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Isoflurane Alters Angiotensin II–Induced Ca^{2+} Mobilization in Aortic Smooth Muscle Cells from Hypertensive Rats

Implication of Cytoskeleton

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Background: **Angiotensin II (AngII) is a potent vasoconstrictor involved in the short-term control of arterial blood pressure. Isoflurane was reported to decrease vascular tone through an alteration of vascular smooth muscle cell vasomotor response to several agonists, but its effect on AngII signaling is not known. On the other hand, vascular response to AngII is altered in hypertension. In this study, the authors tested the hypothesis that (1) isoflurane alters AngII-induced intracellular Ca^{2+} mobilization in aortic vascular smooth muscle cell from Wistar Kyoto and spontaneously hypertensive rats, and (2) this effect could be associated with an alteration of the organization of microtubular network, reported to be involved in AngII signaling.**

Methods: **The effect of 0.5–3% isoflurane was studied (1) on AngII (10^{-6} M)-induced intracellular Ca^{2+} mobilization, intracellular Ca^{2+} release from internal stores, and Ca^{2+} influx in Fura-2 loaded cultured aortic vascular smooth muscle cell isolated from 6-week-old Wistar Kyoto and spontaneously hypertensive rats, using fluorescent imaging microscopy; and (2) on the organization of cytoskeletal elements, using immunofluorescence labeling.**

Results: **In both stains, isoflurane decreased in a concentration-dependent manner AngII-induced intracellular Ca^{2+} mobilization, Ca^{2+} release from internal stores, and Ca^{2+} influx through nifedipine-insensitive Ca^{2+} channels. This effect occurred at a lower concentrations of isoflurane in Wistar Kyoto rats than in spontaneously hypertensive rats. In both strains, the effect of isoflurane on AngII–Ca^{2+} mobilization was abolished by impairment with nocodazole, vinblastine, or paclitaxel of microtubules polymerization. Isoflurane directly altered tubular network organization in a concentration-dependent and reversible manner.**

Conclusions: **Isoflurane decreased AngII-induced Ca^{2+} mobilization at clinically relevant concentrations, suggesting that vascular response to AngII could be altered during isoflurane anesthesia. The hypertensive strain was found less sensitive than the normotensive one. In both strains, the isoflurane effect was associated with a microtubular network interaction.**

THE decrease in vascular tone observed during isoflurane anesthesia has been related in part to an alteration of the vascular smooth muscle cells' (VSMC) vasomotor response to vasoactive substances, including norepinephrine, endothelin, or vasopressin.[1–3] Angiotensin II (AngII) is a potent vasoconstrictor involved in the short-term control of arterial blood pressure during isoflurane anesthesia.[4] The hemodynamic effects of AngII are mediated through binding to angiotensin subtype 1 (AT_1) receptors, which belong to the G protein–coupled receptor family. Activation of AT_1 receptor stimulates phospholipase C to hydrolyze phophatidylinositol 4,5-biphosphate, thereby producing inositol 1,4,5-triphosphate. The latter then binds to its specific receptor located on Ca^{2+} stores to release Ca^{2+} from internal stores, and to activate Ca^{2+} influx from extracellular spaces.[5–7] A normal response of VSMC to AngII may be critical for hemodynamic stability in several conditions encountered during the perioperative period, especially hypovolemia. However, the effect of isoflurane on AngII response is not described.

The present study was undertaken to assess the effect of isoflurane on AngII-induced intracellular Ca^{2+} mobilization and to gain further insight into the cellular mechanisms by which isoflurane could modulate AngII signaling. One mechanism that has been associated with intracellular Ca^{2+} mobilization is cytoskeletal function. We were particularly interested in the effect of isoflurane on microtubular and actin networks because several volatile anesthetic agents have been reported to directly interact with the organization of cytoskeletal elements,[8,9] and both actin and microtubular networks were recently reported to be involved in the transduction of several extracellular signals, including G protein–coupled transduction pathways and Ca^{2+} mobilization from internal store, or cell contraction in response to various agonists.[10–16]

On the other hand, structural and functional abnormalities of the arterial wall have been reported in some experimental models of genetic hypertension and have been associated with an increase in intracellular Ca^{2+} mobilization in VSMC in response to several agonists, especially AngII.[17–23] These abnormalities could partly

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explain the lability of blood pressure observed during general anesthesia in individuals who have essential hypertension.[24,25] For this purpose we compared the effects of isoflurane in VSMC from spontaneously hypertensive rats (SHR) to normotensive control (Wistar Kyoto rats [WKY]).

Material and Methods

Cell Culture

The study was approved by the Institutional Animal Investigation Committee and was conducted following recommendations established by the European Community Guidelines for Animal Ethical Care and Use of Laboratory Animals (Directive 86/609). Young male WKY/NIco rats (mean arterial pressure ± SEM = 98 ± 4 mmHg; n = 30) and SHR/NIco (mean arterial pressure ± SEM = 136 ± 5 mmHg; n = 25) aged 6 weeks were obtained from Iffa-Credo (L'Arbresle, France) and were used throughout the study. Cultured VSMC were obtained by enzymatic digestion as previously described.[16,26] In brief, aortas were incubated for 10 min in Dulbecco's modified Eagle medium (Eurobio, Les Ulis, France) supplemented with glutamine (2 mM), 0.1% bovine serum albumin, penicillin (10 U/ml), streptomycin (100 mg/ml), and collagenase (295 U/ml). After mechanical removal of adventitial and endothelial cell layers, the media was incubated in dissecting solution (20 min, 37°C) to which elastase (90 U/ml) and pronase (0.33 mg/ml) had been added. Cells were then detached by gentle pipetting of the tissue through a large hole Pasteur pipette. The procedure was then repeated twice on undigested tissue.

Isolated cells were seeded at 1.5–2.0 × 10^5 cells/ml into 25-cm^2 flasks in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Eurobio), 2 mM L-glutamine, 25 mM HEPES, pH 7.4, 10 U/ml penicillin, and 100 mg/ml streptomycin, and incubated at 37°C and 5% CO_2 in a humidified incubator. At confluence, secondary cultures were obtained by serial passage after the cells were harvested with 0.5 g/l trypsin and 0.2 g/l EDTA (Sigma-Aldrich, Saint Quentin Falavier, France) and reseeded in fresh Dulbecco's modified Eagle medium containing 10% fetal calf serum. Cells between the third and ninth passage were studied at confluence and were made quiescent by incubation for 48 h in a fetal calf serum–free media (0.5%) before the experiment. At least 10 sets of cells were analyzed at each passage.

Cell Ca^{2+} Measurements

Intracellular Ca^{2+} variations were assessed in single cells using fluorescence imaging as described previously.[16] In brief, cells were loaded with the acetoxymethyl ester form of Fura-2 (5 μM, 30 min at 37°C; Molecular Probe, AA Leiden, The Netherlands). The coverslips were mounted on the stage of a Nikon Diaphot microscope (Nikon, Kawasaki, Japan) fitted with a cooled integrating Coupled Charged Device imaging system (Newcastle Photometric System, Newcastle upon Tyne, United Kingdom), and cells were superfused with Na^+–HEPES solution at 37°C at a flow rate of 1 ml/min. An area on the coverslip chamber containing 10–15 cells was randomly selected with the microscope, and the position of the objective was focused to view the median section of the cells. Cells were illuminated alternately at 350 and 380 nm, and the intensity of emitted light from single cells during a 500-ms period at wavelength greater than 520 nm was measured. The ratio of the light intensities at the two wavelengths, plotted against time, was used to reflect qualitative changes in intracellular Ca^{2+}.[27] Because calibration procedures are prone to error, no attempt was made to calibrate the ratio values.[5,28,29]

Staining of Filamentous Actin and α-Tubulin

To assess the effect of isoflurane on the organization of the cytoskeleton, the actin and microtubular networks were visualized using double indirect immunofluorescence labeling. After two washes in phosphate-buffered saline (Life Technologies, Cergy-Pontoise, France), VSMC were fixed in 4% formaldehyde for 10 min and then permeabilized 10 min with 0.2% Triton X-100 (Sigma-Aldrich, Saint Quentin Falavier, France) at room temperature. Cultures were incubated in phosphate-buffered saline containing 5% bovine serum albumin for 60 min to block nonspecific binding sites. This was followed by overnight incubation at 4°C in a humidified chamber in the presence of monoclonal antibodies directed against α-tubulin (1/200; Sigma-Aldrich) in phosphate-buffered saline containing 2% bovine serum albumin. After washing, horse biotinylated antimouse immunoglobulin G (1/30; Vector Laboratories, Abcys, Paris, France) was added for 60 min at room temperature. Cells were washed again and incubated for 60 min with Streptavidin-Texas red (1/30; Amersham, Bioscience, Orsay, France). This was followed after washes by an incubation with fluorescein isothiocyanate conjugated–phalloidin (1/20; Sigma-Aldrich) for 90 min. After a final wash, coverslips were placed in mounting medium (Fluoprep; Merieux, Lyon, France). Slides were examined with a Leica DMLB fluorescence microscope set for fluorescein and Texas red fluorescence and connected to a Sony 3 CCD DXC 930P color camera (Sony Corp., Tokyo, Japan). The resulting images were printed on an Epson Stylus Photo 890 printer (Seiko Epson Corp., Nagano, Japan). In control experiments, the incubation steps with primary antibodies were omitted. Experiments were performed in triplicate.

Disorganization of Actin or Tubular Networks

A common approach to assess the implication of cytoskeletal elements in cellular response is to hamper the organization of a network using specific agents. Prelim-

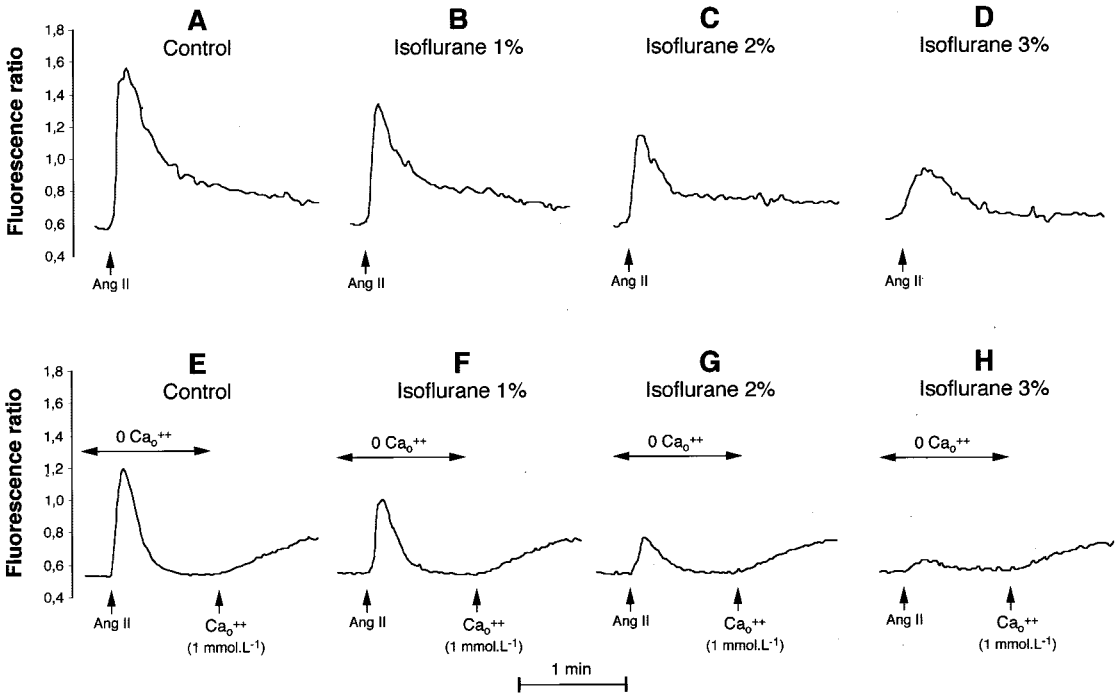

Fig. 1. Representative recordings of the effect of 1 μM angiotensin II (AngII) on intracellular Ca^{2+} increase in a control cell from WKY and in cells incubated in the presence of 1, 2, or 3% isoflurane. In protocols *A–D*, cells were exposed to AngII in the presence of external Ca^{2+} (Ca_o^{2+}). In protocols *E–H*, cells were incubated for 60 s in the nominal absence of external Ca^{2+} and then exposed to Ang II. External Ca^{2+} was reintroduced in the perfusion medium and AngII-induced Ca^{2+} influx was estimated by the time course of Ca^{2+} increase. In recordings *B–D* and *F–H*, cells were incubated in Na^+–HEPES medium containing 1, 2, and 3% isoflurane, respectively (15 min). Ratios of the emission fluorescence (> 520 nm) measured at excitation wavelengths of 350 and 380 nm are shown on the ordinate.

inary experiments were conducted to determine the optimal concentration and time of incubation for each agent to impair network organization without inducing cell shape modification. Cells were incubated 30 min at 37°C in the presence of 5 μM nocodazole, 10 μM vinblastine, 8 μM paclitaxel or 2 μM cytochalasin D and then either stimulated by AngII to assess intracellular Ca^{2+} mobilization or fixed for tubulin and actin immunostaining.

Experimental Procedure

Cells were exposed to AngII, which induced a rapid increase in intracellular Ca^{2+} (figs. 1A–D) followed by a return toward initial values. Ca^{2+} transient was characterized by its amplitude (peak minus basal ratio) and slope of intracellular Ca^{2+} increase. The optimal concentration of AngII that induced a maximal mobilization of Ca^{2+} in both stains was determined by increasing concentration (0.1 nM to 10.0 μM) in both WKY and SHR strains.[16] In accordance with previous studies, this was observed for concentrations greater than 0.5 μM.[19] Subsequently, the effect of AngII was assessed at 1 μM in both strains. AT_1 antagonists CGP-48 933 (100 nM) and CI-996 (100 nM) inhibited the response to AngII in both strains (> 95% inhibition, $P < 0.0001$ for each, results not shown). AngII-induced intracellular Ca^{2+} mobilization was not significantly different in cells from the third to the ninth passage in both strains ($F = 0.26$, analysis of variance; $P =$ not significant [NS]). As previously described, AngII induced a receptor desensitization that precluded repetitive stimulation of the same cell by AngII.[30]

AngII-induced Ca^{2+} mobilization from internal stores was assessed in the absence of external Ca^{2+} (figs. 1E–H). Ca^{2+} transient was characterized by its amplitude (peak minus basal ratio), slope of intracellular Ca^{2+} increase, and total Ca^{2+} released, estimated by area under transient. As previously described, Ca^{2+} mobilization was higher in the SHR than in WKY (table 1).[16,19]

AngII-induced Ca^{2+} influx was estimated after exposure of cells to AngII in the absence of external Ca^{2+}, from the rate of intracellular Ca^{2+} increase following reintroduction of external Ca^{2+} (fig. 1E). In preliminary experiments, intracellular Ca^{2+} changes occurring in nonstimulated cells as a consequence of Ca^{2+} chelation and reintroduction were found to be very low, with a mean of 0.002 ratio unit/min. Ca^{2+} influx elicited by AngII was not significantly altered by nifedipine (5 μM) in either strain (WKY: 98 ± 8% of control values, n = 73; SHR: 93 ± 14% of control values, n = 43; P = NS for each). To assess the role of the Na^+-Ca^{2+} exchanger in AngII-induced Ca^{2+} influx, experiments were performed in the absence of external Na^+, which had been replaced by *N*-methyl-glucamine (NMG; Sigma-Aldrich). Ca^{2+} influx was similar in Na^+–HEPES and NMG–HEPES solution in both WKY (Na^+–HEPES: 0.396 ± 0.024 ratio

Table 1. Characteristics of Ca^{2+} Mobilization Induced by Angiotensin II

	WKY (n = 34)	SHR (n = 40)	*P* Value
Angiotensin II–induced Ca_i^{2+} mobilization from internal stores			
Amplitude (ratio unit)	0.50 ± 0.03	0.76 ± 0.05	< 0.0001
Slope of Ca_i^{2+} increase (ratio unit/min)	6.8 ± 0.7	7.8 ± 0.7	0.37
Total Ca_i^{2+} (ratio unit/s)	9.3 ± 0.6	12.9 ± 1.1	< 0.0001
Angiotensin II–induced Ca^{2+} influx (ratio unit/min)	0.51 ± 0.05	0.51 ± 0.02	0.21

Angiotensin II (1 μM)–induced Ca^{2+} mobilization from internal stores measured in the nominal absence of external Ca^{2+} (Ca^{2+}-free medium), and Ca^{2+} influx measured upon reintroduction of external Ca^{2+} (1 mM). Data are presented as mean ± standard error of the mean. *P* value for comparison of spontaneously hypertensive rats (SHR) *versus* Wistar-Kyoto (WKY) rats. n = number of cells analyzed; Ca_i^{2+} = intracellular Ca^{2+}.

unit/min; NMG–HEPES: 0.402 ± 0.042; n = 109; *P* = NS) and SHR (Na^+–HEPES: 0.481 ± 0.048 ratio unit/min; NMG–HEPES: 0.410 ± 0.040; n = 103; *P* = NS). This suggests that the participation of the Na^+–Ca^{2+} exchanger in Ca^{2+} influx is negligible in our conditions.

Anesthetic Agent

Output from an isoflurane calibrated vaporizer (Fortec 3; Cyprane LTD, Keighley, United Kingdom) was bubbled through 100-ml glass bottles containing either Na^+–HEPES or Ca^{2+}-free Na^+–HEPES solution and allowed to equilibrate for 60 min at 37°C. Air at a flow rate of 1 l/min was used as a carrier gas. Isoflurane (obtained from Abbott Laboratories, Cergy, France) was studied at concentration of 0.5, 1, 2, or 3 vol% in air. No correction was performed to adjust for interspecies variation of anesthetic potency because end-tidal isoflurane requirement to achieve a 1.0 minimal alveolar concentration level of anesthesia has been reported to be very similar in WKY (mean ± SD: 1.20 ± 0.05%) and SHR (mean ± SD: 1.26 ± 0.07; difference NS).[31] Teflon tubing was used to reduce anesthetic agent loss between the bottle and the recording chamber. Aqueous phase concentrations of isoflurane in the solution, sampled at the recording chamber level, were determined by direct spectrometry at three wavelengths (210, 280, and 600 nm) using a variable wavelength spectrometer (model 1050; Hewlett Packard, Les Ulis, France).[32] Isoflurane concentrations measured after 60-min equilibration with the superfusion solution were 0.27 ± 0.05, 0.51 ± 0.08, and 0.68 ± 0.13 mM (mean ± SEM) at 1, 2, and 3%, respectively. To ensure stable isoflurane concentration in the solution at the recording chamber level, the chamber itself was covered by a semiclosed Plexiglass reservoir, continuously aerated by a derivation from the output of the vaporizer. Isoflurane concentration in the gas phase of this reservoir was continuously monitored by an infrared calibrated analyzer (Capnomac; Datex Instrumentarium, Helsinki, Finland).

Fluids and Drugs

The composition of the Na^+–HEPES solution was 140 mM NaCl, 4.5 mM KCl, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 1.0 mM $CaCl_2$, 5.6 mM glucose, and 10 mM HEPES. Ca^{2+}-free Na^+–HEPES solution was made without $CaCl_2$ and with the addition of 1 mM EGTA. NMG–HEPES solution was made by replacing the NaCl with NMG. Penicillin and streptomycin were obtained from Life Technology (Cergy-Pontoise, France). AngII, thapsigargin, cytochalasin D, nocodazole, vinblastine, and paclitaxel (all obtained from Sigma-Aldrich), CI-996 (obtained from Parke-Davis Pharmaceutical Research, Ann Arbor, MI), CGP-48933 (obtained from Ciba-Geigy, Basle, Switzerland), and nifedipine (obtained from Bayer, Puteaux, France) were dissolved in Na^+–HEPES solution before use.

Statistical Analysis

Results are presented as mean ± SEM. The values of parameters of Ca^{2+} mobilization measured in treated cells are expressed as percentage of the control values (untreated cells). Student *t* test for unpaired data were used to compare mean values obtained in control cells with values obtained in treated cells and to compare mean values obtained in WKY with those in SHR. A comparison of the values obtained in cells from passages 3–9 for each strain was performed by analysis of variance with multiple testing according to the Bonferroni method. Statistical analysis were performed using Statview 5.1 software (SAS Institute Inc., Cary, NC). $P < 0.05$ was considered significant.

Results

Effect of Isoflurane on Cell Ca^{2+} Handling

Resting cell Ca^{2+} concentration was not significantly altered by exposure of VSMC to 3% isoflurane for 10 min (WKY: 100 ± 6% of control value, n = 29, *P* = NS; SHR: 103 ± 6%, n = 30, *P* = NS) or for 15 min (WKY: 99 ± 6% of control value, n = 25, *P* = NS; SHR: 105 ± 6%, n = 28, *P* = NS).

To assess the effect of isoflurane on total intracellular Ca^{2+} mobilization, cells loaded with Fura-2 were incubated in the presence of 0.5–3% isoflurane for 15 min and then exposed to AngII in the presence of external Ca^{2+}. In both strains, isoflurane induced a concentration-dependent decrease in intracellular Ca^{2+} release elicited by AngII in the presence of external Ca^{2+} (WKY: figs. 1A–D and table 2; SHR: table 3). This effect was observed at concentration of isoflurane greater than 0.5% for WKY and 1% for SHR.

To assess the effect of isoflurane on AngII-induced intracellular Ca^{2+} release from internal stores, cells were

Table 2. Effects of Isoflurane on Angiotensin II–induced Ca^{2+} Mobilization in Wistar-Kyoto Rats

	Isoflurane (%)			
	0.5	1	2	3
Angiotensin II–induced Ca^{2+} mobilization				
In the presence of external Ca^{2+}				
Amplitude	89 ± 13	63 ± 3‡	54 ± 4‡	49 ± 5‡
Slope	99 ± 13	79 ± 5*	63 ± 6‡	39 ± 5‡
n (cells/coverslips)	42/5	67/6	80/7	58/6
In the absence of external Ca^{2+}				
Amplitude	97 ± 10	72 ± 6†	40 ± 3‡	7 ± 4‡
Slope	82 ± 11	75 ± 5*	28 ± 4‡	4 ± 2‡
Total Ca^{2+}	94 ± 7	77 ± 9	35 ± 4‡	9 ± 4‡
n (cells/coverslips)	65/5	40/6	41/6	26/5
Angiotensin II–induced Ca^{2+} influx	84 ± 12	78 ± 4†	41 ± 4‡	35 ± 4‡
Thapsigarghin-induced Ca^{2+} influx	NM	67 ± 4‡	35 ± 3‡	29 ± 4‡
n (cells/coverslips)	—	62/6	45/6	66/6

Effect of isoflurane (0.5–3%) on angiotensin II (1 μM)–induced intracellular Ca^{2+} mobilization in the presence (Na^+-HEPES medium) and nominal absence of external Ca^{2+} (Ca^{2+}-free medium) and on Ca^{2+} influx induced either by angiotensin II or thapsigarghin in vascular smooth muscle cells from Wistar-Kyoto rats. Results are expressed as percent of control values obtained in nontreated cells.

* $P < 0.05$, † $P < 0.001$, and ‡ $P < 0.0001$ for isoflurane-treated cells *versus* nontreated cells.

n = number of cells/number of coverslips analyzed; NM = not measured.

incubated in the presence of 0.5–3% isoflurane for 15 min and then exposed to AngII in Ca^{2+}-free medium. Ca^{2+} influx was assessed on reintroduction of external Ca^{2+}. In both strains, isoflurane decreased Ca^{2+} release from internal stores and Ca^{2+} influx in a concentration-dependent manner (WKY: figs. 1E–H and table 2; SHR: table 3).

To assess the effect of isoflurane on Ca^{2+} stores operated channels, a depletion of Ca^{2+} intracellular stores was induced by exposing cells to thapsigargin (3 μM), an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} adenosine triphosphatase, in the absence of external Ca^{2+}. In both strains, this induced a transient intracellular Ca^{2+} increase at a slower rate than that observed with AngII. Incubation of VSMC with thapsigargin for 5 min abolished the response to subsequent infusion of AngII in the two strains (results not shown), as previously reported.[19] Furthermore, thapsigargin completely depleted intracellular Ca^{2+} stores because ionomycin addition did not elicit any increase in cell Ca^{2+} (WKY: 98 ± 1% of inhibition, n = 28; SHR: 98 ± 2% of inhibition, n = 25; $P < 0.001$ for each). Reintroduction of Ca^{2+} (1 mM) into the medium induced a Ca^{2+} influx of similar magnitude to that observed after stimulation with 1 μM AngII. Isoflurane at concentration of 1–3% and 2–3% significantly decreased thapsigargin-induced Ca^{2+} influx in WKY (table 2) and SHR (table 3), respectively.

Table 3. Effects of Isoflurane on Angiotensin II–induced Ca^{2+} Mobilization in Spontaneously Hypertensive Rats

	Isoflurane (%)			
	0.5	1	2	3
Angiotensin II–induced Ca^{2+} mobilization				
In the presence of external Ca^{2+}				
Amplitude	104 ± 8	94 ± 3#	86 ± 4#	79 ± 5†#
Slope	103 ± 8	83 ± 8	63 ± 5‡	54 ± 6‡§
n (cells/coverslips)	89/7	49/7	69/5	53/4‖
In the absence of external Ca^{2+}				
Amplitude	102 ± 11	92 ± 5†	61 ± 5‡‖	35 ± 3‡‖
Slope	101 ± 13	84 ± 5	57 ± 4†‖	57 ± 6‡§
Total Ca^{2+}	114 ± 12	89 ± 5	56 ± 5‡‖	30 ± 4‡§
n (cells/coverslips)	32/5	32/4	51/6	35/6‖
Angiotensin II–induced Ca^{2+} influx	89 ± 15	92 ± 5	71 ± 5‡#	30 ± 3‡
Thapsigarghin-induced Ca^{2+} influx	NM	85 ± 6	51 ± 3‡	44 ± 4‡
n (cells/coverslips)	—	62/6	67/6	66/6‖

Effect of isoflurane (0.5–3%) on angiotensin II (1 μM)–induced intracellular Ca^{2+} mobilization in the presence (Na^+-HEPES medium) and nominal absence of external Ca^{2+} (Ca^{2+}-free medium) and on Ca^{2+} influx induced either by angiotensin II or thapsigarghin in vascular smooth muscle cells from spontaneously hypertensive rats. Results are expressed as percent of control values obtained in nontreated cells.

* $P < 0.05$, † $P < 0.001$, and ‡ $P < 0.0001$ for isoflurane-treated cells *versus* nontreated cells; § $P < 0.05$, ‖ $P < 0.001$, # $P < 0.0001$ for spontaneously hypertensive *versus* Wistar-Kyoto rats.

n = number of cells/number of coverslips analyzed; NM = not measured.

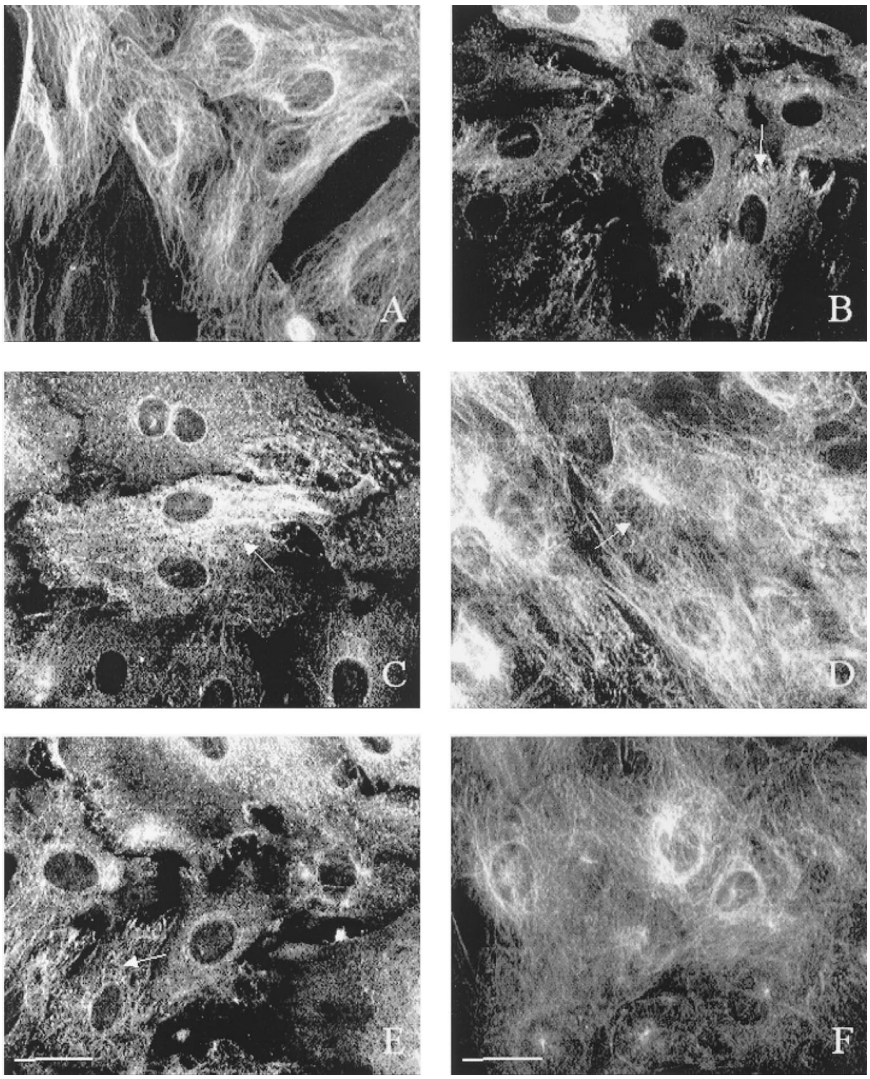

Fig. 2. Immunostaining of α-tubulin network in cells from Wistar Kyoto rats (WKY). (*A*) Control cells; (*B*) pretreatment with nocodazole (5 μM, 30 min) showing the formation of α-tubulin aggregates (*arrow*); (*C–E*) exposure to 1, 2, and 3% isoflurane, respectively (15 min), revealing the alteration of tubular organization (*arrows*); (*F*) exposure to 3% isoflurane (15 min), followed by 60 min of wash out, showing the reversibilty of isoflurane effect. Bar = 100 μm.

Reversibility of Isoflurane Action

To assess the reversibility of the effect of isoflurane, cells were exposed to 3% isoflurane for 15 min and washed out with isoflurane-free Na^+–HEPES solution for another 60-min period. In both strains, the effect of isoflurane on AngII-induced Ca^{2+} mobilization (results not shown) was completely reversible 60 min after anesthetic retrieval.

Interaction between Cytoskeleton Elements and Isoflurane

Cytoskeletal elements have been shown recently to be involved in the Ca^{2+} mobilization induced by AngII.[16] We tested the hypothesis that the inhibitory effect of isoflurane could be linked to an interaction between isoflurane and actin or tubulin networks.

Effect of Disorganizing Cytoskeleton Elements on Isoflurane Action

We first studied the effect of an alteration of cytoskeleton dynamic on the inhibition AngII-induced Ca^{2+} mobilization by isoflurane.

To assess the implication of α-tubulin network, cells were incubated in the presence of nocodazole (5 μM, 30 min at 37°C) and exposed to 2% isoflurane. At this concentration, nocodazole disorganized microtubular network without altering cell shape (WKY: fig. 2, SHR: fig. 3), and, as expected, no differential effect could be observed on actin network with this agent (result not shown). As previously described,[16] pretreatment with nocodazole significantly reduced AngII-induced Ca^{2+} mobilization from internal stores (fig. 4), whereas Ca^{2+} influx was not significantly altered in both strains. In both WKY and SHR, 2% isoflurane had no significant effect on Ca^{2+} mobilization in cells pretreated with nocodazole (fig. 4). Similar results on Ca^{2+} release from internal stores were obtained with vinblastine or paclitaxel, which alter organization of microtubules by different mechanisms, in both WKY and SHR (fig. 4).

The role of actin network was assessed using pretreatment with cytochalasin D at a concentration of 2 μM for 30 min, known to disrupt actin network, with the for-

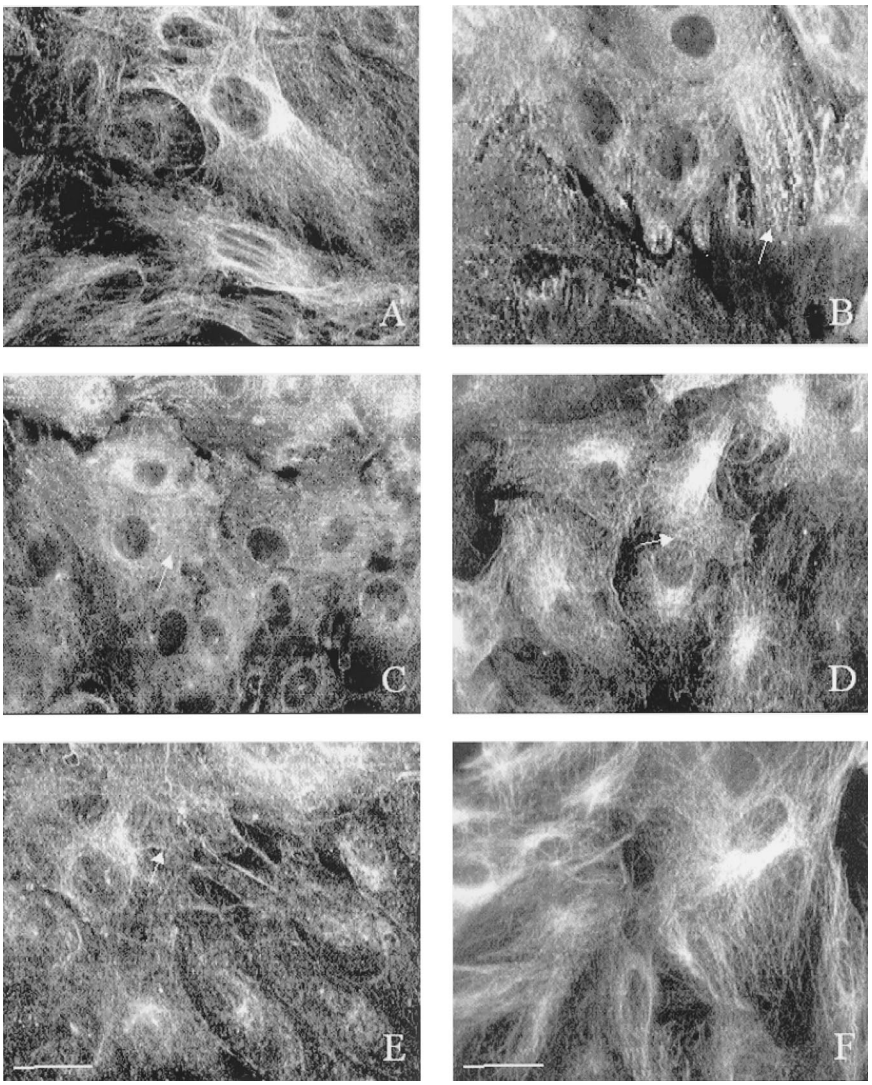

Fig. 3. Immunostaining of α-tubulin network in cells from spontaneously hypertensive rats (SHR). (*A*) Control cells; (*B*) pretreatment with nocodazole (5 μM, 30 min) showing the formation of α-tubulin aggregates (*arrow*); (*C–E*) exposure to 1, 2, and 3% isoflurane, respectively (15 min), revealing the alteration of tubular organization (*arrows*); (*F*) exposure to 3% isoflurane (15 min), followed by 60 min of wash out, showing the reversibilty of isoflurane effect. Bar = 100 μm.

mation of patches scattered throughout the cytoplasm, without altering cell shape (control: fig. 5A [WKY] and fig. 5B [SHR]; cytochalasin D: fig. 5C [WKY] and fig. 5D [SHR]). As previously described,[16] pretreatment with cytochalasin D significantly decreased the AngII-induced intracellular Ca^{2+} transient in the SHR (amplitude: 61 ± 14% of control value; $P < 0.001$), whereas it was not altered in cells from WKY (105 ± 8%; P = NS). Pretreatment with cytochalasin D did not significantly alter the effect of 2% isoflurane in both WKY (44 ± 9% of control; $P < 0.01$) and SHR (56 ± 8% of control; $P < 0.01$).

Effect of Isoflurane on Cytoskeleton Organization

We also tested the hypothesis that the effect of isoflurane on AngII-induced Ca^{2+} mobilization could be linked to an alteration by isoflurane itself of cytoskeletal network organization. To study this, immunofluorescent staining of α-tubulin and actin were performed in VSMC

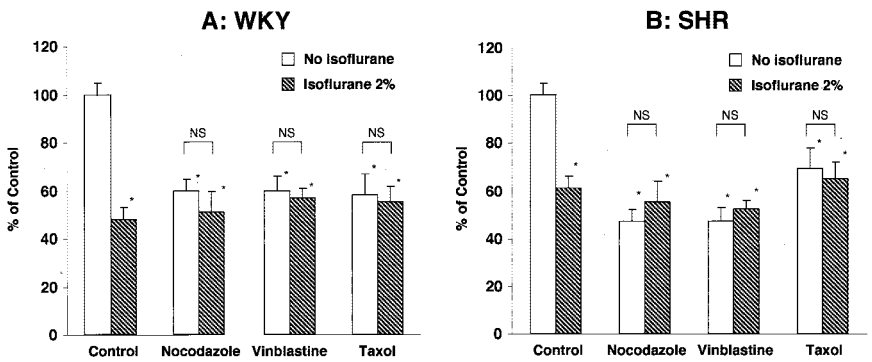

Fig. 4. Effect of nocodazole (5 μM, 30 min), vinblastine (10 μM, 30 min), or paclitaxel (8 μM, 30 min) on the amplitude of angiotensin II–induced Ca^{2+} mobilization in cells from Wistar Kyoto rats (WKY; *A*) and spontaneously hypertensive rats (SHR; *B*) in the presence or absence of 2% isoflurane. Results are expressed as percent of values obtained in control cells. *$P < 0.05$, treated *versus* control cells.

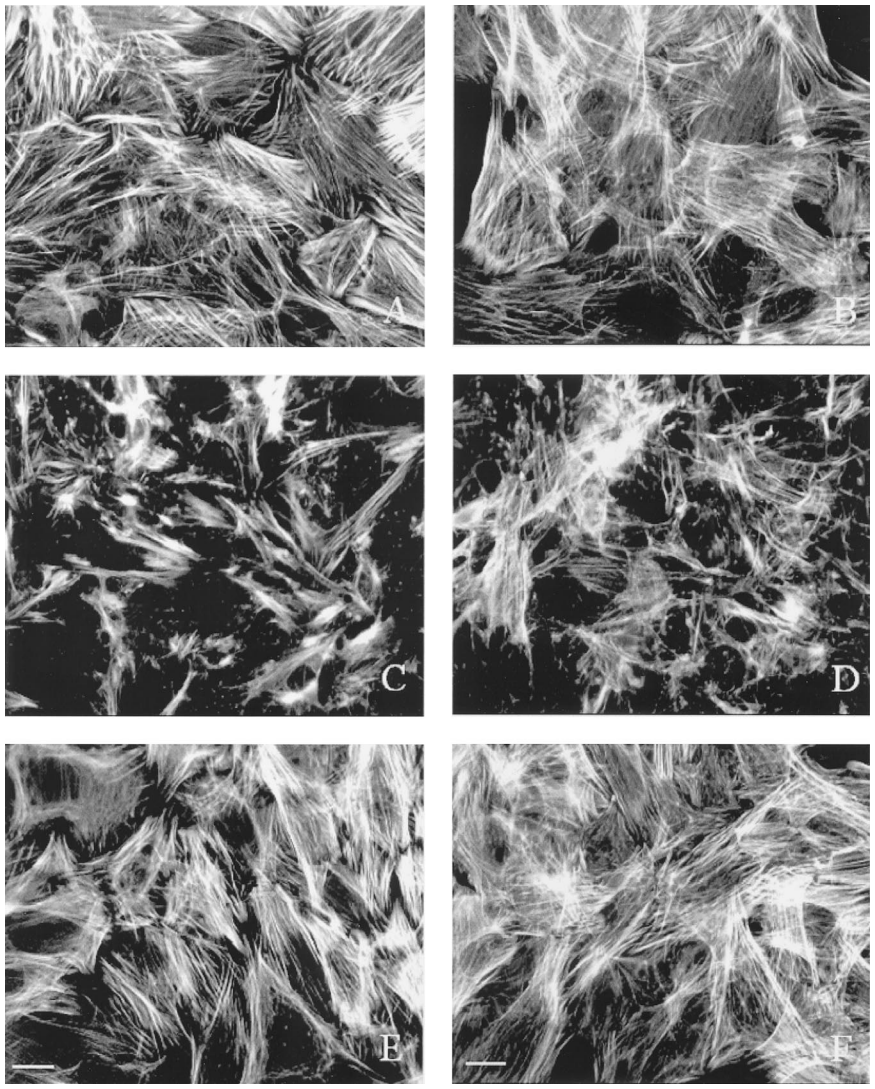

Fig. 5. Immunostaining of actin network, in cells from Wistar Kyoto rats (WKY; *A–C*) and spontaneously hypertensive rats (SHR; *D–F*). (*A* and *D*) Control cells; (*B* and *E*) cytochalasin D (2 μM, 30 min), showing the alteration in actin organization; (*C* and *F*) exposure to 3% isoflurane (15 min), showing the lack of visible effect of isoflurane on actin network.

exposed to 1, 2, and 3% isoflurane for 15 min. In control conditions, α-tubulin network was regular and homogeneous in both WKY (fig. 2A) and SHR (fig. 3A). Isoflurane altered in a dose-dependent manner the tubular organization with formation of α-tubulin aggregates. This effect was observed at a concentration of 1% or greater in the WKY and 2% or greater in SHR (figs. 2 and 3). It is noteworthy that at 3% isoflurane, alteration in α-tubulin networks were similar to that produced by nocodazole (5 μM; figs. 2B and 3B). These alterations were reversible 60 min after anesthetic retrieval (figs. 2F and 3F).

In contrast, isoflurane did not alter actin network organization in both WKY (control: fig. 5A; 3% isoflurane: fig. 5E) and SHR (control: fig. 5B; 3% isoflurane: fig. 5F).

Discussion

The current study demonstrated that clinically relevant concentrations of isoflurane decreased Ca^{2+} mobilization elicited by AngII in both normotensive and hypertensive rat strains. Isoflurane reduced both AngII-induced Ca^{2+} mobilization from internal stores and Ca^{2+} influx through nifedipine-insensitive Ca^{2+} channels. The hypertensive strain was found more resistant to isoflurane than the normotensive one. This effect appeared to be strongly associated with a disorganization of the microtubular network by the anesthetic agent in both strains.

The effect of isoflurane on AngII-induced Ca^{2+} mobilization is consistent with the effect of isoflurane on Ca^{2+} mobilization elicited by other agonists known to stimulate phospholipase C, such as vasopressin.[33] An effect of isoflurane on Ca^{2+} stores *per se* is unlikely, as no significant increase in cell Ca^{2+} could be observed when cells were exposed to isoflurane alone. This is in accord with previous data reported by Akata *et al.*[34] This suggests that the inhibition by isoflurane of AngII-induced Ca^{2+} increase is related to an alteration in the dynamic of Ca^{2+} release elicited by AngII, rather than an effect on Ca^{2+} stores. This effect does not seem to be consequent to an interaction with the AT_1 receptors or

AT_1-related G_{q11} protein, because isoflurane did not affect AngII-induced inositol 1,4,5-triphosphate synthesis or lipophosphatidate signaling.[35,36]

We therefore tested the hypothesis that isoflurane could affect the signaling pathway of AngII downstream of inositol 1,4,5-triphosphate synthesis. Our working hypothesis was that an interaction with microtubules network could be responsible of the inhibition of Ca^{2+} release from internal stores. Previous studies have reported the role of microtubular network in the transduction of extracellular signal in various cell lines. In this regard, AngII-induced Ca^{2+} mobilization in VSMC from both WKY and SHR strains was reported to require an intact microtubular network.[16] On the other hand, Allison *et al.*[8] have shown, using electron microscopy, that short-term exposure of cells to halogenated anesthetics could induce dispersal of the microtubule system and disaggregation of the microtubules.[8] This effect was concentration-dependent and reversible after withdrawal of the agent and was found to be associated with a direct effect on microtubule assembly.[9] More recently, an intact microtubular network was shown to be critical in the process of isoflurane-induced preconditioning in cardiomyocytes.[37] Our results showed that the effect of isoflurane on AngII-induced Ca^{2+} release was abolished in cells pretreated with either nocodazole, vinblastine, or paclitaxel, agents known to alter the micotubular dynamics by different mechanisms. These results, taken together with the direct and reversible effect of isoflurane on microtubular assembly, strongly suggest that the effect of isoflurane on Ca^{2+} release may be related to an interaction with microtubular network, at a level that remains to be determined. This implication of the microtubules in the signaling pathways linking AngII receptor to Ca^{2+} release appears to be similar in both WKY and SHR. In contrast, isoflurane had no visible effect on actin network, and disorganizing the actin network alone did not affect the response to isoflurane in the WKY.

Agonist-stimulated release of intracellular Ca^{2+} from the intracellular stores is accompanied by repletion of the store by Ca^{2+} influx from the extracellular space.[7] It is now recognized that the action of AngII in VSMC involves both voltage-operating channels and voltage-independent channels.[38] The relative contribution of these two pathways to cell Ca^{2+} increase depends on the VSMC type and experimental conditions.[39] The lack of effect of nifedipine in this study suggests that Ca^{2+} influx is mediated by voltage-independent channels, in accordance with previous reports.[6] This is further supported by our observation that agonist-independent depletion of intracellular Ca^{2+} stores with thapsigargin activates a Ca^{2+} entry pathway. This pathway, termed "capacitative Ca^{2+} entry," has been reported in different cell types, including VSMC, is insensitive to nifedipine, and contributes to smooth muscle tone.[39–41] The current results show that isoflurane inhibits both AngII- and thapsigargin-induced Ca^{2+} influx. This is in accord with studies showing that isoflurane inhibited the Ca^{2+} influx elicited by vasopressin or platelet-derived growth factor in A7r5 aortic VSMC and by bradykininin in bovine aortic endothelial cells.[33,42] In contrast, Hirata *et al.*[43] have shown that isoflurane enhanced receptor-operated Ca^{2+} influx in VSMC from rat thoracic aorta submaximally activated by phenylephrine. These discrepencies may be related to the diversity of influx pathways and the complexity of their regulation by signaling elements.

The increase in intracellular free Ca^{2+} is the principal mechanism initiating contraction in VSMC. AngII induces a pharmacomechanical excitation–contraction coupling that occurs without changes of the membrane potential.[44] Several studies have suggested that microtubules can affect the contractile process in several cell types.[10,27] Despite this emerging evidence, the mechanisms of these observations have not been established, and the consequence of the alteration in response to AngII induced by isoflurane on VSMC contraction remains unknown. However, it may be linked to the role of AngII in short-term control of blood pressure during isoflurane anesthesia.[4]

Another main result of this study is the lower sensitivity of the SHR to isoflurane as compared with the WKY strain. This difference cannot be linked to an interspecies variation in anesthetic potency, because anesthetic requirement of isoflurane was shown to be similar in WKY and SHR.[31] In contrast, it could be related to the structural and functional abnormalities of the arterial wall and to the alteration in Ca^{2+} handling observed in VSMC from SHR.[18,20,23] Intracellular Ca^{2+} concentration and Ca^{2+} storage pools in cultured aortic VSMC from SHR were found to be increased during nonstimulated conditions. The response to various agonists, including AngII, is also enhanced in the SHR, as shown in the current study and in that by Cortes *et al.*[19] In this regard, we observed an increase in the amplitude of intracellular Ca^{2+} variation induced by AngII, suggesting an increase in Ca^{2+} mobilization from internal stores in the SHR as compared with WKY, in accordance with previous studies.[17] Furthermore, regulation of AngII signaling pathways of the normotensive and hypertensive strains have been shown to differ in several respects, such as role of tyrosine kinase or mitogen-activated protein kinase, suggesting a signaling pattern characteristic of the hypertensive phenotype.[5,16,45] Whether this difference in sensitivity to isoflurane could be related to the greater instability of arterial blood pressure during isoflurane administration deserves further investigation. However, human hypertension differs in several points from hypertension of SHR, and our results cannot by directly extrapolated to humans with chronic hypertension.

Large conduit arteries do not contribute to peripheral vascular resistance, and the difference between the two strains we reported cannot be directly extrapolated to

the regulation of arterial blood pressure during isoflurane anesthesia. However, Ca^{2+} mobilization elicited by AngII was reported to differ only minimally between aortic and mesenteric vessels.[23] The model of aortic VSMC from SHR strain is of particular interest in hypertension, because the structural and functional changes of arterial wall have been linked to the alteration in arterial blood control and to detrimental consequences of hypertension. Furthermore, studying the effect of isoflurane on AngII-induced signaling pathways in this model could contribute to a better understanding of the molecular mechanisms implicated in the effect of anesthetics.

In conclusion, the current results established that isoflurane altered AngII-induced Ca^{2+} mobilization from internal stores and Ca^{2+} entry through capacitative Ca^{2+} channels in cultured rat aortic VSMC. The hypertensive rats were less sensitive to isoflurane than normotensive ones. This effect of isoflurane was associated to a disorganization of the microtubular network and implicated similar mechanisms in both normontensive and hypertensive strains. These results may prove useful in understanding the alteration in vascular reactivity observed during isoflurane anesthesia.

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