Novel Expression of GABA_A Receptors on Resistance Arteries That Modulate Myogenic Tone

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Abstract

The clinical administration of GABAergic medications leads to hypotension which has classically been attributed to the modulation of neuronal activity in the central and peripheral nervous systems. However, certain types of peripheral smooth muscle cells have been shown to express GABA_A receptors, which modulate smooth muscle tone, by the activation of these chloride channels on smooth muscle cell plasma membranes. Limited prior studies demonstrate that non-human large-caliber capacitance blood vessels mounted on a wire myograph are responsive to GABA_A ligands. We questioned whether GABA_A receptors are expressed in human resistance arteries and whether they modulate myogenic tone. We demonstrate the novel expression of GABA_A subunits on vascular smooth muscle from small-caliber human omental and mouse tail resistance arteries. We show that GABA_A receptors modulate both plasma membrane potential and calcium responses in primary cultured cells from human resistance arteries. Lastly, we demonstrate functional physiolog-

ic modulation of myogenic tone via GABA_A receptor activation in human and mouse arteries. Together, these studies demonstrate a previously unrecognized role for GABA_A receptors in the modulation of myogenic tone in mouse and human resistance arteries.

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Introduction

The prevalence of hypertension in the USA is greater than 30% in the adult population and the health care costs associated with hypertension are astronomical [1]. The direct correlation between hypertension and other comorbid states has made hypertension a risk factor for almost all leading causes of mortality [2, 3] and morbidity [4–6]. As the population of patients with hypertension increases [2], new and effective therapies are needed to treat patients who are failing current therapies for hypertension.

It is well recognized that clinically used GABAergic anesthetics are associated with hypotension due to a decreased systemic vascular resistance. Mechanisms of GABAergic anesthetic-induced hypotension are thought to

include peripheral autonomic ganglion inhibition [7], central paraventricular nucleus inhibition [8], and baroreceptor inhibition [9]. However, these neuronal targets may not provide a complete mechanism of anesthetic-induced hypotension. Furthermore, there has been growing evidence that GABAergic anesthetic-mediated vasorelaxation is due to direct activation of GABA_A receptors on vascular smooth muscle (VSM) cells [10–14]. However, these studies were performed in large arteries or in cell cultures derived from these large vessels, neither of which are the physiologic determinants of blood pressure. The primary determinants of vascular resistance are small resistance arteries. These resistance arteries have not yet been evaluated for their intrinsic GABAergic response on myogenic tone or their GABA_A receptor subunit expression.

GABA_A receptors are ligand-gated, inhibitory chloride channels that are well characterized in the central nervous system (CNS). While their physiology and pharmacology have been extensively characterized in the CNS, they have only recently been appreciated to be functionally expressed on many peripheral cell types, including other non-VSM [15]. Classic GABA_A receptors are heteropentamers containing a combination of 19 possible subunits (α [1–6], β [1–3], γ [1–3], δ , ϵ , θ , π , and ρ [1–3]). GABA_A channels containing the necessary subunits to form a functional channel have been previously described in airway smooth muscle [16]. Furthermore, activation of these GABA_A receptor channels on airway smooth muscle cells increased chloride channel flux and attenuated calcium release into the intracellular compartment, leading to a direct decrease in airway smooth muscle tension [15, 17–23]. Recognizing the vasodilatory effects of GABAergic anesthetics and the growing evidence of direct GABA_A-mediated smooth muscle relaxation, we hypothesize that GABA_A receptors exist on VSM cells of small resistance arteries and have significant effects on resistance arterial tone and function.

Materials and Methods

Materials

Gabazine, NS1619, and KCl were obtained from Sigma (St. Louis, MO, USA). Muscimol, picrotoxin, and bicuculline were obtained from Tocris (Bristol, UK). Propofol was obtained from MP Biomedical (Santa Ana, CA, USA). The fluorescent potentiometric probe (FLIPR) membrane potential assay kit was obtained from Molecular Devices (Sunnyvale, CA, USA). Trypsin 0.05%–EDTA and FURA-2 (calcium dye) were purchased from Invitrogen (Carlsbad, CA, USA). TRIzol reagent was obtained from Ambion (Austin, TX, USA). Immunoaffinity purified primary antibodies

for the GABA_A α 4 subunit (LS-C15) were obtained from Lifespan Biosciences (Seattle, WA, USA).

Animals and Human Tissue

All mouse protocols were approved by the Columbia University Institutional Animal Care and Use Committee. Mice were euthanized with i.p. pentobarbital. Mouse tail arteries (MTA) were obtained from 5- to 10-week-old C57/BL6 wild-type mice of both sexes. The human omental tissue protocol was reviewed by the Columbia University Institutional Review Board (IRB) and was deemed not to be human subject research under 45 CFR 46. Adult human omental samples were received from discarded tissue during colorectal surgeries, and resistance arteries (80–120 μ m) were dissected under a dissecting microscope. This method of procurement used de-identified samples as per IRB review and therefore medical information regarding patients is not available.

Cell Culture

The adventitial layer was removed from human omental arteries (HOA) using forceps under a dissecting microscope. The lumen was opened and the inner endothelial layer was mechanically abraded to remove the endothelium and basement membrane. The remaining muscle layer was then dissociated using a Worthington Biochemical (Lakewood, NJ, USA) Papain Dissociation System with collagenase type 4 (2 units/mL) added during the digestion step. Digestion occurred at 37 °C for 1 h under constant agitation by a rocker as per the manufacturer's protocol, which includes an ova mucoid gradient to isolate smooth muscle from other cell types. After isolation, the cells were grown to confluence in 75-cm² flasks and 96-well black-walled clear bottom plates for fluorescent FLIPR membrane potential assays. All cells were maintained in M199 media (Gibco-ThermoFisher, Waltham, MA, USA) with or without additives (human recombinant fibroblast growth factor 1 ng/mL, human recombinant epidermal growth factor 0.25 ng/mL, insulin 1 μ g/mL, transferrin 0.55 μ g/mL, selenium 0.67 ng/mL, and 10% fetal bovine serum) at 37 °C in 95% air/5% CO₂. Prior to all cell-based assays, primary VSM cells were maintained in serum- and additive-free M199 media (i.e., basal M199 media only) for 24 h prior to experimentation.

Isolation of RNA and RT-PCR

In experiments where RNA was isolated using laser capture microdissection (LCMD), mouse tail and human omental resistance arteries were embedded in OCT compound followed by isopentane/dry ice freezing. Frozen sections of 6 μ m thickness were made under RNase-free conditions and were placed on a single 1-mm PEN-membrane-coated slide (PALM Microlaser Technologies, Westchester, NY, USA) and processed using an LCMD staining kit (Ambion AM1935). Histologic and morphologic-guided laser dissection was performed, harvesting only central portions of smooth muscle to avoid contamination from other adjacent cell types using a PALM MicroBeam laser microscope. RNA was recovered from LCMD samples using an RNAqueous-micro kit (Ambion) according to manufacturer's recommendations (approx. 3 μ g total RNA was recovered per 5 million square microns). Recovered RNA was reverse transcribed into cDNA using a commercially available kit, SuperScript VILO (Invitrogen). LCMD-captured RNA (10 μ L) in sample buffer was reverse transcribed using random hexamer primers at 42 °C for 1 h in 20 μ L, according to the manufacturer's recommendations.

Newly synthesized cDNA (5 μ L) from RNA isolated by LCMD from MTA smooth muscle cells was used in PCR using the Advantage 2 Polymerase Kit (Clontech, Mountain View, CA, USA). Sense and antisense primers (0.4 μ M) were used for corresponding GABA_A channel subunits (Table 1) and were designed within separate exons to span large introns to ensure that contaminating genomic DNA did not yield false positive PCR bands at the molecular size expected for cDNA amplification. Primers targeting GAPDH (Table 1) were used as a relative estimate of RNA quantity between tissue samples and to confirm RNA quality in each sample. All cDNA samples were denatured at 94 °C for 10 s. Annealing and extension temperatures were all 68 °C for 1 min. Each sample underwent 30–40 cycles of amplification in a PTC-200 Peltier thermal cycler (Bio-Rad, Hercules, CA, USA). PCR products were analyzed on a 5% non-denaturing polyacrylamide gel in Tris acetate, EDTA buffer. The gel was stained with ethidium bromide (Molecular Probes, Eugene, OR, USA) and visualized using a gel imager (Biospectra UVP, Upland, CA, USA) and Visionworks software (Biospectra UVP).

Immunohistochemistry for GABA_A α 4 Subunit in HOA

Omental artery tissue samples were dissected and fixed with 5% formalin for 4 h and stored in 70% ethanol. Following serial dehydration through a graded alcohol series, fixed tissues were then embedded in paraffin blocks and serial 6- μ m sections were placed on a glass microscope slide. The slide was then dewaxed and incubated in hydrogen peroxide (30% in PBS) for 30 min. After washing and permeabilization with 0.1% Triton-X, the slide was placed in 10% goat serum in PBS for 2 h at room temperature. Serum was removed and the slide was incubated with an immunoaffinity purified GABA_A α 4 subunit-specific antibody (rabbit; LS-C15, Life-Span BioSciences, Seattle, WA, USA; 1:100) in PBS overnight at 4 °C. A serial section on the same slide was incubated under identical conditions except that the primary antibody was omitted. The slide was again washed and incubated with biotinylated goat anti-rabbit IgG (1:80) in 10% BSA/PBS for 1 h at room temperature. Biotinylated antibodies were then stained and visualized using a VectaStain (Burlingame, CA, USA) ABC kit.

Membrane Potential Measurements Using Fluorescent Potentiometric Dye

Human VSM cells were grown to 100% confluency in 96-well black-walled clear-bottom plates. Cells were made serum- and growth factor-free for 24 h prior to the assay (in basal M199 medium). On the day of the assay, cells were washed with Krebs's buffer (140 mM NaCl/4.7 mM KCl/2.5 mM CaCl₂/1.2 mM MgCl₂/11 mM HEPES/10 mM D-glucose, pH 7.4). FLIPR membrane potential blue dye was dissolved in Krebs buffer at the concentration of 1 vial (approx. 125 mg) in 100 mL. Cells were incubated in 100 μ L of 50% dye for 20 min in a humidified 37 °C cell culture incubator (95% air/5% CO₂). Fluorescence was repetitively measured at 2-s intervals in a prewarmed (37 °C) Flex Station 3 UV spectrophotometer (Molecular Devices) using an excitation wavelength of 530 nm, an emission wavelength of 565 nm, and a cutoff filter of 550 nm. NS1619 100 μ M (potassium channel opener causing hyperpolarization), 40 mM KCl (depolarizing agent), and 10 μ M propofol or 0.1% DMSO control was added at a single concentration using the automated injection feature of the spectrophotometer during continuous fluorescent readings. In some experiments bicuculline was added to the wells manually 5 min before propofol as a pretreatment.

Intracellular Calcium Measurements Using a Fluorescent Ratiometric Calcium Indicator

Primary cultures of HOA smooth muscle cells were grown to full confluence in black-walled, clear-bottomed, 96-well plates. The cells were made serum- and growth factor-free for 24 h prior to the assay (in basal M199 medium). They were washed with 100 μ L (per well) of Hanks' balanced salt solution (HBSS) and then loaded with HBSS (100 μ L/well) containing 2.5 μ M Fura-2 AM, before being incubated for 30 min at 37 °C. The wells were then washed with HBSS and an additional incubation step was performed for 30 min at 37 °C (95% air, 5% CO₂). The cells were then pretreated for 2 min with muscimol 1–100 μ M, propofol 0.1–10 μ M or vehicle (0.1% DMSO) before the addition of 50 nM endothelin-1 by use of the automatic injection feature of the FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) during continuous fluorescent measurements (excitation 340/380 nm and emission 510 nm). Real-time changes in intracellular Ca²⁺ are reported as RFU (relative fluorescence units).

Electrophysiologic Recordings of VSM Using an Automated Patch Clamp System

Human VSM cells were dispersed into Krebs's buffer at a concentration of 5 million cells/mL and cells were made serum- and growth factor-free for 24 h prior to the assay (in basal M199 medium). The cells were loaded into a SyncroPatch 96-well automated patch clamp system (Nanion, Munich, Germany) within a disposable loading plate with a medium resistance pore size. The cells were then placed into the automated patch system and a whole cell configuration with a transient use of seal enhancement buffer. The cells were continuously analyzed for the quality of the seal resistance and capacitance. The cells were then placed under a –80-mV voltage clamp and treated with 100 μ M muscimol. The currents were recorded in 4- or 10-s sweeps at a sample rate of 20 kHz. Pretreatments were programmed into the automation, where 50 μ M picrotoxin was added in the external solution or low chloride solution replaced the internal solution. Treatments were performed on cells that initially responded to muscimol and had seals within >200 M Ω . Internal solution in mM: KCl 110, NaCl 10, EGTA 20, HEPES10 [pH 7.2]. External solution in mM: NaCl 140, KCl 4, MgCl₂ 1, CaCl₂ 2, glucose 5, Hepes 10 (pH 7.4). Seal enhancer solution in mM: NaCl 80, KCl 3, MgCl₂ 10 CaCl₂, Hepes 10 (pH 7.4). Low chloride internal solution in mM: K-gluconate 110, NaCl 10, EGTA 20, HEPES 10 (pH 7.2).

Resistance Artery Myogenic Tone Studies

Arteries from mouse tail or human omentum were dissected, leaving the adventitia and endothelium intact. Mouse arteries of less than 110 microns in diameter were used, and human arteries less than 300 microns in diameter were used. Vessels were cannulated with glass micropipettes (75–100 μ m). The pipettes were mounted within a chamber that was continuously perfused with buffered solution (buffer used in human studies in mM: 118 NaCl, 5.6 KCl, 2.5 CaCl₂, 2.4 MgSO₄, 1.3 NaH₂PO₄, 25 NaHCO₃, and 5.6 glucose, pH 7.4; buffer used in murine studies in mM: 115 NaCl, 2.5 KCl, 2.5 CaCl₂, 2.4 MgSO₄, 1.3 NaH₂PO₄, 25 NaHCO₃, and 5.6 D-glucose, pH 7.4) that was oxygenated (95% O₂/5% CO₂) and temperature controlled (37 °C). The micropipettes were connected to a peristaltic pump manually controlled to provide a constant intraluminal pressure. The wall diameter was monitored by video edge detection, measuring smooth muscle isotonic contraction or

Table 1. Sense and antisense primers

Gene	Access No.	Sequence (5' to 3')	cDNA product	gDNA product
<i>Human GABR subunit RT-PCR primer sequence</i>				
GABRA1	NM_000806	GAAGAGAAAGATTGGCTACTTTGTTATTCAAACAT GAGCGTAAGTGTGTTTTCTTAATAAGAGGAT	371	6,268
GABRA2	NM_000807	TCTGCCCTAATTGAATTTGCAACTGTTAATTACTT CTATTCTGGACATTCTGTCAATTTTGCTAACACTG	295	11,616
GABRA3	NM_000808	CACTTCCATCTCAAGCGAAAAAATTGGCTACTTTGT CCCACGATGTTGAAGGTAGTGCTGGTTTTCT	398	29,255
GABRA4	NM_000809	CAAACCGTATCAAGTGAAACCATCAAATCAAT GCTTAGTGTGGTCATGGTGAGGACAGTTGTTAT	225	9,074
GABRA5	NM_000810	GCAGACGGTGGGCACTGAGAACA GATAAGATCACGGTCATTATGCAGGGAAGGTA	138	2,734
GABRA6	NM_000811	CAGTGACAATATCAAAGCTACTGAACCTTTGGAA AATCCTGCAAATGCAACTGGGAAGAGAA	259	9,556
GABRB1	NM_000812	TCGCACTAGGAATCACGACGGTGCTTA GAGCCACTCGTCTCATTCGGATTT	316	19,063
GABRB2	NM_021911	GCTGCCAGTGCCAACAATGAGAAGA TGGGGGTCCATCGTATACAGAGAGAAA	170	4,571
GABRB3	NM_000814	TCACAACTGTGCTGACAATGACAACCATCAAC TAATTTTGAGCTGTGAAGACCTCCTCCGTAGA	474	13,271
GABRG1	NM_173536	CTTCCCCTATGGAATTCCTGTCCACTGGAATTTT CAGGCACTGCATCTTTATTGATCCAAAAAGACACC	320	6,253
GABRG2	NM_198904	AGGTCTCCTATGTCACAGCGATGGATCTCT GACACTCATAGCCGTACTCTTCATCTCTCTCT	267	4,022
	NM_198903	AGGTCTCCTATGTCACAGCGATGGATCTCT GACACTCATAGCCGTACTCTTCATCTCTCTCT	243	4,022
GABRG3	NM_033223	GACCTGGACAGATAGTCGCCTTCGATTCAACAGCACA TCTGTGGTGTCTTCTGAGGCCCATGAAGTCAAACGTATA	393	153,930
GAPDH	NM_002046	CCAGGGCTGCTTTAACTCTGGTAAAGTGATA CATCGCCCCACTTGATTTTGGAGGGA	213	432
<i>Mouse GABR subunit RT-PCR primer sequence</i>				
GABRA1	NM_010250	CTATGGACAGCCCTCCCAAGATGAACTTA GTGACGAAAATGTCGGTCTTCACTTCAGTTA	147	13,427
GABRA2	NM_008066	GAACAGAGAATCGGTGCCAGCAAGA TGCAAATTCAATTAGGGCAGAGAACACAA	175	32,654
GABRA3	NM_008067	GGTTCATAGCCGTCTGTTATGCCTTTGTA GTTTTCTTTGTTGGAGCTGCTGGTGT	158	10,348
GABRA4	NM_010251	CCTGTGCTGAAGGAGAAACACACAGAA GAATGGATTTGGACTGGAAGCTAAGTG A	210	51,841
GABRA5	NM_176942	TGCTATGCATTTGTCTTCTCTGCTCTGATT GGAGGATGGGTGAGCTTTCCAGTTGTAA	182	4,859
GABRA6	NM_008068	GTCTGAATCCCTGCAAGCAGAGATTGTT TTTAAGATGGGCGTTTCTACTGAGGGCT TT	138	7,544
GABRB1	NM_008069	TGTCGCCAGCATAGACATGGTCTCGGAA TCAGCTACTCTGTGTCAAGGGTCAGAT	138	168,664
GABRB2	NM_008070	GGGTGCCTGACACCTACTTCCTGAATGATA CAGTTTTGTTTCATCCAGTGGATACCGCCTT	175	41,666
GABRB3	NM_008071	TACCACCGTGCTCACCATGACAACCATCAAC TTTGATTTTGAGCTGTGAAGACCTCCTCCGT	475	8,373
GABRD	NM_008072	CCAGTTCACTATCACCAGTTACCGCTTCAC GCGTTCCTCACATCCATCTCTGCCCTT	441	1,292
GABRE	NM_017369	CACATGCTCAATTTTCCAATGGATTCTCACTCTT AGCGTGGCCATGGTGAGCACAGAAGTAC	377	5,545
GABRG1	NM_010252	GGAATACGGAACCTTGCAATATTTTACTAGCAACA CAAACACTGGTAGCCATAATCATCTTCCCTT	175	20,154
GABRG2	NM_008073	GACGCTGTGGATTCTGCTCCTGCTAT CTCTGGAACCTTTGGAGTCAACACCCAT	121	23,665
GABRG3	NM_008074	GCCAGCTGCAACTGCATAACTTCCCT AGTCAAACGTATAGAGCCGCATGAT	156	209,406
GABRP	NM_146017	GAGAACCTGCATTGGAGTGACAACGGTGTTA AGTTGACATTGTACCAGAGATTTCAATGCT	320	1,771
GABRQ	NM_020488	CTGTTCCCTGGATCTGAAAAATTCCTATGGAC TACCCTGGCTGCAGAGGAATCATAGTTCATCCAA	346	1,191
GAPDH	NM_008084	CCGTAGACAAAATGGTGAAGGTCGGTGTGAA CAATGAAGGGGTCGTTGATGGCAACAAT	120	1,954

relaxation (Living Systems, St. Albans, VT, USA). The arteries were exposed to 3 cycles of an acute rapid increase in intraluminal pressures from 0 mm Hg and held for 2 min at a higher intraluminal pressure; 90 mm Hg for mouse and 120 mm Hg for human, at which time the vessel diameter was measured. Once a control myogenic contraction was established, the resistance arteries were exposed to GABA_A modulators and rechallenged for 3 cycles with increasing luminal pressure (0–90 mm Hg for mouse and 0–120 mm Hg for human). The 3rd and 6th myogenic challenge under the control or experimental conditions were compared. Time (buffer) and vehicle controls (DMSO) were performed to verify the consistency between the 3rd and 6th myogenic tone challenge. GABA_A modulators included muscimol (100 μ M; specific GABA_A agonist), gabazine (100 μ M; specific GABA_A antagonist), propofol (0.1 μ M IV anesthetic with GABA_A agonist effects) in 0.1% DMSO. The myogenic tone was measured at maximal constriction within the 5 min of increased intraluminal pressure. Studies were performed with 100 μ M gabazine alone to demonstrate the effects of GABA_A antagonism on baseline tone in MTA. At an intraluminal pressure of 90 mm Hg, diameters before and after 5 min of treatment of gabazine were digitally collected. Pressures of 90 and 120 mm Hg for myogenic tone measurements were chosen based on two reasons. Firstly, these pressures have been demonstrated in multiple prior studies to cause a large magnitude stretch-induced vessel contraction (myogenic tone) [24–27]. Secondly, intraluminal pressures in these higher ranges will achieve the force generation that occurs during hypertension, allowing for an assessment as to whether GABA_A receptors have a significant effect on myogenic tone within hypertension ranges.

Statistical Analyses

Each experimental permutation included intraexperimental vehicle controls. In the fluorescence membrane potential, calcium indicator, and electrophysiology experiments, we employed one-way ANOVA with Bonferroni post test comparisons between appropriate groups. In the myogenic tone studies, a 2-tailed paired Student's *t* test was employed. Data are presented as means $\pm$ SEM. *p* < 0.05 was considered significant in all cases.

Results

RT-PCR Survey of GABA_A Subunits in Human and Mouse Resistance Arterial Vascular Smooth Muscle

To evaluate the expression profile of GABA_A receptor subunits in resistance arteries, MTA smooth muscle samples and HOA samples were analyzed for the expression of mRNAs encoding GABA_A subunits (Fig. 1). LCMD was used to histologically identify and selectively capture vascular smooth muscle (Fig. 1a). From these target tissue samples, RNA was extracted for RT-PCR (reverse transcription polymerase chain reaction). The PCR results demonstrate the expression of mRNA encoding the α 3, α 4, β 2, γ 2, and δ GABA_A subunits in MTA smooth muscle (Fig. 1b; *n* = 3), and α 4, β 2, and δ GABA_A subunits in HOA smooth muscle (Fig. 1c; *n* = 4). All negative control

water blank samples (no input RNA) did not yield any PCR products (data not shown) and all positive control brain (mouse and human, respectively) samples yielded PCR products of the expected molecular size. RT-PCR products using primers against GAPDH demonstrate the relative loading amounts of RNA from vascular smooth samples and brain samples in both human and mouse experiments (Fig. 1b, c). GABA_A subunits α 1, α 2, α 5, α 6, β 1, β 3, γ 1, and γ 3 were not detected in RNA isolated from either mouse tail or human omental arteries (data not shown) despite detecting their expected cDNA products in the brain positive control tissues. To confirm the specificity of GABA_A subunit expression in resistance arterial VSM and to demonstrate the protein expression of the GABA_A α 4 subunit, immunohistochemistry on human omental resistance arteries was performed. A longitudinally sectioned omental artery demonstrating dense brown staining representing GABA_A α 4 protein expression was evident in the smooth muscle layer (Fig. 1d; *n* = 3). The control serial section with primary antibody omitted was devoid of significant brown staining (*n* = number of mice or patients).

GABA_A Receptor Modulation of Plasma Membrane Potential in Arterial Smooth Muscle Cells

To demonstrate the function of GABA_A channels in primary VSM cells, the cells were loaded with the FLIPR potentiometric dye. Propofol (100 nM), an intravenous anesthetic with known GABA_A activity, induced a depolarizing change in membrane potential demonstrated by an increase in FLIPR fluorescence (*p* < 0.01 compared to 0.1% DMSO control; *n* = 4; Fig. 2a, b). Furthermore, the change in membrane potential induced by propofol was inhibited by pretreatment with a GABA_A-specific inhibitor, bicuculline (10 μ M; *p* < 0.05 compared to 100 nM propofol; *n* = 5), indicating that the change in membrane potential induced by propofol was via GABA_A receptor activation. KCl (40 mM) was used as a positive control for depolarization (*p* < 0.01 compared to 0.1% DMSO control; *n* = 8). NS1619 (potassium channel activator; 10 μ M) was used as a hyperpolarization control (*p* < 0.05 compared to 0.1% DMSO control; *n* = 8; Fig. 2a, b). The data were analyzed by comparing the absolute maximal fluorescence change compared to the DMSO vehicle control.

GABA_A Receptor Modulation of Intracellular Calcium in Arterial Smooth Muscle Cells

To demonstrate the effects of GABA_A receptor activation on intracellular calcium responses in cultured hu-

Fig. 1. a Representative images of OCT-embedded MTA sections. The smooth muscle layer was morphologically identified before (left image) RNA was captured from VSM cells using LCMD. The area excised is indicated by the red arrow in the right image. Representative gel images of RT-PCR products from LCMD-excised VSM from MTA (**b**) or HOA (**c**) using primers targeting mRNA encoding different GABA_A subunits. RNA extracted from whole brain from mouse and human, respectively, served as positive con-

trols. Primers targeting GAPDH mRNA demonstrate the relative mRNA input from brain versus VSM. **d** Representative light microscopic images of a longitudinal section of HOA labeled with a primary rabbit antibody targeted against human GABA_A receptor α4 subunit (brown staining; left panel). The VSM layer is denoted by an asterisk. A control serial section of HOA in which the primary antibody was omitted during immunohistochemistry is shown (right panel).

Fig. 2. **a** Aggregate tracing of the change in fluorescence in primary cell cultures of human omental VSM cells loaded with potentiometric dye (FLIPR). Fluorescence was continuously measured as 40 mM KCl, 100 nM propofol, 100 μM NS1619, or 0.1% DMSO was added. **b** Graphical representation of RFU changes during treatments of airway smooth muscle loaded with FLIPR dye. Propofol at 100 nM demonstrated a 36 ± 3.2 ΔRFU (mean ± SEM; ** $p < 0.01$ compared to control; $n = 4$), which was significantly inhibited by 10 μM of bicuculline (GABA_A-specific inhibitor; 18 ± 1.3 ΔRFU; * $p < 0.05$ compared to 100 nM propofol; $n = 3$); 40 mM KCl induced a 53 ± 4.1 ΔRFU (** $p < 0.01$ compared to control; $n = 7$). As a hyperpolarization control, 10 μM NS1619 (potassium channel activator) was used and demonstrated a -27 ± 2.7 ΔRFU (* $p < 0.05$ compared to control; $n = 7$). As a vehicle control, 0.1% DMSO vehicle demonstrated a -2.5 ± 0.66 ΔRFU ($n = 7$). **c** A representative tracing of intracellular calcium fluorescence in freshly dispersed human omental VSM cells preincubated with

calcium ratiometric dye (Fura-2). Cells were stimulated with 50 nM endothelin-1 at 50 s. In cells pretreated with vehicle (0.1% DMSO) for 2 min, endothelin-1 induced an acute increase in calcium-mediated fluorescence that was attenuated by pretreatment with either 100 nM propofol or 100 μM muscimol pretreatment. **d** Graphical representation of ratiometric fluorescence values of Fura-2-loaded human omental resistance arterial smooth muscle cells. Endothelin-1 in the presence of 0.1% DMSO vehicle demonstrated a ratiometric increase of 0.38 ± 0.06 ΔRFU (ex340/380; $n = 3$). Propofol at 10, 1, and 0.1 μM decreased endothelin responses to 0.15 ± 0.10 , 0.09 ± 0.01 , and 0.15 ± 0.03 ΔRFU (ex340/380), respectively (* $p < 0.05$ and ** $p < 0.01$, respectively, when compared to endothelin-1 alone; $n = 4$). Muscimol at 100, 10, and 1 μM decreased the endothelin-1 response to 0.11 ± 0.01 , 0.11 ± 0.01 , and 0.14 ± 0.01 ΔRFU (ex340/380), respectively (* $p < 0.05$ and ** $p < 0.01$, respectively, when compared to endothelin-1 alone; $n = 4$).

Fig. 3. Representative electrophysiologic recordings of human omental vascular cells using an automated patch clamp system. Cells were held in a voltage clamp configuration with a holding potential at -80 mV. Picrotoxin (50 μ M) inhibited muscimol (100 μ M)-induced currents (**a**), and decreasing the chloride internal concentration from 120 to 10 mM also inhibited muscimol-in-

duced currents (**b**). **c** Graphical representation of current induced by muscimol (669 ± 149 pA [mean $\pm$ SEM]; $n = 6$). Muscimol currents were significantly reduced in the presence of picrotoxin (94.7 ± 12.7 pA; $p < 0.05$ compared to muscimol; $n = 3$) and also reduced by low internal chloride concentrations (104 ± 26.2 pA; * $p < 0.05$ when compared to muscimol; $n = 3$).

man resistance arterial smooth muscle cells, the cells were pretreated with the GABAergic anesthetic propofol or the classic GABA_A agonist muscimol before a subsequent challenge with endothelin-1, a known vasoconstrictor. The cells were treated with propofol (0.1 – 10 μ M) or muscimol (1 – 100 μ M) for 2 min before treatment with endothelin-1 (50 nM). Endothelin-1 alone in the presence of 0.1% DMSO vehicle demonstrated a ratiometric increase of 0.38 ± 0.06 Δ RFU (mean $\pm$ SEM; $n = 3$), indicating an increase in the intracellular concentration of calcium (Fig. 2c, d). Pretreatment with propofol at 10 , 1 , and 0.1 μ M decreased endothelin-1-induced calcium responses ($p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively, compared to endothelin-1 alone; $n = 4$; Fig. 2d). The classic GABA_A receptor ligand, muscimol, at 100 , 10 , and 1 μ M decreased the endothelin-1 calcium responses ($p < 0.01$, $p < 0.01$, $p < 0.05$, respectively, compared to endothelin-1 alone; $n = 4$; Fig. 2d).

Electrophysiologic Recordings of Human Arterial Smooth Muscle Cells Using an Automated Patch Clamp System

To quantify the electrophysiologic responses of GABA_A channel activation in human omental resistance arterial smooth muscle cells, electrical current recordings were performed using an automated patch clamp system. Primary cultured cells of resistance arterial smooth muscle demonstrated inward current responses to 100 μ M muscimol (Fig. 3; $n = 6$). A muscimol response was demonstrated in 10% of cells patched in culture. To demonstrate the specificity of the muscimol response, the muscimol-induced currents were inhibited by the GABA_A-specific inhibitor picrotoxin ($p < 0.05$ compared to muscimol alone; $n = 3$; Fig. 3a, c). Furthermore, to demonstrate that the GABA_A response was indeed a chloride current, the internal solution was changed to a low chloride composition (by replacing KCl with K-gluconate) reducing the internal chloride concentration from 120 to 10

Fig. 4. **a** Representative vessel diameter tracings recorded during pressure myography in mouse tail resistance arteries. Pressure was raised from 0 to 90 mm Hg, which induced an initial dilation that induced a contraction (myogenic tone) as demonstrated in the control (buffer solution alone) tracing. Propofol pretreatment inhibited myogenic tone resulting in increased dilation. The GABA_A antagonist gabazine antagonized the propofol dilatory effects, resulting in a control level of myogenic tone. **b** Quantitative analysis of myogenic tone expressed as a change in vessel diameter. In control studies, a 0- to 90-mm Hg intraluminal pressure challenge resulted in a mean (±SEM) diameter change of 26 ± 7.4 μm, while propofol pretreatments blocked myogenic tone, resulting in vaso-dilation and a larger change in vessel diameter (50 ± 5.8 μm [mean ± SEM]; * $p < 0.05$; $n = 3$). **c** Co-pretreatment with propofol and the GABA_A antagonist gabazine resulted in a change in vessel diameter (30 ± 5.8 μm) that was not different from the control (31 ±

10 μm), indicating that the propofol inhibition of myogenic tone is GABA_A mediated (ns; $n = 3$). **d** Representative vessel diameter tracings during pressure myography in MTA (0–90 mm Hg). Muscimol pretreatment inhibited myogenic tone and increased vessel dilation. **e** Quantitative analysis of myogenic tone under control conditions demonstrated a vessel diameter change of 22 ± 5.6 μm. The GABA_A agonist muscimol inhibited myogenic tone, resulting in an increased vessel diameter of 33 ± 7.5 μm (* $p < 0.05$; $n = 3$). **f** Results demonstrating no significant difference (ns; $n = 3$) between 0.1% DMSO (19 ± 3.6 μm) and the control (buffered solution alone, 16 ± 2.8 μm) on myogenic tone generation. **g** Effects of gabazine alone on baseline tone at 90 mm Hg. Gabazine treatment demonstrated no significant change to baseline tone (94.8 ± 11.4 μm) at 90 mm Hg (ns; $n = 3$) when compared to the control (buffered solution alone, 95.1 ± 10.5 μm).

mm. This resulted in an equalization of the electrochemical gradient for chloride and a loss of muscimol-induced current ($p < 0.05$ compared to muscimol alone; $n = 3$; Fig. 3b, c).

GABA_A Ligands Decreased Myogenic Tone in Mouse Tail Resistance Artery

In order to study the physiologic effects of GABA_A activation in intact vessels, resistance artery myogenic tone studies were performed using pressure myography. Under

Fig. 5. a Representative diameter tracings recorded during pressure myography in human omental resistance arteries. Intraluminal pressure was increased from 0 to 120 mm Hg, inducing an initial dilation that induced a control contraction (myogenic tone). Muscimol, a GABA_A-specific agonist, pretreatment inhibited myogenic tone and increased vessel dilation. **b** Quantitative analysis of myogenic tone under control conditions demonstrated a vessel diameter change of $38 \pm 0.7 \mu\text{m}$ (mean $\pm$ SEM). The GABA_A agonist muscimol inhibited myogenic tone, resulting in an increased vessel size ($42 \pm 0.7 \mu\text{m}$; * $p < 0.05$; $n = 3$).

control conditions (buffer alone), mouse tail resistance arteries demonstrated myogenic tone when challenged with an increase in intraluminal pressure from 0 to 90 mm Hg (Fig. 4a). The addition of propofol ($0.1 \mu\text{M}$) to the buffer superfusing the arterial segment decreased myogenic tone, resulting in an increased dilation ($p < 0.05$; $n = 3$; Fig. 4a, b). The inhibition of myogenic tone by propofol was reversed by the addition of $100 \mu\text{M}$ gabazine, a GABA_A-specific inhibitor ($p > 0.05$; $n = 3$), resulting in myogenic tone that was not significantly different from the control (ns; $n = 3$; Fig. 4a, c), indicating that the propofol inhibition of myogenic tone is GABA_A mediated.

To further prove that GABA_A activation reduces myogenic tone, the GABA_A agonist muscimol ($100 \mu\text{M}$) was added to the buffer superfusing the MTA before the change in the intraluminal pressure from 0 to 90 mm Hg. Muscimol inhibited myogenic tone, resulting in an increased diameter change, which was significantly dilated compared to the control (buffered solution alone; Fig. 4d, e; $n = 3$). Time controls were performed to demonstrate a maintenance of myogenic responses between the 3rd rise in intraluminal pressure and the 6th rise in intraluminal pressure. No significant differences were found between the 3rd pressure cycle and the 6th pressure cycle in the presence of 0.1% DMSO vehicle control (Fig. 4f). A gabazine control was performed by measuring the diameter change at 90 mm Hg before and after $100 \mu\text{M}$ gabazine was added. There was no significant change in the plateau phase of the myogenic response after gabazine treatment (Fig. 4g).

The GABA_A Agonist Muscimol Decreased Myogenic Tone in Human Omental Resistance Arteries

To determine whether the effects of GABA_A activation demonstrated in mouse arteries are also present in hu-

man resistance arteries, myogenic tone studies were performed with freshly isolated human omental resistance arteries. Myogenic tone under control conditions (buffered solution alone) demonstrated myogenic function following an increase in intraluminal pressure from 0 to 120 mm Hg. Pretreatment of human vessels with muscimol ($100 \mu\text{M}$) inhibited myogenic tone, resulting in an increased vessel dilation ($p < 0.05$; $n = 3$; Fig. 5a, b).

Discussion

The primary findings of the present study are: (1) GABA_A subunits are expressed on smooth muscle cells from small-caliber murine vessels and human resistance arteries; (2) targeting of GABA_A receptors on human resistance arterial smooth muscle modulates plasma membrane potential; and (3) myogenic tone is inhibited by GABA_A agonists in whole tissue models. In VSM, myogenic tone is a contractile mechanism that is directly controlled by membrane potential and depolarization. When certain small-caliber arteries are faced with increased intraluminal pressure, stretch-induced cation channels depolarize the VSM leading to activation of voltage-dependent calcium channels and TRP channels, resulting in calcium influx and a concomitant increase in intrinsic VSM tone [28–30]. Studies have demonstrated that the amount of depolarization in VSM directly correlates to the amount of calcium influx and the myogenic tone generated [29]. Given this established relationship between membrane depolarization and myogenic tone, we utilized an ex vivo myogenic tone model as the physiologic correlate to explore the influences of GABA_A channel activation on voltage-dependent VSM tone. These studies describe for the first time GABA_A-mediated decreases of myogenic tone in human vessels.

Myogenic tone is a property specific to small resistance arteries and increased myogenic tone is associated with an overall increased contractility of the VSM. When the luminal pressure increases, a myogenic response is initiated, which leads to vessel constriction to maintain a constant flow through the arteries. During pathologic states, such as hypertension, resistance arteries demonstrate an increase in myogenic tone in the cerebral and skeletal muscle vasculature [31–33]. However, the distinct pathophysiologic role of myogenic tone in hypertension is not well defined. Not all animal models of hypertension consistently show enhanced myogenic tone. In the rat model of sodium intake-induced hypertension, there seems to be a decrease in myogenic tone [34]. In contrast, in the spontaneously hypertensive rat model, myogenic tone is increased in certain vascular beds [31–33]. The mechanisms that allow the cardiovascular system to compensate and modulate their myogenic tone may have influence over normal physiology and pathophysiology. By demonstrating the effects of GABA_A activation on myogenic tone, we implicate a possible mechanism of endogenous vascular modulation of myogenic tone since GABA circulates in blood and many peripheral cells are capable of synthesizing GABA. The effect of GABA_A on vascular biology will require further studies in order to fully determine the role that GABA plays in vascular physiology and the potential therapeutic value of GABAergic targeting. In the current study, resistance vessels from human omentum were used as a model of myogenic tone. In order to make further conclusions on blood pressure regulation and the pathogenesis or treatment of hypertension, studies on other vascular tissue from other regions (i.e., mesenteric and renal) will need to be performed, recovered from patients carefully clinically phenotyped with regard to their cardiovascular status.

Since GABA_A ligand selectivity is largely determined by the α subunit composition of the GABA_A receptor, we performed RT-PCR analysis using LCMD to obtain discrete areas of smooth muscle from mouse tail and human omental resistance arteries. We detected mRNA encoding the $\alpha 3$, $\alpha 4$, $\beta 2$, $\gamma 2$, and δ GABA_A subunits in VSM, while mRNA encoding for other GABA_A subunits were not detected ($\alpha 1$, $\alpha 2$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 3$, $\gamma 1$, and $\gamma 3$). These results demonstrate an expression profile of GABA_A α , β , and δ/γ subunits in a sufficient repertoire needed to form a classic functional GABA_A receptor. Data from RT-PCR analysis revealed that the specific α subunit composition of VSM GABA_A ($\alpha 4, \alpha 3$) receptors did not include the predominant subunits found in the

CNS ($\alpha 1, \alpha 2$), but rather an expression profile more consistent with extrasynaptic/peripheral GABA_A receptors [35]. Extrasynaptic GABA_A channels are characteristically responsive to lower concentrations of GABA and display slower desensitization properties (collectively termed “tonic”) [36]. This current study describes the novel mRNA expression profile of the GABA_A receptor subunits on VSM. Further studies need to be performed to further demonstrate the protein expression profile to fully correlate with the mRNA expression profiles and to understand the possible subunit stoichiometry of these expressed subunits.

Primary VSM cells loaded with a potentiometric fluorescent indicator demonstrated an increased fluorescence in the presence of propofol, a widely used clinical GABAergic anesthetic. Propofol had a greater depolarization effect on VSM than a classic GABA_A agonist, muscimol. Previous studies have demonstrated that propofol causes a variety of molecular and physiologic responses in VSM cells other than GABA_A activation, such as increases in smooth muscle TRP channel activation and modulation of VSM calcium at higher concentrations [37, 38]. To further specify and quantify the electrophysiologic response of GABA_A activation on resistance artery physiology, intracellular current recordings were performed using the GABA_A-specific agonist muscimol on individual VSM cells. This demonstrated an inward current at negative membrane potential, and a loss of that current in the presence of a GABA_A-specific inhibitor or a decreased chloride gradient (10 mM chloride in internal solution).

Only 10% of the smooth muscle had responses to GABA agonist exposure in the patch clamp system, while global measurements of the potentiometric indicator in a monolayer of cells revealed a more robust response. This is likely due to the potentiometric indicator allowing for the measurement of fluorescence changes within the entire field of cells in a 96-well plate, while the automated patch system measures a single cell in a dispersed culture. Cells grown to confluence in culture allows for cell to cell interactions such as gap junction formation. This GABA responsive subpopulation may also give rise to the possibility that there is differential expression within the VSM itself.

We further demonstrated that GABA_A activators decreased the calcium responses elicited by endothelin. We demonstrated that at a membrane potential of -80 mV an inward current is elicited by a GABA_A receptor agonist. With an electrochemical equilibrium that is estimated at -40 mV in systemic arteries [39], a tonic chlo-

ride flux through GABA_A receptors would prevent the full activation of membrane potential gated channels, normally around 20 mV, and inhibit calcium entry through voltage-regulated calcium channels. Further studies demonstrating in situ tissue recordings will be needed to fully elucidate the mechanism of GABA_A-mediated loss of myogenic tone.

In conclusion, we have described the subunit expression profile of GABA_A receptors in resistance arterial VSM cells. Furthermore, we demonstrated that the GABA_A α4 subunit protein is contained within the VSM layer of the resistance artery. We elicited a functional GABA_A channel response by demonstrating the ability of the receptor to cause a membrane potential change when activated by a GABA_A-specific agonist. We demonstrated a physiologic response to the membrane potential changes caused by the GABA_A activation in ex vivo myogenic tone studies in human and mouse resistance arteries. These studies indicate that functional GABA_A receptors exist on resistance arteries and may be a viable therapeutic target for the treatment of hypertension.

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Statement of Ethics

All animal studies were approved by IACUC. All human tissue studies were deemed non-human subject research by the IRB of Columbia University.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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Author Contributions

Peter D. Yim wrote the manuscript and was involved in the design, performance, and analysis of all the studies. George Gallos was involved in study design, analysis of data, and editing of the paper. Steven Lee-Kong was involved in the performance and study design of all human tissue studies. Dingbang Xu performed studies, analyzed data, and wrote sections of the manuscript. Dan E. Berkowitz and Charles W. Emala were involved in study design, analysis of data, and editing of the manuscript. William Dan edited the text and figures, as well as performing/designing the experiments. Amy Wu was involved in writing and editing of the figures, as well as performing the experiments.

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