactivation of the EGF receptor.](image)
tion was observed in the same extracts (Fig. 6B). Thus, studies to this point show that 1) both Src and PYK2 phosphorylation are inhibited by either SNAP or NAC pretreatment; 2) NAC but not SNAP inhibited EGF receptor transactivation; 3) only SNAP lowered Ang II-induced increases in intracellular Ca\(^{2+}\); and 4) the transactivation of the EGF receptor appeared to be independent of intracellular Ca\(^{2+}\).

The Effects of NAC May Be Explained by Its Action as a Reducing Agent and the Potential to Reduce Oxidative Stress—It was necessary to pretreat the cells with NAC to show an inhibition of Ang II-induced effects such as EGF receptor phosphorylation (Fig. 7A). Because the pretreatment time was increased from 6 min to 12 h, there was a progressive decrease in the phosphorylation of the EGF receptor by Ang II. In these experiments the medium was routinely changed, removing NAC 1 h prior to treating the cells acutely with agonists, suggesting the effect of NAC was occurring within the cell. In separate experiments, NAC was maintained in the medium during the time Ang II was added, and the results were comparable with those shown in Fig. 7A (data not shown). To gain additional insight into the mechanism by which NAC was having an effect on signaling in the cardiac fibroblast, we focused on its inhibitory effect on EGF receptor phosphorylation. Fig. 7B shows that NAC inhibited transactivation of the EGF receptor by a mechanism independent of changes in intracellular glutathione. In a previous study we established that pretreatment with NAC increased glutathione levels, whereas BSO, an inhibitor of glutathione biosynthesis, produced a marked reduction in intracellular total glutathione when added alone or in combination with NAC (9). NAC pretreatment inhibited EGF receptor phosphorylation in the absence or presence of BSO, whereas BSO treatment alone had no effect on Ang II-induced EGF receptor phosphorylation. Fig. 7C summarizes three separate experiments where Ang II-induced EGF receptor phosphorylation was determined following pretreatment for 12 h with either 1 mM ascorbate, 10 mM NAC, 10 mM DL-cysteine, or 10 mM D-cysteine. The inhibitory effect documented above for NAC also was found for either the DL or D-isomers of cysteine, and in separate experiments (not shown) a similar inhibition of transactivation of the EGF receptor was found when only L-cysteine was added. With any form of cysteine, it was necessary to pretreat the cells for more than 6 h, in contrast to the more rapid effect of NAC. Fig. 7D shows that the effect of NAC could be mimicked by pretreatment with dithiothreitol, a thiol-containing reducing agent, whereas the antioxidant ascorbic acid, which lacks a thiol moiety, had no effect on EGF receptor phosphorylation. Fig. 7E shows that the inability of ascorbate to influence EGF receptor phosphorylation was consistent even when cells were pretreated for periods between 1 and 16 h.

Hydrogen peroxide addition to cells often is used as a model to induce oxidative stress and hydrogen peroxide addition to the cardiac fibroblasts did increase EGF receptor phosphorylation, PYK2, or Src phosphorylation rapidly. However, the time course of the increase in EGF receptor phosphorylation was not as rapid as that for either Src, PYK2, or EGF receptor phosphorylation (Fig. 8A). Src, PYK2, or EGF receptor phosphorylation induced by hydrogen peroxide addition were reduced in a dose-dependent manner by NAC pretreatment (Fig. 8B). The peroxide-induced EGF receptor phosphorylation was inhibited by NAC pretreatment, with more effective inhibition occurring when pretreatment times exceeded 3 h (Fig. 8C), a temporal relationship somewhat longer than that found for the effect of NAC pretreatment on Ang II-induced transactivation of the EGF receptor as was shown in Fig. 7A.
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FIG. 8. NAC inhibits hydrogen peroxide-induced changes in Src and PYK2 phosphorylation and EGF receptor phosphorylation. A, cells were pretreated for the designated time with 1 mM hydrogen peroxide (H2O2), and then cell extracts were analyzed for both the phosphorylated forms of Src and PYK2 and for EGF receptor phosphorylation. B, cells were pretreated for 12 h with 10 mM NAC, the medium changed for 1 h to equilibrate the cells in the absence of NAC, and then H2O2 added for 10 min prior to measuring both the phosphorylated forms of Src and PYK2. To determine changes in the EGF receptor, peroxide treatment was for 30 min. C, cells were pretreated for the designated time with 10 mM NAC and then treated for 30 min with 1 mM H2O2 following a change in the medium to remove excess NAC. Cell extracts were analyzed for EGF receptor phosphorylation and for total amounts of EGF receptor. IB, immunoblotting; IP, immunoprecipitation.

DISCUSSION

These studies document several unique effects of NAC on the signaling pathways initiated by Ang II in cardiac fibroblasts and contrast its effects with those of NO. The biochemical steps examined include the transactivation of the EGF receptor by Ang II, the activation of phospholipase C and subsequent increase in IP3 and intracellular Ca2+, and the Ca2+-dependent phosphorylation of PYK2 and Src. All the above changes are part of the multiple pathways leading to ERK activation by Ang II, and the distinct effects of NAC and NO on these cellular reactions provide an explanation for the additive effects of both NAC and NO on ERK activation we reported previously (9).

We have reported two unique findings related to the Ang II-induced phosphorylation of the EGF receptor: that the effect is apparently independent of acute increases in intracellular Ca2+ and that NAC inhibits the response. With regard to the lack of a dependence on Ca2+, it should be noted that transactivation of the EGF receptor by Ang II has been described in both vascular smooth muscle cells and neonatal cardiac fibroblasts to be dependent on Ca2+ as indicated by its sensitivity to BAPTA treatment, A23187, or calmodulin sensitivity to calmodulin antagonists (7, 8). In contrast, the phosphorylation of the EGF receptor by Ang II in our experimental system was marginally affected by low concentrations (1 μM) of BAPTA and was unaffected by pretreatment with more conventional doses (10–20 μM) of BAPTA. The absence of an essential role for Ca2+ in the EGF receptor transactivation is further supported by the demonstration that the calcium ionophore A23187 failed to induce the phosphorylation of EGF receptor for 1–30 min, whereas it clearly was effective in inducing other Ca2+-dependent processes, such as ERK phosphorylation, within this time frame.

Further evidence that the transactivation between Ang II and the EGF receptor was Ca2+-independent in our experimental model included the observation that the NO donor SNAP, which clearly reduced the acute increase in Ang II-induced intracellular Ca2+, did not affect EGF activation of ERK or transactivation of the EGF receptor by Ang II and the subsequent dependent downstream association of Shc and Grb2 (11). Consistent with our findings, Zwick et al. (18) recently reported that bradykinin-induced EGF receptor transactivation, a G protein-coupled receptor-mediated event, was mediated through a Ca2+/calmodulin-independent signaling pathway in PC12 cells, whereas Ca2+ was implicated when EGF transactivation was induced by potassium. In rat liver epithelial cells, the role of Ca2+ in the activation ERK by both Ang II and EGF was independent of both BAPTA treatment and protein kinase C inhibition (19). In our experimental model, we reported EGF activation of ERK to be independent of acute increases in intracellular Ca2+ and PKC inhibition, whereas Ang II activation of ERK, although independent of protein kinase C inhibition, was dependent upon intracellular Ca2+, whereas transactivation of the EGF receptor clearly was not (11). It may be that differences in cell type, cell phenotype, or the multiple systems that maintain basal or agonist stimulated levels of intracellular Ca2+ will vary among the protocols reported, thereby making Ca2+ sensitivity a variable, rather than an absolute, requirement for EGF receptor transactivation.

NAC inhibited the Ang II-induced EGF receptor phosphorylation, whereas the EGF-stimulated response was unaffected. The ability of NAC to inhibit the transactivation of the EGF receptor represents a unique effect of NAC and could provide insight into the mechanisms involved in G protein receptor-linked transactivation. It is known that the transactivation of the EGF receptor plays a key role in the G protein-coupled receptor-mediated activation of MAP kinases (4) and has been implicated for several hormones and in several cell types (7, 20, 21). We (11) and others (7, 8) have shown that blockade of EGF receptor phosphorylation by the specific tyrphostin AG 1478 also blocked Ang II activation of ERK, thus NAC presumably can influence an intracellular site or sites required for the transactivation process. The inhibition of EGF receptor transactivation by NAC was independent of absolute changes in glutathione based on the lack of effect of BSO and could be mimicked by dithiothreitol pretreatment but not by the antioxidant ascorbate. Those methods were essentially the same as we used previously to demonstrate inhibition of Ang II-mediated ERK activation by NAC (9), implicating the transactivation of the EGF receptor as one potential target site to explain the inhibition of ERK by NAC. Importantly, our protocol involves pretreatment of cells with NAC, followed by removal of exogenous NAC, thus the changes reported represent effects of NAC occurring within the cells.

NAC also inhibited the association of Shc and Grb2 with the EGF receptor, an event known to couple membrane receptors to downstream cascades. However, NAC did not affect the Ang II-induced increases in intracellular Ca2+, clearly distinguishing its effects from that of SNAP, which, although it lowered intracellular Ca2+, did not influence the association of Shc and Grb2. This provides further support for the independent activation of Ang II of either Src or PYK2, contrasted with the transactivation of the EGF receptor and the subsequent Shc-
Grb2 association. Interestingly, neither SNAP nor NAC affected the G protein-linked events leading to phospholipase C activation and increased levels of IP3. In other studies it was suggested that Ang II affected vascular smooth muscle cells through an association of the Ang II receptor with PLC-γ (22) and that both PLC-β1 and PLC-γ were activated by Ang II in vascular smooth muscle cells (17), and EGF was shown to activate PLC-γ in NR6 mouse fibroblasts (23).

Our data implicate Ca2+ regulation as a key site for NO action. NO has been studied extensively with regard to its effects on calcium homeostasis, and the topic has been reviewed recently (24). SNAP (e.g. NO) may attenuate the activation of the nonreceptor tyrosine kinases examined in the present study through its suppressive effect on Ang II-induced increases in intracellular Ca2+. Because there was no effect on IP3 levels, the possibility that NO may regulate either the intracellular response to IP3 or processes of Ca2+ influx and reuptake should be considered. In smooth muscle cells, NO was recently shown to influence the uptake of Ca2+ by its effect on sarcoplasmic reticulum ATPase (25). It is noted that concentrations of Ang II (10−8 M) that produce submaximal increases in IP3 levels at the time point we used (20 s) did produce a maximal response with regard to ERK activation (9). This could be due to the differential sensitivity of signaling cascade components upstream of ERK, combined with the known observations that ERK activation can be caused by diverse signaling pathways initiated by Ang II, including some that are less dependent upon changes in intracellular Ca2+.

The possibility that nonreceptor tyrosine kinases such as PYK2 and Src are responsible for ERK activation has been proposed (12). Paradoxically, both NAC and NO prevented the Ang II-induced phosphorylation of both PYK2 and Src, tyrosine kinases that were sensitive to BAPTA pretreatment in this study. It is not yet clear how NAC pretreatment might influence the EGF receptor by Ang II or other agonists occurs remains unresolved, but our finding with NAC may provide an approach to elucidating these mechanisms and influencing them pharmacologically.

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