S-Glutathiolation of Ras Mediates Redox-sensitive Signaling by Angiotensin II in Vascular Smooth Muscle Cells

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Angiotensin II (AII) increases production of reactive oxygen species from NAD(P)H oxidase, a response that contributes to vascular hypertrophy. Here we show in cultured vascular smooth muscle cells that S-glutathiolation of the redox-sensitive Cys118 on the small GTPase, Ras, plays a critical role in AII-induced hypertrophic signaling. AII simultaneously increased the Ras activity and the S-glutathiolation of Ras (GSS-Ras) detected by biotin-labeled GSH or mass spectrometry. Both the increase in activity and GSS-Ras was labile under reducing conditions, suggesting the essential nature of this thiol modification to Ras activation. Overexpression of catalase, a dominant-negative p47phox, or glutaredoxin-1 decreased GSS-Ras, Ras activation, p38, and Akt phosphorylation and the induction of protein synthesis by AII. Furthermore, expression of a Cys118 mutant Ras decreased AII-mediated p38 and Akt phosphorylation as well as protein synthesis. These results show that H2O2 from NAD(P)H oxidase forms GSS-Ras on Cys118 and increases its activity leading to p38 and Akt phosphorylation, which contributes to the induction of protein synthesis. This study suggests that GSS-Ras is a redox-sensitive signaling switch that participates in the cellular response to AII.

Considerable evidence implicates reactive oxygen species (ROS) as mediators of cellular signaling by several kinds of stimuli, including cytokines, growth factors, hormones (1), or mechanical forces (2). However, the molecular mechanism by which ROS alter cellular signaling is not established. Reactive thiols on Cys residues of select proteins are among the most sensitive sites to be modified by ROS. For example the reactive Cys on phosphatases (3) or protein kinase C (4) can be modified by ROS and change their function. Oxidants react with these redox-sensitive thiols to form thyl radicals that subsequently can react with other thiols to form mixed disulfide bonds (5–7). In mammalian cells GSH is the most abundant low molecular thiol, which as a result is most likely to bind to protein thiols to form mixed disulphides, a process termed S-glutathiolation.

Many previous reports have demonstrated S-glutathiolation of (GSS-) isolated or purified proteins or in cells or tissues exposed to nonspecific thiol oxidants such as diamide or H2O2. A reactive thiol, Cys 118, has been identified in the small GTPase, Ras, which ROS alter cellular signaling is not established. Reactive thiols on Cys residues of select proteins are among the most sensitive sites to be modified by ROS. For example the reactive Cys on phosphatases (3) or protein kinase C (4) can be modified by ROS and change their function. Oxidants react with these redox-sensitive thiols to form thyl radicals that subsequently can react with other thiols to form mixed disulfide bonds (5–7). In mammalian cells GSH is the most abundant low molecular thiol, which as a result is most likely to bind to protein thiols to form mixed disulphides, a process termed S-glutathiolation.

The small GTPase, Ras, modulates diverse signaling pathways, and a reactive thiol, Cys118, has been identified in the GT-binding region of Ras. Previous work shows that Cys118 can be S-nitrosated, leading to an increase in Ras activity and to downstream signaling (9–11). However, Williams et al. (12) found that S-nitrosation itself may not directly change Ras conformation, thus questioning whether this modification could itself explain activation of Ras. S-Glutathiolation of the C-terminal Cys of Ras with diamide has been reported, but there have been no reports regarding S-glutathiolation of Cys118 on Ras affecting its activity.

Angiotensin II (AII) induces vascular smooth muscle cell (VSMC) hypertrophy, proliferation, and migration via ROS formation by stimulation of NAD(P)H oxidase, a process that may contribute to a variety of vascular diseases, such as hypertension, atherosclerosis, and restenosis (13–15). Ushio-Fukai et al. (16, 17) showed that AII-stimulated ROS from NAD(P)H oxidase plays a critical role in the activation of p38, Akt, but not ERK. They also showed that p38 and ERK pathways contribute synergistically to smooth muscle hypertrophy by showing that the MEK inhibitor, PD98059, and the p38 inhibitor, SB203580, each blocked ~40% of the hypertrophic response, whereas together they additively blocked the response. Therefore, AII-induced hypertrophy may be mediated in part by redox-sensitive activation of p38 and Akt and redox-insensitive activation of ERK. In this study we employed AII-stimulated VSMC as a model for examining the relationship between GSS-Ras formation and redox-sensitive hypertrophic signaling with adenovirus-mediated genetic modulation of NAD(P)H oxidase, glutaredoxin-1 (GRX-1) and Cys118 mutation of Ras (C118S Ras). Our studies indicate that oxidant-induced thiol modification of Ras mediates activation of p38 and Akt but not the ERK activation that contributes to the increase in protein synthesis.

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**EXPERIMENTAL PROCEDURES**

**Drugs and Antibodies**—Sulfo-NHS biotin and an EZ-link Ras activation kit were obtained from Pierce. PD-10 desalting columns, protein A-Sepharose, and streptavidin-Sepharose beads were obtained from Amersham Biosciences. Bradford protein assay, Bio-spin 6 column, polyvinylidene difluoride membrane, and other reagents for immunoblotting were obtained from Bio-Rad. Polyethylene glycol was obtained from Aldrich. AG1475 was obtained from Calbiochem (San Diego, CA). Other chemicals were from Sigma. Epiperal growth factor (EGF) and anti-Ras antibody (clone Ras10) were obtained from Upstate Biomedical (Golden, CO). Rabbit anti-mouse IgG antibody was obtained from Jackson ImmunoResearch (West Grove, PA). Anti-phosphorylated total Akt, p38, ERK, and p70S6 kinase antibodies were obtained from Cell Signaling (Beverly, MA). Anti-p47(phox) antibody was obtained from Transduction Laboratories (San Diego, CA). Anti-human catalase antibody was obtained from Novex (San Diego, CA). Rabbit anti-mouse IgG antibody was obtained from American Diagnostica Inc. (Greenwich, CT). Ras cDNA and adenoviral vector were obtained from Clontech (Palo Alto, CA). Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), PENTRD/Topo vector, and Gateway cassette were obtained from Invitrogen.

**Cell Culture**—Rat cultured VSMC were prepared as reported before ([16, 17]). The cells were grown with Dulbecco's modified Eagle's medium with 10% FBS and penicillin/streptomycin and quiescent with 0.1% FBS for 36–48 h before treating with Ali.

**Virus Preparation**—Adenoviral constructs were developed as previously described. The catalase (CATC) and dominant-negative p47(phox) (DN-p47(phox)) (S303A/S304A double mutant, gift from Dr. B. M. Babior (Upstate Biotechnology)) cDNAs were subcloned into the adenoviral shuttle vector (pShuttle), which contains the cytomegalovirus promoter. This shuttle vector was co-transfected with pADeAsy into Escherichia coli, the resultant cosmid was transfected with calcium phosphate into HEK 293 cells, and adenoviral plaques were selected. The adenovirus was propagated in HEK 293 cells and purified via a double cesium chloride gradient. Using the TCID50 method, the viral titer was determined. Similar methods were used for the cloning of β-galactosidase. The adenoviral vector to express GRX-1 was previously reported ([19]). Adenovirus with C118S Ras was made as follows. Ras cDNA was subcloned from Ras expression vector to pENTRD/Topo vector by PCR. Site-directed mutagenesis of C118 was introduced by 18 cycles of PCR subcloned from Ras expression vector to pENTRD/Topo vector by PCR. 

**Introduction**—The method followed those previously reported with some modifications ([6]). Biotinylated GSH ester was made by mixing 25 μM sulfo-NHS-biotin with 25 μM GSH ethyl ester in 50 mM NaHCO3, at pH 8.5 for 2 h followed by the addition of 125 mM NaHCO3, at pH 8.5 for 1 h. Biotinylated GSH ester (250 μM) was preincubated with VSMC in culture for 1 h. At each time point the cells were washed three times with cold phosphate buffer and lysed in buffer (Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 50 μM diethyltriaminopentaacetic acid, 2 mM phenylmethylsulfonyl fluoride) containing 10 mM N-ethylmaleimide to block further thiol reactions. Approximately 1 mg of protein was passed through a PD-10 Sephadex-G25 column to eliminate the excess low molecular weight biotin products. The proteins were mixed with streptavidin-Sepharose beads for 1 h. The beads were washed five times with lysis buffer with 0.1% SDS, and the final precipitate was incubated with 10 μg of elution buffer (lysis buffer + 200 μM DTT) to release S-glutathiolated proteins. After adding Laemmli buffer containing 5% β-mercaptoethanol, GSS-Ras was detected by immunoblotting with monoclonal anti-Ras antibody (UBI, clone Ras10).

**Lecine Incorporation**—The method followed previous reports ([2, 16, 17]). After quiescence, Ali (0.1 μM) was added to VSMC. After 1 h 1 μM/ml of [3H]leucine was added to each dish and incubated for 8 h. The cells were washed five times with phosphate-buffered saline and fixed with 10% trichloroacetic acid for 30 min at 4 °C. After washing with ice-cold water once, protein was isolated with 0.5 M NaOH for 90 min, and radioactivity was counted with a scintillation counter. The values for cells treated with Ali are normalized to the value obtained in untreated cells ([16]).

**Ras Preparation for MALDI-TOF Mass Spectrometry Analysis**—The cells were scraped into lysis buffer containing iodoacetic acid (10 mM), and the proteins were separated with centrifugation (10,000 × g for 15 min). After 1 h the proteins were passed through a PD-10 Sephadex-G25 column to eliminate excess iodoacetic acid. The lysate (2–3 mg of protein) was prewashed with protein A-Sepharose and incubated overnight with monoclonal anti-Ras antibody (25 μg). Thereafter, protein A (~100 μg) was added with rabbit anti-mouse IgG antibody (30 μg). The immunoprecipitate was washed with nonreducing Laemmli buffer (50 μl), and 40 μl of sample was separated by nonreducing SDS-PAGE and stained with Coomassie Blue. Approximately 10 μl of sample was used for immobiloblotting Ras to identify the major band. The Ras protein band was cut from the gel, and protein was digested in the gel with trypsin (Sequencing grade; Sigma) and dried with a Speedvac (Savant, Albertville, MI). GSS-Cys containing peptides of Ras were detected by MALDI-TOF mass spectrometry and analyzed with the SWISS-PROT data base found on the Profound Web site (prowl.rockefeller.edu/cgi-bin/ProFound). Reduced Cys- and GSS-Cys-containing peptides were identified on the basis of the predicted increase in mass (RSSG-C3H7N3O6S). Accepted fit parameters for peptide mass were <100 ppm for monoisotopic mass, <200 ppm for average mass, and up to four trypticcleavages. The data fit well with the protein identified in the data base as gi 131873 sp P20171 RASH RAT TRANSFORMING PROTEIN P21/H-RAS-1 (C-H-RAS).

**Data Analysis**—For all of the experiments employing immunoblots, similar results were obtained in at least three or four separate experiments. The increase in GSS-Ras and Ras activity caused by Ali was confirmed by densitometry (Molecular Analyzer, Hercules, CA), and differences in band densities were analyzed with Student’s t test. p < 0.05 was considered to be statistically significant. Differences in the percentage of increase in [3H]leucine incorporation between untreated and Ali-treated cells were analyzed with Student’s t test. p < 0.05 was considered to be statistically significant.

**RESULTS**

All-induced S-glutathiolation and Thiol-redox-sensitive Activation of Ras in VSMC—All (0.1 μM) increased ROS formation in VSMC as detected by 2,7’-dichlorofluorescein fluorescence (data not shown) as shown previously ([16]). As detected by biotinylated GSH, Ali (0.1 μM) increased GSS-Ras formation (3.6 ± 0.4-fold at 15 min p < 0.05, n = 5; Fig. 1A). GSS-Ras was increased by Ali in a concentration-dependent manner (Fig. 1B). H2O2 (250 μM) also increased GSS-Ras (Fig. 1B). Increased GSS-Ras was noted as early as 5 min, peaked at 30 min, and decreased at 60 min (Fig. 1C). Formation of GSS-Ras preceded the phosphorylation of p38 or Akt but not ERK (Supplemental Fig. 1). This suggests temporal correlation of GSS-Ras formation with activation of p38 and Akt, two downstream mediators previously shown to be involved in Ali-induced protein synthesis.
S-Glutathiolation of Ras by Angiotensin II

Effects of C118S Ras Expression on AII-induced Redox-sensitive Signaling—To test the functional importance of S-glutathiolation, Cys118 on Ras was mutated to serine (C118S Ras), overexpression of GRX-1 markedly decreased phosphorylation of p38 and Akt but was without effect on ERK (Fig. 3B). GRX-1 overexpression also inhibited the induction of protein synthesis (Fig. 3C).

The Identification of GSS-Cys on Ras by AII with MALDI-TOF Mass Spectrometry—To confirm that GSS-Ras is formed by endogenous GSH pools and to identify GSS-Cys sites, Ras was immunopurified from AII-treated VSMC, separated by SDS-PAGE, and trypsinized in gel. The peptides were analyzed by MALDI-TOF mass spectrometry, and reduced HS-Cys and GSS-Cys on Ras were analyzed (Table I). Approximately 60% coverage of the sequence of H-Ras was achieved, and three GSS-Cys sites were identified (Cys80, Cys118, and one of the C-terminal Cys181, Cys184, or Cys186).

Effects of C118S Ras Expression on AII-induced Redox-sensitive Signaling—To test the functional importance of S-glutathiolation, Cys118 on Ras was mutated to serine (C118S Ras), incorporated into an adenoviral vector, and overexpressed in VSMC (16, 17). To determine the importance of the thiol modification of Ras, Ras GTP binding activity was assessed with a GST-Raf pull-down assay. Ras activity was increased 2-fold at 15 min. Importantly, the increase in Ras activity was eliminated by DTT (Fig. 1D), suggesting that, like GSS-Ras formation, activation of Ras by AII is dependent on thiol-redox status.

AII-induced GSS-Ras and Ras Activation Were Mediated by ROS from NAD(P)H Oxidase—A NAD(P)H oxidase inhibitor, diphenyleneiodonium chloride (DPI; 10 μM, 30 min) decreased GSS-Ras as well as the activation of Ras by AII at 15 min (Fig. 2A). Previous reports showed that phosphorylation of the NAD(P)H oxidase subunit, p47phox, also assessed downstream signaling events affected by ROS, including H2O2, which has been implicated in hypertrophic signaling by AII (16, 17), catalase or DN-p47phox (18) was overexpressed in VSMC to decrease the reduction of GSS-Ras formation and the activation of Ras, as well as the induction of protein synthesis by AII. A, an NAD(P)H oxidase inhibitor DPI (10 μM, 30 min) decreased GSS-Ras formation (top row) and Ras activation by AII (middle row; 4.0 ± 0.7 versus 2.5 ± 0.6-fold of Control, without or with DPI, respectively, p < 0.05, n = 4) without change in total Ras (bottom row). B, overexpression of catalase or DN-p47phox decreased GSS-Ras formation (top row) and Ras activation by AII (middle row) without change in total Ras (bottom row). Overexpression of catalase (C) or DN-p47phox (D) also decreased the incorporation of [3H]leucine caused by AII.

Overexpression of GRX-1 decreases GSS-Ras formation, Ras activation, phosphorylation of p38 and Akt, and the induction of protein synthesis by AII. A, overexpression of GRX-1 decreased GSS-Ras formation (top row) as well as the increase in activity of Ras caused by AII (middle row), without change in total Ras (bottom row). B, overexpression of GRX-1 decreased phosphorylation of p38 and Akt but not ERK by AII. C, overexpression of GRX-1 also decreased the incorporation of [3H]leucine caused by AII.

The time course (Fig. 1D) indicated that GSS-Ras formation is reversible, suggesting that an enzymatic process may be involved in the reduction of S-glutathiolated Ras. Thiol-disulfide exchange of GSS-protein mixed disulfides is specifically regulated by the enzyme GRX, which in turn is regulated by GSH and GSH reductase (5, 19). To test the specificity of S-glutathiolation for the activation of Ras, GRX-1 was overexpressed in VSMC. Overexpression of GRX-1 inhibited the increase in Ras activity and GSS-Ras formation caused by AII, indicating the requirement of GSS-Ras formation for the increase in activity (Fig. 3A). Similar to the effects of inhibiting ROS production, expression of GRX-1 markedly decreased phosphorylation of p38 and Akt but was without effect on ERK (Fig. 3B). GRX-1 overexpression also inhibited the induction of protein synthesis (Fig. 3C).

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MALDI-TOF mass spectrometry analysis of GSS-Cys modified peptides of Ras from AII-treated VSMC

| Reduced Cys (-SH, IAA-SH) | Measured mass | Computed mass | Error | Start | End | MetO | IAACYS | Sequence |
|--------------------------|---------------|---------------|-------|-------|-----|------|--------|----------|
| Cys^21                    | 2886.4        | 2886.2        | 80    | 43    | 65  |      |         | QQVIGDETCLLDLIDTAQGEEEYSAMR |
| Cys^118                   | 2876.3        | 2876.3        | -4    | 103   | 128 |      |         | +VRKSDVYPVLFYGLCVEGLAAR7V3R  |
| Cys^181, Cys^184, Cys^186 |              |               |       |       |     |      | ++      | EIRQNHKLNPDESGLGCMCQK  |
| Cys^181, Cys^184, Cys^186 |              |               |       |       |     |      | ++      | QKRLNPDESGLGCMCQK  |

S-Glutathiolated Cys (GSS-Cys)

| GSS-Cys^80                 | 4303.0        | 4303.8        | -11   | 69    | 101 |      |         | DQYMTQEGFLCVPAINNTKSPEDIHQREQIK |
| GSS-Cys^118                | 2707.3        | 2707.1        | 72    | 102   | 123 |      |         | RVKSDVYPVLFYGLCVEGLAAR |
| GSS-Cys^181, -Cys^184, -Cys^186 | 2386.8      | 2386.8        | 13    | 170   | 189 |      |         | KLIIIPDESGLGCMCQK  |
|                            | 2708.5        | 2708.1        | 143   | 170   | 189 |      |         | KLIIIPDESGLGCMCQK  |

**DISCUSSION**

It is now well recognized that AII increases production of oxidants derived from NAD(P)H oxidase, which play an important role in vascular hypertrophy and the pathogenesis of vascular diseases (13–15). Previous reports indicate that the increased oxidants activate p38 and Akt, accounting for the redox-sensitive component of signaling leading to hypertrophy caused by AII (16, 17); however, an upstream direct molecular target for ROS has not been identified. In this study the activation of Ras by S-glutathiolation at Cys^118 was identified to be a critical step in redox-sensitive signaling leading to AII-induced hypertrophy. Overexpression of GRX-1 confirmed the specificity of the activation of Ras by S-glutathiolation as well as its involvement in activating downstream signaling by AII. GSS-Cys^118 was identified with mass spectrometry and overexpression of C118S Ras mutant diminished p38 and Akt phosphorylation and protein synthesis (Fig. 6).

NAD(P)H oxidase is activated at the plasma membrane where we hypothesize it is well situated for ROS generated by it to attack Cys^118 and activate membrane-bound Ras. This hypothesis is supported by the fact that H_2O_2 increased formation of GSS-Ras (Fig. 1B), and either DPI or overexpression of...
catalase inhibited GSS-Ras formation and Ras activation (Fig. 2). These agents also inhibited p38 and Akt phosphorylation and the increase in protein synthesis by AII as shown previously (16, 17), and we further confirmed this by showing similar effects of overexpressing DN-p47\textsuperscript{phox}. Collectively, our data show that ROS from NAD(P)H oxidase are responsible for GSS-Ras formation and activation of Ras by AII, which is responsible for redox-sensitive signaling (p38, Akt) and induction of protein synthesis.

To identify the GSS-Cys site on Ras, we employed mass spectrometry, and three sites were identified (Cys\textsuperscript{80}, Cys\textsuperscript{118}, and one of the C-terminal Cys\textsuperscript{181}, Cys\textsuperscript{184}, or Cys\textsuperscript{186}). Among them, Cys\textsuperscript{118} is located in the GTP-binding region, and S-nitrosation of this Cys is associated with an increase in activity of purified Ras (9–11). We did not detect S-nitrosated Cys\textsuperscript{118} by mass spectrometry, nor is nitric oxide thought to be involved in AII signaling in VSMC. Therefore, it is reasonable to hypothesize that GSS-Ras formation on this Cys is responsible for the increase in Ras activity and for redox-sensitive signaling by AII. The specificity of the involvement of GSS-Ras for redox-sensitive hypertrophic signaling was tested with the overexpression of GRX-1, a cytosolic protein of the thioredoxin superfamily. GRX-1 inhibited Ras activation, p38 and Akt phosphorylation, and induction of protein synthesis by AII (Fig. 3), emphasizing the importance of the GSS-adduct of Ras in mediating redox-sensitive signaling.

It is controversial whether or not ERK activation by AII is redox-sensitive (16, 25), and our data show that AII-induced ERK phosphorylation is much less sensitive to overexpression of catalase, DN-p47\textsuperscript{phox}, and GRX-1, as well as the C118S Ras mutant compared with their effects on phosphorylation of p38 or Akt. Although Ras/Raf/MEK1/ERK has been widely regarded as a major pathway, it is not clear that AII exclusively activates ERK via this pathway in VSMC. Takahashi et al. (26) reported that Ras may not be involved in ERK activation by AII in rat aortic smooth muscle cells. Others showed that Raf activation by AII is not mediated by Ras but rather by protein kinase C (27). These reports may help to explain our findings that GSS-Ras is not required for ERK activation but plays a role in phosphorylation of p38 and Akt. Recent studies showed that transactivation of the EGF receptor may mediate ERK activation by AII (23), and the EGF receptor/Grb/mSOS/Ras is the major pathway by which EGF activates ERK. Therefore, we tested the effects of AG1478, an EGF receptor tyrosine kinase inhibitor, on formation of GSS-Ras and phosphorylation of ERK and Akt. Compatible with previous reports (23, 28), AG1478 partially inhibited ERK and Akt phosphorylation by AII but had no effect on GSS-Ras formation. Therefore, GSS-Ras formation by AII-stimulated ROS apparently is not a consequence of EGF receptor transactivation. Previously, the Ras C118S mutant was shown to have about 75% basal activity (9) and preserved ability to mediate growth factor-dependent mSOS-induced activation (10). However, the mutant prevented redox-dependent PI3 kinase activation. Therefore, GSS-Ras-mediated Akt and p38 phosphorylation and mSOS-dependent activation of Ras via EGF receptor transactivation may be separate signaling pathways and be localized in different cellular compartments. AG 1478 also decreased AII-induced Akt phosphorylation, suggesting that both GSS-Ras and EGF receptor transactivation by AII may contribute to Akt activation.

In previous reports, ischemia-reperfusion increased S-thiolation labeling of Ras in heart (29), and diamide increased GSS-Cys\textsuperscript{118} and Cys\textsuperscript{184} in recombinant Ras (11). These reports did not assess the changes in Ras activity caused by GSS modification. A recent report suggests that S-nitrosation of Ras Cys\textsuperscript{118} itself may not change the structure of Ras but that the increased activity of recombinant Ras was associated rather with the chemical process of S-nitrosation (12). This suggests that redox cycling of this critical thiol, possibly via either S-nitrosation or sulfenilation could be an intermediate in S-glutathiolation, which would be favored because of the abundance of intracellular GSH. Because the glutamate residue of GSH has an extra carboxyl group, S-glutathiolation (but not S-nitrosation or sulfenilation) adds a negative charge to modified proteins that together with the steric effects of the tripeptide might directly change enzyme activity analogous to protein phosphorylation. Although many studies have shown that thiol antioxidants like N-acetylcysteine inhibit redox-sensitive signaling, they could do so by increasing intracellular GSH levels, enhancing the activity of GRX, and affecting S-glutathiolation of key signaling proteins including Ras. A recent reported showed that fluid shear stress up-regulated GSH reductase activity in endothelial cells, inhibiting JNK activation by ROS (24). This result could be explained by the fact that GSH reductase activity couples with GRX for reducing the mixed disulfide. Thus, proteins in various signaling cascades could be reversibly regulated by S-glutathiolation, GRX, and GSH reductase to mediate redox-sensitive signaling in various cell types.

In summary, this study clearly shows, 1) that the ROS-mediated modification of a critical thiol of Ras is important in mediating the actions of AII, 2) that intracellular GSS-Ras formation increases following AII, 3) that GSS-Ras is responsible for a portion of increased Raf-binding activity, 4) that p38 and Akt activation are most affected by GSS-Ras, and 5) that these events contribute to hypertrophic signaling induced by AII.

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