Supporting Information

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SI Text

Materials and Methods. ***Chemicals and solutions.*** L-Arginine, D-Arginine, N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-nitro-L-arginine (NLA), N^{ω}-nitro-D-arginine (NDA), L-phenylephrine hydrochloride, sodium nitroprusside (SNP), miconazole, methyl palmitate (PAME), methyl stearate (SAME), heptadecanoic acid, 16 methyl, methyl ester, nicotine (Nic), and 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-9), strontium nitrate, L-Phe, L-Lys, D-Lys, L-His, phenylephrine HCl, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (carboxy-PTIO), and atropine were from Sigma–Aldrich. Guanethidine was purchased from Novartis (formerly CIBA Pharmaceuticals). Vasoactive intestinal peptide (VIP), [*p*-chloro-D-Phe^{6}, Leu^{17}]-VIP (a VIP receptor antagonist), calcitonin gene-related peptide (CGRP), and CGRP(8–37) (a CGRP receptor antagonist) were purchased from Bachem. Tin protoporphyrin IX (SnPPIX) (a heme oxygenase-1 inhibitor) was purchased from Frontier Scientific. All drugs were dissolved in deionized water (pH 7.4) as stock solutions except palmitic acid methyl ester, stearic acid methyl ester, and heptadecanoic acid, 16 methyl, methyl ester. The fatty acids were dissolved in 100% methanol and added to Krebs' solution and did not precipitate out of solution, indicating that the fatty acids were fully dissolved with a final methanol concentration of <0.1%. These drugs were added as final concentrations into continuously gassed (95% O_2 and 5% CO_2) Krebs' bicarbonate solution (pH 7.4) consisting of 196.87 mM NaCl, 5.16 mM KCl, 1.33 mM $CaCl_2$, 25.56 mM $NaHCO_3$, 1.22 mM $MgSO_4{\cdot}7H_2O$, 1.01 mM dextrose, 0.34 mM EDTA, and 0.28 mM ascorbic acid (1). Calcium-free Krebs' solution was prepared by the removal of 1.33 mM $CaCl_2$ and replaced with the same concentration of strontium nitrate (2).

Decentralization and denervation of the superior cervical ganglion (SCG). Male Sprague–Dawley rats were anesthetized with pentobarbital sodium (Nembutal, 35 mg/kg, i.p.). A midline incision was made on the skin longitudinally on top of the larynx. Blunt dissection technique of the muscle and fat tissues was performed to expose the carotid artery. Between the internal and external carotid arteries (near the bifurcation) is the SCG, which was exposed and severed with scissors either at the preganglionic nerve (decentralization) or postganglionic nerve (denervation) (3). The procedure was performed on both SCG of the rat. Animals were killed 5 days after surgical denervation or decentralization. The completeness of the denervation was confirmed by the disappearance of catecholamine fluorescence in the circle of Willis (data not shown), a result similar to that found previously (3). In addition, the presence of ptosis of the eyelids (3) was already evident 1 day after either surgical denervation or decentralization of the SCG. Furthermore, 5 days after the surgery, the decentralized SCG was visibly reduced in size, and the denervated SCG was enlarged.

Catecholamine fluorescence. The circle of Willis of the anesthetized rats was carefully dissected out, cleaned, and immersed in ice-cold (4 °C) 2% glyoxylic acid (pH 7.4) for 30 min (3). The arteries were cut longitudinally and flattened on the coverslip and dried in a stream of warm air with a hair dryer for 15 min and heated in an oven at 110 °C for 6 min. The specimens were then covered with xylene and examined under a fluorescence microscope (magnification, 20×) and photographed (3). Consistent with our previous findings (3), dense catecholamine fluorescence fibers were present on the circle of Willis of control rats and those after 5-day decentralization of the SCG. Catecholamine fluorescence fibers of the circle of Willis, however, had completely disappeared after 5-day denervation of the SCG ($n > 5$).

SCG cell culture. Primary SCG neuronal cultures (4, 5) were prepared from male Sprague–Dawley rats (4–8 weeks old) killed with sodium pentobarbital. Freshly dissected SCGs were placed in cold Hibernate A (Invitrogen) solution and cut into small pieces. Then, the ganglia were transferred to Mg^{2+}/Ca^{2+}-free Hanks' balanced salt solution (HBSS) containing trypsin (1 mg/mL; Sigma–Aldrich) and collagenase D (1 mg/mL; Roche Diagnostics) and were incubated for 50 min at 37 °C. Cells were released by gentle trituration with a fire-polished glass pipette at the end of the incubation. The cell suspension was centrifuged at 300 × *g* for 5 min. The pellet was gently resuspended in Neurobasal culture medium (Invitrogen) containing B27 (1:50 dilution;Invitrogen), 0.5 mM L-glutamate and nerve growth factor (100 ng/mL; Alomone Laboratories). All media and HBSS contained 100 units/ml penicillin and 100 units/mL streptomycin. The single-cell suspension was plated onto a 4-well culture plate with a 0.3% gelatin-coated (Sigma–Aldrich) glass coverslip (12-mm diameter; Fisher Scientific) in each well and incubated with air containing 5% CO_2 at 37 °C. The growth medium was changed once on day 2. The SCG cells were stained with anti-rabbit neurofilament 200 (Sigma–Aldrich) as a marker of neuronal cells.

Electrophysiology. The SCG neuronal cells were used 2–5 days after plating. The Giga-seal patch-clamp technique was used to record whole-cell currents (4, 5). A glass coverslip containing cultured neurons was transferred from the growth medium to a recording chamber containing the extracellular recording solution [140 mM sodium gluconate, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES (pH 7.35) and 300–310 mOsm] on a phase-contrast microscope (Nikon TMD-Diaphot). Recording electrodes were prepared from capillary glass (PG52151-4; World Precision Instruments). After filling with intracellular solution [140 mM potassium gluconate, 5 mM KCl, 10 mM EGTA, 10 mM HEPES, 2 mM K-ATP, 2 mM $MgCl_2$, and 0.5 mM GTP (pH 7.35)], electrode resistance in the extracellular recording solution was 4–6 MΩ. The holding potential was kept at −60 mV, and currents were recorded by using a WPC-100 patch-clamp amplifier (E.S.F. Electronic). Data acquisition and analysis were digitized (VR-10B; InstruTech) and stored on videotape. For analysis, data were filtered at 2.5 kHz [−3-dB frequency with an 8-pole low-pass Bessel filter (LPF-8; Warner Instruments)] and digitized at 5 kHz. Current amplitudes were measured by using Channel 2 (software kindly provided by Michael Smith, Australian National University, Canberra, Australia).

All FAMEs and fatty acids were dissolved in 100% methanol as stock solution and diluted with 200 μM bovine serum albumin (BSA) (6). A final concentration of BSA was 2 μM with a final methanol concentration of 0.1%. BSA (2 μM) was present in all drug applications, including FAMEs/fatty acids, nicotine, and 0.1% methanol (control). BSA alone did not affect nicotine-induced inward currents. Nicotine (60 μM) was dissolved in extracellular recording solution and was applied for 20 s. FAMEs and fatty acids were incubated for 3 min for their effects on the inward currents. To avoid development of tachyphylaxis by repeated applications of nicotinic agonists, the cells were washed for 3 min with extracellular solution before the next application. The recording chamber was perfused continuously, and cells

were exposed to a constant flow of bath solution between drug applications at 2 mL/min at room temperature into the bath.

Two-electrode voltage clamp for α7-nAChR-expressing oocytes. Stage V or VI oocytes from *Xenopus laevis* were harvested and injected with the α7-nAChR RNA by using a nanoinjector (Drummond) (7). The oocytes were maintained at 18 °C. Membrane currents were recorded 2 days after the injection. The ND96 bath solution (pH 7.5) contained 96 mM, 2 mM KCl, 1.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5.0 mM HEPES. During recording, oocytes were perfused continuously with the bath solution at a rate of 7 mL/min. Stock solution of drugs was diluted into the ND96 solution. PAME and PA were prepared before the experiment in methanol at 0.1 M and then diluted to 0.01 M with BSA solution as described in last paragraph. Solution containing 30 μM PAME flowed continuously into the recording chamber for 2 min by perfusion, while 0.1 mM nicotine and 0.3 mM choline were applied directly onto the oocytes.

Two-electrode voltage clamp for the whole-oocyte recording was performed at room temperature by using an OC-725C amplifier (Warner) (7). The borosilicate glass capillaries (1.5 mm) (World Precision Instruments) were pulled by using a P-97 microelectrode puller (Sutter). When filled with 3 M KCl, the electrodes had 0.1 to 1-MΩ resistance. The membrane potential was held at −60 mV. Data acquisition and analysis were performed with pClamp 9.0 and Digidata 1322A (Axon Instruments). The traces were filtered at 1 KHz and sampled at 2 KHz. The maximum inward current was determined as the current amplitude. To compensate the difference in the α7-nAChR expression level, the data were normalized as percent of the agonist response.

Results. There are advantages to using the superfusing bioassay cascade technique for studying the vascular effects of fatty acids. PAME and SAME are hydrophobic in nature. The vasodilatory property of these FAMEs is difficult to detect in systems such as a submerged tissue bath technique (8). This is because upon application of exogenous PAME and SAME that are dissolved in methanol stock solution, these FAMEs quickly come out of solution as soon as they are added to a "larger" volume of the Krebs' solution in the tissue bath. Accordingly, these FAMEs will not reach the submerged tissues under examination. This problem is avoided by using the superfusion bioassay cascade system, which enables detection of biological activity of the FAMEs by perfusing them directly onto the aortic rings to cause a response. Another major advantage of the superfusion bioassay cascade technique is that it provides the most sensitive assay to analyze response because of small amounts of vasoactive substance (9). This assay also allows simultaneous correlation of a response and concentration of vasoactive substance that causes the response.

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