2-Hydroxyoleic Acid
A New Hypotensive Molecule

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Abstract—Recent studies have shown that diets rich in monounsaturated fatty acids (MUFA s) from olive oil, a natural source of oleic acid, have beneficial effects on blood pressure (BP) in hypertensive patients. With this in mind, we investigated whether a synthetic derivative of the MUFA oleic acid, 2-hydroxyoleic acid (2-OHOA), was capable of regulating the BP of Sprague-Dawley rats. Intraperitoneal and oral administration of 2-OHOA to rats induced significant and sustained decreases in BP in a time-dependent manner. Without affecting heart rate, treatments for 7 days provoked reductions in systolic BP of 20 to 26 mm Hg. At the molecular level, the density of Gαs, but not Gαi, or Gαo, increased in membranes from the hearts and aortas of 2-OHOA–treated rats, whereas in heart membranes, the density of Goq/11 and protein kinase Cα proteins was also augmented. These molecular alterations were reflected in the increase in cAMP levels after Gαs protein and β-adrenergic receptor stimulation. On the contrary, inhibitory hormones reduced adenylyl cyclase activity to the same extent in 2-OHOA–treated rats as in vehicle-treated ones. Our results indicate that cardiovascular tissues from 2-OHOA–treated rats exhibited increased cAMP production in response to Gαs activation, which might be attributed to enhanced expression of Gαs proteins. As a result of this change, a significant reduction in systolic BP was observed. Therefore, BP can be lowered by administration of 2-OHOA, which might represent the first member of a new family of antihypertensive drugs. (Hypertension. 2004;43:249-254.)

Key Words: blood pressure • fatty acids • signal transduction • G proteins • hypotension

Many alterations in the structure and function of the cell membrane have been associated with hypertension. Changes in the plasma or membrane lipid composition have been reported in both hypertensive humans1 and in animal models of hypertension.2,3 These changes have been associated with abnormalities in cation transport systems,4 cytosolic Ca2+ regulation,5 and impaired signal transduction.6 As a result, it has been proposed that modifications of the membrane lipid composition may affect the physical and functional properties of membranes and in consequence, might account for the alterations in signaling implicated in the physiological control of blood pressure (BP). In this context, long-term fatty acid (FA) intake is known to influence both membrane lipid composition and BP. Olive oil, a natural source of the monounsaturated FA (MUFA) oleic acid, has been shown to have beneficial effects on BP (when high doses are consumed for long periods) and is associated with a low incidence of coronary heart disease.7 Olive oil consumption causes favorable changes in plasma lipid and lipoprotein profiles8,9 and also in the phospholipid content and FA composition of both erythrocyte membranes from hypertensive patients and aorta membranes from spontaneously hypertensive rats.10,11 Moreover, a slight reduction in saturated fat intake along with the use of olive oil markedly lowers the drug requirements of hypertensive patients.12 Nevertheless, the mechanisms underlying the cardiovascular benefits of this diet are not fully understood.

Guanine nucleotide regulatory proteins (G proteins) are GTP-binding proteins that play a pivotal role in the control of BP. They are heterotrimeric proteins composed of α-, β-, and γ-subunits. The α-subunit binds and hydrolyzes GTP and also confers the specificity of G proteins for receptors and effectors. G proteins are implicated in the regulation of a variety of signal transduction systems, including the adenylyl cyclase (AC)/cAMP signaling pathway. Interestingly, the AC/cAMP system has been shown to be involved in a variety of cellular functions related to the regulation of BP, including vascular permeability,13 salt and water transport,14 catecholamine release,15 and the regulation of vascular smooth muscle tone16 and heart contractility,17 cAMP being a potent vasodilator.18

The purpose of our study was to evaluate the effects of the MUFA 2-hydroxyoleic acid (2-OHOA), a synthetic derivative of oleic acid, on BP in rats. In addition, we investigated the effects of this compound on cardiovascular signaling to document the molecular mechanisms of 2-OHOA on BP.
Methods

Animal Treatments
Female Sprague-Dawley rats (12 weeks old, 230 to 250 g; Charles River Laboratories, Barcelona, Spain) received either vehicle (10% ethanol IP, n=18) or 2-OHOA (10 or 30 mg/kg IP, n=24) every 8 hours for 7 days. To determine possible toxic effects and the oral effectiveness of 2-OHOA treatments, rats were administered a high oral dose of this compound (600 mg/kg, n=4) every 12 hours for 7 days. BP was measured at least in triplicate 2 hours after drug administration in warmed, restrained, conscious rats by the tail-cuff method with a computerized oscillometric system recorder (Nryptem system 645, Cibertec). This method was chosen because of its noninvasive character, enabling daily measurements with the same rats throughout the entire treatment.19 After the final measurement, the rats were killed by decapitation, and their hearts and aortas were dissected out and immediately frozen in LN2 before being stored at −80°C. All experiments were carried out according to the Institutional Committee of Animal Research (Comissiò de Bioètica de la Universitat de les Illes Balears).

Preparation of Cell Membranes From Left Ventricles and Aortas
Left ventricles and aortas were ground in a mortar with LN2. The tissue powder was then homogenized in a tissue blender (Ultra-Turrax, Janke & Kunkel) containing ice-cold 50 mmol/L Tris-HCl buffer, pH 7.5, with 1 mM EDTA, 2 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 5 mM L-iodoacetamide. The homogenate was centrifuged for 10 minutes at 11000 g and 4°C, and the supernatant was subsequently centrifuged for 20 minutes at 40 000 g. The final pellet was washed in homogenization buffer, resuspended in assay buffer, and used for immunoblotting experiments and the determination of AC activity.

Immunoblot Analysis and Quantification of Specific Immunoreactivity
Quantitative immunoblotting of membrane G proteins and protein kinase (PK) Ca, as well as of total PKA subunits from left ventricles and aortas, was performed as described before.20 Primary polyclonal antibodies anti-Gαs, anti-Gαo, and anti-Gαq/11 were from Santa Cruz Biotechnology, and anti-Gβi/γi, was from New England Nuclear Corp. Primary monoclonal antibodies anti-PKCa, anti-PKAcα, anti-PKAαS, and anti-PKAαT were from BD Transduction Laboratories. Quantification was performed by image analysis by using standard curves with 5 points (ie, total protein loaded vs integrated optical density) of different protein contents loaded on the same gels, as described.20 This quantification procedure was repeated at least 3 times for each sample by running duplicates on different gels. Values from 2-OHOOA-treated rats were normalized to the protein content values of the vehicle-treated rats, which were considered 100%.

AC Assay
For the AC assay, cell membrane preparations were diluted to a protein content of 1 mg/mL with a solution of 10 mmol/L Tris-HCl, pH 7.4, 250 mmol/L sucrose, and 1 mmol/L EGTA. The membrane suspension (40 μL) was added to 210 μL of a solution containing 100 mmol/L Tris-HCl, pH 7.4, 1 mmol/L ATP, 10 mmol/L MgCl2, 1 mg bovine serum albumin, and a creatine phosphokinase–ATP-generating system (consisting of 10 mmol/L sodium phosphocreatine, 8 IU phosphocreatine kinase, and 10 μmol/L GTP[S]) and incubated for 10 minutes at 30°C. The enzymatic reaction was stopped by boiling, and the mixture was immediately centrifuged at 3000g for 15 minutes. The production of cAMP was measured in the supernatant with a [3H]cAMP radioimmunoassay kit (Amersham Pharmacia). Basal AC activity and its responsiveness to activators such as GTP[S] (10 μmol/L), isoprenaline (100 μmol/L), noradrenaline (100 μmol/L), and forskolin (100 μmol/L), as well as to inhibitors such as angiotensin II (Ang II, 10 μmol/L) and the atrial natriuretic factor analogue C-ANF23 (0.1 μmol/L), were investigated in the membranes isolated from left ventricles and aortas. AC activity was calculated as picomoles cAMP formed per milligram protein per minute.

Data Analysis
Data are mean ± SEM from the number of animals indicated (n). Statistical significance was calculated with GraphPad software. Two groups of values were compared by the 2-tailed t test, whereas ≥2 groups of values were compared by 1-way ANOVA followed by Duncan multiple-comparison test. Differences between experimental groups were considered statistically significant at a value of P<0.05.

Results

In Vivo Effects of 2-OHOOA on BP
The effect of 2-OHOOA on BP was investigated by measuring systolic BP (SBP) before and during administration in Sprague-Dawley rats. Treatments with 10 and 30 mg/kg 2-OHOOA IP decreased the SBP with respect to vehicle-treated rats at all time points (Figure 1A). Maximal SBP reductions measured on the last day of treatment with 10 and 30 mg/kg were 26 and 20 mm Hg, respectively (Figure 1A). Hypotensive effects of 2-OHOOA were also observed at times shorter than 24 hours. Administration of a single dose of 10 and 30 mg/kg IP 2-OHOOA induced SBP reductions of 16±4 mm Hg (P<0.05, n=10) and 18±4 mm Hg (P<0.05, n=12), respectively, within 4 hours after compound administration (mean basal SBP was 131±2 mm Hg, n=20). To
study any possible toxicological effects of this compound and its effectiveness after oral administration, 2-OHOA dissolved in soybean oil was administered to another group of rats. Oral administration of 2-OHOA (600 mg/kg every 12 hours) decreased BP when compared with vehicle-treated rats (reductions of 13, 25, and 22 mm Hg after 2, 4, and 6 days of treatment, respectively; Figure 1B). Cardiac rate values were unchanged with respect to those of vehicle-treated rats. Moreover, despite the high 2-OHOA doses administered, no histologic or cytoligic toxicity was detected on autopsy by both optical and electron microscopy (data not shown).

Effects of 2-OHOA Treatment on Cellular Signaling Proteins

To investigate the molecular mechanisms underlying the effects of 2-OHOA on BP, we measured the levels of signaling proteins implicated in the control of BP, such as G proteins, PKCα, and PKA. The concentrations of these proteins in membranes isolated from the left ventricles and aortas of vehicle- and 2-OHOA–treated rats (30 mg/kg IP for 7 days) were determined by quantitative immunoblotting. This analysis showed that the amounts of the AC-stimulatory G protein Gs were increased in cardiovascular tissues from 2-OHOA–treated rats. Levels of the Gαs protein (52 kDa) were increased by 65±14% in heart membranes (Figure 2A) and by 52±12% in aorta membranes (Figure 2B) from 2-OHOA–treated rats when compared with vehicle-treated rats. Likewise, in 2-OHOA–treated rats, the levels of cardiac but not of aortic Gαq/11 protein (42 kDa) were also upregulated by 31±9% with respect to vehicle-treated rats. In contrast, heart and aorta membranes from 2-OHOA–treated rats did not exhibit significant variations in the levels of the AC-inhibitory G proteins Gαi1 (40 kDa; Gαi1 is not expressed in rat heart and aorta21) and Gαo (40 kDa) when compared with vehicle-treated animals (Figure 2).

Finally, the levels of membrane PKCα (Figure 3A), but not the levels of the total catalytic (cat) or regulatory (I and II) PKA subunits (Figure 3B), were increased significantly in left ventricles from 2-OHOA–treated rats (48±5%).

Effect of 2-OHOA Treatment on AC Activity

Having observed that 2-OHOA treatment (30 mg/kg IP for 7 days) increased Gαs protein levels, we set out to determine whether G protein–mediated signaling was also influenced. Thus, AC stimulation, both independent of and dependent on Gs protein–coupled receptors, was studied in cardiovascular tissues from 2-OHOA–treated rats. Exposure to GTPγS (10 μmol/L) stimulated AC activity in left ventricle (Figure 4A) and aorta (Figure 4B) membranes from vehicle- and 2-OHOA–treated rats. However, cAMP accumulation was significantly higher in 2-OHOA–treated rats, increasing by 20±3% in heart membranes and by 76±31% in aorta membranes (Figure 4). β-Adrenergic stimulation of heart and aorta membranes with isoproterenol (100 μmol/L) or noradrenaline (100 μmol/L) increased AC activity >2-fold in both vehicle- and 2-OHOA–treated rats when compared with the basal activity. Nevertheless, stimulation of AC through Gs protein–coupled β-adrenergic receptors was augmented further as a result of 2-OHOA treatment. The production of cAMP stimulated by either adrenergic agonist was significantly greater in 2-OHOA–treated rats when compared with vehicle-treated rats, increasing by 20±5% in heart membranes and by 92±38% in aorta (Figure 4). Thus, it appears that 2-OHOA treatment does indeed increase both receptor-independent and -dependent G-protein activity.

To investigate whether the increase in cAMP levels could be caused by a direct effect of 2-OHOA on the AC enzyme, forskolin-stimulated cAMP accumulation was also studied. In heart membranes, forskolin (100 μmol/L) induced similar levels of cAMP production in vehicle- and 2-OHOA–treated rats (17-fold with respect to basal activity; Figure 5A). In contrast, stimulation of aortic AC by forskolin was increased by 40±8% in 2-OHOA–treated rats when compared with vehicle-treated rats (Figure 5B).

Finally, inhibition of cAMP accumulation through the Gi/o protein–coupled receptors, angiotensin (AT) and ANF receptors, was investigated in vehicle- and 2-OHOA–treated rats. No difference was seen between vehicle- and 2-OHOA–

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**Figure 2.** Effect of 2-OHOA administration on membrane G-protein levels in left ventricle and aorta. Rats received an intraperitoneal injection of either vehicle (10% ethanol) or 2-OHOA (30 mg/kg) every 8 hours for 7 days. Top panels show representative immunoblots for cardiac (A) and aortic (B) G-protein αs (Gs), αq/11 (Gαq/11), αi2 (Gi), and αo (Go) subunits from vehicle- and 2-OHOA–treated rats. Amount of total protein loaded for the immunoreactive bands shown was (in μg) 64 for Gs, 21 for Gα11, 34 for Gi, and 36 for Go. Chemiluminescence bands are representative of 6 or 7 separate experiments. Bottom panels show levels of cardiac (A) and aortic (B) membrane G-protein subunits quantified against standard curves, as described in Methods, and expressed as percentages of vehicle-treated rats (taken as 100%). Columns represent the mean±SEM of 6 or 7 experiments. *P<0.05 compared with vehicle-treated group. **P<0.01.
treated rats in the inhibition of forskolin-stimulated cAMP accumulation by Ang II (10^{-9} M) and C-ANF 4-23 (0.1 \mu M), either in heart (Figure 5A) or aorta (Figure 5B) membranes.

**Discussion**

Cells that participate in cardiovascular regulation might undergo lipid-dependent modifications of their membrane composition that are responsible for the elevation of BP. Indeed, alterations of membrane cholesterol or phospholipid content, phospholipid distribution, the molecular species of particular phospholipid classes, and the degree of FA saturation have all been reported in both hypertensive humans and in experimental animal models of hypertension. Interestingly, we recently demonstrated that in elderly hypertensive patients, alterations in cell membrane lipid levels are also associated with a reduction in the density of signaling proteins involved in the control of BP, such as G proteins and PKC. Therefore, changes in lipid composition of the plasma membrane might alter signaling pathways involved in the control of BP. If this hypothesis were correct, nutritional and pharmacologic interventions aimed at normalizing the abnormal lipid composition of the plasma membrane could prove to be a useful therapy for high BP. In this context, interest in the potential cardiovascular health benefits of dietary MUFAs has increased in recent years. There has been considerable research effort directed toward analyzing the effects of MUFA-rich diets, focusing on plasma lipids and the lipid/FA composition of cell membranes, but the molecular mechanisms underlying its effects have not been investigated yet. However, olive oil, a natural source of the MUFA oleic acid, had moderate beneficial effects on BP, but only when high doses were consumed for several months. We demonstrate for the first time that short-term administration of the synthetic MUFA 2-OHOA, a structural analogue of oleic acid, decreases BP in rats (reductions of up to 26 mm Hg) by modulating G protein–mediated cellular signaling. The decrease in BP induced by 2-OHOA occurred in parallel with an increase in cAMP levels in cardiovascular membranes. Activation of AC and the consequent increase in intracellular cAMP concentrations are well-known mechanisms mediating vasorelaxation via several vasodilators, eg, prostaglandin I_2. Different molecular mechanisms could account for the observed increases in cAMP production, including alterations in the levels or activity of G proteins that link membrane receptors to the AC and/or changes in the activity of this enzyme. We have shown that membranes isolated from the aorta and heart of 2-OHOA–treated rats exhibit increased AC activity in response to β-adrenergic receptor–dependent and –independent stimulation. More-
over, an increase in the levels of Gαs protein was found in cardiovascular tissues after 2-OHOA treatment, and 2-OHOA directly enhanced AC activity in aorta membranes. These results suggest that 2-OHOA induces an increase in cAMP levels, thereby influencing the density/activity of both G proteins and AC. Accordingly, in animal models of hypertension and in response to a number of different hormones, Gs-coupled receptors have been shown to exhibit decreased efficacy and/or potency to increase cAMP concentrations. Furthermore, decreased Gs-protein function has been detected in hypertensive subjects, and lower levels of Gs protein have been found in cardiovascular membranes from 2-OHOA-treated rats, as described in Methods. Values are mean±SEM of 6 to 8 experiments. *P<0.01 compared with vehicle-treated group.

Figure 5. Effect of 2-OHOA administration on AC inhibition of forskolin-stimulated cAMP production in rat heart and aorta. Rats received an intraperitoneal injection of either vehicle (10% ethanol) or 2-OHOA (30 mg/kg) every 8 hours for 7 days. AC activity was determined in the presence of forskolin (100 μmol/L) plus MnCl₂ (10 mmol/L), alone (maximal activity) or in combination with Ang II (10 μmol/L) or C-ANF₄₋₂₃ (0.1 μmol/L) in left ventricle (A) and aorta (B) membranes from vehicle- and 2-OHOA-treated rats, as described in Methods. Values are mean±SEM of 6 to 8 experiments. *P<0.01 compared with vehicle-treated group.

Perspectives
Cardiovascular pathologies are involved in ≈50% of all causes of death in industrialized countries, with hypertension being one of the major risk factors. This reason underlines the actual need for new, potent, and side effect–free treatments for hypertension. The Mediterranean diet, rich in olive oil, leads to a high consumption of its major component, the natural MUFA oleic acid. Clinical trials have actually proven its beneficial effects on BP and have given a possible explanation for the low incidence of cardiovascular diseases in the appropriate countries, although the effects were only convincing when high doses were consumed for longer times. In this study, we evaluated for the first time the in vivo effects of a synthetic derivative of oleic acid on BP. This compound, 2-OHOA, demonstrated improved effectiveness by strongly and rapidly reducing rat BP. The hypotensive effect was accompanied by upregulation of the density of membrane Gs proteins, which in turn led to increased levels of cAMP in cardiovascular tissues and the attenuation of BP. Moreover, the low toxicity of 2-OHOA compared with its natural derivative and the fact that it can be administered orally might allow the development of a novel type of antihypertensive drug, based on an improvement of its natural analogue. Accordingly, our current goal is to evaluate the effects of this new molecule in lowering BP in genetic and experimental animal models of hypertension.

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