Vulgarenol, a Sesquiterpene Isolated from *Magnolia grandiflora*, Induces Nitric Oxide Synthases II and III Overexpression in Guinea Pig Hearts

Leonardo del Valle-Mondragón*, Fermín Alejandro Tenorio-López, Gabriela Zarco-Olvera, and Gustavo Pastelín-Hernández

Departamento de Farmacología, Instituto Nacional de Cardiología "Ignacio Chávez", Juan Badiano # 1, Sección XVI, 14080, Tlalpan, México D. F., Mexico.
Fax: +52 55-55 73-09 26. E-mail: leonardodvm65@hotmail.com

* Author for correspondence and reprint requests

Z. Naturforsch. **62 c**, 725–730 (2007); received April 16, 2007

Vulgarenol, a sesquiterpene isolated from *Magnolia grandiflora* flower petals, decreased coronary vascular resistance in the Langendorff isolated and perfused heart model, when compared to the control group [$(15.2 \times 10^7 \pm 1.0 \times 10^7)$ dyn s cm^{-5} *vs.* $(36.8 \times 10^7 \pm 1.2 \times 10^7)$ dyn s cm^{-5}]. Our data suggest that this coronary vasodilator effect probably involved inducible and endothelial nitric oxide synthase overexpression (6.8 and 4.2 times over control, respectively), which correlated with increases in nitric oxide release [(223 ± 9) pmol mL^{-1} *vs.* (61 ± 11) pmol mL^{-1}] and in cyclic guanosine monophosphate production [(142 ± 8) pmol mg^{-1} of tissue *vs.* (44 ± 10) pmol mg^{-1} of tissue], as compared to control values. This effect was antagonized by 3 μM gadolinium(III) chloride, 100 μM *N*-nitro-L-arginine methyl ester, and 10 μM 1*H*-[1,2,4]oxadiazolo[4,2-*a*]quinoxalin-1-one. Hence, the vulgarenol-elicited coronary vasodilator effect could be mediated by the nitric oxide-soluble guanylyl cyclase pathway.

Key words: Magnolia grandiflora, Coronary Vasodilator Activity, Nitric Oxide Synthases

Introduction

Nitric oxide (NO) is one of the most important endogenous vasodilators. It is generated by the oxidative conversion of L-arginine to L-citrulline by an enzymatic system known as nitric oxide synthases (NOS, E. C. 1.14.13.39), of which at least three isoforms are known and expressed in mammals (Moncada *et al.,* 1991; Stuehr *et al.,* 2004). They are derived from different genes, and are identified as: neural (nNOS, NOS I), inducible (iNOS, NOS II), and endothelial (eNOS, NOS III) (Alexander *et al.,* 2006; Moncada *et al.,* 1997; Moncada and Higgs, 2006). NOS III is a constitutive enzyme expressed in the vascular endothelium, cardiac myocytes, platelets, and megakaryocytes (Alderton *et al.,* 2001). NOS I is also a constitute enzyme found in neural cells, skeletal muscle, neutrophils, pancreatic islets, renal macula densa, endometrium, respiratory and gastrointestinal epithelium (Alderton *et al.,* 2001; Bruckdorfer, 2005). Both enzymes are calcium-calmodulin-system-dependent and are strongly associated with blood pressure regulation (Yang and Ming, 2006). On the other hand, NOS II is an inducible enzyme found in macrophages, platelets, endothelium, hepatocytes, chondrocytes, glial cells, neurons, myocardium, megakaryocytes, respiratory epithelium, and in many other cells as well (Shaul, 2002). All three isoforms are homodimers in their active form, with monomers of a relatively constant molecular mass (160 kDa for NOS I, 135 kDa for NOS II, and 140 kDa for NOS III, respectively) (Alderton *et al.,* 2001). When expression of these enzymes takes place, as the result of several autonomous or induced systemic mechanisms, NO intracellular production rises and, when it diffuses to adjacent cells, activates an enzyme known as soluble guanylyl cyclase (sGC) by exchanging NO with Fe^{2+} of the heme group, thereby increasing its activity. When this occurs, cyclic guanosine monophosphate (cGMP) production is overturned, activating a protein known as cGMP-dependent protein kinase (PKC), which relaxes muscle cells. This mechanism is generally regarded as the NO-sGC pathway (Castro *et al.,* 2006; Garthwaite, 2005; Russwurm and Koesling, 2004) and is responsible for the vasodilator activity shown by several bioactive substances. In addition to its well-known vasoactive properties, NO exerts a number of antiatherogenic effects, including inhibition of platelet

aggregation and adhesion, proliferation of vascular smooth muscle cells, and leukocyte adhesion and migration into the arterial wall (Mason, 2006). Therefore, development of new drugs that act by restoring the NO-cGMP levels within the cardiovascular system can be of great interest in the treatment of pathologies such as hypertension, septic shock, impotence, preclampsia, atherosclerosis, and tissue damage associated with reperfusion.

Magnolia grandiflora L. was typified by Linné in 1759. It is an evergreen tree introduced during the conquest by the Spaniards and is now widely distributed in America. Ethnomedically, *Magnolia grandiflora* extracts have been used since ancient times to ameliorate cardiac pathologies, a practice that remains to this day (Schühly *et al.,* 2001). Our previous study (del Valle-Mondragón *et al.,* 2004) showed that vulgarenol [3,4,9-trihydroxy-9-methyl-3a,5,5a,9,9a,9b-hexahydronaphtho[1,2-*b*]furan-2,6(3*H*,4*H*)-dione] (Fig. 1), a sesquiterpene isolated

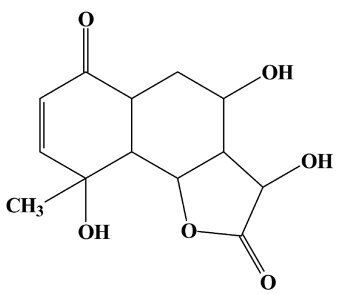

Fig. 1. Chemical structure of vulgarenol [3,4,9-trihydroxy-9-methyl-3a,5,5a,9,9a,9b-hexahydronaphtho[1,2-*b*]furan-2,6(3*H*,4*H*)-dione].

from *Magnolia grandiflora* flower petals, decreased coronary vascular resistance in the Langendorff isolated and perfused heart model. The present study attempts to establish if this coronary vasodilator effect is mediated by the NO-sGC pathway.

Experimental

Chemicals

Ethanol (ACS grade), β-mercaptoethanol, leupeptin hydrochloride, EGTA, Tris-HCl, gadolinium(III) chloride hexahydrate, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), 1*H*-[1,2,4]oxadiazolo[4,2-*a*]quinoxalin-1-one (ODQ), human hemoglobin, Sephadex G-25 (medium), phenylmethanesulfonyl fluoride (PMSF), tergitol (NP-40), and Tween 80 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were from J. T. Baker (Mallinckrodt Baker S. A. de C. V., Xalostoc, Estado de Mexico, Mexico). Sodium dithionite was obtained from Alfa Aesar (Ward Hill, MA, USA). Water was deionized using a Simplicity System (Millipore SAS, Molsheim, France). Monoclonal antibodies raised against NOS II and NOS III (both with a mouse IgG_1 isotype) were obtained from BD Transduction Laboratories (San Jose, CA, USA). All other chemicals were of analytical purity and were used without further purification.

Plant material

Fresh *Magnolia grandiflora* flower petals were collected, identified, and preserved as previously described (del Valle-Mondragón *et al*., 2004).

Extraction and isolation

Petals were ground in a porcelain mortar (Coors, Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The ground material (25 kg) was macerated with 25% aqueous ethanol during one week at 4–8 °C, gauged, and then filtered through a 0.45 μm nitrocellulose membrane filter (Millipore, Billerica, MA, USA). The extract was preserved in amber-glass bottles with polypropylene caps (Pyrex, Corning Incorporated Life Sciences, Acton, MA, USA) at (4 ± 1) °C. High-performance liquid chromatography (HPLC) isolation and purification of vulgarenol were performed with a Beckman System Gold Liquid Chromatographer with a photodiode array detector (PDA) and controlled by the 32 Karat Software Version 7.0 (Beckman Coulter Inc., Fullerton, CA, USA) under the following chromatographic conditions: Phenomenex (Torrence, CA, USA) Lichrosorb RP-18 reversed-phase column (250 × 4 mm i. d.; 5 μm). The mobile phase, consisting of water/methanol/acetonitrile (5:3:1, by volume), pH 6.5 ± 0.1, was filtered through Millipore (Billerica) 0.22 μm nylon filters and degassed by sonication (T490DH instrument, Elma, Singen, Germany) prior to its use. Separation was carried out at room temperature with a flow rate of 1.2 mL min^{-1} and an injection loop of 100 μL. PDA was operated at 254 nm. All samples were microfiltered (Membra-Fil Mixed Cellulose Ester, 0.22 μm, Whatman plc, Middlesex, UK) before injection. The extraction procedure pro-

duced 65.2 mg of the purified compound (yield 0.0026%). Vulgarenol was identified by reported spectral data and comparison with a reference compound (del Valle-Mondragón *et al*., 2004).

Animals

For this study, male guinea pigs (900 – 950 g), bred and maintained at the Animal Facility of the Instituto Nacional de Cardiología "Ignacio Chávez", were used throughout. They were housed under standard conditions of temperature [(25 ± 3) °C], humidity [(50 ± 10)%] and light (12-h light/dark cycle). Animals were fed a standard diet (Certified Guinea Pig Diet LabDiet 5026, PMI Nutrition International, Richmond, IN, USA) and had access to tap water *ad libitum*. All animal procedures were conducted in accordance with our Federal Regulations for Animal Experimentation and Care (SAGARPA, NOM-062-ZOO-1999, Mexico) and were approved by the Institutional Animal Care and Use Committee. During the experiments, all efforts were made to minimize animal suffering.

Biodynamic evaluation of vulgarenol in the Langendorff isolated and perfused heart model and nitric oxide quantification in the perfusion liquid

Biodynamic activity of vulgarenol was evaluated in the Langendorff isolated and perfused heart model, as previously described (del Valle-Mondragón *et al*., 2004; Tenorio-López *et al*., 2006). Guinea pig hearts were perfused with a Krebs-Henseleit (K–H) solution (pH 7.4) with the following composition (in mM): 117.8 NaCl, 1.2 $NaH_2PO_4 \cdot H_2O$, 0.027 Na_2EDTA, 6.0 KCl, 1.6 $CaCl_2 \cdot 2H_2O$, 1.2 $MgSO_4 \cdot 7H_2O$, 24.88 $NaHCO_3$, and 5.55 dextrose, continuously bubbled with 95% O_2/5% CO_2 (Aga Gas S. A. de C. V., Tlalnepantla, Estado de México, México), and kept at 37 °C. K–H solution was continuously perfused at 10 mL min^{-1} using a peristaltic pump (Sigmamotor TM24 · 4, Sigmamotor Inc., Middleport, NY, USA). Under this model, we recorded the left intraventricular pressure of the heart by inserting a latex balloon in the left ventricle, which was connected to a hydropneumatic transducer (Statham Instruments Inc., 7320, Statham Instruments Inc., Hato Rey, Puerto Rico), and the coronary perfusion pressure with a pressure transducer (Gould P23ID, Gould Instruments, Cleveland, OH, USA). Coronary vascular resistance (CVR) was calculated with the following equation (Döring and Dehnert, 1988):

$$\mathrm{CVR} = \left[\frac{\text{coronary perfusion pressure (mmHg)}}{\text{coronary flow (mL min}^{-1})}\right] \left(7.998 \times 10^{6} \frac{\text{dyn s mL}}{\text{mmHg min cm}^{5}}\right) = \left[\frac{\text{dyn s}}{\text{cm}^{5}}\right].$$

Both variables were recorded with a polygraph (Grass 79-D, Grass Instruments Co., Quincy, MA, USA). Cardiac rate was kept constant at 1 Hz by stimulating with a ventricular epicardic pacemaker (Grass-SIU5, Grass Instruments Co). Vulgarenol (5 μM) was infused alone or in the presence of 100 μM L-NAME, an unspecific inhibitor of nitric oxide synthases (Suárez *et al.,* 1999); 3 μM gadolinium(III) chloride, an unspecific blocker of stretch-activated ion channels and calcium influx within the cardiovascular endothelium (Suárez *et al.,* 1999; Nicolosi *et al.,* 2001); or 10 μM ODQ, a soluble guanylyl cyclase selective inhibitor (Isenberg *et al.,* 2006). All the drugs were continuously infused at a rate of 0.3 mL min^{-1} by means of an infusion pump (SP200i, World Precision Instruments Inc., Sarasota, FL, USA) connected to a dispenser (Hamilton, Hamilton Company, Reno, NV, USA) adjacent to the perfusion cannula. Results are expressed in 10^7 dyn s cm^{-5}. Prior to the experiments, 2 μM oxyhemoglobin (OxyHb) was added to the K-H solution. Human OxyHb solutions were prepared by dissolving human hemoglobin crystals in deionized water. Then, the solution was gassed with 95% O_2/5% CO_2 (Aga Gas S. A. de C. V.) for 5 min. Hemoglobin was reduced by a surplus of sodium dithionite. Further on, the solution was gassed for another 30 min with 95% O_2/5% CO_2. The solution was passed through a Sephadex G-25 (medium) column to desalt it (Schulz *et al*., 1999). Analysis of the perfusion liquid was performed using the absorbance difference from 401 to 411 nm ($A_{401}-A_{411}$) (Kelm and Schrader, 1988) in a double beam UV-Vis spectrophotometer (DW2000, SLM Instruments Inc., Urbana, IL, USA). Results are expressed as pmol mL^{-1}.

cGMP quantification in ventricular tissue samples

Left ventricular tissue segments were quickly frozen in liquid nitrogen, weighed and pre-treated according to Hagen *et al*. (2001). The tissue was then homogenized in 5% trichloroacetic acid and the homogenate centrifuged at 1500 rpm per gram

of tissue for 10 min at 4 °C (Sorvall RMC 14, Dupont, Newtown, CT, USA). The supernatant was extracted 4 times with 5 volumes of water-saturated diethyl ether, and then desiccated in an N_2 steam. cGMP content was determined in acetylated samples using an enzyme immunoassay kit (Cyclic GMP EIA Kit, Cayman Chemical, Ann Arbor, MI, USA). Sample analysis was performed at 415 nm on a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Mulgrave, Victoria, Australia), equipped with a microplate module. Results are expressed as pmol mg^{-1} of tissue.

NOS II and NOS III expression analysis by Western blotting

Left ventricular tissue samples were washed with 0.5 M phosphate buffer at pH 7.4. Further on, they were homogenized with a Potter-Elvehjem homogenizer (Daigger, Vernon Hills, IL, USA) in a lysis buffer (50 mM Tris-HCl, 0.1 mM EGTA, 0.1% β-mercaptoethanol, containing 100 mM leupeptin, 1 mM PMSF, 1% NP-40, pH 7.5). After centrifugation at $1000 \times g$ (Spectrafuge 24D, Labnet International Inc., Edison, NJ, USA), the supernatant was boiled in Laemmli loading buffer and separated by SDS-PAGE on a 7.5% acrylamide gel. Proteins were electroblotted onto nitrocellulose, and the membranes were washed in Tris-buffer saline with 0.1% Tween 80 and blocked in 5% defatted milk. Membranes were subsequently incubated with monoclonal antibodies raised against NOS II and NOS III, and the proteins were detected with a horseradish peroxidase-labeled anti-rabbit secondary antibody followed by enhanced chemiluminescence.

Statistical analysis

Data analysis was carried out using SPSS 12.0.1 for Windows software (SPSS Inc., Chicago, IL, USA) with one-way analysis of variance (ANOVA) followed by Student's *t*-test for independent samples. All results are expressed as means ± SEM of 10 independent experiments. A P value < 0.05 was considered as statistically significant.

Results and Discussion

In vitro studies, performed on the isolated and perfused heart model according to Langendorff, showed that 5 μM vulgarenol decreases coronary vascular resistance, when compared to control group [$(15.2 \times 10^7 \pm 1.0 \times 10^7)$ dyn s cm^{-5} *vs.* $(36.8 \times 10^7 \pm 1.2 \times 10^7)$ dyn s cm^{-5}, control group] (Fig. 2), which is correlated with increases in NO release [(223 ± 9) pmol mL^{-1} *vs.* (61 ± 11) pmol mL^{-1}, control group] and cGMP production [(142 ± 8) pmol mg^{-1} of tissue *vs.* (44 ± 10) pmol mg^{-1} of tissue, control group] (Figs. 3 and 4, respectively). This finding suggests that the vulgarenol-elicited coronary vasodilator effect might be mediated by the NO-sGC pathway. In order to test this hypothesis, isolated and perfused guinea

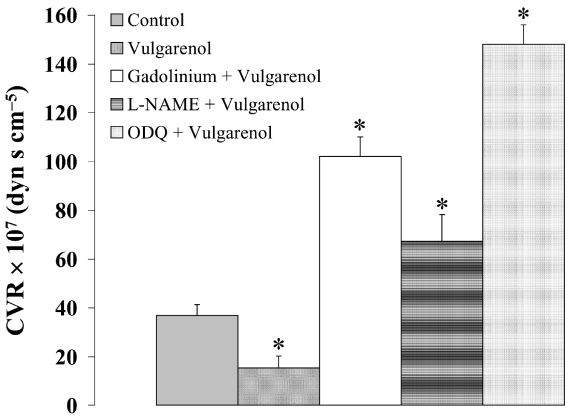

Fig. 2. Vulgarenol effect on coronary vascular resistance (CVR) in isolated and perfused guinea pig hearts according to Langendorff. Values represent means ± SEM; $N = 10$. * $P < 0.05$ *vs.* control group, one-way ANOVA followed by Student's *t*-test.

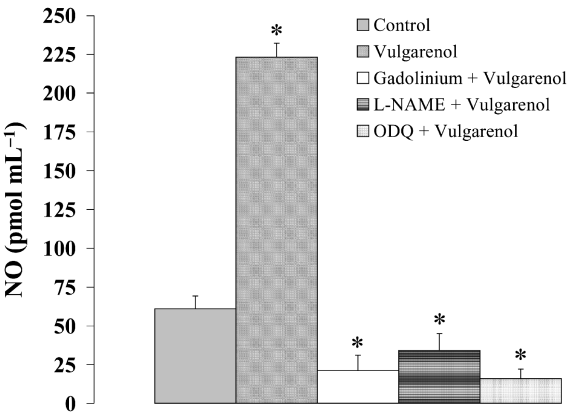

Fig. 3. Vulgarenol-induced nitric oxide production in isolated and perfused guinea pig hearts according to Langendorff. Values represent means ± SEM; $N = 10$. * $P < 0.05$ *vs.* control group, one-way ANOVA followed by Student's *t*-test.

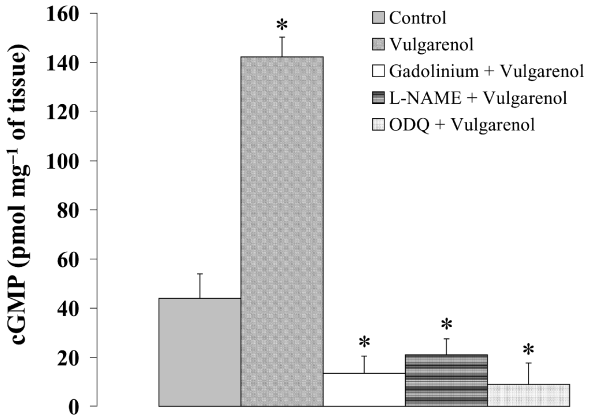

Fig. 4. Vulgarenol-induced cGMP production in isolated and perfused guinea pig hearts according to Langendorff. Values represent means ± SEM; $N = 10$. * $P < 0.05$ *vs.* control group, one-way ANOVA followed by Student's *t*-test.

pig hearts were pretreated with 100 μM L-NAME, 3 μM gadolinium(III) chloride, or 10 μM ODQ. The administration of these blocking agents significantly inhibited the coronary vasodilator effect shown by the administration of 5 μM vulgarenol (Figs. 3 and 4). These results support the fact that vulgarenol-induced vasodilation could be also a calcium-dependent process, due to the fact that the blockade of stretch-activated ion channels also inhibits the Ca^{2+} uptake. This important step for the NOS III activation reverts the vulgarenol-elicited coronary vasodilator effect. In addition, Western blot analysis (Fig. 5) revealed a significant activation of both NOS II and NOS III (6.8 and 4.2 times over control, respectively). Relaxation of smooth muscle cells (SMCs) involves a complex sequence of steps. When NO is produced locally in small amounts, as a result of the oxidative L-arginine pathway catalyzed by the NOS isozymes, it acts as a messenger that crosses cell membranes, binding to the sGC, which in turn produces cGMP and participates in a number of signalling pathways. The rise of the cGMP concentration initiates the reactions that result in smooth muscle relaxation. In addition, our results agree with those obtained with natural products whose vasodilator mechanism probably involves the NO-sGC path-

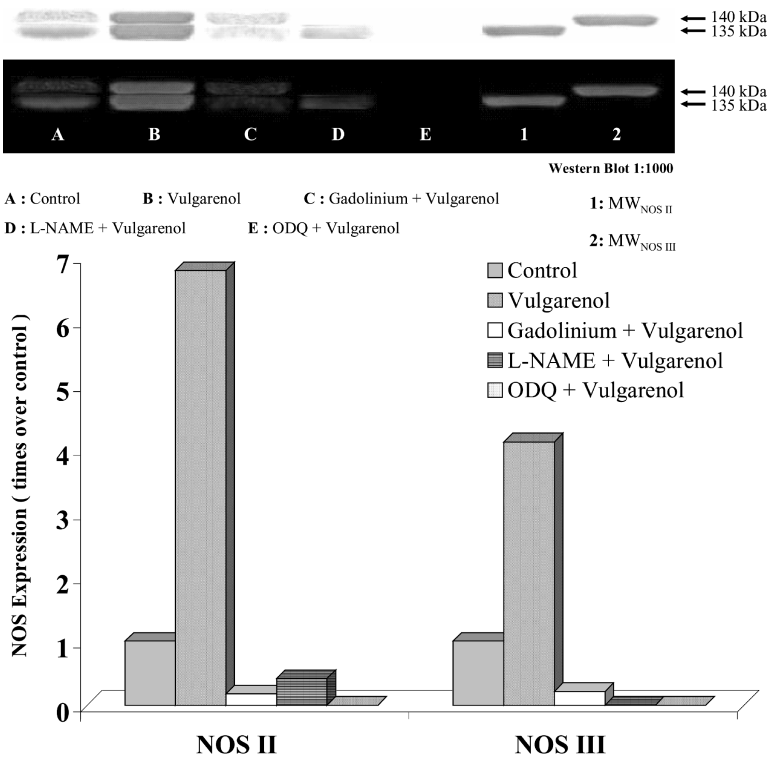

Fig. 5. NOS II and NOS III expression analysis by Western blotting in isolated and perfused guinea pig hearts according to Langendorff.

way. Therefore, further development of vasodilators that act by restoring the NO-cGMP pathway in the vascular system can be of great value for the treatment of several cardiovascular diseases.

Acknowledgements

The authors are grateful to Dr. Ingrid Mascher for her English Language editorial review.

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