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Adrenomedullin Regulates Cellular Glutathione Content via Modulation of γ-Glutamate-Cysteine Ligase Catalytic Subunit Expression

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Adrenomedullin (AM) participates in a wide range of physiological and pathological processes including vasorelaxation, angiogenesis, cancer promotion, and apoptosis. Recently, it has been reported that AM protects a variety of cells against oxidative stress induced by stressors such as hypoxia, ischemia/reperfusion, and hydrogen peroxide through the phosphatidylinositol 3-kinase (PI3K)-dependent pathway. However, the molecular mechanisms underlying the pathway of cell survival against hypoxic injury are largely unknown. In an effort to investigate the survival mechanism against hypoxic injury, we studied the effects of AM on cellular levels of reactive oxygen species, well-known mediators of cell death after oxidative stress, and the mechanism involved in the regulation of reactive oxygen species levels. Here, we show that AM increases γ-glutamate-cysteine ligase (γ-GCL) activity under both hypoxic and normoxic conditions, resulting in an up-regulation of cellular glutathione levels to more than 2-fold higher than basal expression. In addition, we demonstrate that AM induces concentration-dependent expression of the catalytic subunit of γ-GCL (γ-GCLC) at the mRNA and protein levels through the activation of the γ-GCLC promoter fragment sequence from −597 to −320. However, when treated with the PI3K inhibitors, the effects of AM on γ-GCLC expression were completely abrogated, suggesting that a PI3K pathway linked AM with the transcriptional activation of the γ-GCLC promoter. Taken together, our data suggests that AM participates in the regulation of cellular redox status via glutathione synthesis. These results may explain, in part, the mechanism by which AM protects cells against oxidative stress. (*Endocrinology* 147: 1357–1364, 2006)

ADRENOMEDULLIN (AM), INITIALLY identified in human pheochromocytoma (1), is a member of the calcitonin gene-related peptide family. It is synthesized as an inactive, 185-residue precursor protein and released as a mature, 52-residue amide structure peptide (2). Upon secretion, it binds to the G protein-coupled calcitonin receptor-like receptor (CRLR) (3) and exerts autocrine and paracrine effects. Many lines of evidence have shown that AM possesses several other biological effects in addition to its well-known vasoactive activity (4). These include cell proliferation (5), differentiation (6), and oxidative stress-induced cell death inhibition (7). Recently, the inhibition of the redox-sensitive c-Jun amino-teminal kinase (JNK) has been suspected as a putative pathway responsible for the antioxidant effects of AM (8). However, the underlying cellular mechanisms in the regulation of cellular redox status have not yet been clarified.

Glutathione [L-γ-glutamyl-cysteinyl-glycine (GSH)] is an abundant tripeptide thiol involved in the maintenance of cellular redox balance inside mammalian cells (9). It is present in either a reduced or oxidized form, with the reduced form predominating under normal physiological conditions (10). In addition to redox status, imbalance in GSH expression has been implicated in a variety of circumstances, including cancer and neurodegenerative diseases (11). Hence, tight regulation of cellular GSH content is imperative to normal physiological cellular function.

The *de novo* synthesis of GSH is regulated at several levels in cells and requires the consecutive action of two ATP-dependent enzymes: γ-glutamate-cysteine ligase (γ-GCL) and GSH synthase (GS) (10). γ-GCL is the first and rate-limiting enzyme in GSH synthesis; it combines glutamate and cysteine through the γ-carboxyl moiety. Structurally, γ-GCL is a heterodimer composed of a catalytically active heavy subunit (γ-GCLC) and a regulatory subunit (γ-GCLM) (12, 13). γ-GCLM modulates the K_m of the enzyme for glutamate and its sensitivity to feedback inhibition by GSH (14, 15). Increased *de novo* synthesis of GSH is primarily a result of increased synthesis of the γ-GCLC subunit through a combination of increased transcription and mRNA stability (16). The regulation of GSH biosynthesis by γ-GCL in response to various environmental and oxidative stresses is currently the focus of much investigation.

The purpose of this study was to investigate the molecular mechanisms by which AM inhibits hypoxia-induced cell death in association with the redox regulator GSH and its synthetic pathway.

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Abbreviations: AM, Adrenomedullin; AP2, activator protein 2; DCF-DA, 2′,7′-dichlorofluorescin diacetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCF, GC factor; γ-GCL, γ-glutamate-cysteine ligase; γ-GCLC, catalytic subunit of γ-GCL; γ-GCLM, regulatory subunit of γ-GCL; GS, GSH synthase; GSH, L-γ-glutamyl-cysteinyl-glycine; HNE, 4-hydroxynonenal; NADH, reduced nicotinamide adenine dinucleotide; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species.

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Materials and Methods

Cell culture, hypoxic conditions, and reagents

The human type II alveolar epithelial cell line, A549, was purchased from American Type Culture Collection (Rockville, MD) and cultured in RPMI medium containing 10% FBS in a 5% CO_2 atmosphere. For hypoxic conditions, cells in medium degassed with 95% N_2 and 5% CO_2 were transferred to a hypoxic chamber (Ruskinn, Bridgend, UK) equipped with an air lock and continuously gassed with 0.5% O_2 and 5% CO_2 (with the remainder as nitrogen). Human AM and goat antihuman AM antibodies were purchased from Bachem (Bubendorf, Switzerland). 4-Hydroxynonenal and protein kinase inhibitors (LY294002, wortmannin, bisindolylmaleimide, and autocamtide-2) were purchased from Cayman Chemical (Ann Arbor, MI) and Calbiochem (San Diego, CA), respectively. All other reagents were obtained from Sigma-Aldrich, Inc. (St. Louis, MO), unless otherwise specified.

Cell death assay

Hypoxia-induced cell death was evaluated by the morphological observation of pyknotic, shrunken nuclei and fragmented apoptotic bodies after 4′,6-diamidino-2-phenylindole (DAPI) staining. The second method for evaluating damaged cells was performed using the trypan blue exclusion test.

Detection of reactive oxygen species (ROS)

2′,7′-Dichlorofluorescin diacetate (DCF-DA), shown to be relatively specific for hydrogen peroxide, was used to measure ROS. The cells were harvested and suspended in culture medium with 0.2% fetal bovine serum. Twenty micromolar DCF-DA was then added and incubated for 20 min at 37 C. The fluorescent intensity was measured by flow cytometry (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ) or observed using a fluorescence microscope.

Determination of GSH levels and assay for GS and γ-GCL activity

Total intracellular GSH content was measured using an assay kit obtained from OXIS International Inc. (Portland, OR) according to the manufacturer's protocol. GS activity was determined by measuring the formation of ADP in reaction mixtures containing the enzyme and its substrates (17). The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.2), 50 mM KCl, 5 mM L-γ-glutamyl-L-α-aminobutyrate, 10 mM ATP, 5 mM glycine, 20 mM $MgCl_2$, 2 mM EDTA, and extract in a final volume of 0.1 ml. The assay mixture was incubated for 2.5–30 min at 37 C. To measure ADP levels, the reaction mixtures were treated with 0.02 ml 10% sulfosalicylic acid and 0.9 ml of a solution containing 0.5 mM phosphoenolpyruvate, 0.2 mM reduced nicotinamide adenine dinucleotide (NADH), 1 U pyruvate kinase, 40 mM magnesium chloride, 50 mM potassium chloride, and 250 mM potassium phosphate buffer (pH 7.0). The amount of ADP formed was calculated from the observed change in absorbance at 340 nm after the addition of 0.1 ml (1 U) lactate dehydrogenase. Enzymatic activity was measured as change in absorbance per minute per milligram protein. γ-GCL activity was determined as described previously (18). Briefly, enzyme activity was determined at 37 C in reaction mixtures of 1.0 ml containing 100 mM Tris-HCl buffer (pH 8.2), 150 mM KCl, 5 mM ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 2 mM EDTA, 0.2 mM NADH, 17 μg pyruvate kinase, and 17 mg lactate dehydrogenase. The reaction was initiated by adding extract, and the rate of absorbance decrease at 340 nm was monitored. Enzyme-specific activity was measured as micromoles of NADH oxidized per minute per milligram protein; this is equal to 1 IU. For each assay, BSA was added to the cell extracts to determine the specificity of the γ-GCL assay.

Real-time RT-PCR of γ-GCLC and γ-GCLM mRNA

γ-GCLC and γ-GCLM mRNA levels were quantified with real-time PCR using the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. Total RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Carlsbad, CA) with random hexamer priming. The resultant cDNA was amplified using a LightCycler (Roche Diagnostics Ltd., Lewes, UK). Real-time PCR analysis was carried out with the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics) and specific primers for GAPDH, γ-GCLC, and γ-GCLM. The sequences of the gene-specific primers were as follows: γ-GCLC (NM 001498) sense, 5′-AGT TCA ATA CAG TTG AGG-3′, and antisense, 5′-TAC TGA TCC TAT AGT TAT-3′; GCLM (NM 002061) sense, 5′-CAG TTG ACA TGG CCT GTT CAG-3′, and antisense, 5′-TCA AAT CTG GTG GCA TCA CAC-3′; GAPDH sense, 5′-AAC CAT GAG AAG TAT GAC AAC-3′, and antisense, 5′-CTG CTT CAC CAC CTT CTT GAT-3′. The expression level of each mRNA and their estimated crossing points were determined relative to the standard preparation using the LightCycler computer software.

Western blot

A549 cells were harvested and suspended in a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 1.1 mM Na_3VO_4, and 10 mM NaF. Extracted proteins were separated by SDS-PAGE on 12% polyacrylamide gels and electrophoretically transferred onto nylon membranes. The membranes were probed with anti-γ-GCLC or antitubulin antibody (Abcam, Cambridge, UK), followed by incubation with antirabbit IgG horseradish peroxidase-coupled secondary antibody. Detection was performed with an ECL system (Intron, Seoul, Korea).

Promoter assay

The γ-GCLC promoter and its fragments were amplified by PCR from genomic DNA using the upstream oligonucleotides F1 (5′-TTT AAT GAG ATA TTT ACA-3′) (pGL-GCLC-1), F2 (5′-ATG ATA AAT ACA CAC TTT-3′) (pGL-GCLC-2), F3 (5′-TGA GTT CGA CAT TGA TTC-3′) (pGL-GCLC-3), F4 (5′-TCC TGA GCC CCC GTT-3′) (pGL-GCLC-4), and F5 (5′-AGC GGC ACG CGC CTT CTG-3′) (pGL-GCLC-5) and the R1 downstream oligonucleotide (5′-ACT ACA GAG TTA ACA TTT-3′); these were sequenced and cloned into the pGL2 luciferase assay vector (Promega, San Luis Obispo, CA). The luciferase reporter vector was transiently transfected using Transfast (Promega). Luciferase activity was measured on samples containing equivalent amounts of protein using a luminometer (PerkinElmer, Fremont, CA) and luciferase assay reagents (Promega).

Statistical analysis

All data are presented as mean ± SD from three or more independent experiments. Statistical significance was assessed with one-way ANOVA, followed by Dunnett's multiple-range test. P values less than 0.05 were considered statistically significant.

Results

Effects of AM on hypoxia-induced apoptotic cell death

To evaluate the effects of AM on hypoxia-induced cell death, A549 lung epithelial cells were subjected to hypoxia (0.5% O_2) for different time intervals in the presence and absence of 10^{-9} M AM. Hypoxia reduced cell viability, resulting in 38% cell death after 12 h (Fig. 1A). The death induced by 0.5% hypoxic treatment appeared to be characteristic of apoptosis; cells shrunk and their nuclei were condensed or fragmented (Fig. 1B, *left*). However, treatment with 10^{-9} M AM, which was added to culture medium 3 h before hypoxia, decreased the cell death rate to 8% after 12 h of hypoxia (Fig. 1, A and B, *right*). To confirm the effects of AM on hypoxic cell death, cells were treated with the indicated concentration of AM and subjected to hypoxia for 12 h. The rate of cell death decreased in a concentration-dependent manner (Fig. 1C). Our results show that AM suppressed

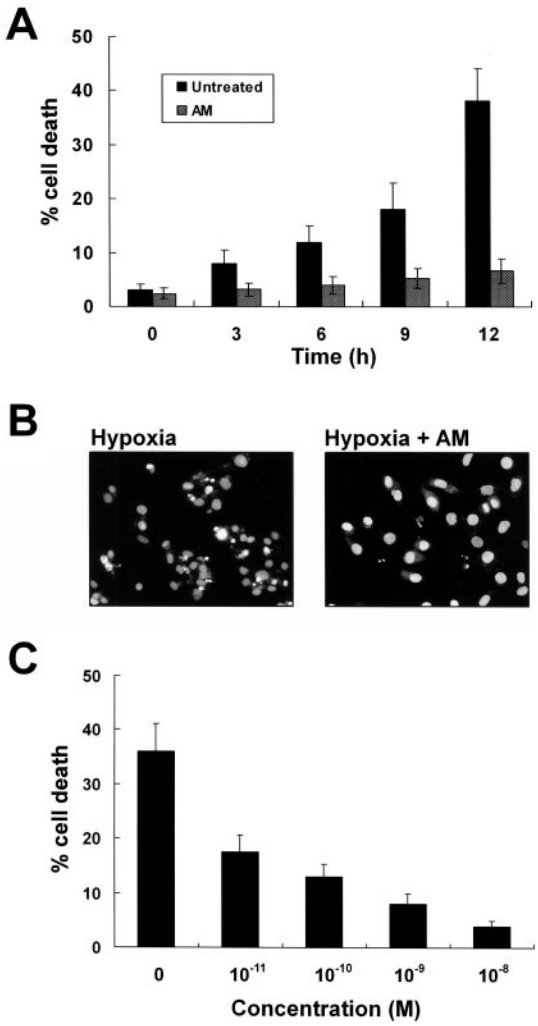

FIG. 1. AM inhibits hypoxia-induced apoptotic cell death. A, A549 cells were exposed to hypoxia (0.5% O_2) for the indicated times in the absence and presence of 10^{-9} M AM (added 3 h before hypoxia). Apoptotic cells with fragmented nuclei or a condensed chromatin pattern were counted under a fluorescent microscope using 4′,6-diamidino-2-phenylindole staining. B, Representative photomicrographs after 12 h of hypoxia. C, Cells were treated with AM of the indicated concentration and subjected to hypoxia (0.5% O_2) for 12 h. Cell death rates were determined in a manner similar to A. Results were analyzed in duplicate; the mean ± SD of three different experiments is shown.

hypoxia-induced apoptotic cell death in a concentration-dependent manner.

AM attenuates hypoxia-induced cellular increases in ROS

Although hypoxic stress signals through a variety of effector molecules, ROS is central in hypoxia-induced cytotoxic injury. The results described above led us to examine whether AM affects ROS levels during hypoxic injury. To this end, A549 cells were exposed to hypoxia for 6 h in the presence and absence of 10^{-9} M AM and the ROS level was determined using DCFH-DA fluorescent dye. Pretreatment of cells with AM 3 h before hypoxia attenuated hypoxia-induced increases in ROS levels (Fig. 2A). Next, we determined whether AM was effective in the suppression of increases in ROS levels by exogenous H_2O_2. A549 cells were pretreated with 10^{-8} M AM for 3 h or untreated and then exposed to 100 nM H_2O_2. As shown in Fig. 2B, AM efficiently suppressed increases in ROS levels by exogenous H_2O_2. However, cotreatment of AM with hypoxia or H_2O_2 was not effective in suppressing ROS increases, suggesting that prior activation of the signaling pathways was needed for ROS scavenging activity of AM (data not shown). Our data may suggest that the increases of intracellular ROS scavenging activity contributed to AM-induced ROS attenuation.

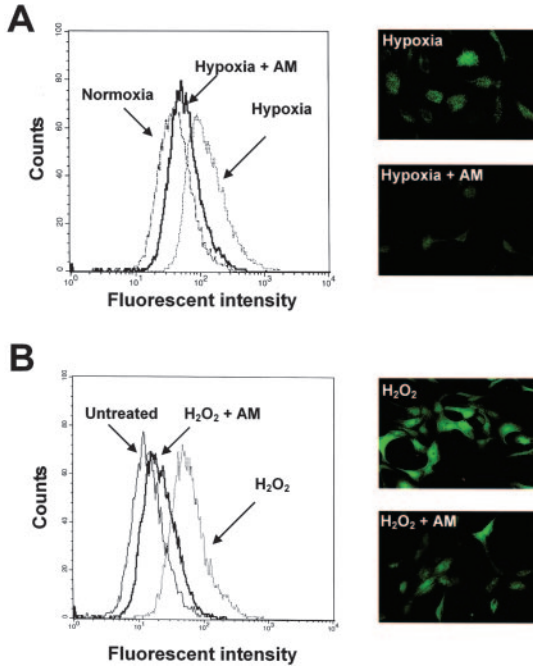

FIG. 2. AM attenuates the increase in ROS by hypoxia or exogenous H_2O_2. A, Cells were cultured in normoxia or hypoxia (0.5% O_2) with or without 10^{-9} M AM for 6 h. ROS levels were measured using flow cytometry after the addition of 20 μM DCFH-DA (*left*). Representative photomicrographs are shown on the *right*. B, Cells were pretreated with 10^{-8} M AM for 3 h or untreated, and then cells were exposed to 100 nM H_2O_2 for 2 h. Determinations of ROS levels were performed after the addition of DCFH-DA using flow cytometry (*left*) and fluorescence microscope (*right*).

Effect of AM on cellular GSH content

GSH is the most abundant low-molecular-weight thiol found in mammalian cells, and it participates in the scavenging of free radicals. Therefore, we next decided to examine whether ROS attenuation driven by AM treatment was associated with cellular GSH content. A549 cells were exposed to hypoxia (2% O_2 for the maintenance of cell viability) or normoxia for different time intervals in the presence and absence of 10^{-9} M AM and were subjected to a measurement of total GSH content. As shown in Fig. 3, AM increased GSH levels by up to 59% over the untreated control cells after 4 h of treatment (H1). Furthermore, the increased levels of GSH were maintained for 6 h against hypoxia in AM-treated cells; on the other hand, GSH levels in untreated cells decreased to 73% of those seen in normoxic cells after 6 h of hypoxia ($P < 0.01$). Cell viability remained over 90% after all hypoxic treatments. Together, our data imply that AM increased GSH

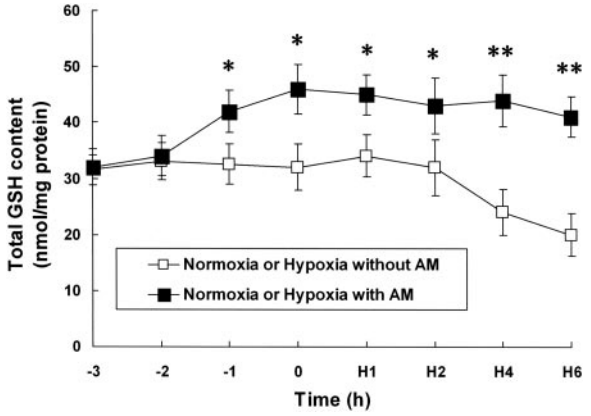

FIG. 3. Effects of AM on total GSH content. Cells were exposed to normoxia or hypoxia (2% O_2) in the absence or presence of 10^{-9} M AM; total cellular GSH equivalents (GSH + GSSG) were then measured. AM was added at −3 h, and cells were exposed to hypoxia at 0 h. Cells showed more than 90% viability after 6 h of hypoxia. Results are the mean ± SD of three different experiments. *, $P < 0.05$; **, $P < 0.01$, compared with untreated cells.

levels (even in normoxic cells) and maintained them against hypoxic stress.

Effect of AM on γ-GCL and GS activity

The synthesis of GSH from its constituent amino acids results from the concerted effort of two ATP-dependent enzymes, γ-GCL and GS (19). To investigate whether the up-regulation of GSH levels by AM resulted from increased activity of GSH synthetic enzymes, we determined the effects of AM on the activity of γ-GCL and GS. Cells were cultured in normoxia or hypoxia (2% O_2) in the presence and absence of 10^{-9} M AM for different time intervals; γ-GCL activities were then determined. As shown in Fig. 4, γ-GCL activity was significantly elevated in AM-treated cells irrespective of oxygen concentration (normoxia or hypoxia) ($P < 0.01$), compared with untreated cells. Cell viability did not affect enzyme activity, because it remained above 90% after all of the treatments. We next determined the activity of GS in association with AM-induced GSH up-regulation. However, a significant increase in GS activity was not noted (data not shown).

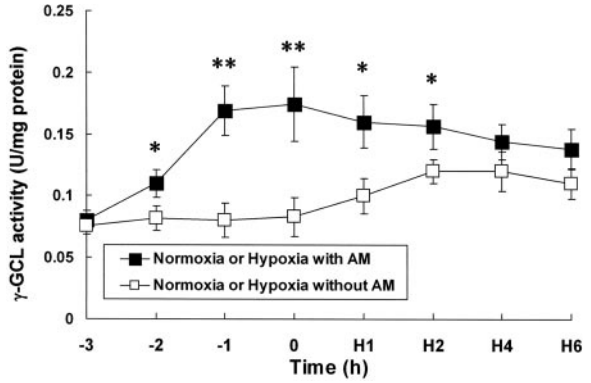

FIG. 4. Regulation of γ-GCL activity by AM. Cells were exposed to normoxia or hypoxia (2% O_2) in the absence or presence of 10^{-9} M AM; γ-GCL activity was then determined. AM was added at −3 h, and cells were exposed to hypoxia at 0 h. Cells were more than 90% viable after 6 h of hypoxia. Results are the mean ± SD of three different experiments. *, $P < 0.05$; **, $P < 0.01$, compared with untreated cells.

Effect of AM on γ-GCL protein and mRNA expression

To investigate the mechanisms involved in the increase in γ-GCL activity, we examined changes in γ-GCLC expression after AM treatment at both the mRNA and protein levels, because γ-GCL activity is regulated by the expression of the catalytic subunit of γ-GCL (20). To determine the quantitative difference in γ-GCLC mRNA expression between AM-treated and untreated cells, we performed real-time PCR. Steady-state levels of γ-GCLC mRNA were determined and normalized to GAPDH. As shown in Fig. 5A, a significant increase in γ-GCLC mRNA levels (relative to the controls) was detected in AM-treated cells in a concentration-dependent manner. 4-Hydroxynonenal (HNE), known to induce γ-GCLC mRNA expression, was used as a positive control (21). Protein levels of γ-GCLC, as detected by immunoblot, were in line with mRNA expression levels (Fig. 5B). Our data indicate that AM increased the expression of both γ-GCLC mRNA and protein.

Effect of AM on γ-GCLC promoter construct-derived luciferase activity

Next, to determine whether the increased γ-GCLC mRNA levels induced by AM reflected mRNA stabilization or increased transcriptional rate, we performed a functional anal-

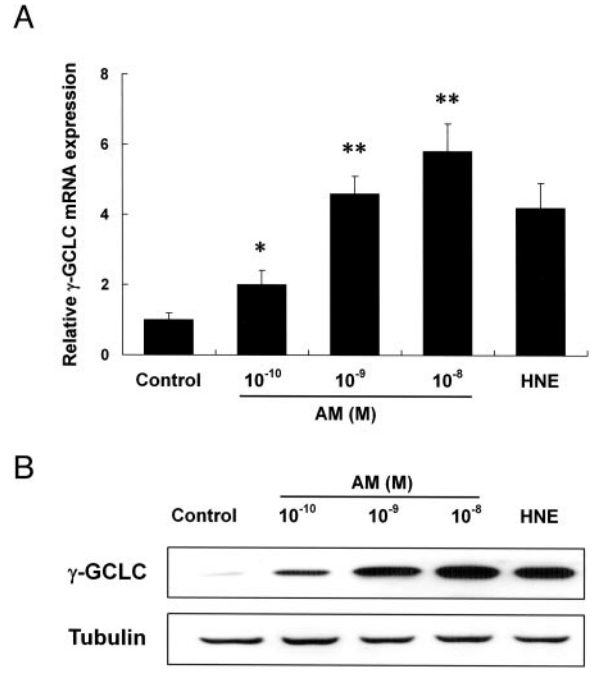

FIG. 5. Effects of AM on γ-GCLC mRNA and protein expression in A549 cells. A, Total RNA was extracted from control cells or cells incubated with various concentrations of AM for 3 h, reverse transcribed, and used in real-time PCR analysis of γ-GCLC mRNA. HNE (20 μM) was used as a positive control. *, $P < 0.05$; **, $P < 0.01$, compared with the control cells. B, Protein was extracted from untreated cells and cells that had been treated with the indicated concentration of AM for 9 h. Western blotting was performed using an antihuman γ-GCLC antibody. Tubulin was used to confirm equal protein loading. HNE lane, positive control.

ysis of partial γ-GCLC promoter fragments subcloned into pGL2 luciferase reporter plasmids. The transcriptional activities of various pGL-Luc reporter plasmids containing successively deleted 5′-flanking sequences of the γ-GCLC gene were determined in AM-treated and untreated cells (Fig. 6A). As shown in Fig. 6B, the transcriptional activities of pGL-GCLC-1, -2, -3, -4, and -5 in AM-treated cells were 4- to 6-fold higher than those observed in untreated cells. However, the transcriptional activity of the reporter plasmid was decreased to the level of pGL-Basic when the sequence from −597 to −320 bp was deleted, suggesting that this sequence was responsible for AM-induced transcriptional up-regulation. This region contains activator protein 2 (AP2), sequence-specific DNA-binding protein 1 (Sp1), metal response element (MRE), and GC factor (GCF) transcriptional factor binding sites. However, deletion of each binding site did not completely abolish luciferase activity, relative to pGL-GCLC-1 (data not shown).

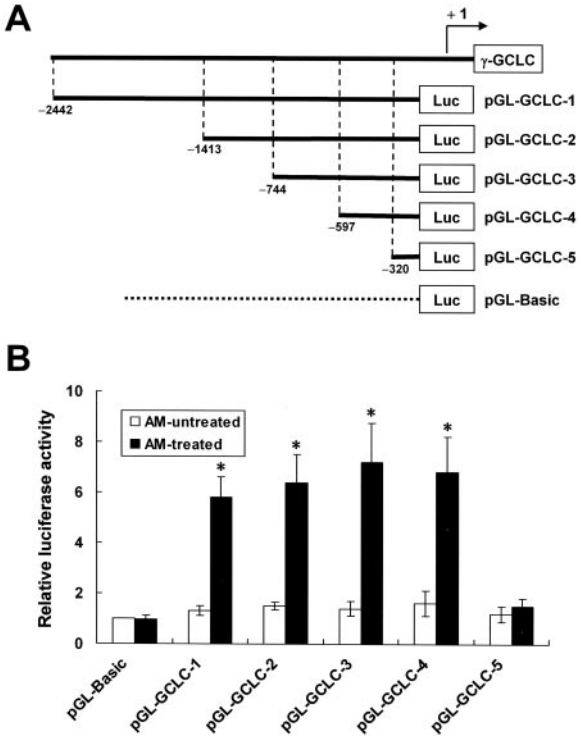

FIG. 6. Transcriptional activity of the 5′-deleted promoter fragments of the γ-GCLC gene in AM-treated cells. A, The 5′-deleted promoter fragment of the γ-GCLC gene was inserted into parental pGL-Basic to construct the indicated luciferase reporter plasmids. *Numbers* represent the nucleotide position from the transcriptional start site of the γ-GCLC gene. B, Cells were transiently transfected with 1 μg of the indicated reporter plasmid, and 24 h after transfection, the cells were untreated (*open bar*) or treated (*closed bar*) with 10^{-9} M AM for 6 h; luciferase activity was then measured. For each construct, the fold increase of luciferase activity is presented. The luciferase activity of untreated cells transfected with pGL-Basic was arbitrarily set to 1. Values are an average of three independent experiments. *, Significant increase over the pGL-Basic or pGL-GCLC-5 transfected cells ($P < 0.01$).

Inhibition of phosphatidylinositol 3-kinase (PI3K) abolishes the effects of AM on γ-GCLC

It has been known that a PI3K-dependent pathway plays a pivotal role in AM-associated cell survival against cytotoxic signals such as ischemia and reperfusion (22). To investigate the signaling pathways responsible for the AM-induced transcriptional up-regulation of γ-GCLC, we treated cells with the various protein kinase inhibitors including PI3K and measured γ-GCLC expression and γ-GCL activity in AM-treated and untreated cells. As shown in Fig. 7A, PI3K inhibitors abolished the increase of γ-GCL activity induced by AM to near basal levels whereas protein kinase A, protein kinase C, and calmodulin kinase inhibitors failed to suppress the γ-GCL activity. In addition, transcriptional induction of γ-GCLC mRNA and protein was suppressed by PI3K inhibitors (Fig. 7, B–D). These results suggest that AM exerted its modulatory effects on γ-GCL through a PI3K-dependent pathway.

Discussion

Induction of intracellular GSH synthesis occurs in response to various cellular stresses, including hypoxia and ischemia. It may play a pivotal role in cellular tolerance to oxidative stress. In the present study, we demonstrated that AM up-regulated cellular GSH contents through the induction of γ-GCL, the regulatory and rate-limiting enzyme in GSH biosynthesis. We also showed that the AM-associated increase in γ-GCL was a result of direct transcriptional activation of the γ-GCLC promoter region via a PI3K-dependent pathway.

There is a growing body of evidence demonstrating that AM exerts a wide range of effects on cell growth and apoptotic death and that these effects are dependent on cell type, stress type, and experimental conditions. Recently, it has been reported that AM may protect against oxidative stress as an endogenous antioxidant (8, 23); furthermore, diverse signaling molecules, such as PI3K and protein tyrosine kinase(s), have been suspected as putative mediators in cell survival pathways (7, 22, 24, 25). However, much remains unknown about the cellular mechanisms underlying oxidation-reduction status regulation by AM.

Although cellular oxidative stresses result in the activation of several signaling pathways, such as c-Jun NH_2-terminal kinase (JNK) and ERK, elevated ROS levels are central in their cytotoxic pathways (26). Our initial data showed that AM rescued cells against hypoxia and suppressed ROS elevation induced by oxidative stress. In line with our data, it has been reported that the degree of ROS elevation induced by hypoxia was higher and resulted in more severe tissue damage in $AM^{+/-}$ mice than in $AM^{+/+}$ mice (27). ROS attenuation by AM raises two possibilities: 1) the direct suppression of ROS production by AM or 2) the activation of intracellular ROS-scavenging systems. Our data showed that AM efficiently suppresses increases in ROS levels caused by exogenous H_2O_2, suggesting that it activates ROS scavenging systems rather than acting as an antioxidant. However, the mechanisms appear to be more complex, because AM acted as an endogenous antioxidant against angiotensin II-induced ROS generation (8). This finding led us to the hypothesis that

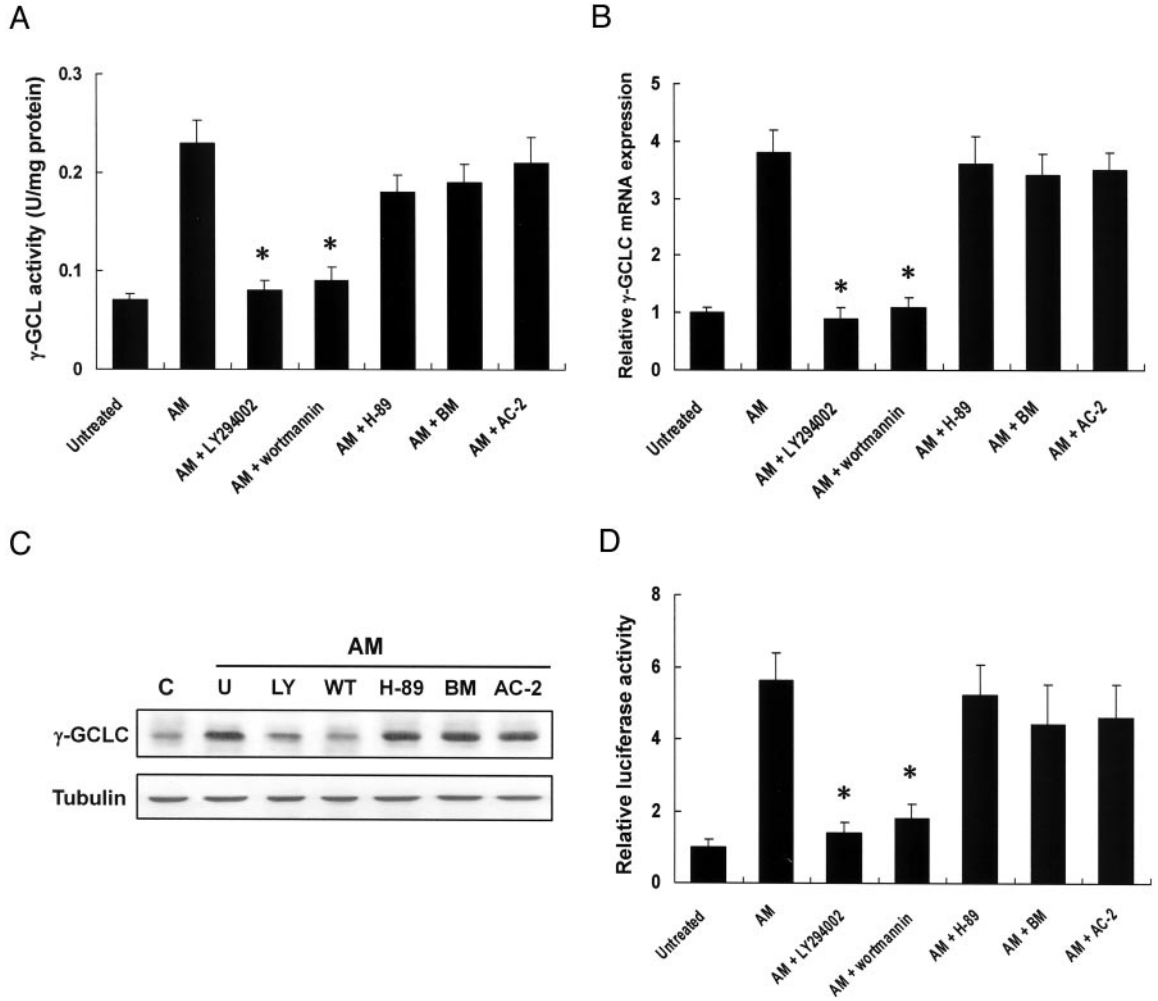

FIG. 7. AM exerts the effects on γ-GCL via a PI3K-dependent pathway. Cells were untreated or treated with 10^{-9} M AM in the presence or absence of 10 μM LY294002 (LY), 1 μM wortmannin (WT), 100 nM H-89, 50 nM bisindolylmaleimide (BM), or 100 nM autocamtide-2 (AC-2). LY294002 and other protein kinase inhibitors were added to the culture medium 6 and 3 h before AM treatment, respectively. All data are averages of three independent experiments or representative blots. A, γ-GCL activity was determined after 3 h of AM treatment. *, $P < 0.01$. B, Real-time PCR analysis for γ-GCLC mRNA. RNA was extracted after 3 h of AM treatment. *, $P < 0.01$. C, Western blot for γ-GCLC. Protein was extracted after 9 h of AM treatment. C, Control cells; U, AM alone. D, Cells were transiently transfected with 1 μg of pGL-GCLC-1 reporter plasmid. Luciferase activity was determined after 6 h of AM treatment. *, $P < 0.01$.

AM may regulate one or more intracellular ROS scavenging systems. To this end, we focused on the GSH system, because GSH is a key regulator of cellular redox balance based on the fact that its cellular concentration is about 500- to 1000-fold higher than other redox systems, such as thioredoxin and nicotinamide adenine dinucleotide phosphate (28).

The precise mechanisms of ROS inhibition by AM have not yet been clarified. Our results from the enzyme assays, real-time PCR, and immunoblots showed that AM elevated cellular GSH levels via an up-regulation of its rate-limiting synthetic enzyme, γ-GCL. This finding suggests that GSH may be one of the candidate molecules that links AM with redox regulation systems. However, there are several reports showing that AM suppresses ROS production through different signaling pathways, such as the activation of the cAMP-protein kinase A pathway in mesangial cells (29) and the inhibition of nicotinamide adenine dinucleotide phosphate oxidase via the nitric oxide-cGMP signaling pathway (30). It is unclear whether these pathways are activated in concert or sequentially, depending on cell type or the severity of oxidative stress. However, the effect of AM on γ-GCL was rapid and potent enough to overcome initial feedback inhibition by GSH under normoxic conditions (Figs. 3 and 4). It may be that AM affects the activity or expression of GSH-modifying enzymes such as GSH reductase or peroxidase, because the function of GSH as an intracellular redox buffer depends on the relative amounts of the reduced and oxidized forms.

We next elucidated the transcriptional regulatory mechanisms by which AM exerted its effects on the induction of γ-GCL. Although it has been reported that γ-GCL activity can be modulated positively or negatively by posttranscriptional mechanisms including phosphorylation, oxidation, and S-nitrosation, increased γ-GCL activity involves a transcriptional component in a majority of documented cases (31). This transcriptional regulation may involve one or both

of the catalytic and modulatory subunits depending on the specific stimuli. However, a variety of stresses, including oxidants, ionizing radiation, and TNF-α, induce catalytic subunit expression (32–34). By using 5′-flanking deletion fragments of γ-GCLC promoter, we showed that a sequence of about 280 bp (from −597 to −320 bp) responded to AM. This region is highly GC rich and contains three AP2 binding sites, two sequence-specific DNA-binding protein 1 (Sp1) binding sites, two metal response element (MRE) binding sites, and two GCF binding sites (35). However, it seems to be unlikely that a single transcriptional binding site in this region is critically responsible for transcriptional up-regulation of the γ-GCLC gene by AM because deletion of each transcriptional binding site in this region decreased luciferase activity to about 80–90% of pGL-GCLC-1 (data not shown). This result suggests that more than one transcriptional factor participates in the up-regulation of γ-GCLC gene in response to AM; multiple positive and negative regulatory elements, such as AP1, AP2, nuclear factor-κB, antioxidant responsive element (ARE), GCF, and electrophile response element (EpRE), contribute to the basal transcriptional activity of the γ-GCLC gene (35).

AM acts as a trigger of multiple signaling cascades, including the cAMP (29), nuclear factor-κB (36), tyrosine kinase (24), protein phosphatase 2A (37), and PI3K/Akt pathways (7, 22). Depending on cell types or experimental conditions, AM may activate or inhibit more than two signaling cascades. For example, AM may either increase or decrease intracellular calcium concentration independent of cAMP (38, 39), and it stimulates MAPK in vascular smooth muscle cells and inhibits MAPK activity in mesangial cells (25, 29). Individually or in concert, these pathways transmit diverse survival signals from AM to target molecules. Our results suggest that a PI3K-dependent pathway plays a pivotal role in the AM-induced transcriptional activation of the γ-GCLC gene even though other signaling cascades are partly involved (Fig. 7). Identification and characterization of the transcriptional factor(s) responsible for γ-GCLC gene induction are in progress.

In conclusion, we have shown that AM participates in the regulation of GSH synthesis by increasing γ-GCL activity via PI3K-dependent transcriptional activation of the γ-GCLC promoter. Our data may explain, in part, the mechanism by which AM rescues a variety of cells from oxidative stress.

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