Angiotensin II Type 1 Receptor
Relationship With Caveolae and Caveolin After Initial Agonist Stimulation

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Abstract—Caveolae are membrane domains that have been implicated in signal transduction, and caveolins are major structural components of these domains. We found that all reported caveolin isoforms (caveolin-1, -2, and -3) were expressed in vascular smooth muscle cells (VSMCs); however, only caveolin-1 mRNA was regulated by angiotensin II (Ang II). Ang II (100 nmol/L) increased caveolin-1 mRNA, with a peak at 2 hours (193 ± 6% of control, \( P < 0.01, n = 4 \)). In contrast, Ang II significantly decreased caveolin-1 protein, with a nadir at 4 hours (64 ± 5% of control, \( P < 0.01, n = 6 \)). \(^{[35]}\)S\)Methionine labeling showed that Ang II increased caveolin biosynthesis (226 ± 33% of control labeling at 4 hours), suggesting that the transient decrease in caveolin protein levels is due to increased degradation. When cells were fractionated with sucrose, on agonist stimulation, AT₁ receptors appeared in fraction 5 where caveolin was fractionated. This migration was blocked by low temperature and treatment with phenylarsine oxide, interventions that interfere with agonist-induced Ang II type 1 (AT₁) receptor sequestration and tonic phase signaling. In addition, caveolin-1 coimmunoprecipitates with AT₁ receptor only on agonist stimulation. These data support the concept that the caveola is a specialized signaling domain in VSMCs that can be dynamically accessed by the AT₁ receptor. Because of the signaling and coupling proteins that are localized in caveolae and because of evidence that these proteins may interact directly with caveolin, caveolin–AT₁ receptor interaction likely represents an important focus for dynamic control of receptor signaling in VSMCs. (Hypertension. 1998;32:459-466.)

Key Words: angiotensin II • signal transduction • receptors, angiotensin

Angiotensin II (Ang II) is a pluripotent hormone in vascular smooth muscle cells (VSMCs).\(^1\) It activates several phospholipases, including phospholipase C-\(\beta_1\) (PLC-\(\beta_1\)),\(^2\) phospholipase D (PLD),\(^3\) cytosolic phospholipase A\(_2\),\(^4\) and PLC-\(\gamma\), the PLC isoform originally associated with activation of receptor tyrosine kinases.\(^5\) In addition to stimulating both influx of calcium and its release from intracellular stores, Ang II activates protein kinase C and mitogen-activated protein kinases and stimulates VSMC hypertrophy and growth.\(^6\) Furthermore, Ang II transmodulates the activity of the platelet-derived growth factor (PDGF)-\(\beta\) receptor by inducing its tyrosine phosphorylation in a manner that is independent of PDGF ligand.\(^7\) The organizing principles controlling the orderly expression of these multiple signaling pathways are incompletely understood.

We have provided evidence for both temporal and spatial components of the organizing scheme regulating Ang II signaling in VSMCs. Thus, Ang II stimulation of diacylglycerol formation is distinctly biphasic, with an initial (<5 second) phase that rapidly desensitizes (~2 minutes) and a subsequent sustained phase that continues for >30 minutes.\(^8\) The initial phase is mediated by PLC-\(\beta\)-activation coupled to \(G_{\text{q}}\), \(G_{\text{q12}}\), and \(G_{\beta\gamma}\) subunits.\(^9\) Sequestration of the Ang II type 1A (AT₁A) receptor into what we postulated to be a discrete signaling domain is an absolute requirement for the development of the sustained phase of diacylglycerol generation,\(^9\) which is mediated by PLD activation with conversion of phosphatidic acid to diacylglycerol by phosphatidic acid phosphohydrolase.\(^10\) PLD activation is dependent on \(\beta\gamma\) derived from \(G_{\text{q12}}\) (Ushio-Fukai M, Alexander RW, Griendling K, unpublished observations, 1998), but the identity of the putative sequestered signaling domain is unknown.

We postulated originally that the tonic-phase signaling domain was the coated pit. The coated pit subsequently has become associated with internalization of phosphorylated, desensitized \(G\) protein–coupled receptors generally, and the \(\beta\)-adrenergic receptor particularly,\(^11\) making it an unlikely candidate for continued signaling. Anderson et al\(^12,13\) have demonstrated the movement of AT₁ receptors after agonist activation to both coated and noncoated pits, with the subsequent formation of large vesicular bodies in VSMCs in culture and in intact aorta. The identity of these noncoated pits and vesicular bodies has not been established, but they bear some structural similarity to caveolae.

Caveolae are small invaginations, located at or near the plasma membrane, that are characterized by the presence of caveolin, a 21- to 24-kDa cytoskeletal protein that exists as several isoforms (caveolin-1, -2, and -3).\(^14\) The caveola is an attractive candidate as the domain in which the tonic-phase Ang II signaling is generated. Recently, it has been reported
that several hormone or growth factor receptors (endothelin-1, PDGF, epidermal growth factor [EGF] and signaling molecules (plasma membrane calcium pump, inositol 3,4,5-triphosphate receptor-like protein, src-related kinases, ras, and ras-1) are localized in these regions, suggesting that caveolae may function as unique cell surface signal transduction domains. This concept is supported by the observation that signal transducing proteins are rapidly recruited into caveolae upon PDGF and EGF stimulation. Furthermore, it has been reported recently that agonist stimulation of the cardiac m3 muscarinic cholinergic receptor promotes its translocation into a low-density gradient fraction, presumably caveolae. Caveolae are also involved in receptor-mediated endocytosis and transcytosis.

The general concept of the caveola as a discrete signaling domain has been challenged, at least in endothelial cells. Thus, an immunologically based approach to isolation failed to confirm that caveolae are strikingly enriched in signaling and coupling molecules. The major impediment to assigning a critical role to caveolae in signal generation appears to be inherent in the difficulty in isolating a purified caveolar fraction. The discrepancies in the accumulated data and their interpretation in endothelial cell “caveolar fractions” illustrate this point. Thus, endothelial caveolar-enriched fractions isolated using traditional cell fractionation approaches are said to be enriched in signaling molecules, whereas fractions derived by antibody-absorption approaches are not. Given these difficulties, providing evidence that the caveola is a signaling domain for the AT1A receptor in VSMCs would appear to require functional as well as cell fractionation data associating the receptor with this structure.

To test the hypothesis that the sequestered signaling domain to which the AT1A receptor in VSMCs moves after initial agonist stimulation is the caveola, we took complementary approaches. First, using gradient centrifugation, we showed that Ang II stimulated movement of the AT1A receptor into a caveolin-enriched membrane fraction. Second, we demonstrated that Ang II stimulated increased turnover of caveolin-1 protein, a finding consistent with agonist-induced increases in internalization and degradation of the caveolar structure. Finally, it was found that anti-AT1A receptor antibody communoprecipitated caveolin-1. These data provide compelling evidence for the stimulated association of the receptor with the caveola, which then is internalized. Inferentially, these events are likely associated with the sustained phase of the Ang II signaling sequence.

Methods

Reagents
Canine caveolin-1 cDNA (Genbank Accession #U47060) and rat caveolin-3 cDNA (Genbank Accession #U31968) were kind gifts from Dr Michael P. Lisanti (Massachusetts Institute of Technology). Losartan was a kind gift from Dr R.D. Smith (DuPont/Merck). Ang II, Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid), genistein, phenylarsine oxide, and DMEM were purchased from Sigma Chemical Co. n-Octylglucoside was purchased from Boehringer Mannheim, and GF109203X was from LC Laboratories. Ionomycin was from Calbiochem. [32P]dCTP, [35S]methionine (1175 Ci/mmol), and Enhanced chemiluminescence (ECL) Western blotting labeling kits were from Stratagene, and the ECL Western blotting detection system was purchased from Amersham Life Sciences. Monoclonal antibodies (mAb) caveolin-1 (clone 2297), anti-caveolin-3 IgG (mAb, clone 26), anti-CD36 IgG (mAb, clone 73), and anti-dynamin IgG (mAb, clone 41) were purchased from Transduction Laboratories. mAb against caveolin (clone Z034) was from Zymed Laboratories Inc. Protein A/G Plus-Agarose and anti-AT1 receptor antibody (N-10) were purchased from Santa Cruz Biotechnology Inc.

Cell Culture
VSMCs were isolated from the thoracic aorta of male Sprague-Dawley rats by enzymatic digestion as described previously and maintained in DMEM supplemented with 10% calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. For studies on the effect of Ca2+ on caveolin-1 regulation, cells were incubated in balanced salt solution (BSS) (mmol/L: NaCl 130, KCI 5, MgCl2 1, CaCl2 1, 5, pH to 7.4 with Tris base) or BSS without calcium for 15 minutes before the addition of Ang II. For experiments, cells between passages 6 and 15 were used at confluence.

RNA Isolation and Northern Blot Analysis
Total RNA was extracted from cells as described previously and separated on a 1.0% agarose gel containing 6.6% formaldehyde. RNA was then transferred to a Nytran membrane and immobilized by UV cross-linking. The expression of caveolin-2 in VSMCs was confirmed by reverse transcription–polymerase chain reaction (RT-PCR) and sequencing. The forward primer was 5′-TCGATCTCAGCTGAGTTT-3′, and the backward primer was 5′-GCTCTGATACAATGGAGCAATGAT-3′, which are identical to nt 100 to 119 and nt 379 to 402 of the human cDNA sequence, respectively. After the first strand, cDNA was synthesized from 1 µg of total rat VSMC RNA using murine leukemia virus reverse transcriptase; PCR amplification was performed with deoxynucleoside triphosphate and Taq DNA polymerase for 30 cycles: 1 minute at 95°C, 2 minutes at 50°C, and 1.5 minutes at 72°C. After purification of the PCR product, cycle sequencing was performed. The deduced amino acid sequence of the RT-PCR product had 97% homology to human caveolin-2, thus, it was identified as rat caveolin-2. The full-length canine caveolin-1 cDNA, rat caveolin-2 RT-PCR product, or full-length rat caveolin-3 cDNA were labeled with [α-32P]dCTP in a random primed reaction using the Prime-It II kit. After UV cross-linking, membranes were prehybridized and hybridized as described previously and washed twice in 1× SSC plus 0.1% SDS at 55°C and once in 0.5× SSC plus 0.1% SDS at 60°C. Autoradiograms were quantified with an imaging densitometer using the Molecular Analyst software (Bio-Rad Laboratories). Band density was normalized to the intensity of ethidium bromide–stained 28S ribosomal RNA after transfer to the membrane.

Protein Purification and Immunoblot Analysis
VSMCs were washed 3 times with ice-cold PBS and lysed with 1 mL Buffer A (mmol/L: HEPES 50, EDTA 5, NaCl 50, pH 7.5) containing 1% Triton X-100, 60 mmol/L n-octylglucoside, protease inhibitors (10 µg/mL aprotinin, 1 mmol/L PMSF, and 10 µg/mL leupeptin), and phosphatase inhibitors (50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and 10 mmol/L sodium pyrophosphate). Extracted protein was quantified by the Bradford assay. Proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Membranes were blocked with PBS containing 5% nonfat dry milk, 2% BSA, and 0.2% Tween 20, incubated with caveolin-1 mAb, washed, and incubated with horseradish peroxidase–conjugated goat anti-mouse antibody. The ECL Western blotting system was used for detection.

Immunoprecipitation
VSMCs were lysed with Buffer A as described above. Samples were then divided into 2 tubes (400 µL each), and immunoprecipitation was performed using Protein A/G Plus-Agarose and either anti-AT1 receptor antibody (3 µg/mL) or preimmune rabbit IgG. The speci-
ficity of this antibody under these conditions has been shown previously by our laboratory. After extensive washing with Buffer A containing both Triton X-100 and n-octylglucoside, samples were separated by SDS-PAGE, transferred to PVDF membranes, and probed with mAb against caveolin-1.

**Biosynthetic Labeling Experiments**

VSMCs were washed with methionine-free DMEM and incubated for 4 hours in this same medium supplemented with 10% calf serum, 2 mmol/L glutamine, and [35S]methionine (100 μCi/mL) in the presence or absence of Ang II (100 nmol/L). Cells were washed in PBS and lysed in Buffer A containing both Triton X-100 and n-octylglucoside. Protein concentration was measured, and 1 mg of each sample was immunoprecipitated with either anti-caveolin antibody (Z034) or mouse IgG. Immunoprecipitated samples were dissolved in 45 μL Laemmli buffer containing 5% β-mercaptoethanol, and 20 μL of each sample was subjected to 12% SDS-PAGE. Gels were incubated with En/Hance and autoradiographed overnight. To determine the radioactive band corresponding to caveolin, duplicate gels were blotted onto PVDF membranes and probed with mAb against caveolin-1. Immunoblots were visualized with ECL and excised from the membrane, and the radioactivity of the bands was quantified by liquid scintillation spectroscopy.

**Cell Fractionation**

Caveolin-enriched membrane fractions were prepared using the method of Li et al with minor modifications. Briefly, cells were washed with ice-cold PBS and scraped into 2 mL of 500 mmol/L sodium carbonate (pH 11.0) with protease inhibitors (0.2 mmol/L PMSF and 20 μg/mL leupeptin). Cells were then subjected to Dounce homogenization (150 strokes) followed by three 20-second bursts of sonication (Micro Ultrasonic Cell Disrupter, Kontes). The homogenate was adjusted to 45% sucrose by mixing with 2 mL of 90% sucrose prepared in MBS (25 mmol/L Mes, pH 6.5, 0.15 mol/L NaCl) and placed at the bottom of an ultracentrifuge tube. A 5% and 35% discontinuous sucrose gradient was formed above (4 mL of 5% sucrose in MBS containing 250 mmol/L sodium carbonate with 0.1 mmol/L PMSF and 10 μg/mL leupeptin). Samples were then centrifuged at 260 000g for 16 to 20 hours at 4°C. Fractions (1 mL) were removed sequentially from the top and designated as fractions 1 through 12. Protein concentration in each fraction was quantified by the Bradford assay.

**Statistical Analysis**

Data are expressed as mean±SEM. Overall statistical significance was assessed by ANOVA on untransformed data, followed by comparison of group averages by contrast analysis, using the SuperANOVA statistical program (Abacus Concepts). A value of P<0.05 was considered to be statistically significant.

**Results**

**Expression of Caveolin Subtypes in VSMCs**

To examine the expression of caveolin isoforms in VSMCs, Northern blot analysis using specific probes for caveolin-1, caveolin-2 (rat RT-PCR product, see Methods), and caveolin-3 was performed. As shown in Figure 1, all 3 isoforms are present in VSMCs.

**Subcellular Distribution of Caveolin and AT<sub>1</sub> Receptor**

Previous reports have suggested that agonist-induced AT<sub>1</sub> receptor internalization occurs via both clathrin-coated vesicles and noncoated vesicles. To provide insight into the membrane fractions with which agonist-stimulated AT<sub>1</sub> receptors are associated, we evaluated receptor distribution in detergent-free sucrose gradient fractions of VSMCs. Equal aliquots of fractionated VSMC homogenates were separated by SDS-PAGE. Coomassie R250 staining showed that most of the cellular protein fractionated in fractions 9 to 12 (Figure 2, top). This pattern did not change substantially after Ang II stimulation. Both caveolin-1 and caveolin-3 predominantly migrated in fractions 5 and 6. (Caveolin-2 could not be detected because of the lack of an available specific antibody.) In contrast, CD36 (an 88-kDa plasma membrane protein), clathrin, and dynamin were located in fractions 11 to 12, fractions 9 to 12, and fractions 9 to 12, respectively. These results indicate that caveolin-1 and caveolin-3 were enriched in fractions 5 and 6, but predominantly in 5, and were well separated from the bulk of cellular proteins and from coated vesicles (Figure 2, bottom). Approximately 2.7% of harvested protein from VSMCs migrated in fraction 5.

To determine whether the AT<sub>1</sub> receptor migrates to caveolin-enriched membrane fractions on Ang II stimulation,
we compared the distribution of AT$_1$ receptors in homogenate fractions from untreated cells and cells stimulated with Ang II (100 nmol/L). In untreated cells, the majority of the AT$_1$ receptors were localized in fractions 9 to 12 (Figure 3A). After 5 minutes of Ang II stimulation, a portion (~20%) of the AT$_1$ receptors appeared in the caveolin-enriched fraction. The distribution of AT$_1$ receptors between the caveolin-enriched and nonenriched membrane fractions was similar after 10 minutes of Ang II. Movement of the receptor into fraction 5 was blocked by the AT$_1$ receptor antagonist losartan (100 μmol/L), indicating that this is a specific, agonist-mediated event. In contrast, Ang II stimulation did not change the location of caveolin-1 or caveolin-3. AT$_1$ receptors migrated to the caveolin-enriched fraction by 1 minute and remained there for as long as 10 minutes (Figure 3B). Ionomycin, which increased caveolin-1 mRNA (see below), however, did not cause migration of AT$_1$ receptors into this fraction (n=2). In cells incubated with 100 nmol/L Ang II at 19°C, movement of the AT$_1$ receptor into the caveolin-enriched fraction was markedly inhibited (Figure 3C, left). Incubation of cells with phenylarsine oxide also slowed the movement of the AT$_1$ receptor into the caveolin-enriched fraction. In previous reports, we showed that these interventions effectively inhibited sequestration of the Ang II–receptor complex.\footnote{Effect of Ang II on Caveolin-1 Levels}

If the agonist-occupied AT$_1$ receptor is internalized via caveolae, Ang II stimulation might result in decreased caveolin protein levels due to degradation subsequent to internalization. To test this hypothesis, we performed immunoblot analysis in cells stimulated with Ang II for prolonged intervals. As shown in Figure 4A, both the α- and β-isofoms of caveolin-1 were detected in VSMCs. Both subtypes of caveolin-1 protein were significantly decreased after 4 and 8 hours of Ang II treatment and returned to the control level by 24 hours (Figure 4A).

This decrease in caveolin protein levels could also be a consequence of reduced transcription or enhanced mRNA degradation. Therefore, we tested the effect of Ang II on caveolin mRNA expression in VSMCs. In VSMCs stimulated with 100 nmol/L Ang II for 1 to 24 hours, Ang II significantly increased caveolin-1 mRNA levels as early as 1 hour, with a peak at 2 hours (Figure 5A), in contrast to its effect on protein levels. The upregulation of caveolin-1 mRNA was clearly dose-dependent (Figure 5B). In contrast, neither caveolin-2 nor caveolin-3 mRNA was significantly regulated by Ang II (data not shown). These results suggest that Ang II does not decrease caveolin transcription; rather it increases caveolin-1 mRNA levels.
mRNA levels, perhaps in a compensatory fashion. They also indicate that Ang II and the AT\(_1\) receptor preferentially associate with the caveolin-1 isoform in VSMCs.

To gain insight into the mechanisms responsible for the regulation of caveolin-1 mRNA by Ang II to determine whether they are distinct from those regulating protein levels, we tested the effect of inhibitors of known signaling pathways of Ang II. Caveolin-1 mRNA upregulation was completely blocked by the specific AT\(_1\) receptor antagonist losartan (10 \(\mu\)mol/L) (losartan + Ang II, 97±2% of losartan alone; \(n=4\)), indicating that this is a specific, AT\(_1\) receptor–mediated event (Figure 6A). Neither the protein kinase C inhibitor GF109203X (3 \(\mu\)mol/L) (GF109203X + Ang II, 194±5% of GF109203X alone; \(n=4\)), the tyrosine kinase inhibitor genistein (100 \(\mu\)mol/L) (genistein + Ang II, 195±6% of genistein alone; \(n=4\)), nor the membrane-permeable, nonenzymatic superoxide scavenger Tiron (10 mmol/L) (Tiron + Ang II, 196±5% of Tiron alone; \(n=4\)) had any effect on Ang II–induced caveolin-1 mRNA upregulation at 2 hours (response to Ang II alone was 193±6% of control at 2 hours, Figure 6A). Removal of extracellular calcium, however, blocked the Ang II–induced upregulation of caveolin-1 mRNA (Figure 6B). Similar results were obtained when EGTA was included in the assay buffer. Under these conditions, the Ang II–induced \(\text{Ca}^{2+}\) transient is markedly reduced, and sustained \(\text{Ca}^{2+}\) influx is completely abol-

Figure 5. Ang II regulates caveolin-1 (Cav-1) mRNA levels. A, Ang II (100 nmol/L) was added to standard culture medium containing 10% calf serum for the indicated times. Top, Representative Northern blot; bottom, line graph summarizing data obtained from 4 experiments. B, Various concentrations of Ang II were added to cultured VSMCs for 2 hours. Top, Representative Northern blot; bottom, line graph summarizing data obtained from 4 experiments. Data are expressed as mean±SEM of the percent increase over the control level. *\(P<0.05\) and **\(P<0.01\) compared with control.

Figure 6. Effect of various inhibitors and calcium ionophore on caveolin-1 (Cav-1) mRNA expression. A, Ang II (100 nmol/L) was added for 2 hours with or without indicated inhibitors. Losartan (10 \(\mu\)mol/L), Tiron (10 mmol/L), GF109203X (GF; 3 \(\mu\)mol/L), and genistein (100 \(\mu\)mol/L) were added to the cells 10 minutes before Ang II (100 nmol/L). This result is representative of 4 experiments. B, VSMCs were incubated with Ang II (100 nmol/L) for 2 hours in BSS buffer with or without calcium (see Methods). Top, Representative Northern blot; bottom, bar graph summarizing data from 4 experiments. Data are expressed as mean±SEM of the percent of the control level of calcium-deprived cells. *\(P<0.05\) compared with nontreated VSMCs. C, Effect of calcium ionophore ionomycin. Ionomycin (15 \(\mu\)mol/L) was added to VSMCs for 2 hours. This result is representative of 6 experiments.

lished (data not shown). These results suggest that \(\text{Ca}^{2+}\) mobilization mediates caveolin-1 mRNA upregulation. To further confirm this hypothesis, we tested the effect of ionomycin (15 \(\mu\)mol/L), a calcium ionophore, on caveolin-1 mRNA levels. As shown in Figure 6C, ionomycin (2 hours) upregulated caveolin-1 mRNA to a similar extent (196±5% of control, \(n=4\)) as did Ang II. Importantly, when cells were treated with ionomycin (15 \(\mu\)mol/L) for 2 hours, caveolin-1 protein levels were also significantly increased (140±10% of control, \(P<0.05\), \(n=3\)) (Figure 4B). These findings suggest that Ang II may have a direct effect to enhance caveolin-1 protein degradation by mechanisms that are distinct from the calcium-dependent mechanisms leading to an increase in mRNA.

To test this hypothesis, we labeled cells with \(^{35}\text{S}\)methionine to study the effect of Ang II on the biosynthesis of caveolin. Although immunoreactive caveolin protein was decreased at 4 hours after Ang II stimulation (Figure 7A, top), the incorporation of \(^{35}\text{S}\)methionine into caveolin was significantly higher than control (Figure 7A, bottom). These results indicate that Ang II stimulation increase de novo caveolin protein synthesis as would be predicted from the increase in mRNA. Therefore, the decrease of immunoreactive protein 4 hours after Ang II stimulation is most likely due
to increased protein degradation because it is internalized with the AT1 receptor, with an associated increase in caveolin-1 turnover.

**Coprecipitation of AT1 Receptor and Caveolin**

The apparent association between AT1 receptor internalization and caveolin turnover suggests that caveolin-1 and the AT1 receptor may become physically associated on agonist stimulation. To assess this possibility, coimmunoprecipitation experiments were performed. Lysates from cells with or without Ang II stimulation were immunoprecipitated with anti-AT1 receptor antibody or nonimmune rabbit IgG. Immunoprecipitates were then subjected to Western blot analysis with an mAb against caveolin-1 (see Methods). Figure 8 shows that caveolin-1 coimmunoprecipitates with the AT1 receptor on agonist stimulation, confirming that the activated AT1 receptor becomes associated with caveolin-enriched membrane domains.

**Discussion**

The present study demonstrates in VSMCs an interaction between the AT1 receptor and caveolae. Ang II stimulates movement of a portion of the AT1 receptors from the general plasma membrane fraction to the caveolin-enriched fractions. Ang II also stimulates a calcium-dependent upregulation of caveolin-1 mRNA, a decrease in caveolin-1 levels, and enhanced synthesis of the protein, providing evidence in aggregate of increased caveolin turnover. Finally, caveolin-1 coimmunoprecipitates with the AT1 receptor. These data provide firm evidence relating the AT1 receptor to the caveolae, both structurally and functionally.

To our knowledge, this is the first report showing that caveolin-1 expression is regulated acutely by hormonal stimulation. Interestingly, only caveolin-1 mRNA, but not that of caveolin-2 or -3, was consistently and substantially regulated by Ang II. These studies also provide some insight into the potential mechanism. The superoxide scavenger Tiron had no effect. Removal of extracellular Ca2+1, but not inhibition of protein kinase C or tyrosine kinases, abolished the ability of Ang II to increase caveolin-1 mRNA. Because ionomycin increases caveolin-1 expression, these data strongly suggest that upregulation of caveolin-1 mRNA is Ca2+1 dependent.

The combination of Ang II-induced increased expression of caveolin-1 mRNA and decreased protein expression suggested increased turnover of caveolin-1. This inference was confirmed by the [35S]methionine-labeling experiments indicating increased Ang II–stimulated caveolin biosynthesis. The decrease of the total caveolin-1 protein level is due most likely to increased degradation. Because caveolae mediate the endocytosis of conformationally modified albumin for delivery to endosomes and lysosomes for degradation,30 it is possible that Ang II stimulation results in internalization of caveolae and translocation to intracellular compartments where the caveolin protein is degraded. AT1 receptors in VSMCs are initially dispersed, but after agonist binding, they rapidly coalesce and are internalized through both noncoated as well as coated pits.12,13 In transfected cells, the AT1 receptor, after agonist stimulation, is internalized preferentially through a noncoated pit mechanism that was speculated to be the caveola.11 Internalized Ang II peptide and peptide fragments can be detected in endosomes using either fluorescence microscopy or fractionation studies.31 Thus, the agonist-occupied AT1 receptor may be internalized, at least in part, via the caveola, and during this process the associated caveolin-1 is at least partially de-
graded. The data showing that the calcium ionophore, which does not induce internalization (Figure 3B), increases both caveolin-1 mRNA and protein are consistent with this formulation. This scenario of agonist-induced AT1 receptor sequestration or internalization by a caveola-dependent mechanism is supported by the observations of agonist-stimulated movement of a portion of AT1 receptors into caveolin-enriched membrane fractions, and of caveolin-1 communoprecipitation with the AT1 receptor. Finally, interventions that interfere with agonist-induced AT1 receptor sequestration (and tonic-phase signaling), low temperature and treatment with phenylarsine oxide, impaired movement of the receptor to the caveolin-enriched membrane fraction. Thus, the available data suggest a model in which the AT1 receptors in VSMCs are dispersed on the cell surface and, after agonist occupation, aggregate in and are internalized with specialized membrane domains represented by, at least in part, caveolae. While the functional significance of this movement and sequestration has not been proven, receptor signal generation is likely to be involved.32

Previous reports indicate enrichment of caveolar membrane fractions in signaling molecules, including G proteins, ras, and src, and in receptors, such as those for PDGF and EGF,15-20 and agonist-induced movement of the m3 muscarinic receptor into caveolin-enriched fractions of cardiac membranes.21 We have confirmed the constitutive presence of G protein subunits (Gq/11, and Gβ), PLC-γ1, and PLD activity in VSMC caveolin-enriched membranes (N.I., K.G., R.W.A., unpublished data, 1998). In addition, we have found that caveolin-1-glutathione S transferase (GST) fusion protein can bind Gq/11 and Gβζ in VSMCs (unpublished data). Together with the present finding that AT1 receptors coprecipitate with caveolin-1, the coprecipitation of G protein subunits with the caveolin-1 fusion protein supports the notion that caveolin may act as an organizing molecule for signal transduction of AT1 receptors. Thus, the movement of AT1 receptors into caveolae may result in specific sets of signals that are components of the complex Ang II signaling sequence. At a superficial level, this could be viewed as being accomplished by simply facilitating the colocalization of agonist-occupied receptor and unique combinations of signaling molecules. The actual situation is likely to be more complex, since caveolin itself may play an active role in controlling signal generation. As noted above, there are several signal-transducing molecules and receptors that directly bind caveolin.13,33 Caveolin may even regulate enzyme activity. For example, caveolin-2 binds Gαq, Gα12, and Gα13, which results in an inactivation of Gq subunits.29 In addition, caveolin-1 interacts directly with the oxygenase domain of endothelial nitric oxide synthase (eNOS), and this interaction of eNOS with GST–caveolin-1 fusion proteins significantly inhibits enzyme catalytic activity.34 Furthermore, the relative levels of caveolin isoforms may affect signal generation, since when different caveolin isoforms are expressed in the same cells, they differ in their functional interactions with heterotrimeric G proteins.17 In this context, the differential regulation of caveolin-1 vis-à-vis caveolins-2 and -3 might have functional significance for signaling in response to Ang II in VSMCs. In summary, these data support the concept that the caveola is a specialized signaling domain in VSMCs that can be dynamically accessed and is likely activated by the AT1 receptor. Thus, this signaling domain could contribute to the temporal and spatial resolution of the extraordinarily complex Ang II signaling sequence into specific, definable components.

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References

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