

New Nutraceutical Combination Reduces Blood Pressure and Improves Exercise Capacity in Hypertensive Patients Via a Nitric Oxide–Dependent Mechanism

Albino Carrizzo, PhD; Ornella Moltedo, MSc; Antonio Damato, BSc; Katuscia Martinello, PhD; Paola Di Pietro, MSc; Marco Oliveti, BSc; Fausto Acernese, PhD; Giuseppe Giugliano, MD; Raffaele Izzo, MD; Eduardo Sommella, PhD; Serena Migliarino, MD; Ornella Piazza, MD; Carmine Izzo, MSc; Nicola Virtuoso, MD; Andrea Strianese, MD; Valentina Trimarco, PhD; Pietro Campiglia, PhD; Sergio Fucile, PhD; Annibale Puca, MD; Bruno Trimarco, MD;* Carmine Vecchione, MD*

Background—High blood pressure (BP) has long been recognized as a major health threat and, particularly, a major risk factor for stroke, cardiovascular disease, and end-organ damage. However, the identification of a novel, alternative, integrative approach for the control of BP and cardiovascular protection is still needed.

Methods and Results—Sixty-nine uncontrolled hypertension patients, aged 40 to 68 years, on antihypertensive medication were enrolled in 2 double-blind studies. Forty-five were randomized to placebo or a new nutraceutical combination named AkP05, and BP, endothelial function, and circulating nitric oxide were assessed before and at the end of 4 weeks of treatment. Twenty-four patients were randomized to diuretic or AkP05 for 4 weeks and underwent a cardiopulmonary exercise test to evaluate the effects of AkP05 on functional capacity of the cardiovascular, pulmonary, and muscular systems. Vascular and molecular studies were undertaken on mice to characterize the action of the single compounds contained in the AkP05 nutraceutical combination. AkP05 supplementation reduced BP, improved endothelial function, and increased nitric oxide release; cardiopulmonary exercise test revealed that AkP05 increased maximum O₂ uptake, stress tolerance, and maximal power output. In mice, AkP05 reduced BP and improved endothelial function, evoking increased nitric oxide release through the PKC α /Akt/endothelial nitric oxide synthase pathway and reducing reactive oxygen species production via NADPH-oxidase inhibition. These effects were mediated by synergism of the single compounds of AkP05.

Conclusions—This is the first study reporting positive effects of a nutraceutical combination on the vasculature and exercise tolerance in treated hypertensive patients. Our findings suggest that AkP05 may be used as an adjunct for the improvement of cardiovascular protection and to better control BP in uncontrolled hypertension. (*J Am Heart Assoc.* 2020;9:e014923. DOI: 10.1161/JAHA.119.014923.)

Key Words: exercise capacity • nitric oxide • vascular biology

High blood pressure (BP) is a growing, worldwide epidemic with an enormous economic burden; as such, the care and prevention of arterial hypertension represent

important challenges for the World Health Organization. Indeed, arterial hypertension is associated with higher cardiovascular morbidity and mortality at any age.¹ A pivotal

From the IRCCS Neuromed, Pozzilli, Italy (A.C., A.D., K.M., S.F., C.V.); Department of Pharmacy, University of Salerno, Fisciano, Italy (O.M., F.A., E.S., P.C.); Department of Medicine and Surgery, University of Salerno, Baronissi, Italy (P.D.P., M.O., O.P., C.I., A.S., A.P., C.V.); Department of Advanced Biomedical Sciences, University Federico II of Naples, Italy (G.G., R.I., B.T.); Department of Clinical and Molecular Medicine, School of Medicine and Psychology, Sapienza University of Rome, Italy (S.M.); Department of Cardiovascular Medicine, A.O.U. Federico II, Naples, Italy (N.V.); Department of Advanced Biomedical Sciences, Federico II University Hospital, Naples, Italy (V.T.); Department of Physiology and Pharmacology, Sapienza University of Rome, Italy (S.F.); Cardiovascular Research Unit, IRCCS MultiMedica, Milan, Italy (A.P.).

Accompanying Data S1, S2, Tables S1 through S6 and Figure S1 are available at <https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.014923>

*Dr Trimarco and Dr Vecchione are co-senior authors.

Correspondence to: Bruno Trimarco, MD, Department of Advanced Biomedical Sciences, University Federico II of Naples, Via Pansini, 5, 80131 Naples, Italy; E-mail: trimarco@unina.it and Carmine Vecchione, MD, Vascular Physiopathology Unit, IRCCS Neuromed, Pozzilli (IS) 86077; University of Salerno, Department of Medicine and Surgery, Via S. Allende, Baronissi 84081. E-mail: cvecchione@unisa.it

Received October 17, 2019; accepted January 6, 2020.

© 2020 The Authors. Published on behalf of the American Heart Association, Inc., by Wiley. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

Clinical Perspective

What Is New?

- This is the first demonstration that supplementation with a novel nutraceutical combination can improve functional capacity and cardiovascular protection in uncontrolled hypertensive patients on antihypertensive medication.
- AkP05 has a direct effect on blood pressure, endothelial function, circulating nitric oxide levels, and exercise tolerance in humans.
- The single components of AkP05 exert synergistic effects in maintenance of the redox axis for nitric oxide bioavailability, modulating oxidative stress status and enzyme activity.

What Are the Clinical Implications?

- This novel nutraceutical combination is able to contribute to the reduction of blood pressure levels and to the improvement of endothelial function and exercise effort beyond classical pharmacological therapy.
- The characterization of the beneficial cardiovascular properties of a combination of multiple naturally derived compounds, named AkP05, may lay the foundation for the development of new, natural adjunct therapies aimed at improving cardiovascular protection and contributing to the achievement of target blood pressure levels.

role in the pathogenesis of this condition has been attributed to endothelial dysfunction related to impairment of nitric oxide (NO) production^{2,3} and to altered functional capacity of the cardiovascular, pulmonary, and muscular systems.⁴ Unfortunately, the pharmacological therapies available for the management of hypertensive patients often prove to be ineffective because patients are often reluctant to undergo multiple-drug regimens, so adherence to therapy is reduced.⁴ Moreover, exercise capacity in hypertensive patients on antihypertensive medication has been reported to be reduced, further contributing to end-organ damage.⁵ Thus, an integrative approach capable of improving cardiovascular protection is urgently needed.

We have recently reported that treatment with a combination of nutraceuticals (a mixture of *Bacopa monnieri*, extract of *Ginkgo biloba* leaves, extract of green tea leaves, and phosphatidylserine, named AkP05, Izzek; Damor Farmaceutici, Italy) is able to simultaneously improve cognitive function and arterial compliance, as assessed by the augmentation index, in a population of hypertensive patients in whom BP was already satisfactorily controlled.⁶

Since impairment of arterial compliance is the main determinant of age-induced increase in BP, our aim was to test the action of the nutraceutical combination AkP05 on the modulation of BP in uncontrolled hypertension. In particular,

we decided to evaluate the cardiovascular effects of supplementing current antihypertensive medication with this new nutraceutical combination, assessing BP homeostasis, endothelial function, NO serum level, and exercise tolerance (by a cardiopulmonary test) in hypertensive patients with poorly controlled BP. The molecular mechanisms recruited by AkP05 and its single components were dissected in experimental models.

Methods

Data supporting the findings of this study are available from the corresponding authors on reasonable request. For detailed methodologies, please, see Data S1. The proof-of-concept study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the University of Salerno, which designed and registered the study protocol (no 390-01587470-30081). Written informed consent was obtained from each subject.

Study Design

We enrolled 69 patients of both sexes, aged between 40 and 68 years (56 ± 7.7), diagnosed with essential hypertension. Only hypertensive patients on pharmacologic therapy and with an unsatisfactory, but stable, BP control were screened for assessment of the exclusion criteria. During the observation period, there were no changes in the pharmacological therapy administered: from enrollment until the end of the study, all patients were given, in addition to their normal therapy, either placebo or AkP05 in 1 study and diuretic or AkP05 in the other, in order to rule out the possibility of interference of potential additional or different antihypertensive drugs on cardiovascular function.

Sample Size Calculation

Primary study/outcome

Based on results of previous observations,⁶ we hypothesized that 50% of the patients treated with AkP05 would reach optimal arterial BP values (mean systolic BP <125 mm Hg; mean diastolic BP <85 mm Hg) with respect to only 10% of patients assuming placebo. Based on this assumption and on a 2:1 randomization, 22 patients would be needed in the active treatment group versus 11 in the placebo group to have a statistical power of 80% ($\alpha=0.05$, 2-tailed). Considering a possible dropout of $\approx 20\%$, the total number of patients enrolled was 45 (30 patients in the active treatment group and 15 patients in the placebo group).

Secondary study/outcome

Since CPET represents a novel outcome of the study, a second group of uncontrolled hypertensive patients were enrolled for the assessment of cardiovascular, pulmonary, and muscular systems. Considering the nature of our proof-of-concept study, we performed a randomization 1:1 in which patients were underwent CPET assessment at time 0 (before assumption) and at 28 days after assumption of AkP05 or Diuretic.

Basing on the effect-size that we considered clinically relevant ($d=0.8$) and a power of 80%, considering that we would like to perform a noninferiority study aimed at evaluate the differences between before and after assumption of the compound, with a 1-tail t test, the estimation of the sample size suggests that 12 patients should be recruited for each experimental group ($N=12$ AkP05; $N=12$ Diuretic). This choice has been verified using G*power software.

Twenty-Four Hour Arterial BP Measurement

Patients enrolled for secondary outcome underwent 24-hour arterial BP measurement before and at the end of AkP05 or diuretic assumption for 4 weeks, with a walk200b arterial BP measurement monitoring device (Holter Recorder, Cardioline, Italy), which registers BP every 15 minutes during the day and every 30 minutes during the night. All subjects carried out normal daily activities.

Cardiopulmonary Exercise Test

All cardiopulmonary exercise tests (CPETs) were performed using a QUARK CPET breath-by-breath metabolic measurement system (Cosmed, Rome, Italy). A physician blind to treatment assignment did all tests. Detailed methodology has been described in Data S1.

Evaluation of Endothelial Function

Endothelial function was assessed via the reactive hyperemia index, using an EndoPAT 2000 device (Itamar Medical, Israel). Measurements were performed according to the manufacturer's instructions and were calculated using a computerized automated algorithm (software version 3.1.2) provided with the device. Briefly, subjects were in a supine position for a minimum of 15 minutes before measurements in a quiet, temperature-controlled ($21-24^{\circ}\text{C}$) room with dimmed lights. The subjects were asked to remain as still and silent as possible during the entire measurement period. Each recording consisted of 5 minutes of baseline measurement, 5 minutes of occlusion measurement, and 5 minutes of postocclusion measurement (hyperemic period). Occlusion

of the brachial artery was performed on the nondominant upper arm. The occlusion pressure (200 mm Hg minimum, 300 mm Hg maximum) was at least 60 mm Hg above the systolic blood pressure (SBP).

Measurements of Serum Nitrite Levels

Nitrite is a central component of the NO cycle. The measurement of nitrite levels in serum was performed with a 280i Nitric Oxide Analyzer (Sievers, Italy). In detail, after withdrawal, blood was rapidly transferred to serum vacutainer tubes and centrifuged within 30 seconds for 5 minutes at 800g for serum separation. Only serum samples without visible hemolysis were used for measurements. Before analysis, a mixture of glacial acetic acid and 1.0 mL of 0.5 mol/L ascorbic acid was added to the purge bath to generate a calibration curve for the nitrite. This method is specific for nitrite, since the reaction mixture does not release NO from any other NO_x metabolites. All samples were thawed in the dark just before analysis and kept on ice until injected. Every 4 injections, the reagents of the purge bath were changed. The concentration of nitrite in the samples was determined using a 280i Nitric Oxide Analyzer (Sievers Instruments).

Experimental Models

All experiments involving animals conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011) and were approved by the IRCCS INM Neuromed review board. Because of the important influence of female hormones and the estrous cycle, in which hormones vary periodically (every 4 days, reducing significantly the reproducibility of the studies), all animal studies were performed on male mice. A detailed description of experimental procedures is provided in Data S1.

Vivo Vascular Reactivity Studies and Staining for Superoxide

Vascular reactivity studies were performed on second-order branches of the mesenteric artery, as previously described.⁷ A detailed description of experimental procedures is provided in Data S1.

Cell Culture and NO Measurement

Commercially available human umbilical vein endothelial cells (HUVECs) or coronary artery smooth muscle cells were purchased from Lonza (Walkersville, MD) and grown in

EGM-2 and SmBM basal medium, respectively. The NO concentration in the supernatant was assayed as nitrite, the stable breakdown product of NO, with a chemiluminescence detector (Sievers 280i NO Analyzer), as previously described.⁸ A detailed description of methodology is provided in Data S1.

Fluorescence Microscopy

Free intracellular calcium concentration ($[Ca^{2+}]_i$) recordings were obtained by time-resolved digital fluorescence microscopy on HUVECs or coronary artery smooth muscle cells loaded with the Ca^{2+} indicator Fura2-AM (excitation, 340 and 380 nm; emission, 550 nm). Briefly, cells were incubated for 45 minutes at 37°C with 1 μ mol/L Fura2-AM, then placed in standard normal external solution (in mmol: NaCl, 140; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 2; HEPES-NaOH, 10; and glucose, 10; pH 7.3), and continuously superfused with a gravity-driven perfusion system. Ca^{2+} transients were elicited by applying phosphatidylserine (PS) (0.4 mg/mL) for 2 minutes. The time courses of Ca^{2+} transients were quantified by measuring at each time point (frequency of acquisition 0.3 Hz) the ratio of fluorescence emission stimulating the dye at 340 and 380 nm (R) in the region of interest surrounding each cell present in the field. The rise in cytosolic Ca^{2+} concentration was expressed as the difference ΔR between the ratio values at peak and resting level.

In Situ NO Measurement

NO production in response to different stimuli, as reported in figure legends, was assessed and imaged using 4-amino-5-methylamino-2',7-difluorofluorescein diacetate (DAF-FM) (Invitrogen, D-23842), as previously described.⁷ Briefly, isolated mesenteric arteries were cleaned of fat and connective tissue and equilibrated for 30 minutes in Krebs solution at room temperature. DAF-FM (5 μ mol/L) was then added to the buffer for 30 minutes. Subsequently, vessels were washed twice with fresh Krebs solution and then stimulated as reported in figure legends. After stimulation, vessels were immediately snap-frozen with OCT embedding compound in isopentane prechilled with liquid nitrogen. Frozen rings were then cut into 10- μ m-thick sections using a CM 1250 cryostat (Leica) and imaged by a fluorescence DS-Ri1 microscope (Zeiss Axiophot 2) with excitation at 495 nm and emission at 515 nm. Subsequently, some sections were counterstained with hematoxylin–eosin and observed under a light microscope.

Protein Extraction and Immunoblot Analysis

Immunoblotting was performed as previously described.⁷ A detailed description of methodology is provided in Data S1.

NADPH Oxidase Activity

NADPH oxidase (NOX) activity in mouse mesenteric arteries was measured in control (untreated) vessels and in vessels treated with either angiotensin II (Ang II; 10^{-6} mol/L) or Ang II plus different compounds preincubated for 1 hour at a dose of 0.4 mg/mL. The chemiluminescence that occurred over the ensuing 5 minutes in response to addition of 100 μ mol/L NADPH was recorded (LS6500 Multipurpose Scintillation Counter; Beckman Coulter, Fullerton, CA). In preliminary experiments, homogenates alone, without addition of NADPH, gave only minimal signals, and NADPH did not evoke lucigenin chemiluminescence in the absence of homogenate. A detailed description of methodology is provided in Data S1.

BP Measurements in Mice

SBP was noninvasively measured in conscious mice with a tail-cuff method, using a BP-2000 instrument (Visitech Systems) as previously described.⁹ Briefly, animals were placed in a holder on a temperature-controlled platform (kept at 37°C), and recordings were performed in steady-state conditions. BP values were averaged from at least 3 consecutive measurements.

The dose administered in vivo by gavage was calculated considering the human equivalent dose based on US Food and Drug Administration recommendations.¹⁰ In particular, considering that 1000 mg of whole AkP05 contains 300 mg *Bacopa monnieri* (BM), 50 mg *Ginkgo biloba* extract (GBE), 25 mg PS, and 40 mg green tea extract (GTE), we administered 150 mg/kg of AkP05 or 46 mg/kg of BM, 7.8 mg/kg of GBE, 4 mg/kg of PS, or 6.3 mg/kg of GTE.

Statistical Analysis

All statistical analyses were performed with Prism 8.01 (GraphPad). Student *t* test (2-tailed) was used to calculate statistical significance of 2 independent groups. Data from all experiments are given as mean \pm SD, except for the vascular reactivity and tail-cuff blood pressure experiments, for which data are given as mean \pm SEM. The *z*-score test for 2 population proportions was used to determine the hypertensives who achieve target blood pressure in placebo and active treatment groups. Comparison between measurements before and after treatments have been performed using paired *t* test, whereas analysis of the differences between AkP05 or Diuretic group was performed using unpaired *t* test (2 tailed). Measured effect size was calculated by G*Power 3.1.9.4. When more than 2 independent groups were compared, we used 1-way ANOVA followed by Tukey's post-hoc test. To analyze the effects of treatments on dose-dependent vasorelaxation, we performed a 2-way repeated-measures ANOVA with Tukey's

post-hoc test for multiple comparisons. A $P < 0.05$ was considered statistically significant.

Results

AkP05 Reduces BP in Hypertensive Patients by Improving Endothelial Function and NO Release

Baseline characteristics and medication regimens of the first study population are reported in Tables S1 and S2: no statistically significant differences were found between the 2 groups. As expected, SBP and diastolic blood pressure in the placebo-treated group remained unchanged throughout the observation period (Figure 1A and 1B). By contrast, AkP05 evoked a significant reduction in SBP, but no significant effect on diastolic blood pressure, by the end of the observation period (Figure 1C and 1D), a finding suggestive of the nutraceutical combination having an additive effect with antihypertensive drugs on the control of hypertension.

A useful noninvasive tool for the evaluation of vascular function, as well as for cardio- and cerebrovascular disease risk assessment, is reactive hyperemia index.¹¹ As expected, the reactive hyperemia index did not change in the placebo group (Figure 1E), but treatment with AkP05 did improve it significantly (Figure 1F). We then measured nitrite levels—a well-recognized biomarker of the state of NO metabolism—in the sera of patients: the level of circulating nitrite was significantly increased in the AkP05-treated group, while remaining unchanged in the placebo group (Figure 1G and 1H). No difference in sodium excretion or serum electrolytes between the 2 groups was detected (data not shown). Of note, Pearson correlation revealed an association between the increase of nitrite and the reduction of SBP in the AkP05-treated group (Figure 1I). This led us to hypothesize that AkP05 ameliorated endothelial function and decreased BP through a NO-dependent-mechanism. Moreover, since 53.3% of the AkP05 group reached the target BP of $\leq 125/85$ mm Hg, versus only 6.66% of the placebo-treated group (Table S3), this strongly supports the efficacy of AkP05 supplementation for better management of BP in hypertensive subjects. In addition, evaluation of the impact of medications on the achievement of target values revealed no differences between mono- or polytherapy when analyzing all patients together, nor between males and females (data not shown). This suggests that improvement of BP control is selectively related to AkP05 treatment in both sexes.

AkP05 Improves Exercise Tolerance and Reduces Cardiovascular Risk

Seeing that no changes were observed in the placebo-treated group regarding BP, NO release, and endothelial function,

subsequent clinical evaluations were not performed on these patients.

A second population of a total 24 patients who were not taking diuretics were then selected and randomized with a 1:1 ratio to take, in addition to their usual medication, either AkP05 or a diuretic (Table S4). These patients ($n=12$ treated additionally with AkP05, and $n=12$ age-matched patients treated additionally with a diuretic) underwent 24-hour arterial BP measurement monitoring and a CPET at baseline and after 4 weeks of treatment. At the end of the treatment period, SBP was significantly reduced in both groups in the morning (between 8 and 10 AM), (AkP05: from 137.3 ± 13.6 to 124.2 ± 14.3 mm Hg, $P < 0.0001$; diuretic: from 140.3 ± 11.68 to 124.8 ± 10.55 , $P < 0.0001$) during the evening (between 8 and 10 PM) (AkP05: from 137.4 ± 17.46 to 127.7 ± 11.3 mm Hg, $P = 0.0003$; diuretic: from 139.3 ± 14.27 to 127.0 ± 10.80 mm Hg, $P = 0.0003$), and night (between 2 and 4 AM) (AkP05: from 121.8 ± 14.1 to 112.0 ± 8.1 mm Hg, $P = 0.0006$; diuretic: from 128.8 ± 14.34 to 120.8 ± 11.5 mm Hg, $P = 0.0028$); diastolic blood pressure was significantly reduced in both groups in the morning between 8 and 10 AM (AkP05: from 90.2 ± 6.2 to 81.5 ± 11.2 mm Hg, $P = 0.0004$; diuretic: from 92.7 ± 7.4 to 84.0 ± 9.5 mm Hg, $P < 0.0003$), but the decrease was not statistically significant at all other times (Figure 1J through 1M).

Of note, CPET revealed there was an increase in maximum VO_2 during exercise and a significant reduction in maximum SBP only in the AkP05-treated group. Moreover, exercise duration and maximum power generated during the test were indicative of the nutraceutical combination inducing significant improvements in both parameters (Table 1), a finding suggestive of it having an important impact on the patients' functional capacity. The hypertensive patients treated with a diuretic also had reduced BP, but there were no changes in other CPET parameters. Moreover, the evaluation between the difference pre/post of AkP05 versus Diuretic group revealed a statistical significance and high effect size for SBP_{MAX} , VO_2/kg_{MAX} , Exercise duration, and maximal power (Table 2).

Thus, improvements in functional capacity of the cardiovascular, pulmonary, and muscular systems may be specifically attributable to the nutraceutical.

AkP05 Directly Evokes Vasorelaxation of Mouse Mesenteric Arteries

Based on the beneficial effects seen in the patients, and given the impossibility of characterizing the molecular mechanisms recruited by AKP05 in humans, we decide to investigate the vascular effects of the nutraceutical combination in experimental models.

We first investigated whether AkP05 modulated vascular function per se, using an ex vivo method in which

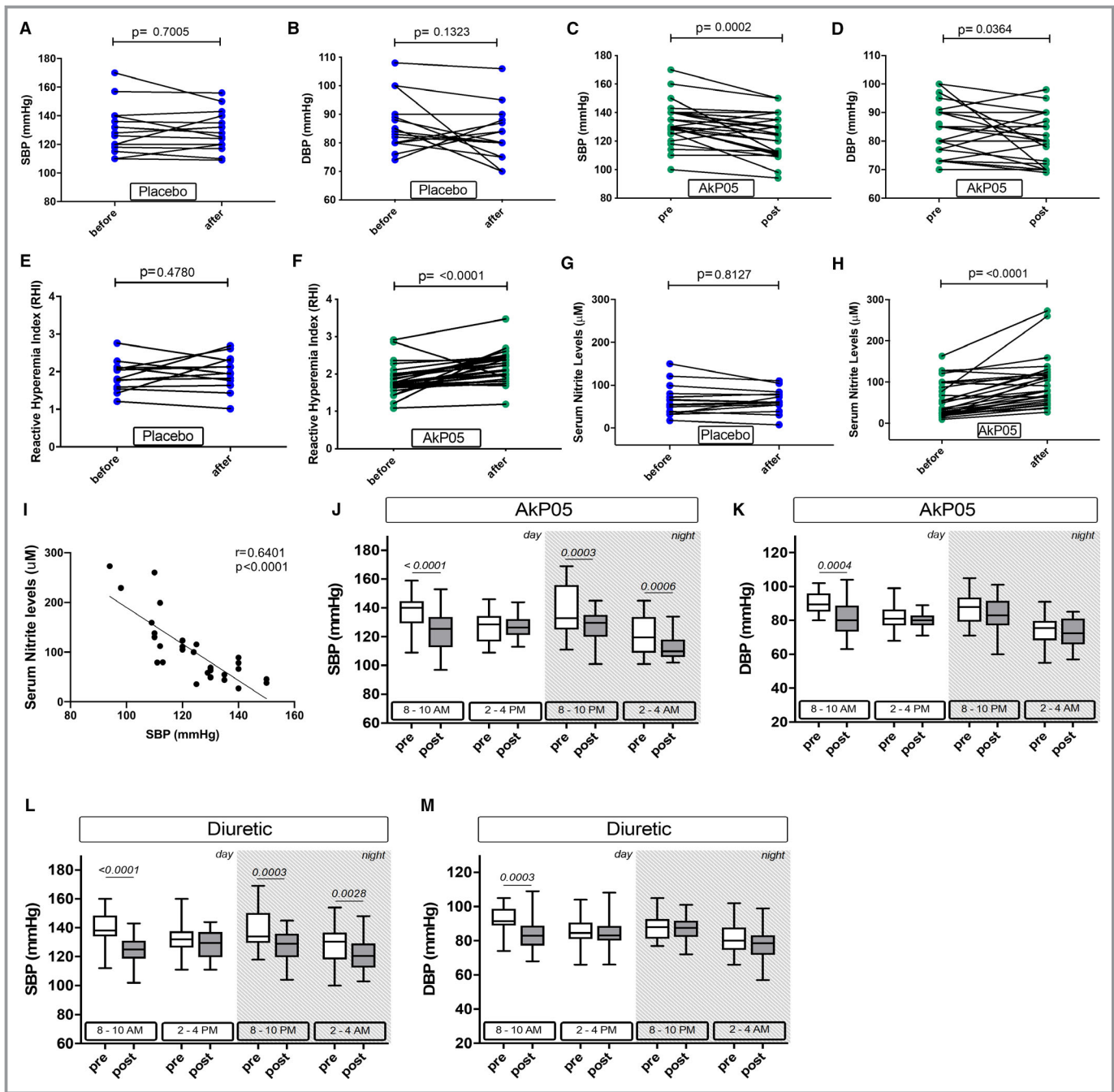


Figure 1. A through D, Systolic and diastolic blood pressure (SBP, DBP) in hypertensive patients before and after treatment with placebo (A,B), and before and after treatment with AkP05 (C and D). E and F, Reactive hyperemia index (RHI) in patients on placebo (E) or AkP05 (F). G and H, Serum concentration of nitrite in hypertensive patients before and after treatment with placebo (G) or AkP05 (H). Dot plots with mean (horizontal line). pre, baseline; post, end of 4 weeks of treatment. I, Correlation between serum nitrite levels and systolic blood pressure in AkP05-treated hypertensive subjects ($n=30$). r =Pearson's correlation coefficient. J through M, 24-hour blood pressure monitoring of hypertensive patients at 2 time points during the day (8–10 AM; 2–4 PM) and night (8–10 PM; 2–4 AM) before and after treatment with AkP05 (J and K) or a diuretic (L and M) for 4 weeks in addition to their usual antihypertensive drug regimen. Box and whisker plots, with the band indicating median, the box indicating first and third quartiles. DBP indicates diastolic blood pressure; SBP, systolic blood pressure.

mesenteric arteries were excised from mice and mounted on a myograph apparatus. Exposure to increasing doses of AkP05 evoked dose-dependent vasorelaxation of arteries, an effect significantly reduced by pretreatment with the

endothelial nitric oxide synthase (eNOS) inhibitor L-nitro-arginine methyl ester (L-NAME) (Figure 2A). In agreement, molecular analysis of the vessels showed clear induction of eNOS phosphorylation on serine¹¹⁷⁷ upon exposure to

Table 1. CPET Parameters Before and After Supplementation of Medication With AkP05 or a Diuretic

Variable	AkP05 Group (n=12)			Measured Effect Size	Diuretic Group (n=12)			Measured Effect Size
	Before	After	P Value		Before	After	P Value	
SBP rest, mm Hg	136.1±10.5	124.6±11.7	0.0016	−1.08	138.33±7.0	127.5±12.6	>0.0001	−1.91
SBP _{MAX} , mm Hg	176.5±18.9	153.8±14.4	0.0031	−0.97	175.6±16.8	182.9±11.4	0.1261	0.35
DBP rest, mm Hg	87.5±11.0	85.0±8.7	0.2030	−0.25	88.3±14.9	87.3±10.9	0.4175	−0.06
DBP _{MAX} , mm Hg	110.7±12.1	103.1±14.5	0.0379	−0.57	112.0±6.6	107.8±11.3	0.1491	−0.32
METs, rest	1.36±0.2	1.52±0.5	0.0947	0.40	1.3±0.4	1.5±0.5	0.1318	0.34
METs, max	8.4±1.8	8.7±1.6	0.0539	0.64	8.1±1.1	8.2±1.6	0.4534	0.03
VO ₂ /kg, rest (mL/min per kg)	4.3±0.7	4.9±1.4	0.0930	0.41	4.2±1.0	4.3±1.0	0.3705	0.10
VO ₂ /kg, MAX (mL/min per kg)	27.6±2.7	31.3±3.2	>0.0001	1.80	28.0±3.3	27.7±3.3	0.1126	−0.37
Exercise duration (min)	12.6±4.3	15.8±4.3	0.0050	0.90	12.3±1.5	12.1±2.0	0.3579	−0.11
Max. power (W)	175.3±33.5	208.3±41.6	0.0219	1.46	176.9±28.1	168.1±32.8	0.0148	−0.72
FVC	4.24±0.6	4.3±0.8	0.2044	0.25	4.4±0.8	4.4±0.8	0.4514	0.04
FEV	3.4±0.6	3.4±0.6	0.3966	−0.08	3.4±0.3	3.3±0.3	0.0646	−0.47

Data are mean±SD. Statistical analysis was performed using paired *t* test between before/after in the same patients' group. Measured effect size was calculated by G*Power 3.1.9.4. CPET indicates cardiopulmonary exercise test; DBP rest, diastolic blood pressure measured at rest; DBP_{MAX}, maximal diastolic blood pressure; FEV, forced expiratory volume in 1 second; FVC, forced vital capacity; Max. power (W), maximal power output; METs_{MAX}, maximal 1 metabolic equivalent; METs rest, 1 metabolic equivalent measured at rest; SBP rest, systolic blood pressure measured at rest; SBP_{MAX}, maximal systolic blood pressure; VO₂/kg_{MAX}, maximal respiratory oxygen uptake; VO₂/kg rest, respiratory oxygen uptake measured at rest.

AkP05 (Figure 2B). Moreover, staining of arteries with DAF-FM demonstrated that NO was released from AkP05-stimulated vessels (Figure 2C).

To investigate the mechanism through which AkP05 modulated NO metabolism, we performed a new set of

ex vivo experiments using pharmacological inhibitors to block well-known signaling pathways leading to eNOS activation.¹² Inhibition of PI3K signaling with LY294002 did not modify AkP05-evoked vasorelaxation (Figure 2D), excluding the involvement of this kinase in the nutraceutical's mechanism of action. By contrast, inhibition of Akt—one of the most important modulators of eNOS—abolished AkP05-evoked vasorelaxation (Figure 2E).

Because classical PKC (protein kinase C) plays an important role in Akt activation,¹³ we then evaluated whether the PKC inhibitor Ro31-8220 also removed AkP05's vasorelaxant ability. Indeed, PKC inhibition eliminated the nutraceutical's dose-dependent vasorelaxation effect (Figure 2E), leading us to explore the sequence of proteins recruited in the pathway. Molecular analysis revealed that PKC inhibition completely blocked AkP05-evoked Akt and eNOS activation, whereas upon Akt inhibition, eNOS continued to be inactive in the face of PKC α activation (Figure 2F). Thus, AkP05 recruits a PKC α /Akt/eNOS intracellular signaling cascade.

Differential Effects of Single Components on Vascular Function

After performing chemical characterization of the nutraceutical by UHPLC-PDA-MS/MS analyses (Data S2, Figure S1 and, Tables S5 and S6), we then set out to identify the

Table 2. Unpaired *t* Test Results on Differences Between Pre/Post Supplementation With AkP05 or Diuretic

Variable	Differences			Measured Effect Size
	AkP05 (n=12)	Diuretic (n=12)	P Value	
SBP rest, mm Hg	−11.3±10.5	−10.8±5.7	0.8859	0.06
SBP _{MAX} , mm Hg	−22.7±23.3	7.1±20.5	0.0030	1.36
VO ₂ /kg, MAX, mL/min per kg	3.74±2.1	−0.29±0.8	<0.0001	2.57
Exercise duration, min	3.18±3.5	−0.15±1.4	0.0060	1.24
Max. power, W	33.08±22.7	−8.75±12.1	<0.0001	2.30

Data are presented as mean of differences±SD. Statistical analysis was performed using unpaired *t* test between mean of the differences before/after in AkP05 vs Diuretic group. Measured effect size for each variable was calculated using G*Power 3.1.9.4 with statistical test "Means: Difference between 2 independent means (2 groups), 2 tails." Max. power (W) indicates maximal power output; SBP_{MAX}, maximal systolic blood pressure; VO₂/kg_{MAX}, maximal respiratory oxygen uptake.

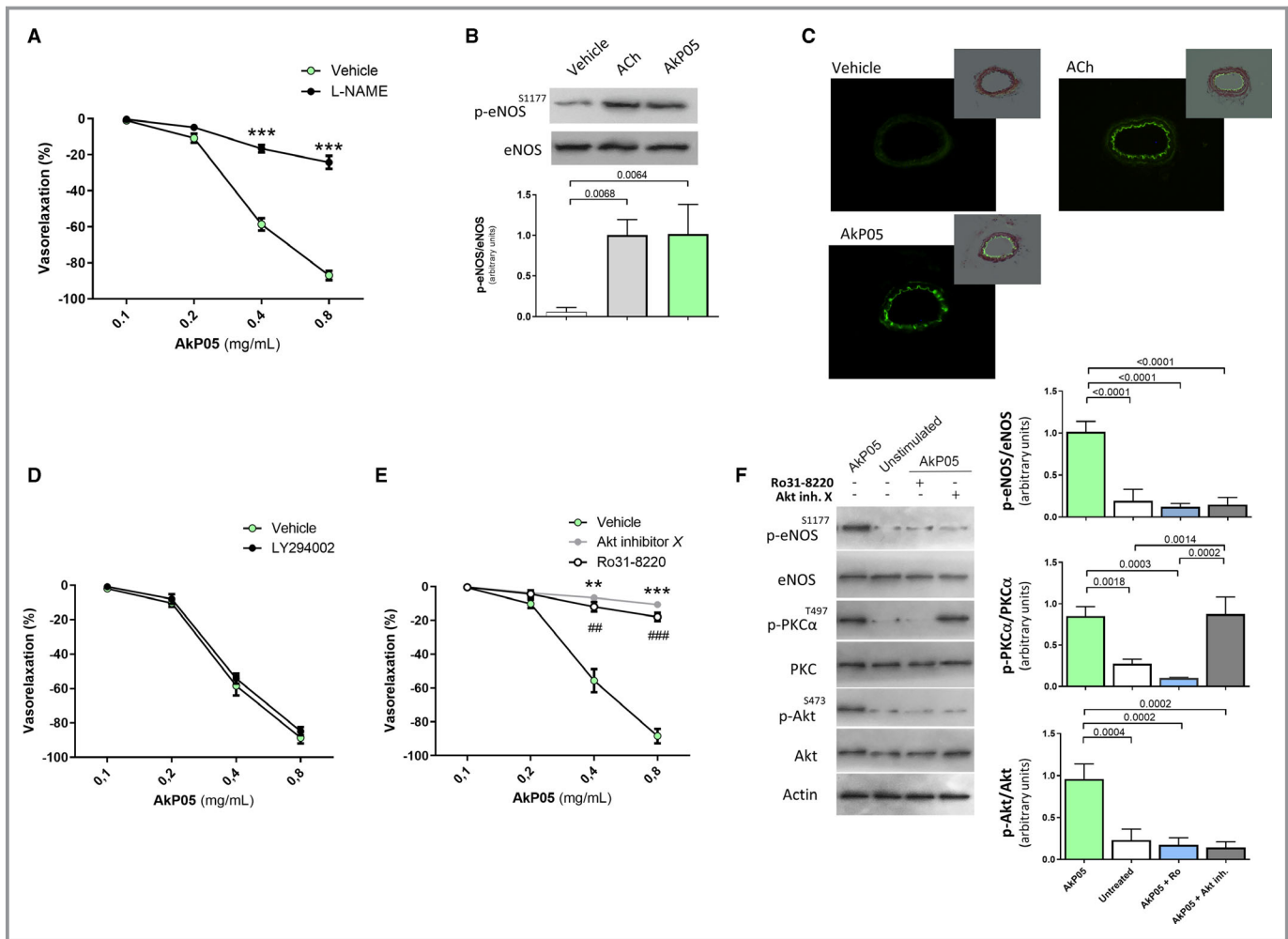


Figure 2. **A**, Dose–response curves of phenylephrine–precontracted mesenteric arteries harvested from C57BL/6 mice to increasing doses of AkP05, in the presence and absence of the eNOS inhibitor L-NAME (300 μmol/L). ****P*<0.001 (*n*=5 for each group). **B**, Representative immunoblot (top) and densitometry (bottom) of p-eNOS (S1177) and total eNOS in mesenteric arteries from C57BL/6 mice, exposed to vehicle, acetylcholine (ACh), or AkP05; (*n*=3 independent experiments). **C**, Representative micrographs of DAF-FM fluorescent signal in mesenteric arteries treated with vehicle, ACh, or AkP05 (*n*=5 for each group). *Inset*: images of the fluorescent signal merged with images of sections counterstained with hematoxylin–eosin. Scale bar, 100 μm. **D** and **E**, Response of phenylephrine–precontracted mesenteric arteries harvested from C57BL/6 mice to increasing doses of AkP05, in the presence of either the PI3K inhibitor LY294002 (**D**), Akt inhibitor X, or the PKC inhibitor Ro31-8220 (**E**). ***P*<0.01; ##*P*<0.01; ****P*<0.001; ####*P*<0.001 (*n*=5 for each group). **F**, Representative immunoblot blots (left) and densitometry (right) of p-eNOS (S1177), total eNOS, p-PKCα (T497), PKC antibody, p-Akt (S473), total Akt, and β-actin in mesenteric arteries from C57BL/6 mice exposed to ACh or AkP05 in the presence of Akt inhibitor X or Ro31-8220; (*n*=3 for each group). eNOS indicates endothelial nitric oxide; L-NAME, L-nitro-arginine methyl ester.

component/s responsible for AkP05’s vascular effects. We found that BM and PS were able to evoke dose-dependent, eNOS-inhibition-sensitive vasorelaxation (Figure 3A and 3B), whereas neither GBE nor GTE modulated endothelial function (Figure 3C and 3D).

These findings led us to quantitatively measure the stable form of NO (nitrite) released from HUVEC cultures exposed to single components. Only in the presence of AkP05, BM, or PS nitrite was released into the supernatant to a comparable extent as that observed with acetylcholine (ACh) (Figure 3E); GTE and GBE did not have any measurable effect on NO

production in HUVECs, as also shown by DAF-FM immunofluorescence signals (Figure 3F).

AkP05 Inhibits Oxidative Stress by Modulating NADPH Oxidase Activity

The level of NO can be modulated by increasing production through activation of eNOS as well as by improving its bioavailability, acting on oxidative stress modulation.¹⁴ In this regard, several nutraceutical compounds have potent antioxidant activities.^{15,16} Thus, we tested the capacity of AkP05 to

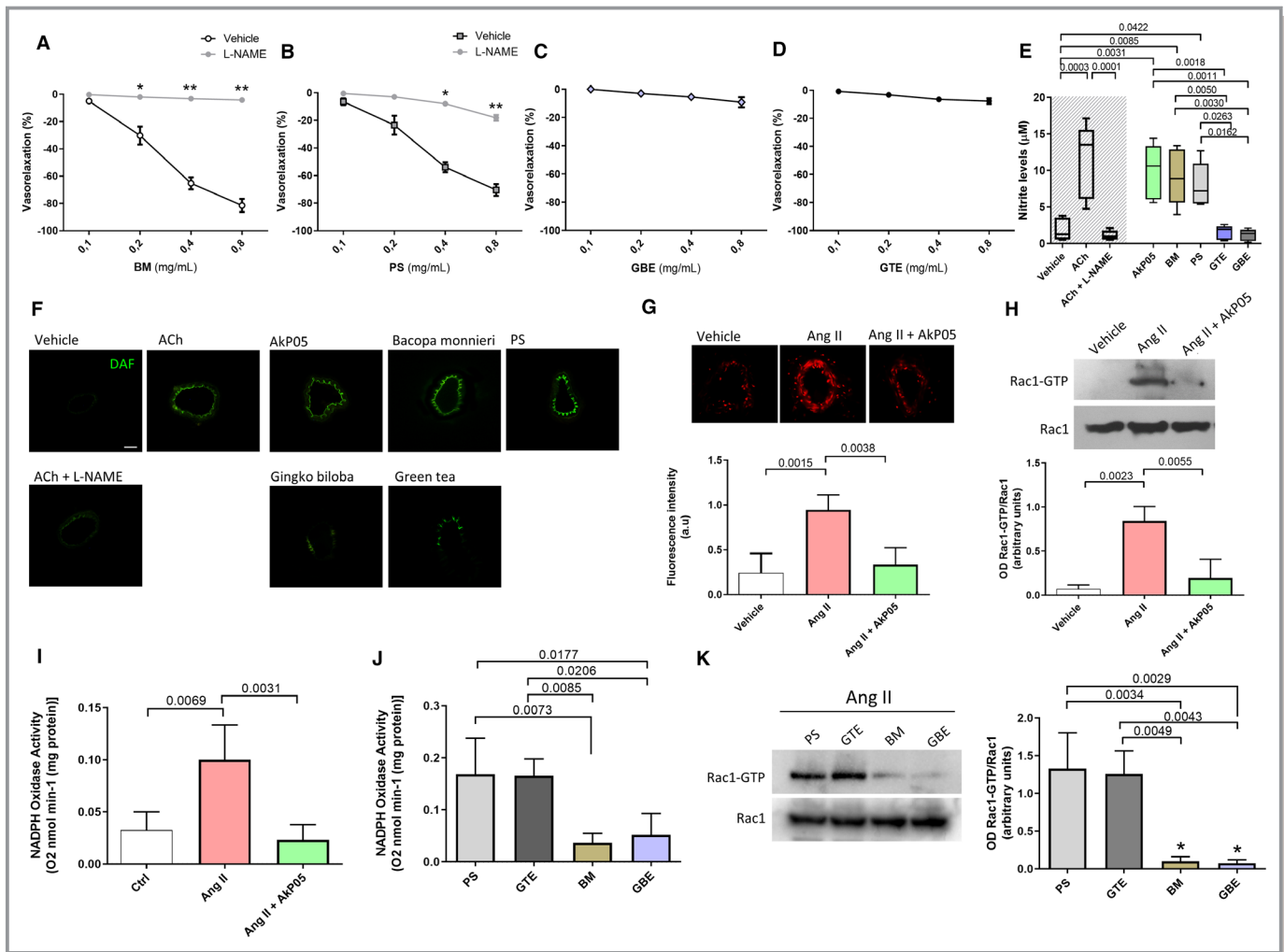


Figure 3. A through D, Dose–response curves of phenylephrine-precontracted mesenteric arteries from C57BL/6 mice to increasing doses of single AkP05 components, in the presence and absence of the eNOS inhibitor L-NAME (300 µmol/L). **P*<0.05; ***P*<0.01 (n=4 for each group). BM, *Bacopa monnieri*; PS, phosphatidylserine; GBE, *Ginkgo biloba* extract; GTE, green tea extract. E, Nitrite levels measured in supernatants from HUVEC cultures treated with vehicle, 100 µmol/L acetylcholine (ACh), ACh plus L-NAME, or the individual components contained in AkP05. Box and whisker representation; (N=5 for each group). F, Representative micrographs of DAF-FM fluorescent signals from mesenteric arteries exposed to vehicle, ACh, ACh plus L-NAME, AkP05, or single components (n=5 for each group). Scale bar, 100 µm. G, In situ detection of superoxide generation (top) and graph of total ROS production (bottom) in C57BL/6 mesenteric arteries exposed to vehicle, angiotensin II (Ang II), or Ang II after pretreatment with AkP05 (0.4 mg/mL for 1 hour), and qualitatively measured with CM-H₂DCF/DA probe. Data are mean±SD; (4 independent experiments). H, Representative immunoblot (top) and densitometry (bottom) of phosphorylation levels of Rac1-GTP and total Rac1 conducted on extracts of mouse mesenteric artery exposed to vehicle, Ang II, or Ang II plus AkP05; (n=3 independent experiments). I and J, The effect of AkP05 (I) and of its individual components (J) on nicotinamide adenine dinucleotide phosphate (NADPH)-induced lucigenin chemiluminescence in C57BL/6 mice mesenteric arteries; (n=4). K, Representative immunoblot (left) and densitometry (right) conducted on extracts of mouse mesenteric artery exposed to Ang II plus BM, PS, GBE, or GTE. Semiquantitative analyses of phosphorylation levels of Rac1-GTP and total Rac1 (n=3 independent experiments). eNOS indicates endothelial nitric oxide; L-NAME, L-nitro-arginine methyl ester; HUVEC, human umbilical vein endothelial cell; ROS, reactive oxygen species.

modulate reactive oxygen species released from vessels after exposure to Ang II, a potent inducer of reactive oxygen species.¹⁷

Pretreatment with AkP05 induced a marked reduction in Ang II-evoked oxidative stress compared with vessels exposed to Ang II alone, as measured qualitatively by DHE (Figure 3G top) and quantitatively by CM-H₂DAF DA (Figure 3G bottom), a

finding demonstrating that the nutraceutical combination has antioxidant properties.

Ang II induces reactive oxygen species production through the modulation of NOX, an enzyme that becomes active after the assembly of different subunits promoted by the small GTPase Rac1. Thus, in order to identify the molecular mechanism through which AkP05 reduces NOX activation,

we performed a pull-down assay on active Rac1. AkP05 significantly reduced Rac1 activation, as shown by reduced Rac1-GTP compared with vessels exposed to Ang II alone (Figure 3H). To translate this result to the functional level, we measured NOX activity, finding a significant reduction of enzymatic activity after exposure to AkP05 (Figure 3I). The evaluation of single components on oxidative stress status revealed that BM and GBE were the only ones capable of reducing Ang II-stimulated NOX activity in our time window (Figure 3J). The evaluation of Rac1 status in the presence of the different components revealed that BM and GBE were specifically able to reduce Rac1-GTP formation, whereas GTE and PS were not (Figure 3K).

AkP05's Single Components Act Through Different Intracellular Mechanisms

Based on our observation of the direct NO-dependent vascular action of the AkP05 components BM and PS, we tried to dissect the specific molecular mechanism recruited. Firstly, we tested the vasorelaxant properties of the components on ex vivo vessels under Akt inhibition. Of note, BM and PS both failed to modulate vascular function under this condition (Figure 4A and 4B), a finding suggesting that Akt represents a common node for the 2 compounds.

To find the upstream modulator of Akt, we tested the effects of BM and PS on vessels in the presence of a potent PI3K inhibitor.⁷ Under this condition, BM and PS continued to evoke dose-dependent vasorelaxation (Figure 4A and 4B), a finding indicating that the kinase was not involved. In contrast, when we inhibited PKC—an important modulator of Akt—the vasorelaxant properties of both components were completely abolished (Figure 4C and 4D).

Among the mechanisms regulating PKC proteins, phospholipase C (PLC) plays a major role through the production of soluble intracellular second messengers.¹⁸ Upon pharmacological inhibition of PLC, the vascular action of BM was completely abolished (Figure 4C), an effect associated with a significant reduction of eNOS activation in vessels treated with inhibitors of PKC or PLC (Figure 4E). In contrast, inhibition of PLC did not alter response to PS (Figure 4D), a finding that led us to explore alternative intracellular signaling pathways involved in PKC α activation.

Intracellular calcium release is the leading mechanism regulating calcium-dependent PKC. In the absence of Ca²⁺ or in the presence of thapsigargin—a noncompetitive inhibitor of sarco/endoplasmic reticulum Ca²⁺ efflux—PS did not evoke dose-dependent vasorelaxation (Figure 4F), nor did it promote activation of PKC α or eNOS (Figure 4G). We then went on to evaluate Ca²⁺ mobilization by PS, measuring [Ca²⁺]_i variations in HUVECs and coronary artery smooth muscle cells. PS

evoked Ca²⁺ transients in all coronary artery smooth muscle cells tested (Figure 4H, *top*), but not in HUVECs (Figure 4H; *bottom*).

Smooth muscle cells and endothelial cells can be electrically and metabolically connected by gap junctions that allow the passage of currents and small signaling molecules, such as Ca²⁺, between cells.^{12,19} Thus, to further investigate the effect of Ca²⁺ transients evoked by PS in smooth muscle cells, we performed vascular reactivity studies in the presence of GAP-27, a mix of small peptides that block gap junctions by inhibiting connexin 43 and 37 (cx43, cx37). In the presence of GAP-27, PS did not evoke any vascular effects, confirming a calcium-dependent mechanism of action (Figure 4I).

Considering the important direct vascular action of the single components of AkP05, we investigated their modulation of BP levels in mice. Following the US Food and Drug Administration recommendations¹⁰ about the human equivalent dose conversion, we administered each component singly for 4 weeks via gavage, with the aim of identifying the cardiovascular active compound in the combination. Surprisingly, none of the individual compounds making up AkP05 was able to exert a hemodynamic effect on its own (Figure 4J).

Synergism of the Single Components Is Needed for AkP05's Hemodynamic Effect

Having seen that none of the individual components was able to modulate BP, we administered the complete nutraceutical combination orally at the human equivalent dose for 4 weeks in order to more reliably reflect the experimental condition applied to humans. Of note, SBP was significantly reduced after 4 hours from administration and continued to remain reduced for the whole observation period of 4 weeks (Figure 5A). This suggested that synergism between components is important for the hemodynamic action of AkP05. Moreover, vascular reactivity studies conducted on mesenteric arteries excised at the end of the treatment period revealed significantly increased Ach-evoked endothelial vasorelaxation (Figure 5B), a finding consistent with the improvement of endothelial function observed in humans.

The results obtained so far suggested that the vascular action of AkP05 is mediated by NO. Thus, to further corroborate our hypothesis, we administered the complete combination in eNOS-knockout mice. As expected, administration of AkP05 did not evoke any hemodynamic effect in eNOS-deficient mice (Figure 5C), strengthening the notion of an NO-dependent action for AkP05 also in vivo. Of note, mesenteric arteries excised at the end of the treatment period from the eNOS-deficient mice displayed altered vasorelaxation (Figure 5D). In addition, whereas serum nitrite was

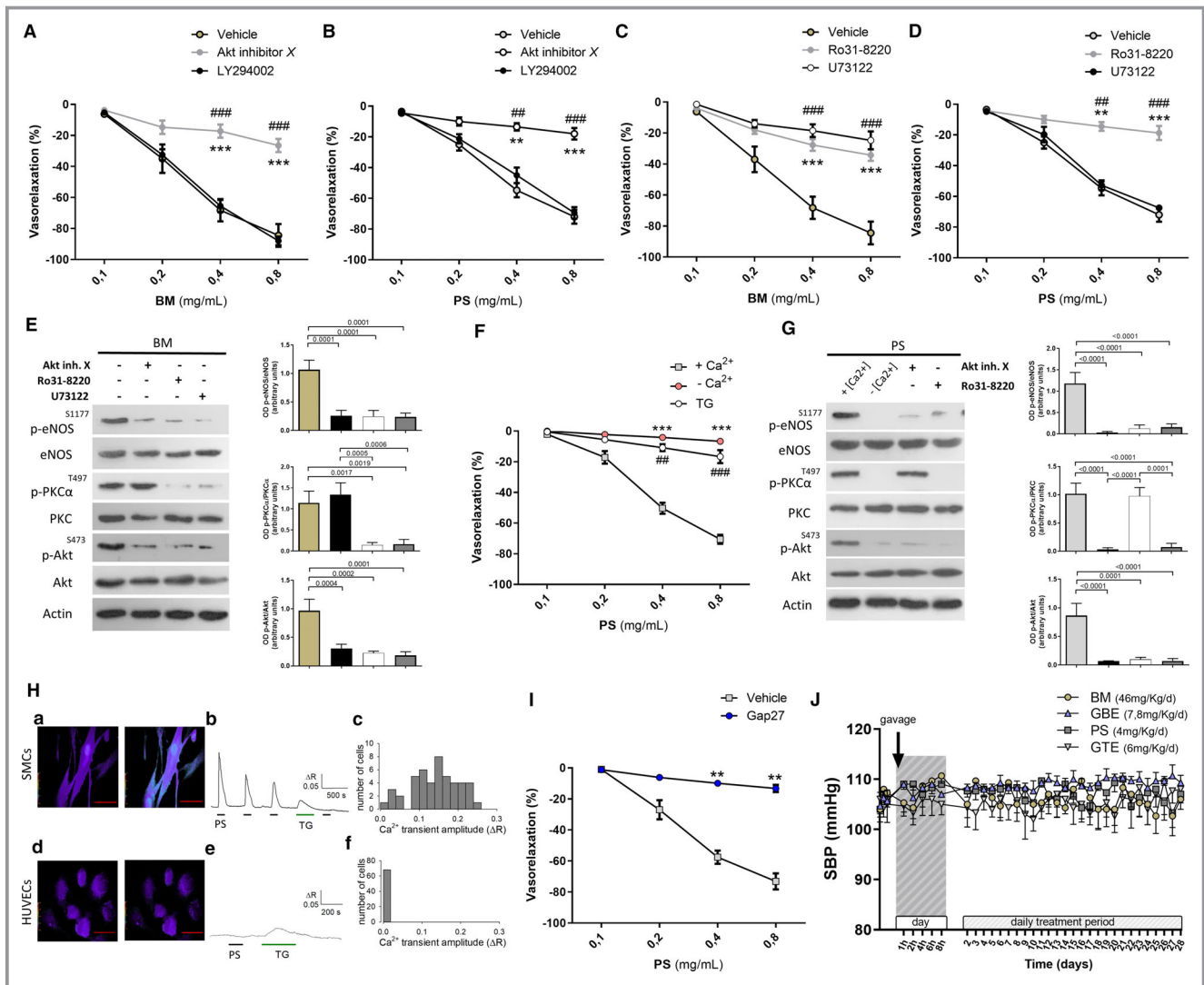


Figure 4. A through D, Dose–response curves of phenylephrine-precontracted mesenteric arteries from C57BL/6 mice to increasing doses of *Bacopa monnieri* (A and C) or phosphatidylserine (B and D) under conditions of either Akt inhibition with inhibitor X or PI3K inhibition with LY294002 (A and B), or PKC inhibition with Ro31-8220 or PLC inhibition with U73122 (C and D). ***P*<0.01, ****P*<0.01; ##*P*<0.01, ###*P*<0.001 (*n*=4 for each group). BM, *Bacopa monnieri*; PS, phosphatidylserine. E, Representative immunoblots (left) and semiquantitative densitometric analysis (right) of protein extracts from mouse mesenteric arteries exposed to BM, for phosphorylation of eNOS on serine 1177, of PKCα on tyrosine 497, and of Akt on serine 473 (3 independent experiments). F, Dose–response curves of phenylephrine-precontracted mesenteric arteries from C57BL/6 mice to increasing doses of PS in normal calcium medium (+ Ca²⁺), in the absence of calcium (– Ca²⁺), or in presence of thapsigargin (TG). ****P*<0.01 vs + Ca²⁺, ##*P*<0.01, ###*P*<0.001 vs + Ca²⁺. (*n*=4). G, Representative immunoblots (left) and semiquantitative densitometric analysis (right) of protein extracts from mouse mesenteric arteries exposed to PS, for phosphorylation of eNOS on serine 1177, of PKCα on tyrosine 497, and of Akt on serine 473 (3 independent experiments). H, Top left, Digital images of the [Ca²⁺]_i level before (left) and after (right) application of 0.4 mg/mL phosphatidylserine (PS) for 2 min to coronary artery smooth muscle cells (CASMCS) loaded with Fura2-AM. [Ca²⁺]_i is quantified as the ratio (R) between fluorescence emissions at 340 and 380 nm (acquisition at 510 nm), and represented as pseudocolors (red line=50 μm). Top middle, Typical time-course of [Ca²⁺]_i changes elicited in CASMCs by PS (horizontal black bar=2 min application) or thapsigargin (TG; 1 μmol/L) (horizontal green bar=5 min application); Top right, Histogram of PS-induced Ca²⁺ transient amplitude distribution in CASMCs (47 cells from 7 fields); Bottom left, Same as in top but performed on human umbilical vein endothelial cells (HUVECs). Please note no change in [Ca²⁺]_i following PS application; Bottom middle, Typical [Ca²⁺]_i time-courses in HUVECs during PS (horizontal black bar) or TG (horizontal green bar) application. Bottom right, Histogram of Ca²⁺ transient amplitude distribution in HUVECs, indicative of no response (68 cells from 10 fields). I, Dose–response curves of phenylephrine-precontracted mesenteric arteries from C57BL/6 mice to increasing doses of phosphatidylserine (PS) in the presence of gap-junction inhibition with GAP-27. **P*<0.05; ***P*<0.01 vs + Ca²⁺. #*P*<0.05, ##*P*<0.01 vs + Ca²⁺ (*n*=6). J, Systolic blood pressure (SBP) in wild-type mice treated with vehicle or the different single components of AKP05 (*n*=3 for each group) at different time-points from first gavage administration (day 1) and after 4 hours from continued daily administration for 4 weeks. eNOS indicates endothelial nitric oxide; PKC, protein kinase C; PLC, phospholipase C.

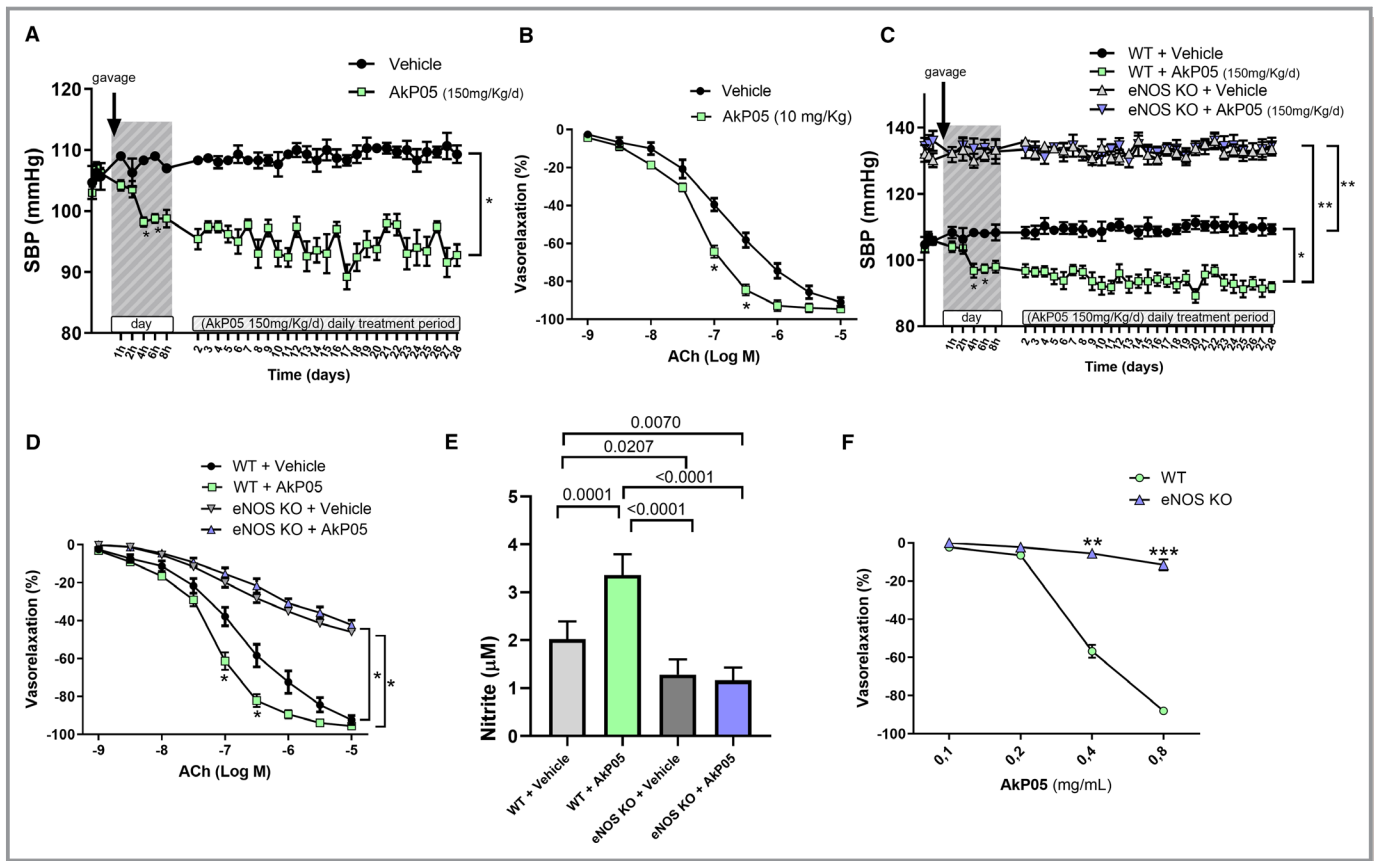


Figure 5. **A**, Time course of systolic blood pressure (SBP) measured noninvasively (**A**) in C57BL/6 mice receiving a daily oral dose of vehicle or AkP05 at different time-points from first gavage administration (day 1) and after 4 hours from continued daily administration for 4 weeks. Arrow indicates start of administration via gavage. Data are mean±SEM (n=5 AkP05; n=3 Vehicle); *P<0.05 vs all. **B**, Dose–response curves to acetylcholine (ACh; 10⁻⁹ mol/L to 10⁻⁵ mol/L) of mesenteric arteries excised from mice at the end of treatment with of vehicle or AkP05; *P<0.05 (n=5 experiments). **C**, SBP in wild-type (WT) and eNOS-knockout (KO) mice during AkP05 or vehicle administration at different time-points from first gavage administration (day 1) and after 4 hours from continued daily administration for 4 weeks. *P<0.05, **P<0.01; (n=5 for each group). **D**, Dose–response curves to acetylcholine (ACh; 10⁻⁹ mol/L to 10⁻⁵ mol/L) of mesenteric arteries obtained from wild-type and eNOS deficient mice at the end of treatment with vehicle or AkP05; *P<0.05 (n=5 independent experiments). **E**, Nitrite concentration in serum collected at the end of 4-week daily treatment in wild-type and eNOS KO mice treated with vehicle or AkP05 (n=5). **F**, Dose–response curves of ex vivo mice mesenteric arteries from eNOS-knockout mice are refractory to the vasorelaxant effect of AkP05. **P<0.01. ***P<0.001 (n=4 for each group).

significantly increased in wild-type mice treated with AkP05, such an increase was not seen in eNOS-deficient mice (Figure 5E). Finally, ex vivo vascular reactivity studies on eNOS-deficient vessels demonstrated an inability of the nutraceutical combination to evoke any direct dose-dependent vasorelaxation in this setting (Figure 5F), a finding proving that eNOS represents the main target of AkP05’s vascular action.

Discussion

Here, we demonstrate that the administration of a novel nutraceutical combination, called AkP05, to hypertensive patients on antihypertensive medication but with unsatisfactory BP control reduces BP, improves endothelial function,

and increases serum NO concentration. These effects are associated with an increase of VO₂ max uptake, stress tolerance, and maximal power output. To the best of our knowledge, this is the first study describing positive actions on the vasculature and exercise tolerance of a nutraceutical preparation in treated hypertensive patients with poor BP control.

Reduced endothelial function represents an early, subclinical stage of vascular alteration that precedes and accompanies the development of hypertension, contributing to increased cardiovascular complications.^{20,21} Downregulated NO, caused by either reduced synthesis or by inactivation because of increased oxidative stress in the hypertensive setting, significantly contributes to endothelial dysfunction and to the rise of BP.²²

Regarding exercise physiology, recent studies have reported that increasing NO production and bioavailability may enhance oxygen and nutrient delivery to tissues, improving exercise tolerance and recovery mechanisms. Based on this concept, we investigated the cardiovascular effects of AkP05 supplementation on these functional aspects, which represent clinically significant parameters defining the health status of hypertensive patients.^{23,24} Over the past decades, CPET has garnered approval for the evaluation of cardiovascular status, exercise capacity, and gas exchange in hypertensive patients, demonstrating that the alteration of these parameters are predictive factors for hypertension progression, end-organ damage, ischemic stroke, myocardial infarction, and total mortality. The good reliability and reproducibility of the test have made it a useful tool for the clinician. Higher maximal SBP and lower oxygen uptake during exercise have been considered powerful predictors of low survival rate in hypertensive patients. On this point, medicated hypertensive patients (regardless of whether disease is controlled or uncontrolled) have an exaggerated SBP increase upon maximal exercise testing,^{5,6} a finding clearly suggesting an inability of common, current antihypertensive drugs to control BP throughout the patient's daily life.

Investigation into the possible beneficial roles of nutraceutical compounds on cardiovascular diseases is increasing, and a number of reports have described the effects produced by the single main compounds making up AkP05. One study assessed the possible cardiovascular action of *Ginkgo biloba* in humans, demonstrating that it had no effect on BP in elderly prehypertensive patients,²⁵ whereas another reported that the administration of *G. biloba* for 3 months led to a significant reduction in SBP and diastolic blood pressure in prehypertensive adults aged 21 to 57 years.²⁶ Thus, *G. biloba* may have different actions in different types of patients. A similar situation has emerged from the analysis of the literature on the effects of green tea in humans, extensively collected in a review by Khalesi et al,²⁷ in which the actions of GTE consumption on BP modulation depended on the population studied and the duration of treatment. In contrast, only a single study has been performed on *Bacopa monnieri* (given at doses from 300 and 600 mg/d) in humans:²⁸ it reported that continuous administration for 12 weeks did not exert any effect on BP. In our proof-of-concept study, we investigated the cardiovascular effect on uncontrolled hypertension of a combination of these natural compounds, which has already been shown to produce beneficial effects on the cognitive performance of hypertensive patients with controlled BP.⁶ We found that the use of AkP05 as an adjunct to therapy for hypertension may significantly aid in reducing maximal SBP response during exercise and in increasing maximal VO₂ consumption, and so may contribute to

cardiovascular risk reduction. Moreover, in agreement with the functional role of NO on exercise tolerance, AkP05-treated patients had increased exercise duration and maximum power generated, a finding suggesting the effectiveness of the nutraceutical combination in inducing adaptations necessary for the improvement of the patients' health status. Of note, in the parallel group of treated hypertensive patients in which AkP05 supplementation was substituted with the administration of a diuretic, there was a significant reduction of BP but no improvement in functional parameters, proving the specificity of AkP05-evoked cardiovascular effects.

To test whether AkP05 exerts per se a direct action on vessels, we focused our attention on experimental models demonstrating both in ex vivo and in vivo studies that the effect of the new nutraceutical combination is mediated by a NO-dependent mechanism since it failed to act in a condition of eNOS deficiency. Of note, daily oral administration for 4 weeks of AkP05 significantly reduced BP during the whole observation period, improved endothelial function, and increased the circulating level of nitrite, findings corroborating what we observed in hypertensive humans. In addition, its effects depend on the combination of the different compounds contained in AkP05, which alone are unable to exert any hemodynamic effect. To clarify this issue, we characterized the vascular action of the individual components on isolated vessels, finding that BM and PS—which are widely used to treat central nervous system disorders, and that promote mental health²⁹ and cognitive performance³⁰—evoked NO release through different molecular mechanisms converging on the PKC α /Akt signaling pathway necessary for phosphorylation of eNOS on its activation site. In particular, whereas BM recruits PLC, PS modulates intracellular calcium mobilization from smooth muscle to the endothelial layer.

A further notable finding emerging from our study on the single components is the antioxidant action exerted by BM and GBE, which blocks the main machinery generating vascular oxidative stress. Specifically, they inhibited activation of Rac-1, a small GTP-binding protein necessary for the activation of NADPH oxidase. In agreement, modulation of the PKC α /Akt/eNOS and Rac-1/NADPH intracellular signaling pathways was crucial for AkP05's action on NO metabolism.

Strong correlation between the level of circulating NO and the modulation of exercise tolerance has been previously reported.³¹ Although it is generally accepted that regular physical activity stimulates NO release, which exerts beneficial effects on the vascular system and contributes to slowing down, suppressing, or even reversing cardiovascular diseases,³² it has been recently demonstrated in a small cohort of patients that assumption of nitrite supplementation for 3 weeks improves vasodilatory reserve and peak oxygen uptake in human subjects.³³ In our present proof-of-concept study, significantly increased NO levels in the serum of

hypertensive patients treated with the nutraceutical combination for 4 weeks led us to speculate that the improvements seen in exercise duration, maximal oxygen uptake, and maximal power output were because of an endogenous mechanism stimulating NO production.

The magnitude of the improvements produced by AkP05 supplementation suggests potential for the clinical management of hypertension. However, it is important to emphasize that although nutraceuticals can complement common anti-hypertensive therapy, they cannot replace it. Nonetheless, based on the efficacy revealed by our study, AkP05 might be considered as an adjunct for the treatment of hypertensive patients, improving both functional capacity and cardiovascular protection.

Study Limitations

Although we firmly believe that AkP05 has potent cardiovascular effects, it is important to emphasize that we have tested its properties only on a small population of treated hypertensive patients: it was difficult to enroll a large number of patients before assessing safety and efficacy of the combination. More studies are therefore needed to fully characterize and corroborate its efficacy in different types of cardiovascular risk patients. Second, we characterized the molecular mechanisms evoked by the single components of AkP05 without identifying the specific active compound/s (peptides or proteins) released following gastrointestinal digestion of the AkP05 tablet. Obviously, in order to improve the combination's effectiveness, more studies are needed to identify the active compounds released from each component. Our study also lacks pharmacokinetic characterization: this would have required serial blood sample collection to characterize the specific properties of AkP05, which would have been difficult to justify for this initial experimentation. Finally, this was a single-center study, which may limit generalizability to other populations. Clearly, future studies are needed to eliminate these limitations.

Perspectives

Although more studies are needed to completely characterize the cardiovascular effects of AkP05, the blood pressure-lowering effect and increased effort tolerance evoked suggest that it could be used in cardiovascular disease prevention. Moreover, the identification of a novel nutraceutical combination able to improve NO production and bioavailability in the hypertensive condition may extend the clinician's armamentarium of approaches for better control of BP.

Disclosures

None.

References

- O'Donnell CJ, Ridker PM, Glynn RJ, Berger K, Ajani U, Manson JE, Hennekens CH. Hypertension and borderline isolated systolic hypertension increase risks of cardiovascular disease and mortality in male physicians. *Circulation*. 1997;95:1132–1137.
- Brandes RP. Endothelial dysfunction and hypertension. *Hypertension*. 2014;64:924–928.
- Hermann M, Flammer A, Luscher TF. Nitric oxide in hypertension. *J Clin Hypertens (Greenwich)*. 2006;8:17–29.
- Chant B, Bakali M, Hinton T, Burchell AE, Nightingale AK, Paton JFR, Hart EC. Antihypertensive treatment fails to control blood pressure during exercise. *Hypertension*. 2018;72:102–109.
- Lim PO, MacFadyen RJ, Clarkson PB, MacDonald TM. Impaired exercise tolerance in hypertensive patients. *Ann Intern Med*. 1996;124:41–55.
- Giugliano G, Salemme A, De Longis S, Perrotta M, D'Angelosante V, Landolfi A, Izzo R, Trimarco V. Effects of a new nutraceutical combination on cognitive function in hypertensive patients. *Immun Ageing*. 2018;15:7.
- Carrizzo A, Conte GM, Sommella E, Damato A, Ambrosio M, Sala M, Scala MC, Aquino RP, De Lucia M, Madonna M, Sansone F, Ostacolo C, Capunzo M, Migliarino S, Sciarretta S, Frati G, Campiglia P, Vecchione C. Novel potent decameric peptide of spirulina platensis reduces blood pressure levels through a PI3K/AKT/eNOS-Dependent Mechanism. *Hypertension*. 2019;73:449–457.
- Cudmore MJ, Hewett PW, Ahmad S, Wang KO, Cai M, Al-Ani B, Fujisawa T, Ma B, Sissaoui S, Ramma W, Miller MR, Newby DE, Gu Y, Barleon B, Weich H, Ahmed A. The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis. *Nat Commun*. 2012;3:972.
- Carrizzo A, Ambrosio M, Damato A, Madonna M, Storto M, Capocci L, Campiglia P, Sommella E, Trimarco V, Rozza F, Izzo R, Puca AA, Vecchione C. Morus alba extract modulates blood pressure homeostasis through eNOS signalling. *Mol Nutr Food Res*. 2016;60:2304–2311.
- Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J*. 2008;22:659–661.
- Michelsen MM, Mygind ND, Pena A, Aziz A, Frestad D, Host N, Prescott E; Steering Committee of the i PS. Peripheral reactive hyperemia index and coronary microvascular function in women with no obstructive CAD: the iPOWER study. *JACC Cardiovasc Imaging*. 2016;9:411–417.
- Spinelli CC, Carrizzo A, Ferrario A, Villa F, Damato A, Ambrosio M, Madonna M, Frati G, Fucile S, Sciacaluga M, Capunzo M, Cali G, Milanese L, Maciag A, Puca AA, Vecchione C. LAV-BPIFB4 isoform modulates eNOS signalling through Ca²⁺/PKC-alpha-dependent mechanism. *Cardiovasc Res*. 2017;113:795–804.
- Yang L, Qiao G, Ying H, Zhang J, Yin F. TCR-induced Akt serine 473 phosphorylation is regulated by protein kinase C-alpha. *Biochem Biophys Res Commun*. 2010;400:16–20.
- Margaritis M, Antonopoulos AS, Digby J, Lee R, Reilly S, Coutinho P, Shirodaria C, Sayeed R, Petrou M, De Silva R, Jalilzadeh S, Demosthenous M, Bakogiannis C, Tousoulis D, Stefanadis C, Choudhury RP, Casadei B, Channon KM, Antoniadis C. Interactions between vascular wall and perivascular adipose tissue reveal novel roles for adiponectin in the regulation of endothelial nitric oxide synthase function in human vessels. *Circulation*. 2013;127:2209–2221.
- Carrizzo A, Puca A, Damato A, Marino M, Franco E, Pompeo F, Traficante A, Civitillo F, Santini L, Trimarco V, Vecchione C. Resveratrol improves vascular function in patients with hypertension and dyslipidemia by modulating NO metabolism. *Hypertension*. 2013;62:359–366.
- Forte M, Conti V, Damato A, Ambrosio M, Puca AA, Sciarretta S, Frati G, Vecchione C, Carrizzo A. Targeting nitric oxide with natural derived compounds as a therapeutic strategy in vascular diseases. *Oxid Med Cell Longev*. 2016;2016:7364138.
- Griendling KK, Ushio-Fukai M. Reactive oxygen species as mediators of angiotensin II signaling. *Regul Peptides*. 2000;91:21–27.
- Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer*. 2007;7:281–294.
- Billaud M, Marthan R, Savineau JP, Guibert C. Vascular smooth muscle modulates endothelial control of vasoreactivity via reactive oxygen species production through myoendothelial communications. *PLoS ONE*. 2009;4:e6432.
- Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation*. 2006;113:1708–1714.

21. Carrizzo A, Lenzi P, Procaccini C, Damato A, Biagioni F, Ambrosio M, Amodio G, Remondelli P, Del Giudice C, Izzo R, Malovini A, Formisano L, Gigantino V, Madonna M, Puca AA, Trimarco B, Matarese G, Fornai F, Vecchione C. Pentraxin 3 induces vascular endothelial dysfunction through a P-selectin/matrix metalloproteinase-1 pathway. *Circulation*. 2015;131:1495–1505; discussion 1505.
22. Lembo G, Iaccarino G, Vecchione C, Rendina V, Parrella L, Trimarco B. Insulin modulation of beta-adrenergic vasodilator pathway in human forearm. *Circulation*. 1996;93:1403–1410.
23. Dekleva M, Lazic JS, Pavlovic-Kleut M, Mazic S, Stevanovic A, Soldatovic I, Markovic-Nikolic N, Beleslin B. Cardiopulmonary exercise testing and its relation to oxidative stress in patients with hypertension. *Hypertens Res*. 2012;35:1145–1151.
24. Wensel R, Opitz CF, Anker SD, Winkler J, Hoffken G, Kleber FX, Sharma R, Hummel M, Hetzer R, Ewert R. Assessment of survival in patients with primary pulmonary hypertension: importance of cardiopulmonary exercise testing. *Circulation*. 2002;106:319–324.
25. Brinkley TE, Lovato JF, Arnold AM, Furberg CD, Kuller LH, Burke GL, Nahin RL, Lopez OL, Yasar S, Williamson JD; Ginkgo Evaluation of Memory Study Investigators. Effect of Ginkgo biloba on blood pressure and incidence of hypertension in elderly men and women. *Am J Hypertens*. 2010;23:528–533.
26. Kalus JS, Piotrowski AA, Fortier CR, Liu X, Kluger J, White CM. Hemodynamic and electrocardiographic effects of short-term Ginkgo biloba. *Ann Pharmacother*. 2003;37:345–349.
27. Khalesi S, Sun J, Buys N, Jamshidi A, Nikbakht-Nasrabadi E, Khosravi-Boroujeni H. Green tea catechins and blood pressure: a systematic review and meta-analysis of randomised controlled trials. *Eur J Nutr*. 2014;53:1299–1311.
28. Peth-Nui T, Wattanathorn J, Muchimapura S, Tong-Un T, Piyavhatkul N, Rangseekajee P, Ingkaninan K, Vittaya-Areekul S. Effects of 12-week bacopa monnieri consumption on attention, cognitive processing, working memory, and functions of both cholinergic and monoaminergic systems in healthy elderly volunteers. *Evid Based Complement Alternat Med*. 2012;2012:606424.
29. Kamkaew N, Scholfield CN, Ingkaninan K, Maneesai P, Parkington HC, Tare M, Chootip K. Bacopa monnieri and its constituents is hypotensive in anaesthetized rats and vasodilator in various artery types. *J Ethnopharmacol*. 2011;137:790–795.
30. Downey LA, Kean J, Nemeš F, Lau A, Poll A, Gregory R, Murray M, Rourke J, Patak B, Pase MP, Zangara A, Lomas J, Scholey A, Stough C. An acute, double-blind, placebo-controlled crossover study of 320 mg and 640 mg doses of a special extract of Bacopa monnieri (CDRI 08) on sustained cognitive performance. *Phytother Res*. 2013;27:1407–1413.
31. Tsukiyama Y, Ito T, Nagaoka K, Eguchi E, Ogino K. Effects of exercise training on nitric oxide, blood pressure and antioxidant enzymes. *J Clin Biochem Nutr*. 2017;60:180–186.
32. Nosarev AV, Smaglyi LV, Anfinogenova Y, Popov SV, Kapilevich LV. Exercise and NO production: relevance and implications in the cardiopulmonary system. *Front Cell Dev Biol*. 2014;2:73.
33. Zamani P, Tan V, Soto-Calderon H, Beraun M, Brandimarto JA, Trieu L, Varakantam S, Doulias PT, Townsend RR, Chittams J, Margulies KB, Cappola TP, Poole DC, Ischiropoulos H, Chirinos JA. Pharmacokinetics and pharmacodynamics of inorganic nitrate in heart failure with preserved ejection fraction. *Circ Res*. 2017;120:1151–1161.

Supplemental Material

Data S1.

Supplemental Materials and Methods

Study design

The study was conducted in accordance with the guidelines of the declaration of Helsinki and was approved by the Ethic Committee of CE Campania Sud, Italy, which registered the study protocol (n° 390-01587470-30081). Written informed consent was obtained from each subject. Systolic and diastolic BP were measured by standard sphygmomanometer after 5 min in the supine position, according to the guidelines of the European Society of Hypertension/European Society of Cardiology. Subjects of both sexes with diagnosis of essential hypertension were screened for the following exclusion criteria: previous cardiac or cerebrovascular event; heart failure; diabetes mellitus; history of atrial fibrillation or other severe arrhythmias; chronic kidney disease (defined as serum creatinine levels >1.4 mg/dL); pre-existing psychiatric disorders; neurodegenerative diseases such as multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, early onset/genetic Alzheimer's disease, neuromuscular pathologies, epilepsy; dementia. In addition, patients requiring any pharmacological treatment beyond anti-hypertensive drugs, pregnant women, and women planning to conceive were also excluded from the study.

We performed two different studies in humans.

The first was a randomized double blind placebo controlled study with a 4 week follow-up during which forty-five treated hypertensive patients with stable, unsatisfactory control of blood pressure were randomized to receive placebo or one pill of a nutraceutical preparation (Akp05, 1gr., Izzek®, Damor Farmaceutici, Italy) containing dry plant extract of 300 mg *Bacopa Monnieri* + 50 mg dry extract of *Ginkgo biloba* leaves + 25mg phosphatidylserine + 40 mg dry extract of green tea leaves. The company also produced the placebo, similar in appearance and organoleptic properties to the nutraceutical preparation.

Since there is no gold standard measurement system that fulfils the criteria for optimal medication-adherence monitoring, in this study, for monitoring of the medication compliance, we relied on patient self-reporting. For study drug monitoring, we delivered to each patient a container containing the number of exact capsules to be used for the entire duration of the treatment. Furthermore, they were required to return the container at the end of the treatment period.

Blood pressure, non-invasive endothelial function, as assessed reactive hyperemia index (RHI), and serum nitric oxide concentration were measured in control conditions and at the end of the follow-up period.

The second outcome was a 1:1 randomized since it represents a novel outcome of the study. For this outcome twenty-four patients who were not taking diuretics participated in a second study conducted according to a single blind active controlled protocol. They were randomized to take, in addition to their medication, either one pill of AkP05 or one pill of diuretic (Chlorothiazide, 1gr/day) and underwent 24-hour ambulatory blood pressure monitoring and a cardiopulmonary exercise test (CPET) at baseline and after 4 weeks of treatment.

Cardiopulmonary exercise test

All subjects were instructed to refrain from alcohol consumption and strenuous exercise for 12 h prior to exercise testing. Before starting the tests, the gas analyzer was calibrated using air and a gas mixture of known composition (16% O₂ and 5% CO₂) (Cosmed). Each patient was then acclimated to the

Ergo-Select 100 cycloergometer (Ergoline, Italy). ECG was performed with a QUARK CEPT ECG (Cosmed). Respiratory gas exchange data was measured continuously, and the hardware was set to “facemask” use. After recording baseline activity at 0 W for 30 s, patients underwent a pre-exercise phase for 1 min at 6 W. Subsequently, subjects started the exercise phase, using a ramp protocol with an increment of 1 W every 6 s. The maximal exercise test lasted until inability to maintain the cycling cadency or cessation due to substantial fatigue. The highest systolic BP (SBP), one metabolic equivalent (MET), and VO₂ peak reached during the exercise phase of the incremental test were considered as the maximal values. At the end of the active-exercise phase, patients continued the activity at for 1 min at 50 W, and starting from the 2nd min, power was reduced to 20 W until complete recovery of physiological parameters.

Preparation of extracts

The AkP05 powder or its single components (namely, *Bacopa monnieri* (BM), *Ginkgo biloba* leaves (GBE), extract of green tea leaves (GTE), and phosphatidylserine (PS)) was first ground with a top ball mill (Galena, Italy) to reduce particle size. Powder of each compound and of AkP05 were lyophilized to a particle size of 45 µm with an LS 13320 Tornado Dry Powder System with an Aqueous Liquid Module (Beckman Coulter). After, the powder (30 g) was extracted with 240 mL of 0.1 M sodium phosphate buffer (ratio w/v of 1:16), with 3 freeze-thaw cycles of 4 h (from -20°C to 37°C); after every cycle, the suspension was sonicated for 30 min at 550 W power and then centrifuged (× 6400 rpm for 25 min at 4°C). The supernatant was removed and the pellet retreated with a fresh solution. Finally, all supernatants were pooled and lyophilized. Approximately 15 g for each compound was obtained and diluted in Krebs buffer solution prior to use.

For analytic evaluation of the compounds contained within AkP05, 903.9 mg of the nutraceutical combination was extracted with 5 mL of a methanol:water mixture (80:20, v/v). First, the sample was vigorously mixed for 1 min on a vortex, then phytochemical extraction was accomplished by maceration in an ultrasonic bath for 1 h at 25 °C. Subsequently, samples were centrifuged for 5 min at 5000 rpm, at 25 °C. The operation was repeated twice. The supernatants were pooled, dried under reduced pressure, and dissolved in a methanol:water mixture (80:20, v/v) to obtain a concentration of 15 mg/mL, which was filtered with 0.45 µm filters and then injected.

UHPLC-PDA-MS/MS parameters

UHPLC-PDA-MS/MS analyses were performed on a Nexera UHPLC (Shimadzu, Milan, Italy) coupled to a hybrid Ion Trap-Time of Flight mass spectrometer LCMS-IT-TOF (Shimadzu, Kyoto, Japan). The separation was performed on a KinetexTM C18 150 mm × 2.1 mm, 2.6 µm column protected with a security Guard UltraTM C18 cartridge. The mobile phases were: A, 0.1 % acetic acid in water; B, acetonitrile plus 0.1 % acetic acid. The gradient was 0-15 min, 5-25 B%, 14-22 min, 25-70%B, 22-24 min, 70-95%B, hold for 0.50 min, return to the initial condition in 0.01 min. Column oven: 40°C. Injection volume: 2 µL. Flow rate: 0.5mL/min. PDA parameters were: lamp D2, sampling rate 12.5 Hz, time constant 0.160s. Chromatogram extracted at 254, 280, and 330 nm. The MS detection was operated in negative mode by an ESI source. Flow rate from the LC was split 50:50 prior the source. ESI source parameters were: interface and CDL temperatures 250°C, nebulizing and drying gas 1.5 and 9.5 L/min. Probe voltage: -3.5 kV. MS1 range: 150-1500 m/z. MS/MS was performed in data-dependent mode. Identification of compounds was performed by accurate MS and MS/MS spectra by comparison with literature and molecular formulas provided by the software Formula Predictor (Shimadzu). Quantitation of main compounds was performed by external

calibration employing 5 standards with 5 concentration levels (1, 5, 10, 15, 25 µg/mL). Three replicates of each LC run were carried out.

Experimental models

All experiments involving animals conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011) and were approved by the IRCCS INM Neuromed review board (n° 1070/2015 PR). Male C57BL/6 mice (25±0.9 g body weight) were bred in our animal facility. Male eNOS knockout mice (24±0.9 g body weight) were obtained from Charles River Laboratories. All animals were randomly divided into control and treated groups. All efforts were made to minimize the number of animals used and their suffering. Mice were fed standard chow and water ad libitum. C57BL/6 and eNOS deficient mice were 8 weeks old when used in this study. In vivo administration of AkP05 or single components was performed by gavage. Nobody animal showed adverse events or death during treatments.

Ex vivo vascular reactivity studies and staining for superoxide

Vasorelaxation was expressed as percent reduction of phenylephrine-induced contraction. To evaluate the vascular function of AkP05 or its single components, we exposed precontracted vessels to increasing doses (0.1 mg/mL to 0.8 mg/mL) of each test substance.

To characterize intracellular signaling, some myograph-mounted mesenteric arteries were pre-treated with the following before data for dose-response curves were obtained: 10 µM of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitor LY294002 (Selleckchem, Cat No.S1105) for 1 h; 2 µM of the AKT inhibitor Akt Inhibitor X (Sant Cruz Biotechnology; sc-203811) for 1 h; 300 µM of the eNOS inhibitor N ω -nitro-L-arginine methyl ester (L-NAME) (Sigma-Aldrich, N5751) for 30 min; 2.5 µM of the PLC inhibitor U73122 (Selleckchem, S8011) for 1 hour; 2 µM of the PKC inhibitor Ro31-8220 (abcam, ab120374); or with 200 µM of the selective connexin-43 and connexin-37 inhibitor Gap27 (apexbt, A1045). Vasomotor response was quantified by a second individual who was blind to the genotype of the animal and/or the hypothesis that was being tested for each group. In all vascular experiments, precontraction was obtained by exposure to increasing doses of phenylephrine (10⁻⁹ to 10⁻⁶ M) in order to obtain a similar level of pre-contraction equal to 80% of initial KCl-induced constriction.

Dihydroethidium (DHE, Life Technologies) was used to evaluate production of reactive oxygen species (ROS) in mouse mesenteric arteries, as previously described.¹ Briefly, vessels were incubated with 5 µM of DHE for 20 min and subsequently observed under a fluorescence microscope (Zeiss). Images were acquired by a digital camera system (Olympus Soft Imaging Solutions). A second, estimation of total ROS production in mouse vessels was performed with the membrane-permeable fluorescent probe 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCF/DA) (Invitrogen). After treatment, vessels were incubated with Krebs solution containing 5 µM CM-₂DCFDA for 30 min at 37°C, and then washed two times with PBS prior to fluorescence measurement using a fluorescence microplate reader (TECAN infinite 200 Pro) with 495 nm excitation and 520 nm emission.

Ex vivo vascular reactivity studies and staining for superoxide

Vascular reactivity studies were performed on second-order branches of the mesenteric artery, as previously described.² Quantification of vasomotor response and blood pressure, and molecular analyses were performed by a second individual who was blind to the genotype of the animal and/or

the hypothesis that was being tested for each group. Briefly, vessels were isolated and dissected from fat and connective tissue in ice-cold Krebs solution and gassed with 95% O₂ and 5% CO₂. Subsequently, arteries were mounted on a wire myograph in organ chambers with Krebs solution and treated with increasing concentrations of phenylephrine (10⁻⁹ to 10⁻⁶ M) in order to obtain a similar level of pre-contraction in each ring (80% of initial KCl-induced contraction). Caution was taken to avoid endothelium damage, and functional integrity of the endothelium was confirmed by the vasodilation response to acetylcholine (10⁻⁹ to 10⁻⁶ M).

Cell culture and NO measurement

Commercially available human umbilical vein endothelial cells (HUVECs) or coronary artery smooth muscle cells (CASMCs) were purchased from Lonza (Walkersville, MD, USA) and grown in EGM-2 and SmBM basal medium, respectively. Cells were used within passage five and at 70% confluence for the following sets of experiments. Total nitrite content in the supernatants of HUVEC cultures was measured in an additional experiment. Cells were grown in 100 mm dishes and supernatants collected after stimulation with acetylcholine as control (100 μM for 15 min) or with compounds as reported in figure legends. In some experiments, HUVECs were pretreated for 30 min with 300 μM L-NAME, a well-characterized eNOS inhibitor, to conform the detection of NO. The NO concentration in the supernatant was assayed as nitrite, the stable breakdown product of NO, by a chemiluminescence detector (Sievers 280i NO Analyzer), as previously described.³

Protein extraction and immunoblot analysis

For total protein extraction, mesenteric arteries were lysed in a buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.5), 2 mmol/L EDTA, 1% v/v NP-40, 0.5 % w/v deoxycholate, 10 mmol/L NaF, 10 mM sodium pyrophosphate, 2 mmol/L PMSF, 2 g/ml leupeptin, and 2 g/ml aprotinin (pH 7.4). Lysates were incubated on ice for 15 min and then centrifuged at 38000 x g for 30 min at 4 °C to collect the supernatant. Protein concentration was measured using a dye-binding protein assay kit (Bio-Rad) and read at the spectrophotometer at a wavelength of 595 nm. Immunoblotting was performed as previously described, using the following antibodies: mouse anti-AKT (Santa Cruz, sc-55523; 1:1000); rabbit polyclonal anti-phospho-AKT serine 473 (Cell Signaling, 9271; 1:1000); mouse monoclonal anti-β-actin (Santa Cruz, sc-47778 (C4); 1:2000); mouse monoclonal anti-phospho-eNOS serine 1177 (Enzo Life Sciences, ALX-804-396-C100; 1:600); rabbit monoclonal anti-eNOS (Cell Signaling, 9570; 1:800); mouse monoclonal anti-PKC antibody [M110] (abcam, 23511); and Anti-PKC alpha (phospho T497) antibody (ab76016). Secondary antibodies (1:3000) were purchased from Amersham Life Sciences (GE Healthcare). Bands were visualized with enhanced chemiluminescence (ECL, Amersham Life Sciences), according to the manufacturer's instructions. Immunoblotting data were analyzed using ImageJ software (developed by Wayne Rasband, NIH, USA) to determine density of the bands.

Rac1-GTP pull-down assay

Pooled mouse mesenteric arteries treated with different compounds (Akp05 or single components) at the concentration of 0.4 mg/mL and stimulated with angiotensin II (10⁻⁵ M) for 1 hour, were lysed in a buffer containing NP-40 equipped with the kit. P21-binding domain (PBD) of p21-activated protein kinase (PAK) bound to agarose beads was added, and active Rac1, binding PAK1, was separated by repetitive centrifugation and washing. After, the specimens were boiled in Laemmli buffer, subjected

to SDS-PAGE, and Rac was quantified by Western blot analysis. In detail, Rac1-GTP was detected with the monoclonal antibodies anti-Rac1-GTP γ (1:800; STA-401-1, Cells Biolab Inc.), and total Rac1 with monoclonal anti-Rac1 (1:1000; Abcam). Immunoblotting data was analyzed using ImageJ software to determine density of the bands. The amount of Rac1-GTP was normalized to the total amount of Rac1.

Protein extraction and immunoblot analysis

For total protein extraction, mesenteric arteries were lysed in a buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.5), 2 mmol/L EDTA, 1% v/v NP-40, 0.5 % w/v deoxycholate, 10 mmol/L NaF, 10 mM sodium pyrophosphate, 2 mmol/L PMSF, 2 g/ml leupeptin, and 2 g/ml aprotinin (pH 7.4). Lysates were incubated on ice for 15 min and then centrifuged at 38000 x g for 30 min at 4 °C to collect the supernatant. Protein concentration was measured using a dye-binding protein assay kit (Bio-Rad) and read at the spectrophotometer at a wavelength of 595 nm.

NADPH oxidase activity

NADPH oxidase activity in mouse mesenteric arteries was measured in control (untreated) vessels and in vessels treated with angiotensin II (10^{-6} M), or angiotensin II plus different compounds pre-incubated for 1 h at the dose of 0.4 mg/mL. Vessels were placed in chilled, modified Krebs/HEPES buffer containing (in mmol/L) NaCl, 99.01; KCl, 4.69; CaCl₂, 1.87; MgSO₄, 1.20; K₂HPO₄, 1.03; NaHCO₃, 25.0; Na HEPES, 20.0; and glucose, 11.1, pH 7.4. Periadventitial tissue was carefully removed, and the vessels were repeatedly washed to remove adherent blood cells. Vessel homogenate was prepared in 50 mmol/L of phosphate buffer containing 0.01 mmol/L EDTA. The homogenate was then subjected to low-speed centrifugation (1000 g) for 10 min to remove unbroken cells and debris. Supernatants (40 μ L) were added to glass scintillation vials containing 5 μ mol/L lucigenin in 1 mL of phosphate buffer. The chemiluminescence that occurred over the ensuing 5 min in response to addition of 100 μ mol/L NADPH was recorded (LS6500 Multipurpose Scintillation Counter; Beckman Coulter, Fullerton, CA, USA). In preliminary experiments, homogenates alone, without addition of NADPH, gave only minimal signals, and NADPH did not evoke lucigenin chemiluminescence in the absence of homogenate.

Data S2.

Supplemental Results

Chemical characterization of the nutraceutical compound

The resulting MS data (**Table S5 & Figure S1**) highlighted the presence of compounds belonging to the three matrices. In particular, catechins and procyanidin derivatives, typical of green tea, were eluted in the first part of the LC run; glycosylated flavonoids and ginkgolids, typical of *G. Biloba*, in the middle; and saponins characteristic of the *Bacopa* extract were the last compounds to be eluted. Phosphatidyl serine was not detected, probably under the LOQ of the MS method. The most abundant compounds were apigenin and kaempferol aglycones.

Quantitation of main compounds was performed by external calibration employing 5 standards (**Table S6**) with 5 concentration levels (1, 5, 10, 15, 25 µg/mL), three replicates of each LC run was carried out.

Table S1. Baseline characteristics of hypertensive patients of first study.

Variable	Placebo group (n=15)	AkP05 group (n=30)	<i>p</i>
Clinical characteristics			
Age (yrs)	57.1±6.4	57.5±9.5	0.8643
Males, n (%)	12 (80)	21 (70)	NS
Height (cm)	169.3±9.7	169±7.6	>0.9999
Weight (kg)	74.8±11.4	79.6±15.6	0.3000
Body mass index (kg/m ²)	26.11±3.3	27.7±4.1	0.2154
SBP (mmHg)	130.5±16.9	133.5±12.5	0.5094
DBP (mmHg)	86.60±9.6	85.1±10.4	0.6429
Heart rate (bpm)	68.87±7.1	67.2±8.1	0.5126
Laboratory characteristics			
Total cholesterol (mg/dl)	201.6±24.2	199.8±23.8	0.8133
HDL cholesterol (mg/dl)	48.0±8.5	49.3±9.4	0.6559
LDL cholesterol (mg/dl)	132.6±26.9	127.1±24.6	0.4978
Triglycerides (mg/dl)	115.9±42.7	122.5±36.9	0.5965
Uric acid (mg/dl)	5.6±1.0	5.4±0.8	0.5452
Serum creatinine (mg/dl)	0.94±0.14	0.98±0.16	0.3112
Glycemia (mg/dl)	96.9±11.8	93.7±9.0	0.3228
Medication, n (%)			
ACE-inhibitors	2 (13.3)	4 (13.3)	
AT II receptor blockers	7 (46.6)	17 (56.6)	
Beta-blockers	3 (20)	7 (23.3)	
Calcium antagonists	4 (26.6)	9 (30)	
Diuretics	5 (33.3)	10 (33.3)	

Data are mean±SD. ACE, angiotensin-converting enzyme; AT II, angiotensin II; SBP, systolic blood pressure; DBP, diastolic blood pressure; NS, not statistically significant. Statistical analysis has been performed using unpaired t-test (two-tails).

Table S2. Pharmacological therapies of patients.

Medications	Placebo group (n=15)	AkP05 group (n=30)
ACE-inhibitors	2	2
Zofenopril		
AT II receptor blockers		
Irbesartan 150mg	1	2
Lortaan 50	0	1
Olpress 20	2	3
Ratacand 16	1	0
Beta-blockers		
Dilatrend 25	3	2
Lobivon 5	0	2
Calcium antagonists		
Amlodipina 10	0	2
Norvasc 5	2	2
Diuretics		
Moduretic	2	1
Esidrex	2	0
Diuretics + ACE-inhibitors		
VASORETIC	0	2
AT II receptor blockers + Calcium antagonists		
GIANT 40/5	1	1
PRITOR 80; Amlodipina 10	0	1
Diuretics + AT II receptor blockers		
Olmegan 40/12.5	0	3
Coaprovel 300/12.5	1	1
Corixil 160/12.5	0	1
AT II receptor blockers + Beta-blockers		
OLPRESS 40; Dilatrend 25 ½ cp	2	0
Beta-blockers + Calcium antagonists		
Norvasc 5 ½ cp; Lobivon 5	0	2
AT II receptor blockers + Beta-blockers + Calcium antagonists		
BIVIS 40/10; Dilatrend 25 ½ cp	1	2
Diuretics + AT II receptor blockers + Beta-blockers		
Olmegan 40/12.5; Lobivon 5	0	1
Diuretics + AT II receptor blockers + Calcium antagonists		
Plaunazide 20/25; Norvasc 5 ½ cp	0	1

Table S3. Statistical analysis of hypertensives that achieve target blood pressure in placebo and active treated groups.

	PLACEBO N=15 (%)	AKP05 N=30 (%)	<i>P</i>
Target BP <130/80 mmHg (overall)	1 (6.66)	16 (53.3)	0.002
Target BP <130/80 mmHg (male)	1 (8.33)	10 (47.6)	0.021
Target BP <130/80 mmHg (female)	0	6 (66.6)	0.045

Data are presented as absolute number and percentage (%) of positive patients referred to each sample's population. Statistical analysis was performed using Z Score Calculator for 2 Population Proportions.

Table S4. Baseline characteristics of patients undergoing cardiopulmonary exercise test.

Variable	Supplementation with AkP05 (n=12)	Supplementation with a diuretic (n=12)
Age (yrs)	54.1±7.3	53.7±6.7
Males (%)	6 (50)	6 (50)
Height (cm)	173.8±7.4	170.7±5.6
Weight (kg)	84.3±15.2	84.0±15.3
Body mass index (kg/m ²)	26.8±6.4	27.1±6.0
Medication, n (%)		
ACE inhibitors	2 (16.6)	1 (8.33)
AT II receptor blockers	4 (33.3)	3 (25)
Beta-blockers	3 (25)	4 (33.3)
Calcium antagonists	3 (25)	4 (33.3)

Data are mean±SD. ACE, angiotensin-converting enzyme; AT II, angiotensin II.

Table S5. UHPLC-PDA-MS/MS characterization of AkP05.

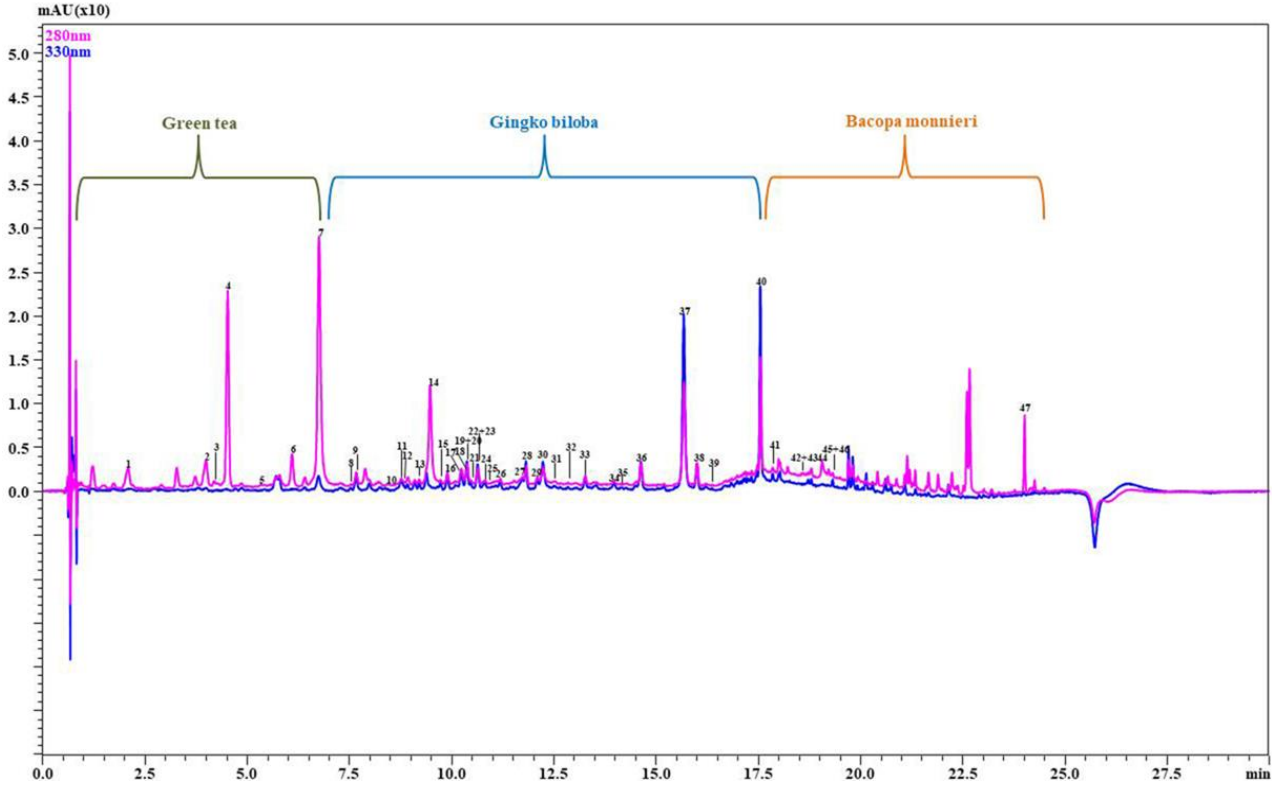
Peak	t _r	Compound	[M-H] ⁻	[MS/MS]	Error (ppm)	Molecular Formula
1	2.11	(-) Epigallocatechin	305.0635	219.0298 125.0421	-10.42	C ₁₅ H ₁₄ O ₇
2	4.00	Gallocatechin	305.0637	219.0695 125.0042 179.0259 2210374	-9.83	C ₁₅ H ₁₄ O ₇
3	4.20	Catechin	289.0688	211.3420 245.0626 161.0527	-6.92	C ₁₅ H ₁₄ O ₆
4	4.54	Unknown	451.1254	281.0680 453.1331 433.1206	1.77	C ₂₁ H ₂₄ O ₁₁
5	5.74	Procyanidin B1	577.1348	289.0643	-2.53	C ₂₇ H ₃₀ O ₁₅
6	6.11	(-) Epicatechin	289.0698	245.0844 161.0427	-6.92	C ₁₅ H ₁₄ O ₆
7	6.75	Epigallocatechin-3-gallate	457.0781	169.0117 193.0212 305.0606	1.09	C ₂₂ H ₁₈ O ₁₁
8	7.50	Kaempferol-3-O-rutinoside	593.1497	285.0377	-2.53	C ₂₇ H ₃₀ O ₁₅
9	7.65	Yinxingestin	491.1224	329.0854	5.90	C ₂₃ H ₂₄ O ₁₂
10	8.49	Ginkgolide C	439.1254	383.1312 259.1273	0.01	C ₂₀ H ₂₄ O ₁₁
11	8.75	Myricetin-3-O-rutinoside	625.1426	316.0179	2.56	C ₂₇ H ₃₀ O ₁₇
12	8.76	Myricetin-3-O-glucoside	479.0829	316.0224		C ₂₁ H ₂₀ O ₁₃
13	9.25	Quercetin-3-O-2'',6''-dirhamnosylglucoside	755.2128	300.0226 757.2132	11.65	C ₃₃ H ₄₀ O ₂₀
14	9.49	Unknown	441.0836	289.0677 169.0081	4.99	C ₂₂ H ₁₈ O ₁₀
15	9.75	Apigenin-7-O-glucoside	431.1006	311.0544 283.0611	5.10	C ₂₁ H ₂₀ O ₁₀
16	9.95	Quercetin-3-O-rhamnosylhexoside-7-O-glucoside	771.2056	301.0306	1.04	C ₂₆ H ₄₄ O ₂₆
17	10.25	Kaempferol-3-O-2'',6''-dirhamnosylglucoside	739.2138	284.0349 255.0276	6.36	C ₃₃ H ₄₀ O ₁₉
18	10.28	Kaempferol-3-O-rhamnosylhexoside-7-O-glucoside	755.2080	285.0292	5.60	C ₃₃ H ₄₀ O ₂₀
19	10.39	Quercetin-3-O-galactoside	463.0848	301.0309		C ₂₁ H ₂₀ O ₁₂
20	10.40	Rutin	609.1461	301.0326	0.01	C ₂₇ H ₃₀ O ₁₆
21	10.41	Quercetin-3-O-glucoside	463.0868	301.0324	-3.02	C ₂₁ H ₂₀ O ₁₂
22	10.49	Isorhamnetin-3-O-2'',6''-dirhamnosylglucoside	769.2234	314.0357 299.0338	4.78	C ₃₄ H ₄₂ O ₂₀
23	10.66	Kaempferol-3-O-hexoside	447.0938	285.0365	1.12	C ₂₁ H ₂₀ O ₁₁
24	10.75	Patuletin-3-O-rutinoside	639.1600	331.1402	5.16	C ₂₈ H ₃₂ O ₁₇

				330.0459 271.0189		
25	10.81	Patuletin-7- O - glucoside	493.1049	330.0375 343.0946	5.15	C ₂₂ H ₂₂ O ₁₃
26	11.20	Kaempferol-3-O-2''- glucosyl-6'- rhamnosylglucoside	755.2079	285.0346 593.1459	5.16	C ₃₃ H ₄₀ O ₂₀
27	11.75	Quercetin-3-O-2''- glucosylrhamnoside	609.1463	300.0239 271.0217 255.0223	6.89	C ₂₇ H ₃₀ O ₁₆
28	11.84	Kaempferol-3-O- neohesperidoside	593.1523	285.0391 255.0296 129.5690	2.02	C ₂₇ H ₃₀ O ₁₅
29	12.13	Kaempferol-3-O-hexoside	447.0924	285.0378	-2.01	C ₂₁ H ₂₀ O ₁₁
30	12.29	Isorhamnetin-3-O- dihexoside	623.1588	315.0467 300.0259	-4.81	C ₂₈ H ₃₂ O ₁₆
31	12.50	Syringetin-3-O-2''- glucosylrhamnoside	653.1710	345.0595	-1.99	C ₂₉ H ₃₄ O ₁₇
32	12.87	Ginkgolide J	423.1291	367.1366 261.1321 395.1071	1.18	C ₂₀ H ₂₄ O ₁₀
33	13.29	Kaempferol-3-O- glucosylrhamnoside	593.1492	284.0250	-1.01	C ₂₇ H ₃₀ O ₁₅
34	13.37	Isorhamnetin-7-O-glucoside	477.1034	314.9209	4.92	C ₂₂ H ₂₂ O ₁₂
35	14.00	Unknown	445.0769	269.0405 436.7879 356.9776	11.46	C ₂₈ H ₁₄ O ₆
36	14.65	Quercetin-3-O-p- coumaroyldiglucoxyde	755.1893	609.1425	0.66	C ₃₆ H ₃₆ O ₁₉
37	15.70	Kaempferol	285.0391	287.0569 229.0408	-4.21	C ₁₅ H ₁₀ O ₆
38	16.02	Kaempferol-p- coumaroyldiglycoside	739.1965	593.1451	11.50	C ₃₆ H ₃₆ O ₁₇
39	16.25	Quercetin-p- coumaroyldiglucoxyde	755.1879	609.1358 300.0234	3.89	C ₃₆ H ₃₆ O ₁₉
40	17.50	Apigenin	269.0432	271.0409 145.0323 225.0450	-8.55	C ₁₅ H ₁₀ O ₅
41	17.88	Bacoside A ₃	973.5042	913.4650 791.3708	6.21	C ₄₇ H ₇₆ O ₁₈
42	18.76	p-glc-Glu-ara	987.5243	941.4830	7.33	C ₄₇ H ₇₄ O ₁₉
43	18.80	Bacopaside II	927.5036	633.3889 795.4484	8.30	C ₄₇ H ₇₆ O ₁₈
44	19.10	Deoxybacopaside I	959.4397	827.3751	5.79	C ₄₆ H ₇₂ O ₁₉ S
45	19.27	Bacopaside A oxy-p-2- glucosyl-malonylpentoside	369.1192	177.0226 371.1151 139.1229	5.20	C ₁₄ H ₂₆ O ₉ S
46	19.28	Bacopaside N1	795.4608	633.3852	9.05	C ₄₂ H ₆₈ O ₁₄
47	24.00	Bacopaside I	977.4548	845.3959	12.99	C ₄₆ H ₇₄ O ₂₀ S

Table S6. Quantification of the most abundant peaks.

Compound	R²	µg/mg	CV%
Apigenin	0.996	0.14 ± 0.01	2%
Kaempferol	0.999	0.25 ± 0.01	1%
Rutin	0.999	0.09 ± 0.01	3%
Kaempferol-3-<i>O</i>-glucoside	0.999	0.05 ± 0.01	3%
(-) Epicatechin	0.999	0.06 ± 0.03	3%

Figure S1. AKP05 profile with UHPLC-PDA-MS/MS peak assignment.



Supplemental References:

1. Schiattarella GG, Carrizzo A, Iardi F, Damato A, Ambrosio M, Madonna M, Trimarco V, Marino M, De Angelis E, Settembrini S, Perrino C, Trimarco B, Esposito G and Vecchione C. Rac1 Modulates Endothelial Function and Platelet Aggregation in Diabetes Mellitus. *J Am Heart Assoc.* 2018;7. doi: 10.1161/JAHA.117.007322.
2. Carrizzo A, Conte GM, Sommella E, Damato A, Ambrosio M, Sala M, Scala MC, Aquino RP, De Lucia M, Madonna M, Sansone F, Ostacolo C, Capunzo M, Migliarino S, Sciarretta S, Frati G, Campiglia P and Vecchione C. Novel Potent Decameric Peptide of *Spirulina platensis* Reduces Blood Pressure Levels Through a PI3K/AKT/eNOS-Dependent Mechanism. *Hypertension.* 2019;73:449-457.
3. Cudmore MJ, Hewett PW, Ahmad S, Wang KQ, Cai M, Al-Ani B, Fujisawa T, Ma B, Sissaoui S, Ramma W, Miller MR, Newby DE, Gu Y, Barleon B, Weich H and Ahmed A. The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis. *Nat Commun.* 2012;3:972.