Antihypertensive action of 2-hydroxyoleic acid in SHRs via modulation of the protein kinase A pathway and Rho kinase

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Abstract **Olive oil consumption leads to high monounsaturated fatty acid intake, especially oleic acid, and has been associated with a reduced risk of hypertension. However, the molecular mechanisms and contribution of its different components to lower blood pressure (BP) require further evaluation. Here, we examined whether a synthetic, non-β-oxidation-metabolizable derivative of oleic acid, 2-hydroxyoleic acid (2-OHOA), can normalize BP in adult spontaneously hypertensive rats (SHRs) and whether its antihypertensive action involves cAMP-dependent protein kinase A (PKA) and Rho kinase, two major regulators of vascular smooth muscle contraction. Oral administration of 2-OHOA to SHRs induced sustained systolic BP decreases in a time-dependent (1–7 days) and dose-dependent (100–900 mg/kg every 12 h) manner. After 7 days of treatment with 2-OHOA (600 mg/kg), the systolic BP of SHRs was similar to that of normotensive Wistar Kyoto rats, returning to its initial hypertensive level after withdrawal of 2-OHOA. This treatment strongly increased the protein expression of the catalytic and regulatory RIα and RIIα PKA subunits as well as PKA activity in aortas from SHRs. Consistently, administration of the PKA inhibitor 8-bromo adenosine-3′,5′-cyclic monophosphorothioate, Rp isomer, to 2-OHOA-treated SHRs induced a pronounced reversal (up to 59%) of the antihypertensive effect of 2-OHOA. Additionally, 2-OHOA completely reversed the pathological overexpression of aortic Rho kinase found in SHRs, suppressing the vasoconstrictory Rho kinase pathway.**—Alemany, R., O. Vögler, S. Terés, C. Egea, C. Baamonde, F. Barceló, C. Delgado, K. H. Jakobs, and P. V. Escribá. **Antihypertensive action of 2-hydroxyoleic acid in SHRs via modulation of the protein kinase A pathway and Rho kinase.** ***J. Lipid Res.*** **2006.** 47: **1762–1770.**

Supplementary key words aorta • fatty acids • hypertension • signal transduction • cAMP-dependent protein kinases • spontaneously hypertensive rats

The Mediterranean diet is characterized by a high consumption of virgin olive oil, which contains a great proportion of the natural MUFA oleic acid (~80%). Epidemiological studies suggest that this diet provides cardiovascular benefits, including the reduction of blood pressure (BP), both in normal and hypertensive subjects (1, 2). Moreover, it has been shown that long-term intake of high doses of olive oil has an inverse association with the risk of developing hypertension (3, 4). 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 (5–8), but the specific molecular mechanisms underlying the beneficial effects of oleic acid and related compounds are still not completely understood.

The increase of BP in essential hypertension is associated with increased peripheral vascular resistance, which is caused by functional and structural alterations of resistance arteries (9, 10). During agonist-induced contraction of vascular smooth muscle cells, phosphorylation of myosin light chain (MLC) is a crucial step for force development (11). In this process, the extent of MLC phosphorylation depends on the equilibrium between the activity of Ca^{2+}-calmodulin-dependent MLC kinase and the MLC phosphatase. Moreover, contraction can also occur independently of changes in intracellular Ca^{2+} by regulation of the activity of MLC phosphatase, a phenomenon called Ca^{2+} sensitization (12). Activation of G_s-coupled receptors (e.g., β_2-adrenergic and prostacyclin receptors) induces smooth muscle relaxation through stimulation of adenylyl cyclase (AC) and an increase in cAMP levels. Subsequent activation of cAMP-dependent protein kinase A (PKA) phosphorylates pivotal regulatory proteins, which then act to decrease intracellular Ca^{2+} levels, finally leading to vasodilation (13). Furthermore, PKA also

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mediates Ca^{2+}-desensitizing effects in smooth muscle by blocking the inhibitory phosphorylation mechanism for MLC phosphatase (14). Accordingly, defects in receptor systems that mediate vasorelaxation via the activation of PKA are considered to play an important role in the pathogenesis and maintenance of increased peripheral vascular resistance, which is characteristic for the hypertensive state in humans (15–17) and in adult spontaneously hypertensive rats (SHRs) with established hypertension (18, 19).

Alterations in the Rho A/Rho kinase pathway, leading to the inhibition of MLC phosphatase, also play a central role in the agonist-induced Ca^{2+} sensitization and hypercontraction of smooth muscle found in hypertensive rats (20) and humans (21). Expression and activity of Rho kinase were found augmented in blood vessels of SHRs (22, 23), and administration of the Rho kinase inhibitor Y-27632 reduced BP in various rat models of hypertension without affecting normal BP (24). In addition, the Rho kinase inhibitor fasudil increased forearm blood flow and decreased forearm vascular resistance in hypertensive patients more strongly than in normotensive humans (21), clearly demonstrating that Rho A/Rho kinase-mediated signaling is involved in the pathogenesis of hypertension.

In this work, we examined for the first time whether the MUFA 2-hydroxyoleic acid (2-OHOA), a synthetic derivative of oleic acid with a hydroxyl group on the α-carbon that avoids its rapid degradation through the β-oxidation pathway (25), in fact exhibits antihypertensive properties in the adult SHR, an animal model with established hypertension, compared with its normotensive counterpart, the Wistar Kyoto (WKY) rat, and whether the potential mechanisms mediating its antihypertensive action involve PKA and Rho kinase.

METHODS

Animals, treatments, and BP measurements

Male SHRs and age-matched normotensive WKY rats (16 weeks old, 250–300 g) were obtained from Charles River Laboratories (Barcelona, Spain). The rats were kept at a constant temperature (24 ± 1°C) with a 12 h dark/light cycle. SHRs and WKY rats were randomly assigned to a control group (vehicle; n = 25) or a 2-OHOA group [racemic mixture of (*R*)- and (*S*)-2-OHOA (Avanti Polar Lipids); 100–900 mg/kg, resuspended in water; n = 38]. Rats were treated orally by gavage every 12 h for 7 days. Body weight was recorded and BP was measured at least in triplicate 6 h after drug administration in warmed, restrained conscious rats, using the tail-cuff method with a computerized oscillometric system recorder (Nyprem system from Cibertec, Barcelona, Spain) (26). Eighteen hours after the last treatment, rats were euthanized by decapitation, and their aortas were removed and immediately frozen in liquid nitrogen and stored at −80°C. Another group of SHRs received a single intraperitoneal injection of 10–30 mg/kg 8-bromo adenosine-3′,5′-cyclic monophosphorothioate, Rp isomer (Rp-8-Br-cAMP) (Biolog, Bremen, Germany), a specific membrane-permeant inhibitor of PKA (27), after 7 days of treatment with vehicle (n = 9) or 2-OHOA (600 mg/kg every 12 h; n = 11). Subsequent changes in systolic BP were monitored during 180 min. All experiments were carried out according to the Institutional Committee of Ethics in Animal Research (Comissió de Bioètica de la Universitat de les Illes Balears).

Preparation of aortic homogenates and membranes

Frozen aortas were pulverized in liquid nitrogen and then homogenized in a buffer containing ice-cold 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM PMSF, and 5 mM iodoacetamide (1:10, w/v) in a tissue blender (Ultra-Turrax; Janke and Kunkel). The homogenate was centrifuged for 5 min at 200 *g* and 4°C. An aliquot of 200 μl of the resulting supernatant was solubilized for 10 min at 75°C upon addition of SDS (1% final). Then, it was mixed with electrophoresis loading buffer, boiled for 5 min, and used for immunoblotting experiments. The remaining supernatant was subsequently centrifuged for 20 min at 40,000 *g* and 4°C. The resulting pellet (membrane) was washed once in homogenization buffer, resuspended in assay buffer, and used for the determination of AC activity.

AC activity assay

Basal and forskolin (10 μM)-stimulated AC activities were determined in aortic membranes as described previously (28).

Immunoblot analysis and quantification of PKA subunit and Rho kinase levels

Quantitative immunoblotting of PKA subunits and Rho kinase in aortic homogenates was performed as described elsewhere (28). Primary monoclonal anti-PKAcatα, anti-PKARIα, anti-PKARIIα, anti-PKARIIβ, and anti-Rho kinase II antibodies were from BD Biosciences Transduction Laboratories (Heidelberg, Germany). Quantification was performed by image analysis, using standard curves with four points (i.e., total protein loaded vs. integrated optical density) of different protein contents loaded on the same gels as described (29). This quantification procedure was repeated at least three times for each sample on different gels. Values were normalized to the protein content of the vehicle-treated WKY rats (taken as 100%).

PKA activity assay

Frozen aortas were pulverized in liquid nitrogen and then homogenized in ice-cold 50 mM Tris-HCl, pH 7.5, 50 mM 2-mercaptoethanol, 10 mM EGTA, 5 mM EDTA, 1 mM PMSF, and 10 mM benzamidine (1:10, w/v). The homogenate was then sonicated on ice four times for 10 s and centrifuged for 10 min at 8,000 *g* and 4°C. An aliquot of 12 μl of the resulting supernatant was mixed with 25 mM Tris-HCl, pH 7.0, 3 mM $MgCl_2$, 0.1 mM ATP, 0.5 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol, in the absence (blank) or presence of 2 μM cAMP, in a total volume of 120 μl. PKA activity was measured using a nonradioactive ELISA kit (Medical and Biological Laboratories, Ltd., Nagoya, Japan), which measures the phosphorylation of the pseudosubstrate, N-RFARKGS(P)LRQKNV-C, where S(P) indicates the serine residue phosphorylated, according to the manufacturer's instructions.

Data analysis

Data are means ± SEM from the number of animals indicated (n). The statistical significance was calculated using GraphPad Software. One-way (where indicated) or two-way ANOVA followed by Bonferroni's test was used for statistical evaluations. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effects of 2-OHOA on BP in SHRs and WKY rats

Oral administration of 2-OHOA (600 mg/kg every 12 h) induced a time-dependent reduction of BP in SHRs. The

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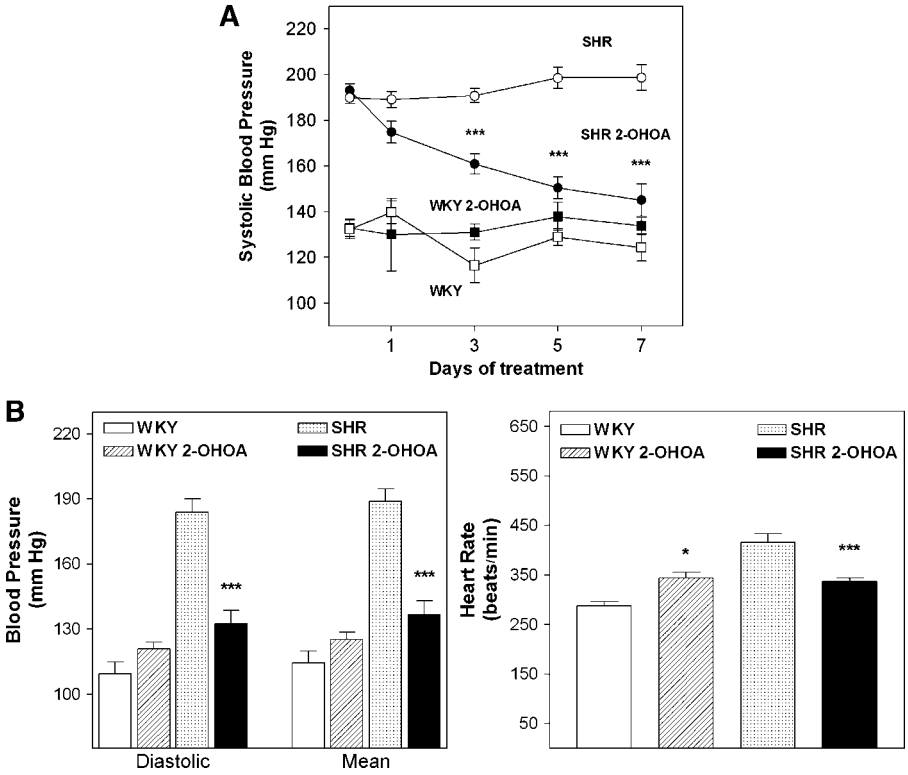

Fig. 1. Effects of 2-hydroxyoleic acid (2-OHOA) treatment (600 mg/kg every 12 h) on blood pressure (BP) and heart rate in spontaneously hypertensive rats (SHRs) and Wistar Kyoto (WKY) rats. A: Time course of the systolic BP in vehicle-treated SHRs (SHR; n = 11), 2-OHOA-treated SHRs (SHR 2-OHOA; n = 15), vehicle-treated WKY rats (WKY; n = 8), and 2-OHOA-treated WKY rats (WKY 2-OHOA; n = 7). *** $P < 0.001$ versus vehicle-treated SHRs (one-way ANOVA followed by Bonferroni's test). B: Diastolic and mean BP and heart rate values after 7 days of treatment. * $P < 0.05$, *** $P < 0.001$ versus vehicle-treated WKY rats and SHRs. Data shown are means ± SEM.

effect of 2-OHOA on systolic BP reached statistical significance after the 3rd day of treatment and had its maximal effect after 1 week (reductions of 30, 48, and 54 mm Hg after 3, 5, and 7 days of treatment, respectively, compared with vehicle-treated SHRs) (**Fig. 1A**). Diastolic BP was equally diminished, with reductions of 29 ± 4 ($P < 0.001$), 48 ± 4 ($P < 0.001$), and 51 ± 6 mm Hg (Fig. 1B) after 3, 5, and 7 days of treatment, respectively, compared

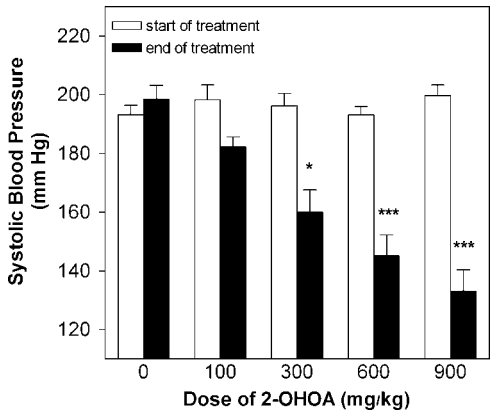

Fig. 2. Dose-dependent effects of 2-OHOA on systolic BP in SHRs. SHRs received an oral dose every 12 h for 7 days of either vehicle or different doses of 2-OHOA (100–900 mg/kg). Systolic BP values were measured at the beginning and end of the treatment. Columns represent means ± SEM of 3–15 animals per group. * $P < 0.05$, *** $P < 0.001$ versus the vehicle-treated SHR group (0 dose) at the end of the treatment (one-way ANOVA followed by Bonferroni's test).

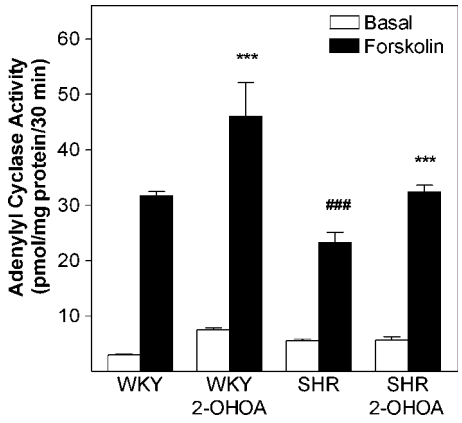

Fig. 3. Effects of 2-OHOA treatment (600 mg/kg every 12 h) for 7 days on adenylyl cyclase (AC) activities in SHRs and WKY rat aortas. AC activities were determined in the absence and presence of forskolin (10 μM) plus $MnCl_2$ (10 mM) in aortic membranes from vehicle- and 2-OHOA-treated rats. Columns represent means ± SEM of five to seven animals per group. Forskolin-stimulated AC activity was reduced significantly in vehicle-treated SHRs compared with vehicle-treated WKY rats ($^{###}P < 0.001$). *** $P < 0.001$ versus vehicle-treated WKY rats and SHRs.

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with vehicle-treated SHRs [mean diastolic BP in vehicle-treated SHRs was 177 ± 3, 187 ± 5, and 184 ± 6 mm Hg (Fig. 1B) at 3, 5, and 7 days, respectively]. In contrast to SHRs, 2-OHOA did not have any effect on BP in normotensive WKY rats (Fig. 1A). After treatment of SHRs for 7 days, 2-OHOA reduced systolic BP (−54 mm Hg, −27 ± 4%) (Fig. 1A), diastolic BP (−51 mm Hg, −28 ± 3%), and mean BP (−52 mm Hg, −28 ± 3%) (Fig. 1B) to values close to those measured in normotensive WKY rats. Oral administration of 100, 300, 600, and 900 mg/kg 2-OHOA for 7 days decreased systolic BP in SHRs by 16, 39, 53, and 65 mm Hg, respectively, compared with vehicle-treated SHRs (**Fig. 2**). At the end of the treatment, systolic BP of SHRs treated with 600 and 900 mg/kg was similar to that of normotensive WKY rats. Heart rate was significantly higher in vehicle-treated SHRs than in WKY rats (Fig. 1B). After 2-OHOA treatment (600 mg/kg every 12 h) for 7 days, the heart rate decreased significantly in SHRs, whereas it increased in WKY rats (Fig. 1B). Together, these results indicate that 2-OHOA administration is able to reduce BP to normotensive levels in SHRs without inducing hypotension in WKY rats. Treatment with 2-OHOA also reduced body weight in SHRs and WKY rats by ~13.1 ± 0.7% and 10.5 ± 1.0%, respectively, which was mainly caused by a reduction of fat depots. However, the antihypertensive effect of 2-OHOA does not seem to be directly related to its ability to reduce body weight. Food restriction of untreated SHRs leading to a similar body weight loss as 2-OHOA treatment (12.2 ± 0.3%) did not affect their BP (200 ± 6 mm Hg before and 192 ± 5 mm Hg after food restriction).

Effect of 2-OHOA on AC activity in aorta

Increases of intracellular cAMP levels are involved in the vasorelaxant effect of a variety of vasoactive agonists, such as prostaglandins and β-adrenergic agonists (19). Therefore, AC activities were measured in aortic membranes from vehicle- and 2-OHOA-treated SHRs and WKY rats. In aortic membranes from vehicle-treated SHRs, forskolin (10 μM)-stimulated AC activity was lower (27 ± 6%) compared with that in WKY rats (**Fig. 3**). Upon 7 days of treatment with 2-OHOA (600 mg/kg every 12 h), forskolin-stimulated AC activity was increased (by 39 ± 5%). In aortic membranes from WKY rats, 2-OHOA treatment resulted in increased basal (by 153 ± 12%) and forskolin-stimulated (by 45 ± 19%) AC activities. These data indicate that 2-OHOA treatment induces an enhance-

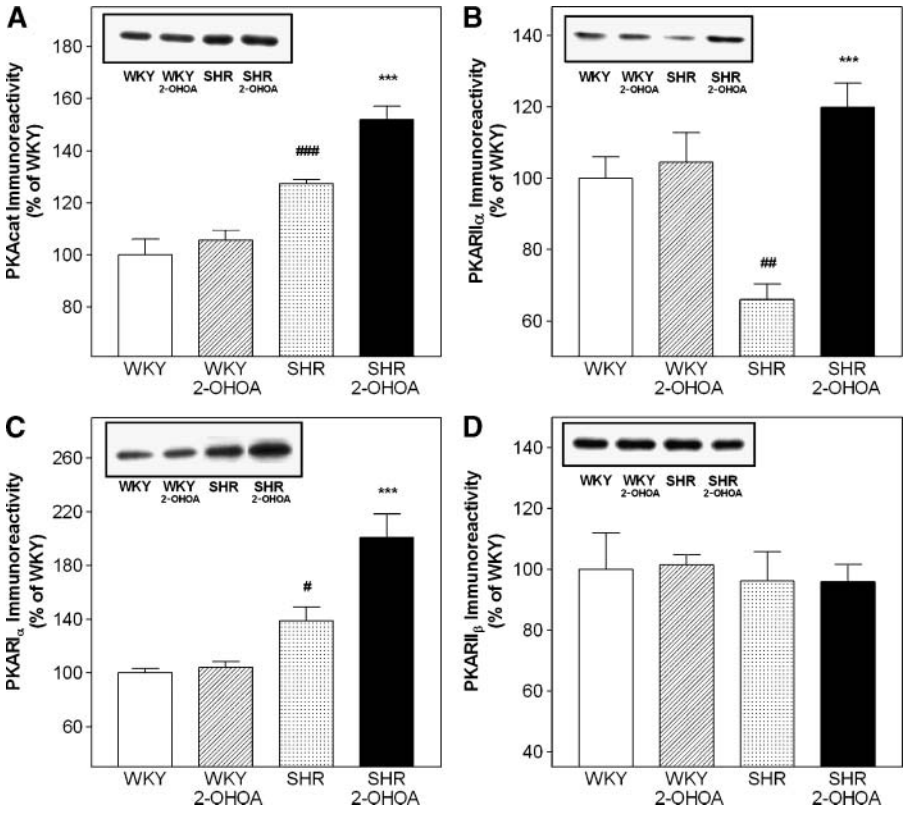

Fig. 4. Effects of 2-OHOA treatment (600 mg/kg every 12 h) for 7 days on protein kinase A (PKA) subunit levels in aortas from SHRs and WKY rats. Insets show representative immunoblots for levels of catalytic (PKAcatα) (A) and regulatory IIα (PKARIIα) (B), Iα (PKARIα) (C), and IIβ (PKARIIβ) (D) PKA subunits. The amount of total protein loaded was 30 μg. Inmunoreactive bands are representative of five to nine animals per group. Columns show levels (means ± SEM) of aortic PKA subunits quantified against standard curves and normalized to the protein content of vehicle-treated WKY rats (taken as 100%). PKAcatα and PKARIα levels were increased, whereas PKARIIα was decreased, in vehicle-treated SHRs compared with vehicle-treated WKY rats ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$). *** $P < 0.001$ versus vehicle-treated SHRs.

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ment of aortic AC activity in both rat strains, which on the other hand responded very distinctly to this agent in terms of BP and heart rate changes.

Effects of 2-OHOA on the density and activity of PKA in aorta

As the vasodilatory action of cAMP is mediated by PKA (13), we next studied the effect of 2-OHOA on both the expression and activity of PKA isoenzymes in aortas from vehicle- and 2-OHOA-treated SHRs and WKY rats. The concentrations of the catalytic (catα) and regulatory (RIα, RIIα, and RIIβ) PKA subunits in aorta were determined by quantitative immunoblotting. This analysis showed that the amounts of aortic PKAcatα and PKARIα subunits were significantly higher (27 ± 2% for PKAcatα and 39 ± 10% for PKARIα) in vehicle-treated SHRs compared with normotensive WKY rats (**Fig. 4A, C**). Moreover, in vehicle-treated SHRs, lower PKARIIα (decrease of 34 ± 4%) and unchanged PKARIIβ subunit levels were found (Fig. 4B, D). Treatment of SHRs with 2-OHOA (600 mg/kg every 12 h) for 7 days further increased the levels of aortic PKAcatα (19 ± 3%) and PKARIα (45 ± 13%) compared with vehicle-treated SHRs (Fig. 4A, C). Likewise, the level of PKARIIα, but not PKARIIβ, in aortas from 2-OHOA-treated SHRs was also upregulated (increase of 82 ± 7%) (Fig. 4B, D). In agreement with these protein expression data, we observed a significant increase of PKA activity (30 ± 5%) in aortas from 2-OHOA-treated SHRs compared with vehicle-treated SHRs (**Fig. 5**). In contrast to SHRs, 2-OHOA treatment had no effect on the density of any of the PKA subunits studied (Fig. 4) or PKA activity (Fig. 5) in aortas from WKY rats. Thus, 2-OHOA treatment specifically increased the expression of PKA subunits and PKA activity in aortas from SHRs.

In vivo effect of the PKA inhibitor Rp-8-Br-cAMP on the antihypertensive effect of 2-OHOA in SHRs

To demonstrate that the augmented expression and activity of vascular PKA induced by 2-OHOA treatment in SHRs is in fact involved in the observed antihypertensive action, the effect of the specific membrane-permeant PKA inhibitor Rp-8-Br-cAMP on systolic BP was investigated in vivo. For this purpose, SHRs treated for 7 days either with vehicle (control) or 2-OHOA (600 mg/kg every 12 h) received a single intraperitoneal injection of 10, 20, or 30 mg/kg Rp-8-Br-cAMP, and subsequent changes in systolic BP and heart rate were

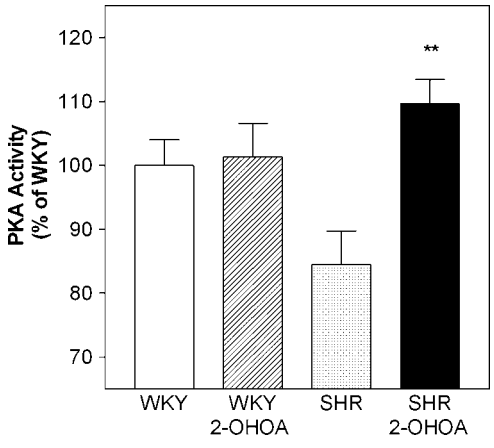

Fig. 5. Effects of 2-OHOA treatment (600 mg/kg every 12 h) for 7 days on PKA activity in aortas from SHRs and WKY rats. PKA activity was expressed as a percentage of activity in vehicle-treated WKY rats (taken as 100%). Columns represent means ± SEM of four animals per group. ** $P < 0.01$ versus vehicle-treated SHRs.

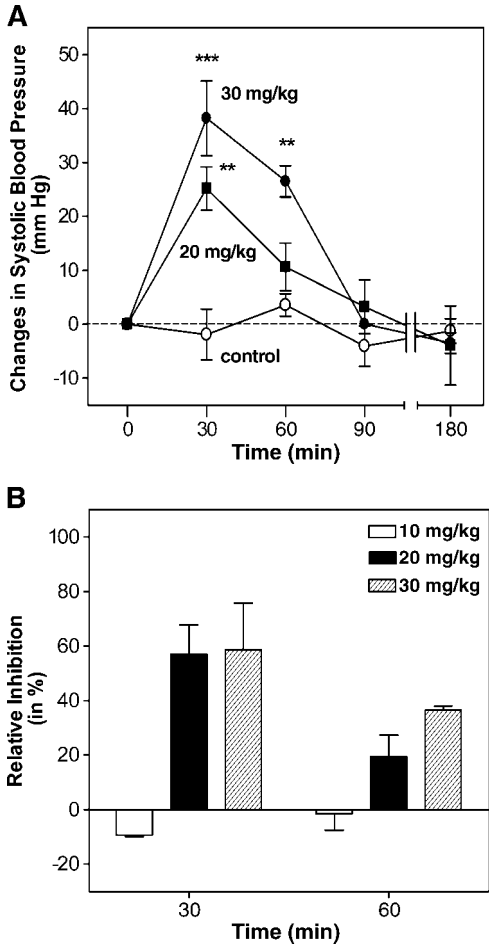

Fig. 6. Effects of acute administration of the PKA inhibitor 8-bromo adenosine-3′,5′-cyclic monophosphorothioate, Rp isomer (Rp-8-Br-cAMP), on systolic BP in vehicle-treated (control) and 2-OHOA-treated SHRs. A: Time course of the changes in systolic BP values (expressed in mm Hg) after acute administration of various doses of Rp-8-Br-cAMP. SHRs treated with vehicle (control; n = 9) or 2-OHOA (600 mg/kg every 12 h; n = 11) for 7 days received one intraperitoneal injection of Rp-8-Br-cAMP at 10, 20, or 30 mg/kg, and BP values were monitored during 180 min. Changes in systolic BP were calculated as the difference between basal systolic BP values (0 min) and the values after 30, 60, 90, and 180 min of Rp-8-Br-cAMP administration. Because Rp-8-Br-cAMP did not affect systolic BP in vehicle-treated SHRs at any dose or time studied, the values shown are means ± SEM (n = 3) of the changes registered after the administration of 30 mg/kg. Mean basal systolic BP values in vehicle- and 2-OHOA-treated SHRs were 206 ± 4 mm Hg (n = 9) and 135 ± 7 mm Hg (n = 11), respectively. ** $P < 0.01$, *** $P < 0.001$ versus vehicle-treated SHRs (control) (one-way ANOVA followed by Bonferroni's test). B: Relative inhibition of the antihypertensive effect of 2-OHOA in SHRs at 30 and 60 min after administration of Rp-8-Br-cAMP. Doses of 20 and 30 mg/kg Rp-8-Br-cAMP significantly reversed the systolic BP reduction induced by 2-OHOA. Data shown are means ± SEM.

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monitored during 180 min. As described above (Fig. 1), treatment of SHRs for 7 days with 2-OHOA strongly reduced systolic BP (−67 mm Hg; n = 11). Administration of Rp-8-Br-cAMP to 2-OHOA-treated SHRs at doses of 20 and 30 mg/kg, but not 10 mg/kg, induced a pronounced rebound of systolic BP at 30 min after compound administration, with increases of 25 ± 4 and 38 ± 7 mm Hg, respectively (**Fig. 6A**), without affecting heart rate (**Table 1**). The relative reversal of systolic BP reduction induced by 2-OHOA amounted to 57 ± 11% and 59 ± 17% at 20 and 30 mg/kg Rp-8-Br-cAMP, respectively (Fig. 6B). The BP-increasing effect of Rp-8-Br-cAMP was maintained for 60 min with the dose of 30 mg/kg (+27 ± 3 mm Hg) and disappeared later, with systolic BP values returning to initial levels at 90 min (Fig. 6A). In contrast to 2-OHOA-treated SHRs, administration of Rp-8-Br-cAMP affected neither systolic BP (Fig. 6A) nor heart rate (Table 1) in vehicle-treated SHRs at any dose or time studied. These results clearly demonstrate the physiological involvement of PKA in the antihypertensive effect of 2-OHOA in SHRs.

Effects of withdrawal of 2-OHOA on BP and the density of aortic PKA in SHRs

We next studied the posttreatment evolution of BP and aortic PKA levels in SHRs after withdrawal of 2-OHOA. Treatment of SHRs for 7 days with 2-OHOA (600 mg/kg every 12 h) induced a time-dependent reduction of systolic BP (reductions of 33 and 59 mm Hg after 3 and 7 days of treatment, respectively, compared with basal systolic BP values) (**Fig. 7A**). At the end of treatment, withdrawal of 2-OHOA led to a time-dependent increase of systolic BP (increases of 31, 53, 63, and 74 mm Hg at 3, 5, 7, and 9 days after 2-OHOA withdrawal, respectively), returning to their initial hypertensive state after ~5 days (Fig. 7A). The concentrations of the PKAcatα, PKARIIα, and PKARIα subunits in aortic homogenates were quantified by immunoblotting after 9 days of 2-OHOA withdrawal. This analysis showed that the amounts of these PKA subunits in aortas from SHRs after withdrawal of 2-OHOA were comparable to the PKA levels found in vehicle-treated SHRs (Fig. 7B). Together, these results demonstrate that the antihypertensive effect of 2-OHOA is reversible, with BP levels returning to their usual hypertensive values, which are accompanied by aortic PKA levels similar to those found in control SHRs.

Effects of 2-OHOA on the density of Rho kinase in aorta

Moreover, we studied the effect of 2-OHOA on the expression of Rho kinase II, which plays a key role in the pathogenesis of hypertensive vascular disease (21, 22). The levels of Rho kinase in aortas from vehicle- and 2-OHOA-treated SHRs and WKY rats were determined by quantitative immunoblotting. This analysis showed that the levels of aortic Rho kinase were significantly higher (60 ± 15%) in vehicle-treated SHRs compared with normotensive WKY rats (**Fig. 8**). Treatment of SHRs with 2-OHOA (600 mg/kg every 12 h) for 7 days decreased aortic Rho kinase expression to levels close to those measured in normotensive WKY rats (Fig. 8). In contrast to SHRs, 2-OHOA treatment had no effect on the density of Rho kinase in aortas from WKY rats. Thus, 2-OHOA treatment specifically decreased the high expression of Rho kinase in aortas from SHRs.

DISCUSSION

Recent epidemiological studies in Mediterranean areas have shown an inverse association between olive oil consumption, a natural source of the MUFA oleic acid, and the risk of hypertension (3, 4). However, oleic acid and other fatty acids are good cellular fuels, and they can be degraded through β-oxidation when imported into the mitochondria. This metabolism impairs the potential pharmacological use of fatty acids, although it does seem that oleic acid-rich oils (e.g., olive oil) had a moderate beneficial effect on BP, but only when high doses were consumed for several months (30, 31). Nevertheless, the specific molecular mechanisms and the contribution of the different components of olive oil to its beneficial effect on BP are not fully understood (1, 2). Thus, to overcome this problem, we have synthesized a new oleic acid analog with a hydroxyl group on the α-carbon, 2-OHOA. This modification of the fatty acid avoids its direct metabolism via the rapid β-oxidation pathway and only allows a basal metabolism by passing first through the slow and noninducible α-oxidation pathway (25). For this reason, its use as a source of energy would be decreased and its cell/organ availability increased. Indeed, the presence of this additional hydroxy group markedly increased the biological activity of 2-OHOA with respect to oleic acid in human adenocarcinoma cells (32), demonstrating the efficiency of this modification. We have recently shown that 2-OHOA has low histological toxicity in several rat organs (29) and is able to induce decreases in systolic BP in normotensive Sprague-Dawley rats (28), but it was completely uncertain whether this new molecule is indeed able to decrease

TABLE 1. Effects of acute administration of the protein kinase A inhibitor Rp-8-Br-cAMP on heart rate values in vehicle- and 2-OHOA-treated SHRs

	Vehicle-Treated SHRs					2-OHOA-Treated SHRs				
Rp-8-Br-cAMP (mg/kg)	0 min	30 min	60 min	90 min	180 min	0 min	30 min	60 min	90 min	180 min
10	370 ± 13	403 ± 21	438 ± 16	408 ± 9	394 ± 22	361 ± 31	369 ± 23	358 ± 19	352 ± 13	370 ± 13
20	419 ± 13	391 ± 20	357 ± 6	383 ± 8	416 ± 39	341 ± 17	325 ± 25	333 ± 24	312 ± 15	347 ± 34
30	360 ± 28	383 ± 16	399 ± 6	340 ± 9	344 ± 24	349 ± 18	347 ± 14	345 ± 9	348 ± 4	346 ± 5

2-OHOA, 2-hydroxyoleic acid; Rp-8-Br-cAMP, 8-bromo adenosine-3′,5′-cyclic monophosphorothioate, Rp isomer; SHR, spontaneously hypertensive rat. SHRs treated with vehicle (n = 9) or 2-OHOA (600 mg/kg every 12 h; n = 11) for 7 days received one intraperitoneal injection of Rp-8-Br-cAMP at doses of 10, 20, or 30 mg/kg, and heart rate values were monitored during 180 min. Values are expressed in beats per minute and represent means ± SEM of three to four animals per group.

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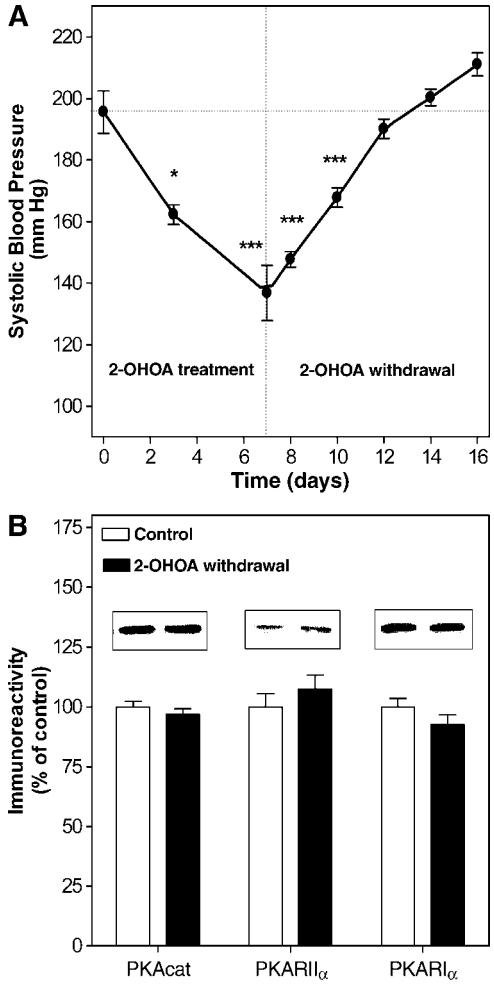

Fig. 7. Effects of withdrawal of 2-OHOA on systolic BP and aortic PKA subunit levels in SHRs. A: Time course of the systolic BP (expressed in mm Hg) during 2-OHOA treatment and 2-OHOA withdrawal. Systolic BP in SHRs was measured at 0, 3, and 7 days of treatment with 2-OHOA (600 mg/kg every 12 h) and at 1, 3, 5, 7, and 9 days after 2-OHOA withdrawal. Mean basal systolic BP values at time 0 were 196 ± 7 mm Hg (n = 6). * $P < 0.05$, *** $P < 0.001$ versus mean basal systolic BP values (one-way ANOVA followed by Bonferroni's test). B: PKA subunit levels in aortas from SHRs after 2-OHOA withdrawal. Insets show representative immunoblots for levels of aortic PKAcatα, PKARIIα, and PKARIα subunits after 9 days of 2-OHOA withdrawal. Inmunoreactive bands are representative of six animals per group. Columns show levels of aortic PKA subunits quantified against standard curves and normalized to the protein content of vehicle-treated SHRs (control) (taken as 100%). Data shown are means ± SEM.

high BP in established hypertension. Therefore, we applied 2-OHOA to adult SHRs with established hypertension and analyzed the cardiovascular responses of these rats compared with normotensive WKY rats. Oral treatment of SHRs induced a time- and dose-dependent sustained reduction of systolic and diastolic BP in SHRs. In contrast, 2-OHOA treatment had no effect on BP in normotensive WKY rats. After 7 days of treatment with 2-OHOA (600 mg/kg every 12 h), BP values of SHRs were close to those in WKY rats. Thus, 2-OHOA is in fact an effective antihypertensive agent.

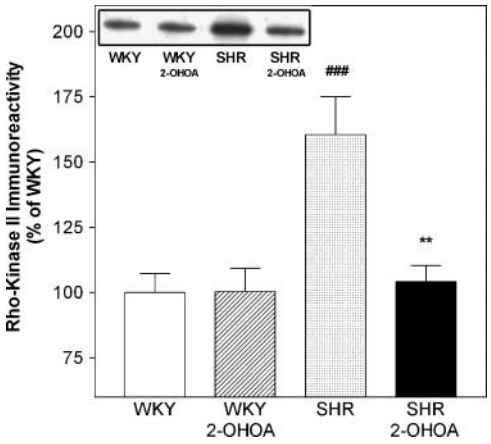

Fig. 8. Effects of 2-OHOA treatment (600 mg/kg every 12 h) for 7 days on Rho kinase II levels in aortas from SHRs and WKY rats. The inset shows a representative immunoblot for 8–11 animals per group (6 μg of total protein). Columns show levels of aortic Rho kinase quantified against standard curves and normalized to the protein content of vehicle-treated WKY rats (taken as 100%). Rho kinase II levels in aortas from vehicle-treated SHRs were increased compared with vehicle-treated WKY rats ($^{###} P < 0.001$). ** $P < 0.01$ versus vehicle-treated SHRs. Data shown are means ± SEM.

The reasons why 2-OHOA decreased systolic BP in normotensive Sprague-Dawley rats (28) but not WKY rats are not clear at present. Possible explanations are the different rat strains (Sprague-Dawley vs. WKY) used in the two studies and the different genders (female Sprague-Dawley vs. male WKY rats). In fact, in the studies on female Sprague-Dawley rats, the decrease in systolic BP induced by 2-OHOA was not associated with changes in heart rate (28), whereas in WKY rats, heart rate was increased significantly by treatment with 2-OHOA (Fig. 1B). This higher cardiac rate could be considered a compensatory mechanism to avoid BP reduction in these rats. However, this does not exclude the possibility that WKY rats may exhibit further mechanisms to counteract a hypotensive effect of 2-OHOA.

In this study, we found an enhanced AC responsiveness to forskolin in aortas from both SHRs and WKY rats, suggesting that 2-OHOA may increase the sensitivity to endogenous vasodilators acting via the AC/cAMP pathway. Treatment of 2-OHOA clearly restored forskolin-stimulated AC activity in SHRs to the level found in normotensive vehicle-treated WKY rats. Although forskolin-stimulated AC activity in 2-OHOA-treated WKY rats was further enhanced compared with that in vehicle-treated WKY rats, no hypotensive effect could be observed. In this regard, high levels of cAMP in heart are able to exert positive chronotropic effects and might be responsible for the above-mentioned increase of heart rate in 2-OHOA-treated WKY rats, a possible compensatory mechanism avoiding BP reduction in these rats. We next studied whether the effector system of cAMP (i.e., PKA) was altered by 2-OHOA treatment. Treatment of WKY rats with 2-OHOA had no effect on the expression of PKA subunits or PKA activity in aorta. In contrast, the expression of PKAcatα and the regulatory subunits RIα and RIIα was increased significantly in aortas from SHRs treated with 2-OHOA. In agreement

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with these protein expression data, PKA activity was upregulated in aortas from 2-OHOA-treated SHRs. Hypertension in SHRs is characterized by an impaired G_s-coupled receptor-induced vasorelaxation that is mediated by cAMP and PKA in smooth muscle (33–35). Thus, the enhanced AC responsiveness, together with the specific increase in PKA subunit expression observed only in 2-OHOA-treated SHRs, very likely explains the BP decrease in these hypertensive rats. To corroborate this hypothesis, we examined the effect on BP of a specific membrane-permeant inhibitor of PKA, the cAMP analog Rp-8-Br-cAMP (27), that was intraperitoneally administered in a single dose at the end of 2-OHOA treatment. In vehicle-treated SHRs, administration of Rp-8-Br-cAMP (10–30 mg/kg) altered neither systolic BP nor heart rate. In contrast, the acute administration of Rp-8-Br-cAMP to 2-OHOA-treated SHRs produced a pronounced reversal (up to 59% at 30 mg/kg Rp-8-Br-cAMP) of the antihypertensive action of 2-OHOA, strongly supporting the notion that augmented PKA expression and activity is one of the main molecular mechanisms responsible for the BP reduction induced by 2-OHOA in SHRs (Fig. 6A, B). On the other hand, withdrawal of 2-OHOA in SHRs resulted in a regression of decreased BP values to hypertensive levels, which was accompanied by aortic PKA levels comparable to those found in vehicle-treated SHRs, indicating that the antihypertensive effect of 2-OHOA is reversible.

It is known that Rho kinase, a pivotal regulatory protein of BP, is pathologically overexpressed in SHRs, thereby contributing to the high BP observed in this animal model of hypertension (22). For this reason, we investigated whether Rho kinase also could be implicated in the molecular mechanism by which 2-OHOA reduces BP in this rat strain. As reported previously, we found that expression of Rho kinase II in aorta from SHRs was increased significantly with respect to normotensive WKY rats. Administration of 2-OHOA decreased this overexpression of Rho kinase in SHRs to a level observed in normotensive WKY rats, whereas it did not alter the expression of Rho kinase in WKY rats. These results indicate that 2-OHOA normalizes supranormal Rho kinase expression rather than downregulates Rho kinase expression by a general inhibitory transcriptional mechanism. Thus, these data suggest that the molecular mechanisms by which 2-OHOA achieves a normalization of increased BP in SHRs involve, in addition to the upregulation of the vasodilatory PKA pathway, a downregulation of the vasoconstrictory Rho kinase.

Although these data do not exclude additional mechanisms involved in the antihypertensive action of 2-OHOA, such as direct effects of the fatty acid on Ca^{2+} or K^+ channels and contractile proteins, these are rather unlikely. First, such direct effects should also lead to vasodilation and a decrease in BP in WKY rats. Second, a direct inhibitory effect of 2-OHOA on proteins involved in vasoconstriction should result in a more rapid decrease in BP. In contrast, the decrease in BP caused by 2-OHOA, even at the highest effective dose, developed rather slowly, over days. It cannot be completely excluded that this time frame could reflect the necessity of reaching sufficiently high intracellular concentrations of the compound to achieve its effects, including rapid mechanisms, such as kinases or phosphatases. However, it is also in agreement with an altered expression of proteins, as observed here for PKA subunits and Rho kinase and reported previously for $G\alpha_s$ (28). In agreement with our observations, during the past two decades it has become evident that fatty acids can act as signaling molecules involved in the regulation of gene expression, although all known target genes to date mainly encode proteins with roles in fatty acid transport or metabolism (36). Further investigations will provide more insights into the transcriptional and posttranscriptional mechanisms by which 2-OHOA is able to control specifically the expression of PKA and Rho kinase. As a result, the development of derivatives of oleic acid could represent a novel pharmacological approach to treat hypertension by controlling the vascular expression of BP-regulating proteins.

We conclude from our study that the MUFA 2-OHOA is an effective antihypertensive agent, which decreases high BP in SHRs to normotensive values. The sustained antihypertensive action of 2-OHOA is apparently caused by an upregulation of the vasodilatory AC/cAMP/PKA pathway and a downregulation of the vasoconstrictory Rho kinase pathway.

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REFERENCES

1. Martinez-Gonzalez, M. A., and A. Sanchez-Villegas. 2004. The emerging role of Mediterranean diets in cardiovascular epidemiology: monounsaturated fats, olive oil, red wine or the whole pattern? *Eur. J. Epidemiol.* **19:** 9–13.
2. Perez-Jimenez, F. 2005. International conference on the healthy effect of virgin olive oil. *Eur. J. Clin. Invest.* **35:** 421–424.
3. Psaltopoulou, T., A. Naska, P. Orfanos, D. Trichopoulos, T. Mountokalakis, and A. Trichopoulou. 2004. Olive oil, the Mediterranean diet, and arterial blood pressure: the Greek European Prospective Investigation into Cancer and Nutrition (EPIC) study. *Am. J. Clin. Nutr.* **80:** 1012–1018.
4. Alonso, A., and M. A. Martinez-Gonzalez. 2004. Olive oil consumption and reduced incidence of hypertension: the SUN study. *Lipids.* **39:** 1233–1238.
5. Herrera, M. D., C. Perez-Guerrero, E. Marhuenda, and V. Ruiz-Gutierrez. 2001. Effects of dietary oleic-rich oils (virgin olive and high-oleic-acid sunflower) on vascular reactivity in Wistar-Kyoto and spontaneously hypertensive rats. *Br. J. Nutr.* **86:** 349–357.
6. Pagnan, A., R. Corocher, G. B. Ambrosio, S. Ferrari, P. Guarini, D. Piccolo, A. Opportuno, A. Bassi, O. Olivieri, and G. Baggio. 1989. Effects of an olive-oil rich diet on erythrocyte membrane lipid composition and cation transport systems. *Clin. Sci.* **76:** 87–93.
7. Ruiz-Gutierrez, V., C. M. Vazquez, and C. Santa-Maria. 2001. Liver lipid composition and antioxidant enzyme activities of spontaneously hypertensive rats after ingestion of dietary fats (fish, olive and high-oleic sunflower oils). *Biosci. Rep.* **21:** 271–285.
8. Vicario, I. M., M. D. Malkova, E. K. Lund, and I. T. Johnson. 1998. Olive oil supplementation in healthy adults: effects in cell membrane FA composition and platelet function. *Ann. Nutr. Metab.* **42:** 160–169.

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9. Clark, J. F., and G. Pyne-Geithman. 2005. Vascular smooth muscle function: the physiology and pathology of vasoconstriction. *Pathophysiology.* **12:** 35–45.
10. Dao, H. H., R. Essalihi, C. Bouvet, and P. Moreau. 2005. Evolution and modulation of age-related medial elastocalcinosis: impact on large artery stiffness and isolated systolic hypertension. *Cardiovasc. Res.* **66:** 307–317.
11. Somlyo, A. P., and A. V. Somlyo. 2000. Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J. Physiol.* **522:** 177–185.
12. Somlyo, A. P., and A. V. Somlyo. 2003. Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol. Rev.* **83:** 1325–1358.
13. Bonnevier, J., R. Fassler, A. P. Somlyo, A. V. Somlyo, and A. Arner. 2004. Modulation of Ca^{2+} sensitivity by cyclic nucleotides in smooth muscle from protein kinase G-deficient mice. *J. Biol. Chem.* **279:** 5146–5151.
14. Wooldridge, A. A., J. A. MacDonald, F. Erdodi, C. Ma, M. A. Borman, D. J. Hartshorne, and T. A. Haystead. 2004. Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of serine 695 in response to cyclic nucleotides. *J. Biol. Chem.* **279:** 34496–34504.
15. Tantini, B., A. Manes, E. Fiumana, C. Pignatti, C. Guarnieri, R. Zannoli, A. B. Md, and N. Galie. 2005. Antiproliferative effect of sildenafil on human pulmonary artery smooth muscle cells. *Basic Res. Cardiol.* **100:** 131–138.
16. Escribá, P. V., J. M. Sanchez-Dominguez, R. Alemany, J. S. Perona, and V. Ruiz-Gutierrez. 2003. Alteration of lipids, G proteins, and PKC in cell membranes of elderly hypertensives. *Hypertension.* **41:** 176–182.
17. Yoshikawa, H., K. Fukuda, Y. Wanaka, K. Kasamatsu, A. Baba, I. Nishio, and Y. Masuyama. 1994. Deficient activity of stimulatory nucleotide-binding regulatory protein in lymphocytes from patients with essential hypertension. *Am. J. Hypertens.* **7:** 713–716.
18. Bohm, M., P. Gierschik, A. Knorr, K. Larisch, K. Weismann, and E. Erdmann. 1992. Role of altered G-protein expression in the regulation of myocardial adenylate cyclase activity and force of contraction in spontaneous hypertensive cardiomyopathy in rats. *J. Hypertens.* **10:** 1115–1128.
19. Feldman, R. D., and R. Gros. 1998. Impaired vasodilator function in hypertension: the role of alterations in receptor-G protein coupling. *Trends Cardiovasc. Med.* **8:** 297–305.
20. Satoh, S., R. Kreutz, C. Wilm, D. Ganten, and G. Pfitzer. 1994. Augmented agonist-induced Ca^{2+}-sensitization of coronary artery contraction in genetically hypertensive rats. Evidence for altered signal transduction in the coronary smooth muscle cells. *J. Clin. Invest.* **94:** 1397–1403.
21. Masumoto, A., Y. Hirooka, H. Shimokawa, K. Hironaga, S. Setoguchi, and A. Takeshita. 2001. Possible involvement of Rho-kinase in the pathogenesis of hypertension in humans. *Hypertension.* **38:** 1307–1310.
22. Mukai, Y., H. Shimokawa, T. Matoba, T. Kandabashi, S. Satoh, J. Hiroki, K. Kaibuchi, and A. Takeshita. 2001. Involvement of Rho-kinase in hypertensive vascular disease: a novel therapeutic target in hypertension. *FASEB J.* **15:** 1062–1064.
23. Ito, K., Y. Hirooka, K. Sakai, T. Kishi, K. Kaibuchi, H. Shimokawa, and A. Takeshita. 2003. Rho/Rho-kinase pathway in brain stem contributes to blood pressure regulation via sympathetic nervous system: possible involvement in neural mechanisms of hypertension. *Circ. Res.* **92:** 1337–1343.
24. Uehata, M., T. Ishizaki, H. Satoh, T. Ono, T. Kawahara, T. Morishita, H. Tamakawa, K. Yamagami, J. Inui, M. Maekawa, et al. 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature.* **389:** 990–994.
25. Foulon, V., M. Sniekers, E. Huysmans, S. Asselberghs, V. Mahieu, G. P. Mannaerts, P. P. Van Veldhoven, and M. Casteels. 2005. Breakdown of 2-hydroxylated straight chain fatty acids via peroxisomal 2-hydroxyphytanoyl-CoA lyase: a revised pathway for the alpha-oxidation of straight chain fatty acids. *J. Biol. Chem.* **280:** 9802–9812.
26. Pfeffer, J. M., M. A. Pfeffer, and E. D. Frohlich. 1971. Validity of an indirect tail-cuff method of determining systolic arterial pressure in unanesthetized normotensive and spontaneously hypertensive rats. *J. Lab. Clin. Med.* **78:** 957–962.
27. Schwede, F., E. Maronde, H. Genieser, and B. Jastorff. 2000. Cyclic nucleotide analogs as biochemical tools and prospective drugs. *Pharmacol. Ther.* **87:** 199–226.
28. Alemany, R., S. Teres, C. Baamonde, M. Benet, O. Vogler, and P. V. Escriba. 2004. 2-Hydroxyoleic acid: a new hypotensive molecule. *Hypertension.* **43:** 249–254.
29. Martinez, J., O. Vogler, J. Casas, F. Barcelo, R. Alemany, J. Prades, T. Nagy, C. Baamonde, P. G. Kasprzyk, S. Teres, et al. 2005. Membrane structure modulation, protein kinase C alpha activation, and anticancer activity of Minerval. *Mol. Pharmacol.* **67:** 531–540.
30. Corrocher, R., A. Pagnan, G. B. Ambrosio, S. Ferrari, O. Olivieri, P. Guarini, A. Bassi, D. Piccolo, A. Gandini, and D. Girelli. 1992. Effects induced by olive oil-rich diet on erythrocytes membrane lipids and sodium-potassium transports in postmenopausal hypertensive women. *J. Endocrinol. Invest.* **15:** 369–376.
31. Ruiz-Gutierrez, V., F. J. Muriana, A. Guerrero, A. M. Cert, and J. Villar. 1996. Plasma lipids, erythrocyte membrane lipids and blood pressure of hypertensive women after ingestion of dietary oleic acid from two different sources. *J. Hypertens.* **14:** 1483–1490.
32. Martinez, J., A. Gutierrez, J. Casas, V. Llado, A. Lopez-Bellan, J. Besalduch, A. Dopazo, and P. V. Escriba. 2005. The repression of E2F-1 is critical for the activity of Minerval against cancer. *J. Pharmacol. Exp. Ther.* **315:** 466–474.
33. Anand-Srivastava, M. B. 1988. Altered responsiveness of adenylate cyclase to adenosine and other agents in the myocardial sarcolemma and aorta of spontaneously-hypertensive rats. *Biochem. Pharmacol.* **37:** 3017–3022.
34. Ruan, X., C. Chatziantoniou, and W. J. Arendshorst. 1999. Impaired prostaglandin E(2)/prostaglandin I(2) receptor-G(s) protein interactions in isolated renal resistance arterioles of spontaneously hypertensive rats. *Hypertension.* **34:** 1134–1140.
35. Shimizu, E., M. Ohyanagi, M. Masutani, and T. Iwasaki. 2002. A decrease in the amount and function of the stimulatory GTP-binding protein in the small resistance arteries of spontaneously hypertensive rats. *Hypertens. Res.* **25:** 743–749.
36. Pegorier, J. P., C. Le May, and J. Girard. 2004. Control of gene expression by fatty acids. *J. Nutr.* **134 (Suppl. 9):** 2444–2449.

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