

# The Role of Hydrogen Peroxide in the Contractile Response to Angiotensin II

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## ABSTRACT

In the last years, reactive oxygen species (ROS) have been proposed as mediators of proliferative/hypertrophic responses to angiotensin II (Ang II), both in vivo and in vitro. However, the hypothesis that the Ang II-dependent cell contraction could be mediated by ROS, particularly  $H_2O_2$ , has not been tested. Present experiments were devoted to test this hypothesis and to analyze the possible mechanisms involved. Catalase (CAT) prevented the increased myosin light chain phosphorylation and the decreased planar cell surface area (PCSA) induced by 1  $\mu$ M Ang II in cultured rat vascular smooth muscle cells (VSMC). This preventive effect of CAT was also detected when 1  $\mu$ M platelet-activating factor (PAF) was used as a contractile agonist instead of Ang II. Similar results were found when using horseradish peroxidase as an  $H_2O_2$  scavenger or cultured rat mesangial cells. In vascular smooth muscle cells, CAT modified

neither the binding of labeled Ang II nor the Ang II-induced inositol 1,4,5-trisphosphate ( $IP_3$ ) synthesis. However, it completely abolished the Ang II-dependent calcium peak, in a dose-dependent fashion. CAT-loaded cells (increased intracellular CAT concentration over 3-fold) did not show either a decreased PCSA or an increased intracellular calcium concentration after Ang II treatment. Ang II stimulated the  $H_2O_2$  synthesis by cultured cells, and the presence of CAT in the extracellular compartment significantly diminished the Ang II-dependent increased intracellular  $H_2O_2$  concentration. The physiological importance of these findings was tested in rat thoracic aortic rings: CAT prevented the contraction elicited by Ang II. In summary, present experiments point to  $H_2O_2$  as a critical intracellular metabolite in the regulation of cell contraction.

In recent years, increasing attention has been paid to reactive oxygen species (ROS) or to the intracellular redox status as possible mechanisms of cytosolic signal transduction (Palmer and Paulson, 1997; Finkel, 1999; Sattler et al., 1999). This aspect of ROS function has been particularly studied in the field of cell proliferation, and it is well documented that ROS may directly induce the proliferation of some cell types (Duque et al., 1993; Nishio and Watanabe, 1997; Tatla et al., 1999), because they trigger intracellular mechanisms involved in cell cycle activation (Lo and Cruz, 1995; Gonzalez-Rubio et al., 1996; Brar et al., 1999). Furthermore, recent work from Sundaresan et al. (1995) has clearly outlined the dependence of the proliferating effect of platelet-

derived growth factor on an increased intracellular hydrogen peroxide ( $H_2O_2$ ) synthesis, thus supporting the importance of ROS as intracellular mediators of proliferating signals.

Angiotensin II (Ang II) may act, in the vascular wall, as a crucial hypertrophic/hyperplastic effector in pathophysiological conditions. These actions of Ang II have been related to a peptide-dependent increased ROS synthesis. In some forms of hypertension, it has been possible to detect an increased oxidative stress, which seems to depend on Ang II overproduction. Moreover, the hypertrophic effect of the peptide on vascular cells may be prevented by antioxidant treatments. Specific studies directed to assess the specific pathway of ROS synthesis activated by Ang II point to  $H_2O_2$  as the main ROS involved in the hypertrophic response and to the NADH/NADPH oxidase system as the primary target for peptide action (Rajagopalan et al., 1996; Laursen et al., 1997; Ushio-Fukai et al., 1998; Zafari et al., 1998).

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**ABBREVIATIONS:** ROS, reactive oxygen species; Ang II, angiotensin II; MLC, myosin light chain; VSMC, vascular smooth muscle cell(s); CAT, catalase; HP, horseradish peroxidase; DMEM, Dulbecco's modified Eagle's medium;  $IP_3$ , inositol 1,4,5-trisphosphate; MC, mesangial cells; PCSA, planar cell surface area; KRH, Krebs-Ringer-HEPES; DCHF, 2',7'-dichlorodihydrofluorescein; DA, diacetate; RTARs, rat thoracic aortic rings; PAF, platelet-activating factor; ACh, acetylcholine; MLC-P, myosin light chain phosphorylation.

The importance of ROS as possible mediators of the Ang II actions has been widely tested in the field of the hypertrophic/hyperplastic responses. However, the hypothesis that ROS may also be involved in the genesis of other effects of Ang II has not been systematically analyzed. Smooth muscle cell contraction is a rather well-defined cellular phenomenon that involves the interaction of agonists with specific membrane receptors, the activation of phospholipase C, the induction of a rapid increase in intracellular calcium, the activation of myosin light chain (MLC) kinase (and probably protein kinase C), and finally, the phosphorylation of the 20-kDa regulatory unit of myosin (Somlyo and Somlyo, 1994; Stockand and Sansom, 1998). No previous reports suggest that ROS could be involved in the modulation of this complex mechanism. Taking into account the previously defined relationships between Ang II and ROS, as well as the importance of these metabolites as mediators of cell responses, the present experiments were done to test the hypothesis that ROS, particularly H<sub>2</sub>O<sub>2</sub>, could be involved in the regulation of the contractile response elicited by Ang II in vascular smooth muscle cells (VSMC). Because this was the case, additional experiments were performed to explore more precisely the mechanisms of this regulation. Finally, the physiological importance of these findings was assessed in an *ex vivo* model of vascular contraction.

## Experimental Procedures

**Materials.** Collagenase types IV and IA, angiotensin II, leupeptin, catalase (CAT), horseradish peroxidase (HP), aminotriazole, platelet-activating factor, acetylcholine, Fura-2-acetoxymethylester, and myosin light chain standard, were purchased from Sigma (St. Louis, MO). Pansorbin was from Calbiochem-Novabiochem Corp. (La Jolla, CA). Acrylamide, bisacrylamide and Coomassie Blue R-250 were from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium, RPMI 1640, fetal calf serum, trypsin-EDTA (0.02%), and penicillin-streptomycin were purchased from Biowhittaker (Walkersville, MD). Culture plates were from Nunc (Kamstrup, Denmark). [<sup>32</sup>P]orthophosphate and [<sup>3</sup>H]inositol 1,4,5-trisphosphate (IP<sub>3</sub>) were purchased from New England Nuclear (Wilmington, DE). X-OMAT films were from Kodak (Rochester, NY). Low- and high-molecular-weight standards were from Pharmacia Biotech (Uppsala, Sweden). [<sup>125</sup>I]-Angiotensin II was from Amersham International (Buckinghamshire, UK). Plates and electrophoresis equipment were from Bio-Rad (Richmond, CA). Dr. Christine A. Kelley, from the National Institutes of Health (Bethesda, MD), kindly provided the anti-platelet myosin antibody. All other reagents were of the highest commercially available grade.

**Cell Culture.** VSMC were obtained from thoracic aortas of Wistar rats by methods described previously (Chamley-Campbell et al., 1979). Briefly, Wistar rats (125–200 g) were sacrificed and exsanguinated. Thoracic aortas were removed, cleaned of surrounding tissues, dissected into small strips, and incubated in DMEM/Ham's F-12 medium with collagenase type IV at 37°C for 45 min. The digested strips were seeded onto 100-mm diameter dishes and maintained in 10 ml of DMEM/Ham's F-12 medium with 10% fetal calf serum, at 37°C, in a humidified atmosphere of 5% CO<sub>2</sub>. Confluent cultures were serially passaged by trypsinization (trypsin-EDTA). The cells were used between the third and fifth passages. Cells exhibited characteristics of VSMC (Chamley-Campbell et al., 1979). In some experiments and to increase the intracellular concentrations of CAT, 24-h serum-deprived cells were incubated with 1500 U/ml CAT for 24 h at 37°C (Sundaresan et al., 1995).

Rat mesangial cells (MC) were cultured using standard techniques (Duque et al., 1992). Kidneys were removed under ether anesthesia

from Wistar rats weighing 100 to 150 g. The glomeruli were isolated by successive mechanical sieving (150 and 50 μm), treated with collagenase type IA, and plated in plastic culture flasks. The culture medium consisted of RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (1 mM), penicillin (0.66 μg/ml), and streptomycin sulfate (60 μg/ml) and was buffered with HEPES, pH 7.2. Culture media were changed every 2 days. Studies were performed in primary cultures on days 20 to 22. The identity of the cells was confirmed by morphological and functional criteria (Duque et al., 1992).

**Analysis of Cell Contraction.** The analysis of cell contraction was performed by measuring MLC phosphorylation and/or by studying the changes in planar cell surface area (PCSA) (Duque et al., 1992; García-Escribano et al., 1993).

Phosphorylation of MLC was determined after immunoprecipitation and SDS-polyacrylamide gel electrophoresis protein separation, as reported previously (García-Escribano et al., 1993). Briefly, after labeling the cells with 50 μCi/ml neutralized, carrier-free sodium [<sup>32</sup>P]orthophosphate (3 h, 37°C), incubations were performed under the conditions detailed elsewhere (see figure legends). Thereafter, the incubation media were removed, and cells were precipitated with ice-cold ethanol. After solubilizing the proteins with a pyrophosphate buffer (100 mM NaF, 8 mM sodium pyrophosphate, 250 mM NaCl, 5 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, and 1% Nonidet P-40), the samples were centrifuged, the supernatants were collected and incubated with human anti-platelet myosin antibody at 4°C for 90 min, and Pansorbin was used to precipitate the immuno-linked MLC. This fraction was separated by 12% SDS-polyacrylamide gel electrophoresis, the gel was frozen and exposed to X-OMAT films, the phosphorylated MLC were identified on the autoradiographs, and the absorbance of the 20-kDa band was measured by videodensitometry (Bio-Rad Laboratories). Results were calculated in arbitrary density units and corrected for the protein content of the sample (Bradford, 1976).

To determine the changes in PCSA, cells were grown in 20-mm plates, and they were studied before confluence. In every experiment, cells were washed twice, discarding the culture medium, and placed in Tris-glucose buffer (20 mM Tris, 130 mM NaCl, 5 mM KCl, 10 mM sodium acetate, and 5 mM glucose, pH 7.45) containing 2.5 mM CaCl<sub>2</sub>. Cells were incubated under different experimental conditions (see figure legends). While incubations were performed, cells were observed under phase contrast with an inverted PFX model TMS-F photomicroscope (magnification, 150×; Nikon, Tokyo, Japan). Photographs of the same cells were taken under the various experimental conditions. Every cell with a sharp margin suitable for the planimetric analysis was considered, and 6 to 12 cells were analyzed per photograph. PCSA was determined by computer-aided planimetric techniques (Duque et al., 1992; García-Escribano et al., 1993). Measurements were performed by two different researchers in a blind fashion. The intraobserver and interobserver variations were 2 and 5%, respectively.

**Angiotensin II-Labeled Binding Assay.** For binding assays (Diez-Marqués et al., 1995), VSMC were washed twice with Dulbecco's modified Eagle's medium and incubated for 3 min with Dulbecco's modified Eagle's medium containing 3 mM EDTA at room temperature. After removing this medium, cells were incubated for 3 min at 37°C, 1 ml of Dulbecco's modified Eagle's medium was added, and cells were gently scraped and centrifuged at 800 rpm. Supernatants were removed and the cells were resuspended in assay buffer (20 mM Tris-HCl, 5 mM glucose, 130 mM NaCl, 5 mM KCl, and 10 mM sodium acetate, pH 7.4), with a protein concentration of 0.5 μg/μl. Binding experiments were performed by adding a fixed amount of [<sup>125</sup>I]-Ang II (0.15 nM) to 125 μg of cell protein, in either the absence or the presence of unlabeled Ang II (10<sup>-4</sup> M), for variable incubation times. The free radioactivity was separated from the bound radioactivity by centrifugation at 11,000g for 2 min, and the resultant pellet was washed three times with ice-cold 0.15 M NaCl. The radioactivity was counted in a Kontron gamma counter (Kontron Instruments AG, Zurich, Switzerland). Nonspecific binding was less than 10% of the

total binding in all experiments. This nonspecific binding component was subtracted from the total bound radioactivity to obtain the corresponding specific binding.

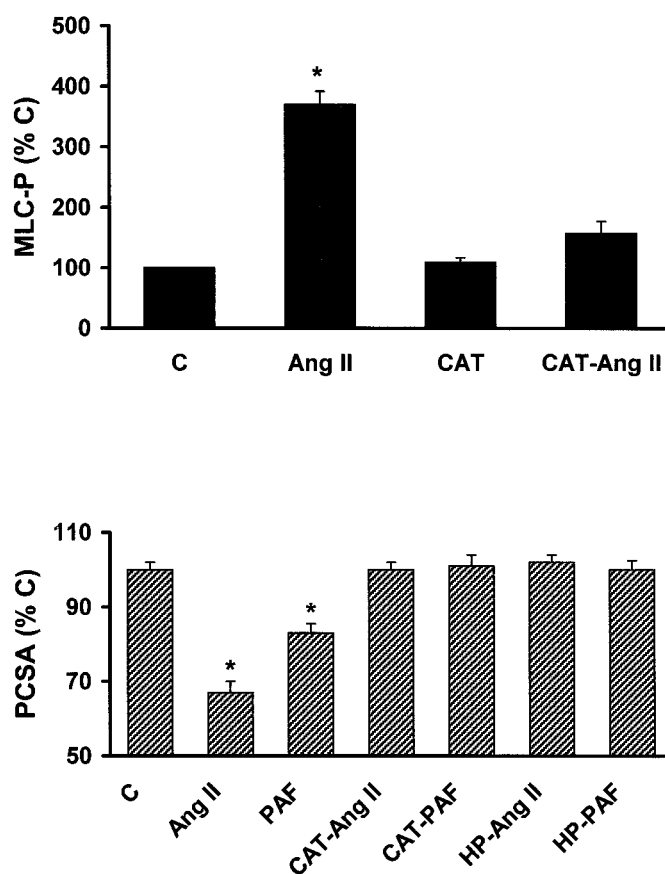
**Analysis of Endogenous Inositol 1,4,5-Triphosphate Synthesis.** For measuring the  $\text{IP}_3$  synthesis, VSMC were washed twice and placed in DMEM/Ham's F-12 medium. Experimental incubations (see figure legends) were performed for 30 s, and the reaction was stopped by aspirating the medium and adding 0.5 ml of 0.5 M trichloroacetic acid. Cells were scraped and centrifuged at 2000g for 15 min at 4°C. The pellet was mixed in 250  $\mu\text{l}$  of 1 M NaOH and stored for protein analysis (Bradford, 1976). The supernatant was extracted four times with 1.25 ml of water-saturated diethylether. The cell extract was neutralized by adding 20  $\mu\text{l}$  of 500 mM Tris-HCl, pH 8.4. The specific binding of [ $^3\text{H}$ ]IP $_3$  to a preparation of bovine cerebellar membranes was used as a radioreceptor assay to determine the endogenous IP $_3$  levels by the method of Bredt et al. (as described by Izquierdo-Claros et al., 1997). Bovine cerebellar membranes were prepared by homogenizing bovine cerebella in a cold buffer A (50 mM Tris-HCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol, pH 7.7) to obtain a protein concentration of 4 mg/ml (Bradford method). These membranes (50  $\mu\text{g}$ /tube) were added to tubes containing 25  $\mu\text{l}$  of [ $^3\text{H}$ ]IP $_3$  (5 nCi/tube) and 50  $\mu\text{l}$  of unknown samples or standard samples containing IP $_3$  (0.005–5  $\mu\text{M}$  in buffer A, pH 8.6) or IP $_6$  (1%, w/v, in buffer A, pH 8.6) to define nonspecific binding. All tubes were incubated for 10 min at 4°C. Separation of bound and free IP $_3$  was achieved by centrifugation at 10,000g for 5 min. After aspiration of the supernatant, 50  $\mu\text{l}$  of 0.15 M NaOH were added to each tube, and the pellet was dissolved. The radioactivity was determined by liquid scintillation counting. The IP $_3$  content was determined by comparing the extent of the inhibition of [ $^3\text{H}$ ]IP $_3$  binding with a calibration curve obtained with known amounts of IP $_3$ . Nonspecific binding was about 13% of the total binding.

**Measurement of Cytosolic Free Calcium Concentration.** For calcium measurement, cells were plated onto 12-mm glass coverslips in 24-well culture dishes. When cells reached confluence, the culture medium was carefully removed, and cells were washed with a Krebs-Ringer-HEPES (KRH) solution (125 mM NaCl, 5 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 2 mM  $\text{CaCl}_2$ , 6 mM glucose, and 25 mM HEPES, pH 7.4) and then incubated with 5 mM Fura-2-acetoxymethyl ester in KRH with 2% BSA (30–40 min, 37°C). Thereafter, coverslips were removed from the plate and placed in fresh KRH. Fluorescence measurements were performed essentially as described by López-Ongil et al. (1999), by placing the glass coverslip in a diagonal position inside a standard 1-cm square cuvette containing 1.5 ml of KRH. The cuvette was placed in a fluorometer (LS50B; Perkin Elmer Cetus, Norwalk, CT) for the continuous recording of the fluorescence signals at excitation and emission wavelengths of 340/380 and 500 nm, respectively. Thereafter, the different reagents were added (see figure legends). Rapid mixing of the agents was achieved by continuous stirring with a magnetic bar placed at the bottom of the cuvette in the center of a 5-mm length of plastic tubing, which prevented the movement of the coverslip. At the end of each measurement, 0.1% Triton X-100 and 10 mM EGTA were sequentially added to obtain maximal and minimal Fura absorbance, respectively. The cytosolic free calcium concentration was calculated according to the method of Grynkiewicz et al. (1985), assuming a  $K_d$  value for Fura-2-calcium interaction of 225 nM.

**Measurement of the Hydrogen Peroxide Synthesis.**  $\text{H}_2\text{O}_2$  synthesis by VSMC was measured by two methods. First, 2',7'-dichlorodihydrofluorescein (DCHF) diacetate (DA) was used as a fluorescent probe for intracellular hydrogen peroxide measurement (López-Ongil et al., 1998). DCHF-DA diffuses readily to the intracellular compartment, where it is desacetylated to the non-membrane-permeable DCHF. Then, during the cellular production of  $\text{H}_2\text{O}_2$ , DCHF is oxidized and emits a fluorescent signal. The method was essentially the same described for calcium (see above), with two differences: VSMC were loaded with 20  $\mu\text{M}$  DCHF-DA, and excitation and emission wavelengths were 488 and 525 nm, respectively.

The fluorescent signal was registered as a function of the time. Second, the phenol red method (Ruiz-Torres et al., 1997) was used to assess the release of hydrogen peroxide by VSMC and MC to the incubation media. Cells were incubated in 2 ml of phosphate buffer containing 0.28 mM phenol red sodium salt and 50  $\mu\text{g}/\text{ml}$  type II horseradish peroxidase, in the presence of Ang II. Thereafter, incubation media were collected, their pH was adjusted to 12.5, and absorbance was read at 610 nm. Concentrations were calculated by using a standard curve of hydrogen peroxide.

**Studies in Rat Thoracic Aortic Rings.** For every experiment, one male Wistar rat aged approximately 6 weeks was anesthetized with sodium pentobarbital, and the thoracic aorta was removed, freed of adhering fat and connective tissues, and cut into ring segments of 3 mm length with parallel razors. Two stainless steel wires were inserted into the lumen of the rat thoracic aortic rings (RTARs). One wire was connected to a force-displacement transducer F30 (Hugo Sachs Elektronik, Freiburg, Germany), and the other was anchored to a plastic holder. The holders were placed in a 20-ml



**Fig. 1.** Changes in myosin light chain phosphorylation (MLC-P) and PCSA in cultured vascular smooth muscle cells. Top, MLC-P. Cells were incubated with 80 U/ml catalase for 10 min, and then 1  $\mu\text{M}$  angiotensin II was added for 30 min. Results are the means  $\pm$  S.E.M. of the densitometric analysis of five different experiments (duplicates), and they are expressed as percentages of the control (C) values ( $1.83 \pm 0.21$  cpm/ $\mu\text{g}$  of protein). Bottom, morphological changes. Cells were incubated with 80 U/ml catalase or 36 U/ml horseradish peroxidase for 10 min, and then 1  $\mu\text{M}$  angiotensin II or platelet-activating factor was added for 30 min. Results are the means  $\pm$  S.E.M. of five different experiments, and they are expressed as percentages of basal PCSA. Top and bottom: C, control cells. Ang II, cells incubated with angiotensin II. CAT, cells incubated with catalase; PAF, cells incubated with platelet-activating factor; CAT-Ang II, cells incubated with catalase plus angiotensin II; CAT-PAF, cells incubated with catalase plus platelet-activating factor; HP-Ang II, cells incubated with horseradish peroxidase plus angiotensin II; HP-PAF, cells incubated with horseradish peroxidase plus platelet activating factor. \* $P < .05$  versus the other groups in both panels.



organ bath (Schuler type 809, Hugo Sachs Elektronik) at 37°C containing oxygenated (95% CO<sub>2</sub> and 5% O<sub>2</sub>) Krebs-bicarbonate solution of the following composition: 118.3 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25.0 mM NaHCO<sub>3</sub>, 11.1 mM glucose, and 2.5 mM CaCl<sub>2</sub>. Isometric tension changes were measured by the transducers, and the data were collected by a control computer and displayed and analyzed with the ACAD software (Hugo Sachs Elektronik).

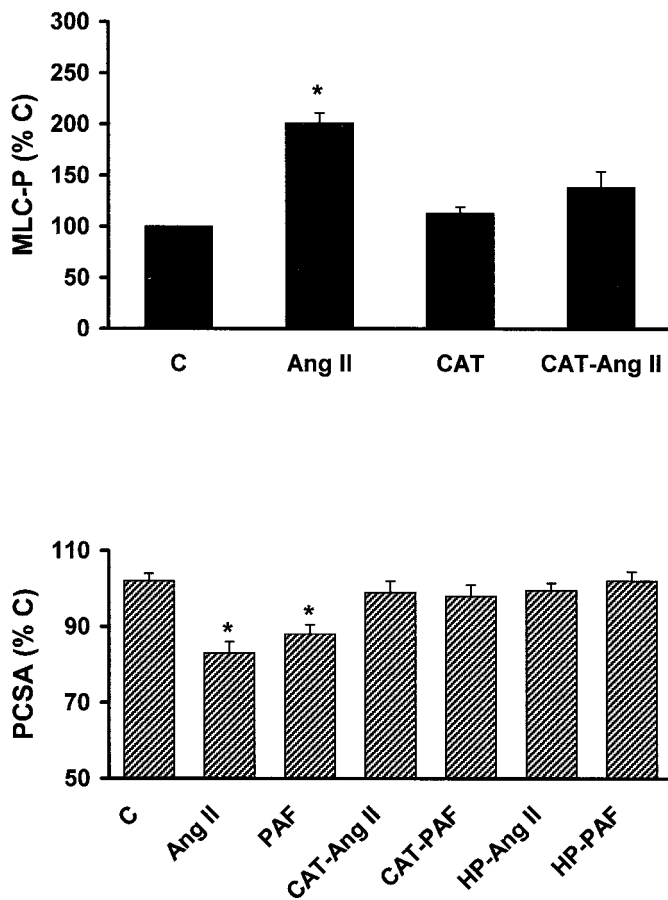
Before the actual experiments, and as described elsewhere (Reynolds and Mok, 1990), the RTARs were stretched progressively and exposed repeatedly to 40 mM KCl to induce contraction at each level of stretching, until a maximal contractile response to 40 mM KCl was obtained. This basal tension was considered to indicate the optimal point on a length-tension curve (~2 g of basal tension). After setting the optimal basal tension, RTARs were allowed to equilibrate for 90 min before addition of test compounds. Dose-response curves to the contractile agents Ang II or platelet-activating factor (PAF) were constructed in the absence and in the presence of different concen-

trations of either CAT or acetylcholine (ACh). Thorough washings were performed, and a stabilization period of 60 min was allowed between dose-response curves. Control experiments were performed with the contractile agonists alone, to check the stability of the RATR responsiveness with time. In all cases, fresh stock solutions were prepared daily in Krebs-bicarbonate buffer.

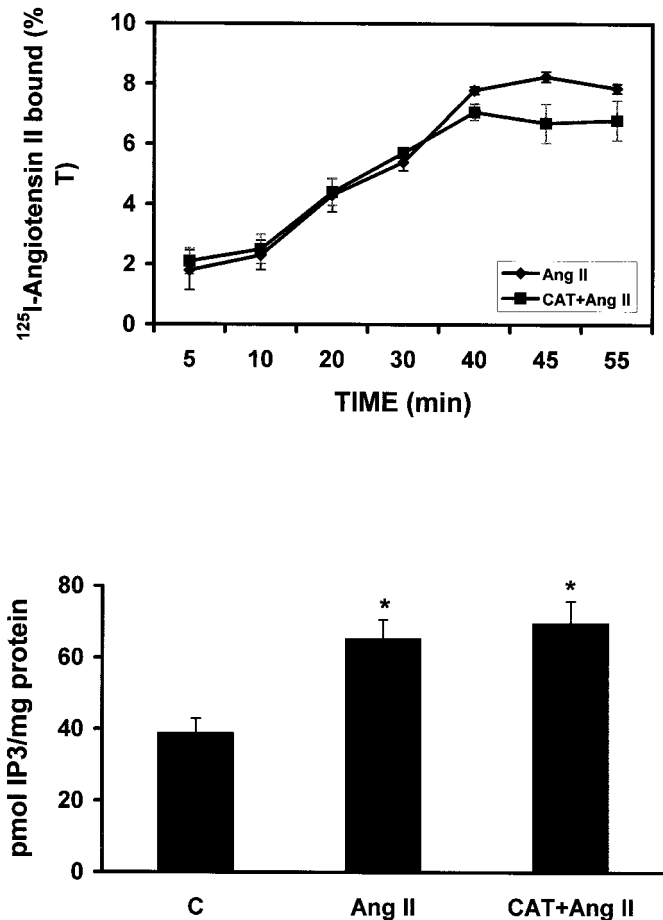
**Statistical Analysis.** In every case, the data shown are the means  $\pm$  S.E.M. of a variable number of experiments (see *Results* and figure legends), and in some cases they are expressed as percentages of the control values. Because the number of data in each distribution was never over 10, nonparametric statistics, particularly Friedman's test, were selected to compare the different groups of results. *P* values < .05 were considered statistically significant.

## Results

In the present experiments, the contractile ability of Ang II in cultured cells was tested by measuring both the phosphorylation of MLC and the changes in PCSA. As shown in Fig. 1, top, Ang II increased the incorporation of phosphate in the 20-kDa regulatory subunit of myosin in VSMC. This fact can



**Fig. 2.** Changes in MLC-P and PCSA in cultured rat mesangial cells. Top, MLC-P. Cells were incubated with 80 U/ml catalase for 10 min, and then 10 nM angiotensin II was added for 30 min. Results are the means  $\pm$  S.E.M. of the densitometric analysis of five different experiments (duplicates), and they are expressed as percentages of the control values ( $1.53 \pm 0.15$  cpm/ $\mu$ g of protein). Bottom, morphological changes. Cells were incubated with 80 U/ml catalase or 36 U/ml horseradish peroxidase for 10 min, and then 10 nM angiotensin II or platelet-activating factor was added for 30 min. Results are the means  $\pm$  S.E.M. of five different experiments, and they are expressed as percentages of basal PCSA. Top and bottom: C, control cells; Ang II, cells incubated with angiotensin II; CAT, cells incubated with catalase; PAF, cells incubated with platelet-activating factor; CAT-Ang II, cells incubated with catalase plus angiotensin II; CAT-PAF, cells incubated with catalase plus platelet-activating factor; HP-Ang II, cells incubated with horseradish peroxidase plus angiotensin II; HP-PAF, cells incubated with horseradish peroxidase plus platelet-activating factor. \**P* < .05 versus the other groups in both panels.



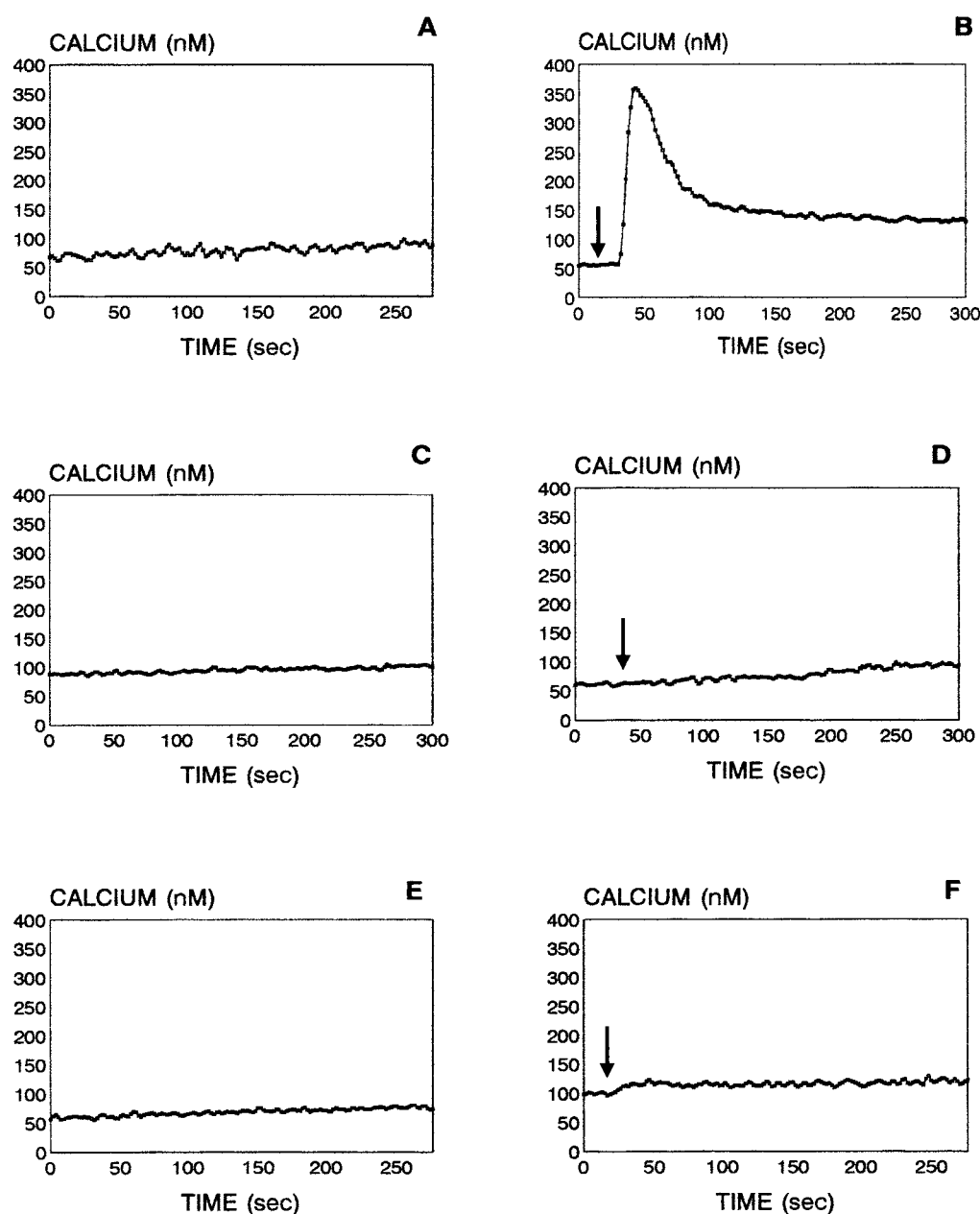
**Fig. 3.** Effect of CAT on the binding of radiolabeled Ang II (top) and the Ang II-dependent IP<sub>3</sub> synthesis (bottom) in vascular smooth muscle cells. In the binding experiments, VSMC were incubated for variable times with labeled angiotensin II (0.15 nM), with (CAT + Ang II) or without (Ang II) 80 U/ml catalase. Results shown are the means  $\pm$  S.E.M. of four experiments (duplicates), and they are expressed as percentages of total counts. There were no statistical differences between the two binding curves. The IP<sub>3</sub> synthesis was measured after 30 s of incubation in control conditions (C), in the presence of 1  $\mu$ M angiotensin II, and in the presence of 1  $\mu$ M angiotensin II plus 80 U/ml catalase (CAT + Ang II). Results shown are the means  $\pm$  S.E.M. of four experiments (duplicates). \**P* < .05 versus C.

be considered as an adequate marker of cell contraction, especially taking into account that Ang II at the same concentration significantly reduced the PCSA of these cells (Fig. 1, bottom). The presence of CAT in the incubation media completely abolished the Ang II-induced changes in MLC phosphorylation and PCSA (Fig. 1). Aminotriazol, a catalase inhibitor (Brenneisen et al., 1997), induced a minimal reduction of VSMC PCSA and enhanced the cell contraction elicited by low Ang II concentrations (values of PCSA after 30 min of incubation: control cells,  $101 \pm 2\%$ ; cells incubated with 5 mM aminotriazol,  $92 \pm 3\%$ ,  $P < .05$  versus control cells; cells incubated with 1 nM Ang II,  $93 \pm 2\%$ ,  $P < .05$  versus control cells; cells incubated with 5 mM aminotriazol plus 1 nM Ang II,  $83 \pm 2\%$ ,  $P < .05$  versus the other groups. Data are the means  $\pm$  S.E.M. of four experiments, and they are expressed as percentages of the initial cell surface.

To test whether the contraction blockade detected in the

presence of CAT was only observed when the contractile agonist was Ang II, we studied cell contraction in VSMC preincubated with CAT and then treated with another well known vasoconstrictor agent, PAF (Rodriguez-Barbero et al., 1995). As shown in Fig. 1, bottom, the reduction in PCSA elicited by PAF completely disappeared after CAT treatment. The reduction observed in PCSA, both in presence of Ang II and PAF, was also completely reversed by pretreating the cells with another hydrogen peroxide scavenger such as horseradish peroxidase (Fig. 1, bottom).

Comparable results were obtained in other kinds of contractile cells of renal origin [e.g., mesangial cells (Duque et al., 1992)]. As shown in Fig. 2, top, the increased MLC phosphorylation elicited by Ang II in these cells completely disappeared after CAT incubation. The analysis of the changes in PCSA (Fig. 2, bottom) revealed that both CAT and horse-



**Fig. 4.** Analysis of the calcium response to Ang II in presence of CAT in vascular smooth muscle cells. Representative experiments, showing the changes in the intracellular calcium concentration with time are shown in the six panels. A, control cells; B, cells incubated with 1  $\mu$ M angiotensin II; C, cells incubated with 80 U/ml catalase; D, cells pretreated for 10 min with 80 U/ml catalase and then 1  $\mu$ M angiotensin II; E, cells loaded with catalase (see under *Experimental Procedures*); F, cells loaded with catalase and then treated with 1  $\mu$ M angiotensin II.

radish peroxidase fully prevented the reduction in PCSA induced by Ang II and PAF.

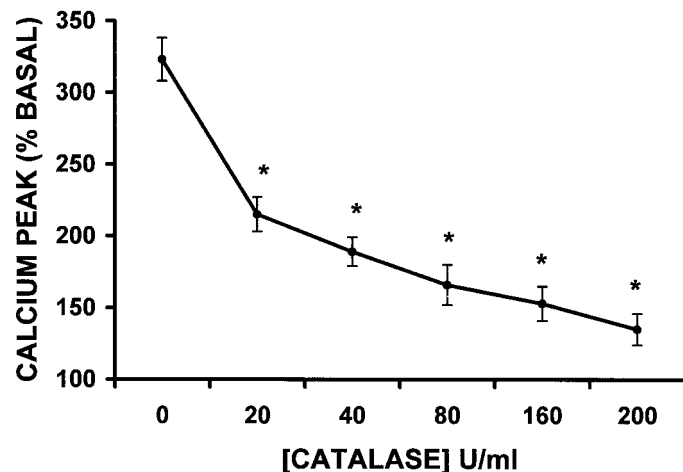
To evaluate the possible influence of CAT on the mechanisms involved in the development of the Ang II-related VSMC contraction, the binding of the peptide to its receptor was studied in control conditions and in the presence of CAT. No changes were detected in the peptide binding to the cells when CAT was present in the incubation media (Fig. 3, top). Moreover, CAT was unable to prevent the phospholipase C activation induced by Ang II in these cells, because stimulated IP<sub>3</sub> synthesis was unaffected by CAT treatment (Fig. 3, bottom). In contrast, the presence of CAT in the incubation media completely abrogated the increased intracellular calcium concentration observed after Ang II treatment of cells. Calcium results are included in Fig. 4. This figure shows that Ang II elicited a characteristic augmentation of intracellular calcium (Fig. 4B). CAT abolished the intracellular calcium movements observed in cells incubated with Ang II (Fig. 4D), whereas CAT itself did not modify calcium concentration (Fig. 4C). The CAT-dependent inhibition of the calcium peak was dose dependent, as depicted in the Fig. 5.

The experiments mentioned previously were performed by adding CAT to the incubation media. To test the importance of intracellular CAT, cells were loaded with CAT, following a procedure described previously (Sundaresan et al., 1995), and extracellular CAT was removed. Basal intracellular CAT activity in VSMC was  $15.6 \pm 4.0$  mKat/mg of protein, and after loading, it increased to  $54.7 \pm 17.5$  mKat/mg. Loaded cells failed to reduce their PCSA (Fig. 6, top), as well as to show an increased intracellular calcium concentration (Fig. 4F; Fig. 6, bottom), after Ang II treatment.

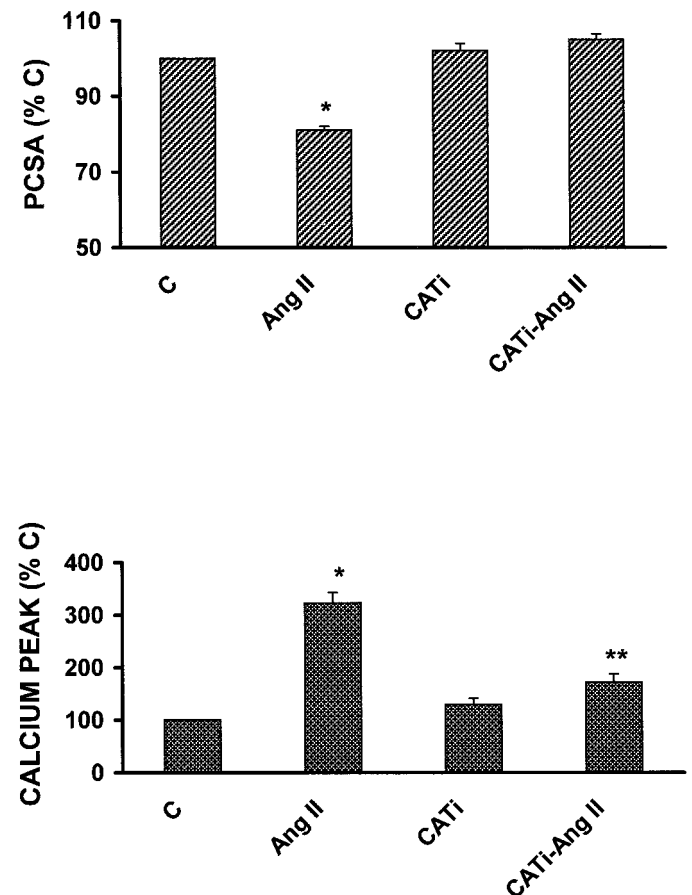
The ability of Ang II to induce the synthesis of hydrogen peroxide was tested. Changes in the intracellular hydrogen peroxide concentration in VSMC were assessed with the fluorescent probe DCHF. Ang II ( $1 \mu\text{M}$ ) induced a very quick (the effect started within 4–6 s after Ang II addition) and transient (maximal peak amplitude about 3 s) increase in the intracellular hydrogen peroxide concentration (Fig. 7A), which was dose-dependent (Fig. 7B). The presence of CAT in

the extracellular compartment significantly diminished the maximal peak amplitude of the Ang II-induced fluorescent signal (under 0.4 s) (Fig. 7C), as well as the area under the curve of this signal (Fig. 7D). When the release of hydrogen peroxide to the extracellular medium was measured by the phenol red method, it was also possible to detect an increased synthesis of this metabolite in both VSMC and MC treated with Ang II (Fig. 8).

Finally, experiments in RTARs were performed to assess the physiological importance of these in vitro observations. Preliminary experiments were performed to exclude the possibility that CAT could act as a direct contractile or relaxing agent on RTARs. To test this hypothesis, the enzyme was added directly to RTARs in a cumulative manner. No changes were detected in the muscular tension at any of the doses tested ( $0.1 \text{ nM}$  to  $10 \mu\text{M}$ ) (data not shown). The effect of CAT on the dose-response curve of Ang II or PAF was tested by preincubating the RTARs for 10 min with different concentrations of CAT before addition of any of the contractile agonists. Preincubation with CAT elicited a clear dose-dependent inhibitory effect on Ang II-induced contractions that was significant at the concentrations of  $0.5$



**Fig. 5.** Quantitative analysis of the dose-response CAT-dependent inhibition of the quick, angiotensin II-induced calcium peak in vascular smooth muscle cells. Cells were preincubated for 10 min with variable concentrations of catalase, and the calcium peak elicited by  $1 \mu\text{M}$  angiotensin II was measured. Data are expressed as percentages of the basal intracellular calcium concentration ( $90 \pm 7 \text{ nM}$ ), and they are expressed as the means  $\pm$  S.E.M. of 10 different experiments. \* $P < .05$  versus 0.



**Fig. 6.** Changes in the response of cultured vascular smooth muscle cells incubated with Ang II after cell loading with CAT. Cells were incubated with  $1500 \text{ U/ml}$  CAT for 18 h, and then CAT was removed from the incubation media. Top, changes in the PCSA. Results are the means  $\pm$  S.E.M. of five different experiments, and they are expressed as percentages of basal PCSA. Bottom, changes in the intracellular calcium peak. Results shown are the means  $\pm$  S.E.M. of five experiments, and they are expressed as percentages of the control value ( $81 \pm 6 \text{ nM}$ ). Top and bottom: C, control cells; Ang II, control cells treated for 30 min with  $1 \mu\text{M}$  Ang II; CATi, control, catalase-loaded cells; CATi + Ang II, catalase-loaded cells treated for 30 min with  $1 \mu\text{M}$  Ang II. \* $P < .05$  versus the other groups in both panels.

and 1  $\mu\text{M}$  (Fig. 9). The highest dose of catalase tested (1  $\mu\text{M}$ ) induced a reduction of  $40.34 \pm 5.32\%$  in the maximal contraction induced by Ang II (Fig. 9). In addition, some experiments were performed in which vehicle alone was added to test the possibility that Ang II underwent some kind of spontaneous desensitization or tachyphylaxis during the long-term experiments. The results showed that there was no displacement of the dose-response curves for Ang II within the duration of the experiment (data not shown). Similar results were observed when PAF was used as the contractile agonist. The maximal inhibition reached was  $28.89 \pm 5.56\%$ . For comparison, parallel experiments were performed in which ACh was used instead of CAT. ACh proved to be similarly potent as CAT at inhibiting Ang II-induced contractions but more potent when PAF was used as the contractile agent in this experimental model. The maximal inhibitions reached with ACh were  $35.9 \pm 7.2\%$  with Ang II and  $40.5 \pm 0.6\%$  with PAF.

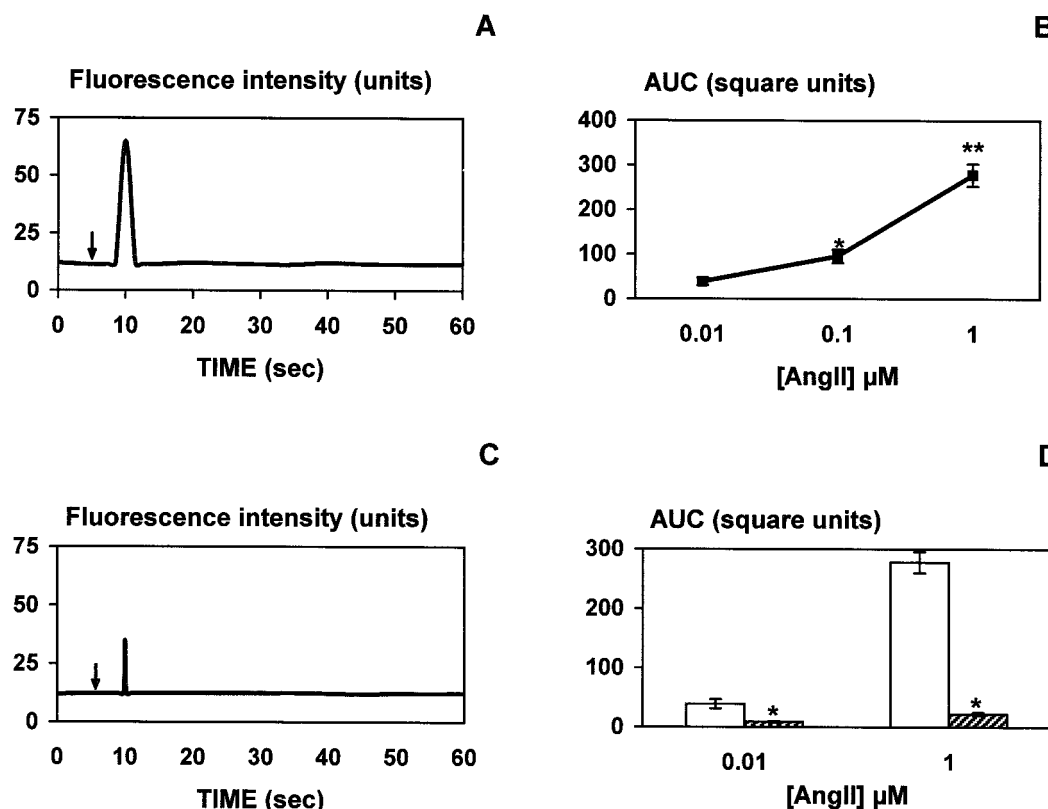
## Discussion

The present experiments clearly demonstrate that CAT completely blocks the increased MLC phosphorylation and the reduction of PCSA elicited by Ang II in cultured VSMC. Because these two parameters are considered good markers of cell contraction (Anderson et al., 1981; Simonson and Dunn, 1986), it can be proposed that CAT prevents VSMC contraction in the presence of Ang II. Moreover, this inhibitory ability does not seem to be restricted just to a vasoconstrictor agonist or a particular cell type, because similar results were obtained in other contractile cells and with other vasoconstrictor mediators. Consequently, the ability of CAT to block cell contraction can be considered a more generalized phenomenon, probably concerning different contractile cells

and various vasoconstrictor mediators. Note that experiments were performed with different Ang II concentrations in VSMC and MC, because it is well known that contractile responses diminish after serial passages (Gunther et al., 1992). Moreover, it must be stressed that experiments were focused on hydrogen peroxide and not on the superoxide anion, because a contractile effect for the latter has not been described previously (Duque et al., 1992).

The mechanisms involved in the CAT-dependent blockade of Ang II-induced VSMC contraction were analyzed by studying some of the initial events that trigger cell contraction. Some of these initial events include the interaction of the agonist with its receptor, the activation of phospholipase C with the subsequent  $\text{IP}_3$  release, and the  $\text{IP}_3$ -dependent quick release of the stored calcium to the cytosol (Somlyo and Somlyo, 1994; Stockand and Sansom, 1998). The inhibitory effect of CAT seems to take place at this last point, because it did not modify either the binding of Ang II to its receptor or the Ang II-induced  $\text{IP}_3$  synthesis, but it abrogated the rapid increase of calcium concentration observed after Ang II treatment. However, whether CAT blocks the interaction of  $\text{IP}_3$  with its receptors or whether it prevents the subsequent calcium release induced by this interaction cannot be concluded from these experiments.

The best-recognized action of CAT is its ability to remove hydrogen peroxide (Baud et al., 1992), and this property of CAT raises the critical question proposed by the present results. Because CAT blocks the Ang II-dependent cell contraction, it can be hypothesized that this blockade depends on hydrogen peroxide removal or, in other words, that  $\text{H}_2\text{O}_2$  generation is necessary for the contraction of the cells after Ang II exposure. Different arguments support this hypothesis. First, horseradish peroxidase, a protein that shares with



**Fig. 7.** Changes in the intracellular concentration of hydrogen peroxide in VSMC incubated with angiotensin II. Cells were loaded with DCHF, and fluorescence changes, reflecting the changes in the intracellular hydrogen peroxide concentration with time, were registered. A, cells incubated with 1  $\mu\text{M}$  angiotensin II. The arrow shows the Ang II addition. B, dose dependence of the angiotensin II-dependent changes in the intracellular hydrogen peroxide concentration (\* $P < .05$  versus 0.01  $\mu\text{M}$ . \*\* $P < .05$  versus 0.1  $\mu\text{M}$ ). C, cells pretreated with 80 U/ml catalase and then 1  $\mu\text{M}$  angiotensin II. The arrow shows the Ang II addition. D, cells pretreated with 80 U/ml catalase and then 0.01 or 1  $\mu\text{M}$  angiotensin II. The hatched bars represent the cells pretreated with catalase (\* $P < .05$  versus the cells without catalase). AUC, area under the curve. Results shown are representative (A and C) or are the means  $\pm$  S.E.M. (B and D) of four different experiments.



CAT only the ability to remove H<sub>2</sub>O<sub>2</sub>, also prevented cell contraction. Second, the synthesis of H<sub>2</sub>O<sub>2</sub> by VSMC and MC increased after Ang II treatment. H<sub>2</sub>O<sub>2</sub> augmented in both culture media and cell cytosols, because it diffuses freely through plasma membranes. Third, it has been demonstrated previously that H<sub>2</sub>O<sub>2</sub> may also act as a contractile agonist in different cell types (Duque et al., 1992; Yang et al., 1998). Finally, the contractile effect of Ang II was enhanced by the intracellular CAT blockade with aminotriazol. Consequently, it is highly probable that the inhibitory effect elicited by CAT could be the consequence of the hydrogen peroxide removal.

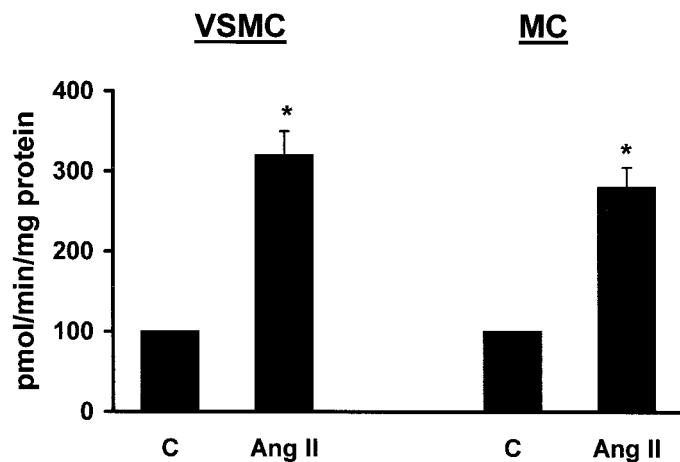
Alternative explanations for the inhibitory effect of CAT and HP are improbable. It can be argued that a complete prevention of cell contraction and calcium increases could be observed if these enzymes act as calcium chelators or channel blockers. However, these properties are not supported by the present results. In the experiments in which CAT was added to the extracellular compartment, a partial blockade of cell contraction could have been expected, because it has been described for calcium deprivation or verapamil. However, the complete prevention of cell contraction and the totally

blunted quick calcium response are not typical features of calcium deprivation or verapamil (de Arriba et al., 1988; Roe et al., 1989). On the other hand, a calcium chelator added to the intracellular compartment, as could have been the case after loading the cells with CAT, would have diminished the basal intracellular calcium concentration in the CAT-loaded cells, which was not the case. Some intracellular channel blockers, such as 8-(*N,N*-diethylamino)octyl 3,4,5-trimethoxybenzoate, may blunt the quick calcium release from the intracellular stores (de Arriba et al., 1988), but they must reach the intracellular compartment, and CAT does not permeate cell membranes after 30 min of incubation.

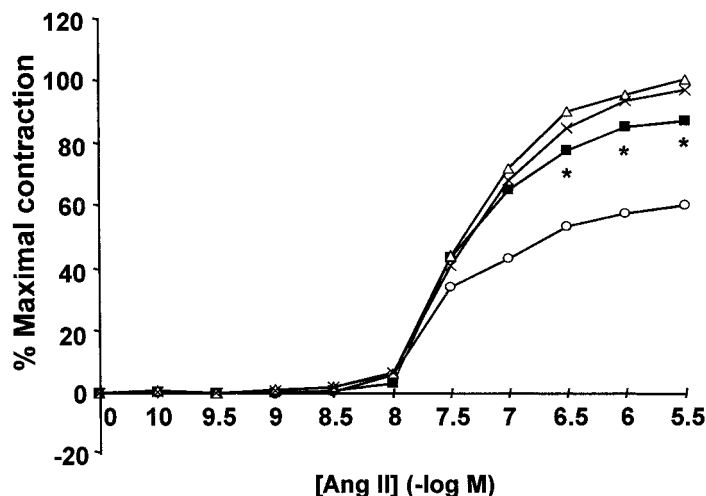
The source of H<sub>2</sub>O<sub>2</sub> after Ang II stimulation remains undefined. Previous studies strongly support the idea that the NADH/NADPH oxidase system may be the target for an increased hydrogen peroxide production after Ang II treatment (Ushio-Fukai et al., 1998). However, it has also been demonstrated that increased activities of phospholipase A<sub>2</sub> and phospholipase C are readily detected after Ang II treatment (Schlondorff et al., 1987; Heagerty and Ohanian, 1993), and synthesis of ROS may be linked to the increased metabolism of arachidonic acid (Baud et al., 1992). Moreover, the blockade of phospholipase A<sub>2</sub> or the lipoxygenase pathway prevents the Ang II-induced contraction of afferent arterioles (Imig and Deichman, 1997). Thus, the activation of these enzymes could also explain the rapid increase of H<sub>2</sub>O<sub>2</sub> in VSMC incubated with Ang II, but a more detailed analysis must be performed.

Interestingly, recent results suggest that not only is the Ang II-induced cell contraction dependent on the classical pathways described previously (Somlyo and Somlyo, 1994; Stockand and Sansom, 1998) but it may also depend on newly described mechanisms of intracellular signal transduction, such as the activation of extracellular signal-regulated kinases (Touyz et al., 1999). The relationships between these two pathways have not been extensively described, but the Ang II-induced synthesis of hydrogen peroxide could play a central role, because it is well known that this ROS may also activate extracellular signal-regulated kinases (Sabri et al., 1998).

Some attention must be paid to the physiological interpretation of these *in vitro* results. A major criticism of cell experiments is the possibility that cultured cells do not at all reproduce the changes observed *in vivo*. To counter this prob-



**Fig. 8.** Hydrogen peroxide synthesis by cultured VSMC or MC. Cells were incubated for 15 min with angiotensin II (1  $\mu$ M in VSMC and 10 nM in MC), and the hydrogen peroxide synthesis was measured by the phenol red method. C, control cells; Ang II, cells incubated with angiotensin II. Results are the means  $\pm$  S.E.M. of four different experiments (duplicates). \**P* < .05 versus C.



**Fig. 9.** Effects of CAT on the Ang II and PAF-induced contraction of RTARs. The RTARs were pretreated with different concentrations of CAT and, subsequently, cumulative concentrations of Ang II or PAF were added to the organ bath. The CAT concentrations provided correspond to 50, 250, and 500 U/ml, respectively. Results are the means  $\pm$  S.E.M. of four to six preparations. \**P* < .05 versus vehicle.



lem, the ability of CAT to prevent the agonist-elicited vascular contraction was tested in aortic rings. As observed in cells, CAT also prevented the contraction induced by Ang II and PAF in isolated vascular structures, thus supporting the physiological importance of cell results.

In conclusion, the present experiments point to hydrogen peroxide as a critical intracellular metabolite in the regulation of cell contraction. Although the mechanism has been particularly tested in VSMC treated with Ang II, it could also take place in other cell types and in the presence of different contractile agonists.

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