N-Acetylcysteine Decreases Angiotensin II Receptor Binding in Vascular Smooth Muscle Cells

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Antioxidants seem to inhibit angiotensin II (Ang II) actions by consuming stimulated reactive oxygen species. An alternative hypothesis was investigated: Antioxidants that are also strong reducers of disulfide bonds inhibit the binding of Ang II to its surface receptors with consequent attenuation of signal transduction and cell action. Incubation of cultured vascular smooth muscle cells, which possess Ang II type 1a receptors, with the reducing agent n-acetylcysteine (NAC) for 1 h at 37°C resulted in decreased Ang II radioligand binding in a concentration-dependent pattern. NAC removal restored Ang II binding within 30 min. Incubation with n-acetylsertine, a nonreducing analogue of NAC, did not lower Ang II binding, and oxidized NAC was less effective than reduced NAC in lowering Ang II binding. NAC did not decrease Ang II type 1a receptor protein content. Other antioxidants regulated Ang II receptors differently: α-lipoic acid lowered Ang II binding after 24 h, and vitamin E did not lower Ang II binding at all. NAC inhibited Ang II binding in cell membranes at 21 or 37 but not 4°C. Dihydrolipoic acid (the reduced form of α-lipoic acid), which contains free sulfhydryl groups as NAC does, decreased Ang II receptor binding, and Ang II–stimulated inositol phosphate formation was decreased by preincubation with NAC (1 h) or α-lipoic acid (24 h) but not vitamin E. In conclusion, certain antioxidants that are reducing agents lower Ang II receptor binding, and Ang II–stimulated signal transduction is decreased in proportion to decreased receptor binding.

The octapeptide angiotensin II (Ang II) contributes to maintenance of blood pressure (BP) and, in excess, causes hypertension. Binding of Ang II to specific cell surface receptors initiates a signal transduction cascade that culminates in contraction, hypertrophy, and sodium reabsorption. Most Ang II actions are transduced through the Ang II type 1 (AT₁) receptor. Recent studies have demonstrated that reactive oxygen species (ROS) are important mediators of Ang II action. Ang II activates enzyme systems that create ROS (1–3), and ROS are formed in response to Ang II in a time- and concentration-dependent pattern (4–8). ROS are intermediates in the Ang II signal transduction cascade (9–12), antioxidants block Ang II–stimulated signal transduction (13–15), and ROS mediate Ang II–related vascular diseases (16–20).

The mechanisms by which antioxidants interfere with Ang II signal transduction have been only partially elucidated. The prevailing hypothesis is that antioxidants consume Ang II–stimulated ROS and thus prevent distal Ang II signaling. In this study, we addressed antioxidant interaction with the initial segment of the Ang II signal transduction cascade, the Ang II surface receptor. Ang II–stimulated signal transduction would be altered if antioxidants regulate Ang II surface binding, because second messenger responses to Ang II are tightly coupled to Ang II surface receptor density (21–25).

The literature regarding regulation of Ang II receptor binding by antioxidants is limited and conflicting. Because the chemical reducing agent dithiothreitol lowers binding capability of AT₁ receptors (26,27), presumably via interaction of its sulfhydryl groups with disulfide bonds in the ligand-binding moiety of the receptor, the antioxidant/reducing agent n-acetylcysteine (NAC) might act similarly. However, in cultured vascular smooth muscle cells (VSMC), incubation with NAC at 4°C did not alter Ang II radioligand binding (28), and the antioxidant vitamin E, which is not a reducing agent, decreased AT₁ receptor mRNA expression in peritoneal macrophages (29). Alternatively, ROS have been shown to decrease Ang II receptor mRNA levels and Ang II receptor density in VSMC (28,30). Antioxidants might reverse this mechanism, resulting in increased Ang II receptor mRNA levels and receptor density.

Because of this conflicting literature, we performed studies in cultured VSMC to determine whether antioxidant compounds decrease Ang II surface receptor binding. We used antioxidants that are potent chemical reducing agents (NAC), that can be converted to reducing agents (α-lipoic acid), and that are not reducing agents (vitamin E).

Materials and Methods

Cultured Cells

Most experiments were performed on VSMC, which contain Ang II AT₁a receptors. Under pentobarbital anesthesia, aortas from 150-g male
Sprague-Dawley rats were partially digested, adhered to culture flasks, and covered with minimal essential medium that contained 10% newborn calf serum, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated in humidified 5% CO2/95% air atmosphere until confluent. Medium was changed every 5 d, and cells were passaged every 7 to 10 d. Studies were performed on cells in passages 3 to 12. Cells exhibited characteristic stellate morphology and stained for α-smooth muscle actin (31). Endothelial cell contamination was minimal (25). For this and other animal studies, we followed National Institutes of Health guidelines, and they were approved by our Institutional Animal Care and Use Committee.

Human embryonic kidney (HEK) cells were transfected with DNA coding for an AT1a receptor–green fluorescence protein (GFP) fusion protein (AT1aR-GFP). DNA was transfected with Lipofectamine (Invitrogen, Carlsbad, CA), using 9 μg of plasmid DNA per 100-cm2 flask or 1 μg per cell culture well in 80% confluent cells. DNA for AT1aR-GFP was generated as described (32). The plasmid pCa18b, containing the rat AT1a receptor cDNA, was amplified by PCR and cloned into pCR II-Topo (Invitrogen). The insert was excised by XhoI/BamHI and subcloned into the expression vector pEGFP-N3 (Clontech, Palo Alto, CA) to form the AT1aR-GFP construct. Fidelity of expression was verified by sequencing. Transfected cells were selected with F12 medium that contained 400 μg/ml G418.

Radioligand Binding

Binding studies were performed on confluent VSMC in duplicate wells of 24-well plates. Binding buffer consisted of 50 mM Tris, 100 mM NaCl, 5 mM KCl, 5 mM MgCl2, 0.25% BSA, and 0.5 mg/ml bacitracin (pH 7.4). Incubation volume was 0.3 ml. Single concentration Ang II receptor binding studies were performed by adding 50 fmol of 125I–Ang II (New England Nuclear, Boston, MA) to all wells and 1 μM unlabeled Ang II to certain wells (to determine nonspecific binding, <15% of total). Competition binding studies were performed by adding 125I–Ang II (50 fmol) to all wells and 6 concentrations of unlabeled Ang II (0.5 nM to 10 μM) to various wells. Studies were performed at 4°C for 90 min to obtain binding equilibrium without receptor internalization (33). Free hormone was removed by washing three times with cold saline. Cells were solubilized with 0.1% SDS/0.1 N NaOH, and gamma radioactivity was counted. Receptor density and binding affinity were determined by Scatchard analysis. Ang II binding studies were also performed on VSMC membranes. Membranes were prepared from VSMC with a polytron and aliquotted into duplicate test tubes (3 to 5 mg of cell protein per test tube), and bound hormone was isolated from unbound hormone with a suction manifold. Methods for arginine vasopressin radioligand binding were the same as those described above for Ang II.

Inositol Phosphate Formation

VSMC in six-well plates were incubated with 5 to 10 μCi of [3H]inositol/well in inositol-deficient growth medium for 24 h. Steady-state uptake of [3H]inositol after 24 h was 300,000 to 500,000 cpm/well. After exposure to effectors, reactions were terminated with 20% TCA. Protein precipitates were discarded, and supernatants were extracted three times with diethyl ether. The upper ether phase was discarded. Samples were adjusted to pH 7 and transferred to AG1-X8 anion exchange resin columns (Bio-Rad, Hercules, CA). Radioactivity elutable with water and 5 mM sodium borate–60 mM sodium formate was discarded. Total inositol phosphates were eluted with 1.0 M ammonium formate in 0.1 M formic acid. Fractions were counted in a scintillation counter.

Immunoblotting

VSMC proteins were solubilized and subjected to SDS-PAGE under reducing conditions. After semidry transfer to polyvinylidene difluoride membranes, blocking buffer (5% defatted dried milk in 10 mM Tris, 150 mM NaCl, 1% Tween-20 [pH 8.0]) was added. Membranes were incubated overnight with primary antibody in blocking buffer. Membranes were washed, exposed to secondary antibody for 1 h, and then washed again. Immunoreactive bands were visualized by the CDP Star chemiluminescent method (New England Biolabs, Beverly, MA). Bands were scanned and quantitated with ScanAnalysis densitometry software (Elsevier/BIOSOFT, Ferguson, MD).

Immunoblotting of AT1a receptors was performed with 1:1000 dilution of a polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Specificity of the antibody was confirmed by us in preliminary studies: Rat VSMC, which contain AT1a receptors, displayed specific 125I–Ang II binding, inositol phosphate responses to Ang II, and reproducible bands on immunoblot, whereas A7r5 cells, which do not contain AT1a receptors, displayed no specific 125I–Ang II binding, no inositol phosphate responses to Ang II, and no bands on immunoblot.

Secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase at 1:1000 dilution. Immunoblotting of AT1aR-GFP was performed with 1:500 dilution of a mAb against GFP (BD Biosciences, Palo Alto, CA), and the secondary antibody was goat anti-mouse IgG conjugated to alkaline phosphatase at 1:1000 dilution.

Oxidation of NAC

NAC was oxidized by bubbling the reduced NAC stock solution with air for 1 h at room temperature, and analysis by atmospheric pressure chemical ionization mass spectrometry was performed. A single peak with a molecular weight of 163.9 was observed in the reduced stock sample, whereas the 1-h oxidation period resulted in >90% loss of the 163.9 peak and the appearance of multiple new peaks of larger and smaller sizes.

Protein Content

Cellular protein was measured by the Lowry method (34).

Statistical Analyses

Data were expressed as mean ± SEM. Comparisons of two group means were performed by unpaired t test, and comparisons of three or more group means were performed by ANOVA. Differences in slopes were assessed by trend analysis. Significance was assigned to the 0.05 level.

Results

Ang II Binding in Intact VSMC

Incubation with NAC for 1 h resulted in concentration-dependent decreases in 125I–Ang II surface binding (Figure 1) in a protocol that consisted of exposure to NAC at 37°C followed by radioligand binding at 4°C. When NAC exposure was extended beyond 1 h, decreases in binding persisted (Figure 2). NAC at 1 mM, a concentration achievable in blood after intravenous administration (35), lowered Ang II binding modestly (12%; Figure 2). Removal of NAC allowed recovery of Ang II binding within 30 min (Figure 2). Inclusion of 10 mM NAC in binding buffer at 4°C (rather than a 37°C preincubation) failed to lower Ang II binding (102 ± 4% of binding from VSMC unexposed to NAC; n = 6), as others have reported (28). To determine whether other antioxidants decrease Ang II binding, we exposed VSMC to vitamin E (nonreducing) or α-lipoic acid (be-
comes a reducing agent when modified intracellularly) before $^{125}$I–Ang II binding. Vitamin E did not alter Ang II binding after brief or prolonged incubations (Figure 1). In contrast, $\alpha$-lipoic acid or its reduced derivative dihydrolipoic acid that contains sulfhydryl groups elicited concentration-dependent decreases in Ang II surface receptor binding; longer incubations were required for $\alpha$-lipoic acid, whereas dihydrolipoic acid was effective after shorter exposures (Figure 3). To determine the specificity of NAC targets, we also studied arginine vasopressin radioligand binding. In contrast to Ang II binding, arginine vasopressin binding was not decreased by NAC at 37°C (5% of control [1 mM NAC], 6% of control [2 mM NAC], 6% of control [5 mM NAC], 7% of control [10 mM NAC]; n = 5).

Mechanisms of NAC Effect on Ang II Binding

Because NAC is acidic (it must be neutralized before use), acidity of the medium could have attenuated ligand–receptor interaction and lowered $^{125}$I–Ang II binding. After 1 h of incubation with vehicle or 10 mM NAC at 37°C, neither color (pH indicator) nor pH of the medium was different between treatments. NAC did not induce dehiscence of VSMC from the culture plate, because cell protein content ($\mu$g/well) was not different after NAC (222 ± 16) or vehicle (204 ± 9; n = 6 wells).

Scatchard plots of binding data after 10 mM NAC for 1 h at 37°C revealed no change in slope with decrease in x intercept (Figure 4), suggesting that NAC lowered surface receptor density rather than binding affinity. N-acetylserine, identical to NAC except for oxygen in place of sulfur, did not decrease Ang II binding (Table 1), demonstrating the importance of the sulfur atom rather than the general structure of NAC in decreasing the level of functional AT$_{1a}$ receptors. That oxidized NAC was much less effective than reduced NAC in lowering Ang II binding (Table 1) highlights the reducing properties of NAC. We also studied the ability of NAC to alter binding of Ang II to a fusion protein that consisted of the coding region (but not the regulatory region) of the AT$_{1a}$ receptor and GFP (AT$_{1a}$R-GFP), shown previously to bind Ang II and transduce its signal (32). Treatment of HEK cells that were transfected with DNA for AT$_{1a}$R-GFP with NAC for 1 h at 37°C resulted in concentration-dependent decreases in Ang II radioligand binding (99 ± 4% of control [1 mM NAC], 89 ± 5% of control [5 mM NAC], 70 ± 9% of control [10 mM NAC]; n = 6; P < 0.05, for 5 and 10 mM NAC compared with control). NAC did not lower Ang II receptor protein content as demonstrated by immunoblotting for AT$_{1a}$ receptors in VSMC and for AT$_{1a}$R-GFP in transfected HEK cells (Figure 5).
Ang II Binding in Membranes
When NAC was added to VSMC membranes, Ang II binding was decreased when experiments were performed at warm temperatures (markedly at 37°C, less pronounced at 21°C) but not at all at 4°C (Figure 6A). In similar experiments with VSMC membranes at 37°C, dihydrolipoic acid, which contains sulfhydryl groups, effected concentration-dependent decreases in Ang II binding, but α-lipoic acid, which does not contain sulfhydryl groups, did not alter Ang II binding (Figure 6B).

Effects on Signal Transduction
Cells were exposed to antioxidants under conditions used earlier in this study: 10 mM NAC for 1 h, 1 mM α-lipoic acid for 24 h, and 100 μg/ml vitamin E for 24 h. Then 100 nM Ang II was added for 1 min to stimulate inositol phosphates. Ang II stimulated inositol phosphates by 92% in the absence of antioxidants. Antioxidant conditions that resulted in decreased Ang II binding (NAC for 1 h or α-lipoic acid for 24 h) resulted in less Ang II–stimulated inositol phosphate formation, whereas a condition that did not alter Ang II binding (24 h of exposure to vitamin E) did not lower Ang II–stimulated inositol phosphate formation (Figure 7).

Discussion
Because ROS are second messengers for Ang II action (1–15), inactivation of ROS by antioxidants within the cell should interrupt the Ang II signal transduction cascade and attenuate Ang II action. However, we considered that antioxidants might also regulate Ang II action by another mechanism: Altering surface receptor binding for Ang II, the initial step in Ang II signaling.

Certain antioxidants that are also potent chemical reducers of disulfide bonds via their free sulfhydryl groups might interact with the disulfide bonds of the Ang II receptor, alter its tertiary structure, and inhibit Ang II–AT1a receptor interactions. The AT1a receptor contains two disulfide bonds in the extracellular region of the receptor: C18-C274 and C101-C180 (36–39). Two of the antioxidants used in our study, NAC and dihydrolipoic acid, can reduce disulfide bonds. Vitamin E and α-lipoic acid do not contain sulfhydryl groups, although the latter can be reduced to dihydrolipoic acid after cellular uptake. This may explain why α-lipoic acid was delayed in its ability to decrease...
Ang II binding compared with dihydrolipoic acid in intact VSMC (Figure 3) and why vitamin E did not decrease Ang II binding in any time frame (Figure 1). Consistently, dihydrolipoic acid but not \( \text{N} \)-acetyl-lipoic acid inhibited Ang II receptor binding in isolated membranes, presumably because broken cell preparations lack the biochemistry to convert \( \text{N} \)-acetyl-lipoic acid to dihydrolipoic acid. NAC or dihydrolipoic acid may mimic dithiothreitol, another potent reducing agent, in regulating Ang II binding; it is well documented that dithiothreitol inhibits binding of Ang II to AT1 receptors (26,27). The inability of \( \text{N} \)-acetylserine, which is structurally similar to NAC but contains a hydroxyl in the place of the sulfhydryl group, to decrease Ang II binding (Table 1) strongly suggests that the sulfhydryl group is important in inhibiting Ang II binding. Furthermore, oxidized NAC, whose free sulphhydryl groups have been modified, weakly lowered Ang II binding, whereas reduced NAC, whose sulphhydryl groups are intact, was more potent.

If NAC alters Ang II receptor tertiary structure, then binding affinity rather than apparent receptor density might be expected to be decreased. However, our results demonstrate the opposite (Figure 4). This result remains unexplained. It is interesting that at 4°C, NAC did not alter Ang II binding in intact cells (data in text) or in membranes (Figure 6). The effect of cold temperature on reduction of disulfide bonds by sulfhydryl groups is uncertain. The temperature effect might also be consistent with an effect of NAC on membrane fluidity and alterations of the physical state of Ang II receptors. It is most interesting that the Ang II AT1a receptor, which has four extra-

Table 1. Mechanisms of NAC effects on Ang II binding

<table>
<thead>
<tr>
<th>Experimental Maneuver</th>
<th>Ang II Binding (% of Control)</th>
<th>Comparison</th>
<th>( P ) Value</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>8</td>
</tr>
<tr>
<td>10 mM n-acetylserine</td>
<td>98 ± 4</td>
<td>With control</td>
<td>NS</td>
<td>8</td>
</tr>
<tr>
<td>10 mM reduced NAC</td>
<td>32 ± 2</td>
<td>With control</td>
<td>&lt;0.01</td>
<td>4</td>
</tr>
<tr>
<td>10 mM oxidized NAC</td>
<td>66 ± 8</td>
<td>With reduced NAC</td>
<td>&lt;0.05</td>
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\( ^{a} \)Antioxidant incubations were performed for 1 h at 37°C in vascular smooth muscle cells. NAC, \( \text{N} \)-acetylcysteine; Ang II, angiotensin II.
cellular cysteine residues and two extracellular disulfide bonds, was inactivated by NAC as evidenced by decreased Ang II radioligand binding, whereas the arginine vasopressin V1a receptor, which has only one extracellular cysteine residue and thus no extracellular disulfide bonds (40), was unaffected by NAC with regard to arginine vasopressin radioligand binding.

It is unlikely that the antioxidants that decrease Ang II binding do so by reducing AT1a receptor mRNA transcription, although we must consider this possibility, because ROS have been shown to downregulate Ang II receptors by this mechanism (28,30). The time course for the NAC effect on Ang II binding is very rapid (Figure 2) and thus unlikely to involve regulation of AT1a receptor mRNA levels. Similarly, NAC decreased Ang II binding in HEK cells bearing AT1aR-GFP, even though its DNA lacks a 5′ regulatory region (32). In addition, immunoblotting data (Figure 5), demonstrating an unchanged amount of Ang II receptor protein, are also not consistent with a gene regulation mechanism. It is inconceivable that NAC or dihydrolipoic acid can regulate Ang II receptors via modulation of AT1a receptor mRNA levels in a broken cell preparation (Figure 6).

We dissociated antioxidant effects of these compounds from their regulatory effects on Ang II receptor binding in a preliminary way. Both NAC (5) and vitamin E (41,42) have been shown to consume Ang II–stimulated ROS, yet these compounds regulate Ang II binding in distinctly different ways: NAC downregulated Ang II binding robustly, whereas vitamin E did not regulate Ang II binding at all (Figure 1).

Our study confirms that changes in Ang II receptor binding correlate well with changes in Ang II–stimulated signal transduction. In intact VSMC, NAC for 1 h decreased Ang II receptor binding (Figure 1) more than α-lipoic acid for 24 h (Figure 3), and incubation with vitamin E did not reduce Ang II binding (Figure 1). Consistently, NAC for 1 h reduced Ang II–stimu-
lated inositol phosphate formation more than α-lipoic acid for 24 h, and vitamin E did not inhibit Ang II–stimulated inositol phosphate formation (Figure 7). These data are consistent with previous data in demonstrating that Ang II surface receptor density is coupled closely with Ang II–stimulated cell responses and argue against the existence of spare Ang II receptors on the surface of VSMC (21–25).

NAC and α-lipoic acid both are in clinical use at present, the former to treat acetaminophen toxicity (43,44) and to provide prophylaxis against contrast-induced acute renal failure (45) and the latter for diabetic neuropathy (46,47). Our results raise the possibility that these agents may ameliorate renal and vascular diseases mediated by Ang II, especially if they mimic dithiothreitol in achieving the favorable combination of lower density is coupled closely with Ang II–stimulated cell responses in myogenic cells.

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