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Original Research Communication

Effects of Thiol Antioxidants on Hepatocyte Growth Factor Signaling in Cardiac Myocytes

KAZUMI KITTA,[1] REGINA M. DAY,[2] JANE REMEIKA,[1] JEFFREY B. BLUMBERG,[1] and YUICHIRO J. SUZUKI[1]

ABSTRACT

We describe here novel antioxidant-sensitive events in which activation kinetics are delayed, leading to inhibition of cell signaling. Hepatocyte growth factor (HGF) transiently phosphorylated p44/42 mitogen-activated protein kinase (MAPK) with a peak at 3–5 min in HL-1 adult cardiac myocytes. Pretreatment of cells with thiol antioxidants, *N*-acetylcysteine or α-lipoic acid attenuated MAPK phosphorylation induced by a 3-min incubation with HGF. However, kinetic analysis revealed that the apparent inhibition of HGF signaling was due to a delay in the activation because HGF phosphorylated MAPK with a peak at 5–7 min in cells treated with thiol antioxidants. This 2-min delay in HGF activation of MAPK resulted in >5-min delay in phosphorylation of MAPK targets such as p90RSK and GATA-4. Hydrogen peroxide did not mimic HGF signaling, and HGF did not induce reactive oxygen species production. Thus, in cardiac myocytes, thiol antioxidants delay HGF-mediated MAPK activation and suppress subsequent signaling events via reactive oxygen species-independent mechanism. Antioxid. Redox Signal. 3, 911–918.

INTRODUCTION

THE ACTIVITY of hepatocyte growth factor (HGF) was first demonstrated in the sera of normal and partially hepatectomized rats (10, 19), and was found to be a potent mitogen of hepatocytes (11). Earlier findings that HGF expression is increased after liver injury induced by CCl_4 (7) suggest that HGF may be an oxidative stress-inducible factor. Similarly, in a rat model of myocardial ischemia and reperfusion, HGF expression was enhanced (12). Ueda *et al*. (21) demonstrated that gene transfection of HGF attenuated reperfusion injury in the rat heart. In humans, serum HGF levels are elevated after acute myocardial infarction (9, 16).

Although the biological actions of HGF on cardiac myocytes appear to have physiological and clinical importance, HGF signal transduction mechanisms in cardiac myocytes have not been documented. We recently found that HGF can phosphorylate p44/42 mitogen-activated protein kinase (MAPK) and GATA-4 in cardiac myocytes (8). In Sarcoma 180 and Meth A cells, Arakaki *et al*. (2) reported that reactive oxygen species (ROS) are involved in the mechanism of HGF signal transduction. Therefore, we tested the hypothesis that HGF may activate

[1]Antioxidants Research Laboratory and Cell and Molecular Nutrition Program, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, and Tufts University School of Nutrition Science and Policy, Boston, MA 02111.
[2]Pulmonary and Critical Care Division, New England Medical Center, and Tufts University School of Medicine, Boston, MA 02111.

MAPK signaling via ROS generation. Our results showed that pretreatment of HL-1 cells with thiol antioxidants, *N*-acetylcysteine (NAC) or α-lipoic acid (αLA), blocked HGF signaling. However, this apparent inhibition of signaling events was due to a delay in HGF-induced MAPK kinase (MEK)–MAPK activation exerted by thiol antioxidants through an ROS-independent mechanism.

MATERIALS AND METHODS

Cell culture

HL-1 adult mouse atrial myocytes that retain differentiated phenotype and contractile activity (4) were obtained from Dr. William Claycomb (Louisiana State University, New Orleans, LA, U.S.A.). Cells were maintained in EXCELL 320 medium (JRH Biosciences, Lenexa, KS, U.S.A.) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, U.S.A.), 10 μg/ml insulin (Life Technologies), 20 μg/ml endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY, U.S.A.), 1 μM retinoic acid (Sigma Chemical Company, St. Louis, MO, U.S.A.), 100 μM norepinephrine (Sigma), 1% nonessential amino acid supplements (Life Technologies), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Sigma) in plastic dishes, coated with 12.5 μg/ml fibronectin and 0.02% gelatin, in a 5% CO_2 atmosphere at 37°C. Cells were replenished with fresh media every 2–3 days. For treatment, cells were starved in EXCELL 320 medium supplemented only with nonessential amino acid, penicillin, streptomycin, and amphotericin B for 18 h and treated with human recombinant HGF or H_2O_2. In some experiments, cells were pretreated with NAC, *R*,*S*-αLA, L-ascorbic acid, or angiotensin II (Ang II). These reagents were purchased from Sigma and were dissolved in culture media without the use of organic solvents as vehicles.

Western blot analysis to determine MEK, MAPK, and p90 kDa ribosomal S6 kinase (p90RSK) phosphorylation

To prepare lysates, cells were washed in phosphate-buffered saline and solubilized with 50 m*M* HEPES solution (pH 7.4) containing 1% (vol/vol) Triton X-100, 4 m*M* EDTA, 1 m*M* sodium fluoride, 0.1 m*M* sodium orthovanadate, 1 m*M* tetrasodium pyrophosphate, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Following centrifugation in a microfuge, protein concentrations in the supernatant were determined (3). Equal protein levels (10 μg) of cell lysates were electrophoresed through reducing (5% β-mercaptoethanol) sodium dodecyl sulfate (SDS) polyacrylamide gels (10%) and electroblotted onto nitrocellulose membranes. Completion of protein transfer from gels to the membranes was checked by staining the gels with Coomassie Blue R-250. The membranes were incubated with the rabbit polyclonal IgG for phospho-specific p44/42 MAPK (Thr^{202}/Tyr^{204}) (New England Biolabs, Beverly, MA, U.S.A.), phospho-specific MEK (MEK1/2) ($Ser^{217/221}$) (New England Biolabs), phospho-specific p90RSK (Ser^{381}) (New England Biolabs), p44/42 MAPK (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), or MEK1 (ZYMED Laboratories, South San Francisco, CA, U.S.A.). Levels of proteins and phosphoproteins were detected with horseradish peroxidase-linked secondary antibodies and ECL System (Amersham Life Science, Arlington Heights, IL, U.S.A.). Equal loading of the samples onto the gels and equal transfer of the proteins to the membranes were confirmed by staining the membranes with Coomassie Blue R-250.

Electrophoretic mobility shift assay (EMSA) to determine GATA-4 DNA binding activity

To prepare nuclear extracts, cells were washed in phosphate-buffered saline and incubated in 10 m*M* HEPES (pH 7.8), 10 m*M* KCl, 2 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.1 m*M* PMSF, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 m*M* sodium fluoride, 0.1 m*M* sodium orthovanadate, and 1 m*M* tetrasodium pyrophosphate for 15 min at 4°C. IGEPAL CA-630 (Sigma) was then added at a final concentration of 0.5% (vol/vol). Samples were mixed vigorously and centrifuged. Pelleted nuclei were resuspended in 50 m*M* HEPES (pH 7.8), 50 m*M* KCl, 300 m*M*

NaCl, 0.1 m*M* EDTA, 0.1 m*M* PMSF, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 m*M* sodium fluoride, 0.1 m*M* sodium orthovanadate, 1 m*M* tetrasodium pyrophosphate, and 1% (vol/vol) glycerol, then mixed for 20 min, and centrifuged for 5 min. The supernatants were harvested, and then stored at −80°C after protein concentrations were determined (3).

To perform EMSA, binding reaction mixtures containing 2 μg of protein of nuclear extract, 1 μg of poly(dI-dC)·poly(dI-dC), and ^{32}P-labeled double-stranded oligonucleotide probe containing consensus GATA sequence (5′-CAC TTG ATA ACA GAA AGT GAT AAC TCT-3′) in 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* dithiothreitol, 10% (vol/vol) glycerol, and 20 m*M* Tris-HCl (pH 7.5) were incubated for 20 min at 25°C. Electrophoresis of samples through a native 6% polyacrylamide gel was followed by autoradiography.

Western blot analysis to determine GATA-4 phosphorylation

SDS–polyacrylamide gel electrophoresis gels (10%) to separate phosphorylated from unphosphorylated GATA-4 contained an acrylamide/bis ratio of 30:0.165. For western blot analysis, gels were electroblotted onto polyvinylidene difluoride membranes, and the rabbit polyclonal IgG for GATA-4 (H-112) (Santa Cruz Biotechnology) was used at a concentration of 1 μg/ml. In these experiments, nuclear extracts isolated as described above were loaded onto a reducing SDS-polyacrylamide gel at a protein concentration of 20 μg.

Intracellular H_2O_2 measurement

Intracellular levels of H_2O_2 and other peroxides were detected by monitoring oxidation of 2′,7′-dichlorofluorescin to 2′,7′-dichlorofluorescein (DCF). Serum-starved HL-1 myocytes were washed twice with Hanks' balanced salt solution and treated with 10 μM 2′,7′-dichlorofluorescin diacetate for 30 min. Cells were then washed three times with Hanks' balanced salt solution and treated with HGF or H_2O_2. DCF fluorescence was monitored using a PerSeptive Biosystems CytoFluor Fluorescence Multi-well Plate Reader at excitation and emission wavelengths of 480 nm and 530 nm, respectively.

Statistical analysis

Means ± SE were calculated, and statistically significant differences between two groups were determined by the Student's *t* test at $p < 0.05$.

RESULTS

Effects of thiol antioxidants on HGF signaling for MEK-MAPK activation

HGF (10 ng/ml) phosphorylated p44/42 MAPK at threonyl and tyrosyl residues in HL-1 cardiac myocytes as monitored by western blot analysis using the phospho-specific antibody. Phosphorylation of MAPK at specific threonyl and tyrosyl residues results in the activation of kinase activity, which, in turn, elicits serine, threonine phosphorylation of target molecules (13). HGF-induced phosphorylation of MAPK was rapid and transient, with the peak occurring at 3–5 min (Fig. 1A). Control experiments showed that all the lanes had comparable levels of p44/42 MAPK proteins (data not shown).

To determine the effects of antioxidants on HGF signaling for p44/42 MAPK activation, HL-1 myocytes were pretreated with 5 m*M* NAC for 4 h before the treatment with HGF. As shown in bracketed portions of Fig. 1A, NAC significantly attenuated p44/42 MAPK phosphorylation induced by treating cells with HGF for 3 min. However, kinetic analysis revealed that this apparent inhibitory effect of NAC was due to a delay in the activation of MAPK. Figure 1A shows the typical kinetics of MAPK activation with the peak at 3–5 min in untreated HL-1 cells (open circles in the line graph of Fig. 1A). NAC-treated cells exhibited a similar degree of HGF-induced MAPK phosphorylation, but with a slightly slower kinetics with the peak at 5–7 min (filled triangles).

The delay in HGF-induced MAPK phosphorylation was also elicited by another thiol antioxidant, αLA (100 μM) (Fig. 1B). In contrast, a nonthiol antioxidant ascorbic acid (100 μM) did not cause the delay (Fig. 1C).

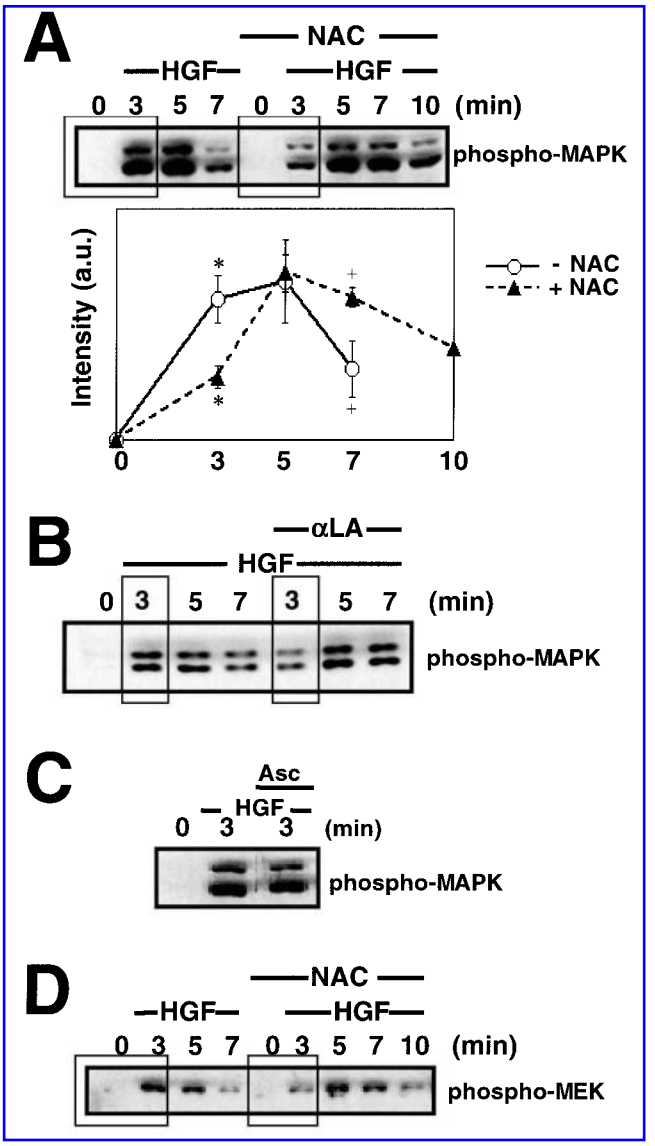

FIG. 1. Effects of thiol antioxidants on HGF-induced MAPK activation. HL-1 cardiac myocytes were pretreated with (**A** and **D**) NAC (5 m*M*), (**B**) αLA (100 μ*M*), or (**C**) ascorbic acid (Asc; 100 μ*M*) for 4 h, and then treated with HGF (10 ng/ml) for the durations indicated. Cell lysates were prepared and subjected to western blot analysis using the antibodies for (A–C) phospho-specific p44/42 MAPK and (D) phospho-specific MEK. Control experiments showed that all the lanes had comparable levels of MAPK and MEK proteins (data not shown). The line graph shows means ± SE of the density of p42 MAPK phosphorylation in untreated (○) and NAC-treated (▲) cells as determined by densitometry analysis. Values with the same symbol (* and +) are significantly different from each other at $p < 0.05$.

In the classical MAPK pathway, in response to various stimuli, MEK elicits phosphorylation of regulatory sites within the p44 and p42 MAPK molecules (13). MEK is activated in response to phosphorylation at serine residues by members of the MAPK kinase kinase superfamily. As shown in Fig. 1D, MEK was activated by HGF with a peak at 3–5 min in untreated cells. Pretreatment with NAC caused a delay in MEK phosphorylation, with the peak shifted to 5–7 min. Control experiments using the MEK antibody determined that all the lanes had comparable levels of MEK protein (data not shown). These results suggest that the target of thiol antioxidants to delay MAPK activation is upstream of MEK.

Effects of thiol antioxidants on HGF signaling for p90RSK and GATA-4 phosphorylation

p90RSK, also known as the MAPK-activated protein kinase-1, was among the first substrates of p44/42 MAPK to be discovered (5). Treatment of HL-1 cardiac myocytes with HGF for 3, 5, or 7 min elicited serine phosphorylation of p90RSK (Fig. 2). In NAC-treated cells, comparable phosphorylation of p90RSK did not occur in response to treatment of cells with HGF for up to 7 min (Fig. 2). Strong activation of p90RSK was observed at 10 min after HGF stimulation in NAC-treated cells. Therefore,

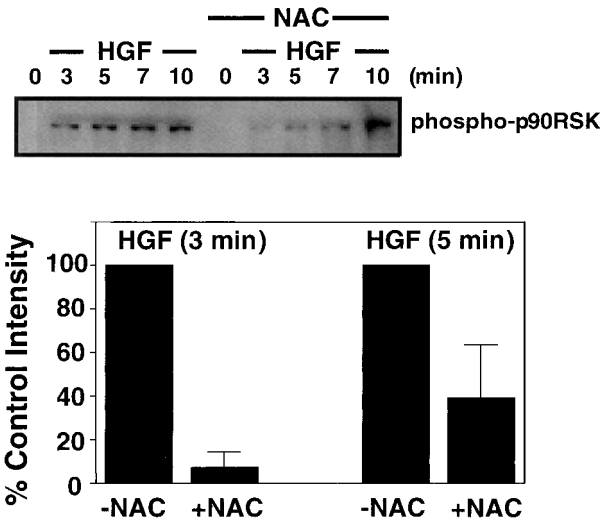

FIG. 2. Effects of NAC on HGF-induced p90RSK activation. HL-1 cardiac myocytes were pretreated with 5 m*M* NAC for 4 h, and then treated with HGF for the durations indicated. Cell lysates were prepared and subjected to western blot analysis using the antibody for phospho-specific p90RSK. The bar graph shows means ± SE of density of phospho-p90RSK bands as determined by densitometry analysis. Band intensities of NAC-treated samples (+NAC) are expressed as % of untreated samples (−NAC) at 3 or 5 min of HGF treatment.

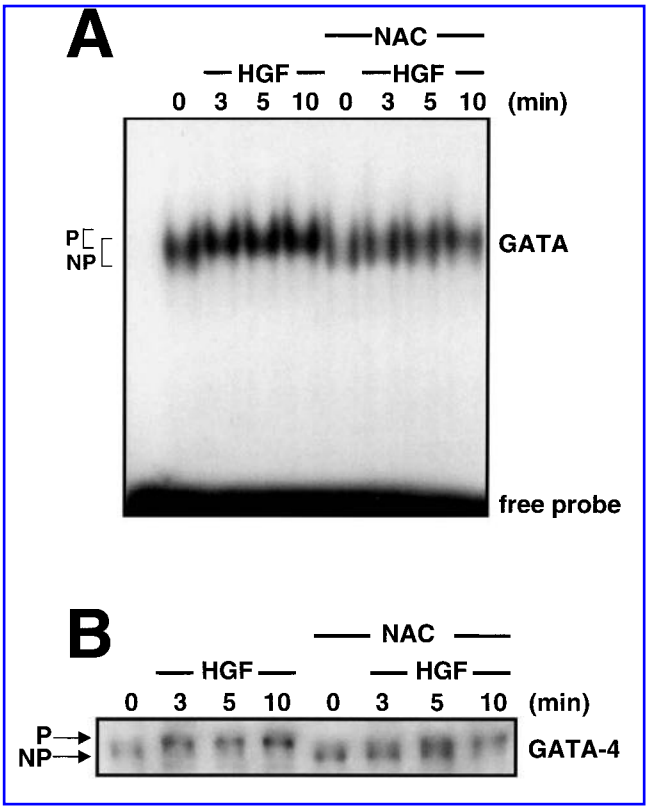

FIG. 3. Effects of NAC on HGF-induced GATA-4 phosphorylation. HL-1 cardiac myocytes were pretreated with NAC (5 m*M*) for 4 h, and then treated with HGF for the durations indicated. Nuclear extracts were prepared and subjected to (**A**) EMSA using ^{32}P-labeled oligonucleotide containing GATA consensus sequence or (**B**) western blot analysis using the GATA-4; antibody. P, phosphorylated GATA-4; NP, nonphosphorylated GATA-4.

NAC-mediated 2-min delay of MAPK activation resulted in >5 min-delay in p90RSK phosphorylation.

We recently found that the activation of p44/42 MAPK pathway by HGF can also lead to the phosphorylation of GATA-4 transcription factor in HL-1 cardiac myocytes (8). This rapid phosphorylation of GATA-4 by HGF can be visualized as a slight upward shift of electrophoretic mobility in native or SDS–polyacrylamide gel electrophoresis. As shown in Fig. 3A, EMSA experiments showed constitutive activity of a GATA binding protein that has been determined to be GATA-4 by supershift experiments. Treatment of cells with HGF caused a rapid upward shift of the GATA-4 band within 3 min. This upward shift was determined to be due to phosphorylation of GATA-4 via the MEK-MAPK pathway (8). In NAC-pretreated cells, the upward shift was not induced by treatment with HGF for up to 5 min (Fig. 3A). By 10 min after HGF treatment, GATA-4 was fully phosphorylated.

Phosphorylation of GATA-4 can also be visualized by western blot analysis (8). As shown in Fig. 3B, GATA-4 exists as two species with different electrophoretic mobility, including a lower unphosphorylated and higher phosphorylated bands. Treatment of cells with HGF caused a rapid upward shift of the lower band, indicating phosphorylation of GATA-4. HGF completely shifted the band to a single upper band within 3 min. In NAC-treated cells, a complete upward shift did not occur within 5 min of HGF stimulation (Fig. 3B). At 10 min, phosphorylation was noted, indicating that NAC caused a delay of GATA-4 phosphorylation. Similar results were obtained when cells were pretreated with αLA (100 μM) before HGF treatment. These results suggest that alterations of MAPK activation kinetics by thiol antioxidants markedly influence the resultant outcomes.

Role of ROS in HGF signaling

To determine whether ROS activate MAPK, we treated HL-1 myocytes with H_2O_2. As shown in Fig. 4, treatment with H_2O_2 (200 μM)

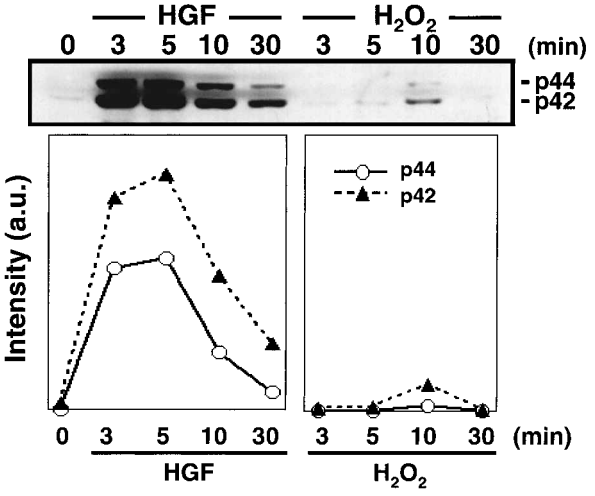

FIG. 4. Effects of H_2O_2 on MAPK activation. HL-1 cardiac myocytes were treated with HGF (10 ng/ml) or H_2O_2 (200 μM) for the durations indicated. Cell lysates were prepared and subjected to western blot analysis using the antibody for phospho-specific p44/42 MAPK. Results of densitometry analysis of p44 (○) and p42 (▲) MAPK are shown in the line graph.

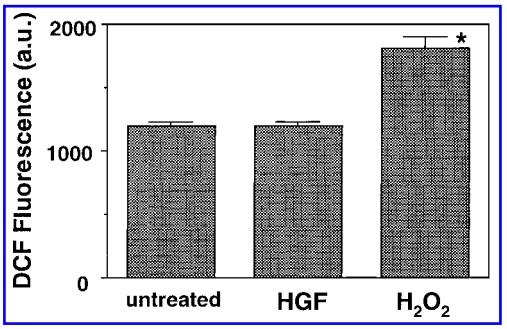

FIG. 5. Effects of HGF on ROS generation. HL-1 cardiac myocytes were loaded with 2′,7′-dichlorofluorescin diacetate, and intracellular generation of H_2O_2 and other ROS was detected by observing DCF fluorescence in untreated cells and cells treated with HGF (10 ng/ml) for 5 min. The figure also shows control experiments where cells were treated with H_2O_2 (20 μM). Values represent means ± SE ($n = 3$). *Value is significantly different from the value from untreated cells at $p < 0.05$.

caused transient phosphorylation of MAPK with the peak at 10 min. However, this activation was much weaker and slower compared with p44/42 MAPK phosphorylation observed in response to HGF.

Intracellular generation of H_2O_2 and other ROS was monitored using DCF fluorescence assays. As shown in Fig. 5, HGF did not induce ROS generation. Thus, ROS do not appear to serve as second messengers for HGF signaling.

Role of refractory/adaptive mechanism

Thiol-containing compounds have been shown to induce immediate-early activation of p44/42 MAPK (18). In HL-1 myocytes, we found that NAC (5 m*M*) also activated p44/42 MAPK (Fig. 6A). This raises a question whether the delay of HGF signaling in NAC-treated cells may have resulted from the cells being in a refractory and/or adaptive state for MAPK signaling even after 4 h of NAC addition. To test this hypothesis, cells were treated with Ang II to elicit immediate-early activation of MAPK (Fig. 6B). We found that pretreatment with Ang II for 4 h did not influence subsequent HGF signaling for MAPK activation (Fig. 6C). Thus, the effects of thiol antioxidants to delay HGF signaling do not appear to involve a mechanism where the initial treatment with thiols triggered a refractory period or adaptation to modulate subsequent HGF signaling.

DISCUSSION

HGF has been shown to be released in response to myocardial ischemia–reperfusion injury (12) and acute myocardial infarction (9, 16). Serum HGF increased 10-fold to ~3 ng/ml in a rat model of ischemia–reperfusion and >30-fold to 10–15 ng/ml in response to acute myocardial infarction in humans. HGF has been postulated to serve as a cardioprotective factor in the heart (17). During early cardiogenesis, HGF and its receptor are expressed in cardiac myocytes (14), suggesting the possible role of HGF signaling in heart development. We recently found that HGF activated a signal transduction pathway leading to phosphorylation of MEK, p44/42 MAPK and GATA-4 in cardiac myocytes (8).

Arakaki *et al*. (2) reported that ROS are involved in HGF signaling for apoptosis in Sarcoma 180 and Meth A cells. As ROS can acti-

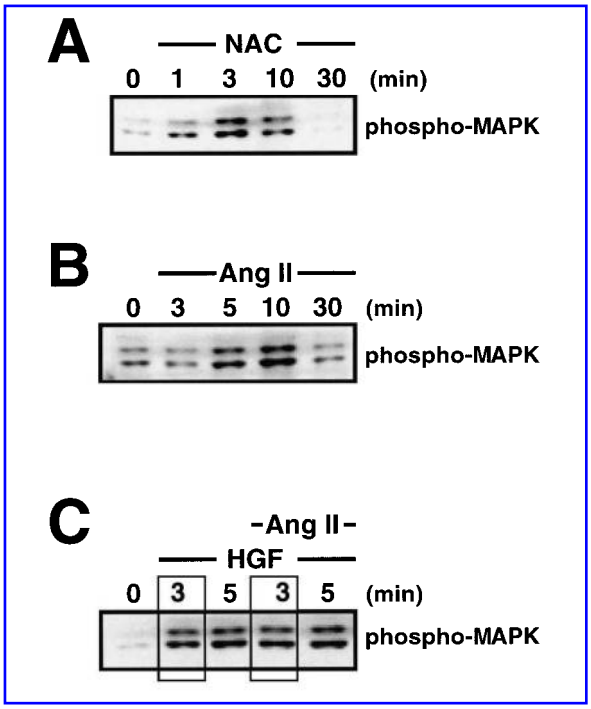

FIG. 6. Effects of Ang II on HGF-induced MAPK activation. (**A** and **B**) HL-1 cardiac myocytes were treated with NAC (5 m*M*) or Ang II (1 μM) for the durations indicated. (**C**) HL-1 cells were pretreated with Ang II (1 μM) for 4 h and then treated with HGF (10 ng/ml). Cell lysates were prepared and subjected to western blot analysis using the antibody for phospho-specific p44/42 MAPK.

vate MAPK in cardiac myocytes (1, 15), we hypothesized that HGF may activate p44/42 MAPK through ROS generation. Pretreatment of cells with thiol antioxidants did not block HGF-mediated MAPK activation. Although the activation was delayed for ~2 min, HGF elicited MAPK phosphorylation in antioxidant-treated cells as strongly as in untreated cells. H_2O_2 only weakly activated MAPK, and the kinetics of H_2O_2-mediated MAPK activation was slower than that achieved by HGF. HGF did not induce ROS generation in HL-1 cardiac myocytes. Similarly, HGF did not generate ROS in either A549 epithelial cells or bovine pulmonary artery endothelial cells (R.M. Day, unpublished results). Thus, HGF does not appear to utilize ROS as second messengers to activate p44/42 MAPK. We also observed that phosphorylation of MAPK targets, p90RSK and GATA-4, were suppressed in antioxidant-treated cells. Without rigorous kinetic studies, one may conclude that antioxidants inhibited HGF-induced phosphorylation of p90RSK and GATA-4, and therefore that ROS are involved in HGF signaling. However, our data indicate that the effects of thiol antioxidants are not through their ROS-scavenging abilities.

The mechanism of delay in HGF-signaling for MAPK activation is unknown and is expected to be complex. Our data indicate that the target of thiol antioxidants is located upstream of MEK (Fig. 7). The c-Met HGF receptor is a heterodimer composed of 50-kDa α- and 140-kDa β-chains, and both subunits have a number of cysteine residues in their extracellular domains (6); thus, c-Met may be under redox regulation. However, the delay in signal transduction by thiol antioxidants appears to be a more general phenomenon because we found that MAPK activation induced by endothelin-1 was also delayed in response to NAC pretreatment in HL-1 myocytes (unpublished results). Also, in NIH/3T3 cells, pretreatment with homocysteine caused a delay in Ang II signaling (20). Thus, a downstream converging point of signaling cascades activated by HGF, endothelin-1, and Ang II may be affected by thiol-containing compounds. As mammalian cardiac myocytes are subjected to frequent signal transduction cycles for excitation–contraction coupling (*i.e.*, 60–500 times/min depending on the species), a 2-min delay in signaling could cause significant impact.

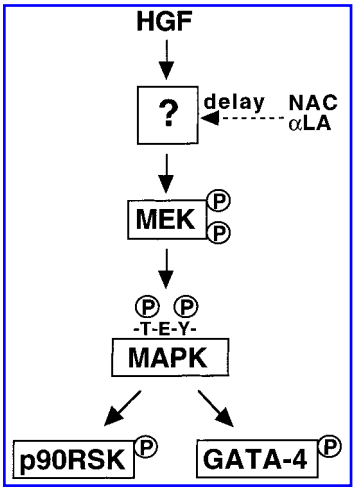

FIG. 7. Scheme of signal transduction pathway for HGF-induced MAPK activation and proposed location of the target for thiol antioxidants to cause a delay. HGF activates serine phosphorylation of MEK1/2 through a yet unknown mechanism. This, in turn, elicits threonine and tyrosine phosphorylation of p44/42 MAPK at -T-E-Y- sequence, leading to activation. Activated MAPK gets translocated and phosphorylates target molecules such as p90RSK and GATA-4.

ACKNOWLEDGMENTS

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ABBREVIATIONS

Ang II, angiotensin II; DCF, 2′,7′-dichlorofluorescein; EMSA, electrophoretic mobility shift assay; HGF, hepatocyte growth factor; αLA, α-lipoic acid; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NAC, *N*-acetylcysteine; PMSF, phenylmethylsulfonyl fluoride; p90RSK, p90 kDa ribosomal S6 kinase; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

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Address reprint requests to:
Dr. Yuichiro J. Suzuki
Antioxidants Research Laboratory
USDA Human Nutrition Research Center on Aging at Tufts University
711 Washington Street
Boston, MA 02111

E-mail: ysuzuki@hnrc.tufts.edu

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