Novel Potent Decameric Peptide of *Spirulina platensis* Reduces Blood Pressure Levels Through a PI3K/AKT/eNOS-Dependent Mechanism

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See Editorial Commentary, pp 291–293

Abstract—Considered as a superfood of the future, *Spirulina platensis* matrix has been extensively used because of its beneficial effect on the management of cardiovascular diseases. However, its nutraceutical properties, bioactive compounds, and molecular mechanisms are unknown. Here, we demonstrate that *S platensis* matrix processed in vitro by simulated gastrointestinal digestion induces direct endothelial nitric oxide (NO)-mediated vasorelaxation of resistance vessels in mice. To gain insight into the bioactive compounds responsible for this effect, we used a complex multistep peptidomic approach to fractionate the crude digest: of the 5 peptide fractions identified (A–E), only fraction E evoked vasorelaxation. High-resolution mass spectrometry–based screening revealed in E the presence of 4 main peptides (SP3–SP6 [spirulina peptides]), of which only SP6 (GIVAGDVTPI) exerted direct endothelium-dependent vasodilation of ex vivo vessels, an effect occurring via a PI3K (phosphoinositide-3-kinase)/AKT (serine/threonine kinase Akt) pathway converging on NO release. In vivo, administration of SP6 evoked a significant hemodynamic effect, reducing blood pressure, an action absent in eNOS (endothelial NO synthase)-deficient mice. Of note, although lower doses of SP6 had no hemodynamic effects, it still enhanced endothelial NO vasorelaxation. Finally, in an experimental model of arterial hypertension, SP6 exerted an antihypertensive effect, improving endothelial vasorelaxation associated with enhanced serum nitrite levels. Based on our results, this novel decameric peptide may extend the possible fields of application for spirulina-derived peptides and could be developed into a promising nonpharmacological approach for the containment of pathologies associated with vascular NO misregulation. **(*Hypertension*. 2019;73:449-457. DOI: 10.1161/HYPERTENSIONAHA.118.11801.)** ● **Online Data Supplement**

Key Words: blood pressure ■ endothelium ■ peptides ■ spirulina ■ vasodilation

The pharmacological properties of natural compounds have garnered increasing attention in the field of alternative and coadjuvant therapeutic approaches to cardiovascular diseases.[1] Currently, the food industry is focused on bioactive molecules contained in foods or natural matrices, which, besides their nutritional value, can bring health benefits, especially in the treatment of chronic diseases.[2] Moreover, these compounds are characterized by fewer side-effects in comparison with pharmacological therapies, so consumers tend to prefer their use for health promotion.

The dried biomass of the cyanobacterium *Spirulina platensis*, known as spirulina, is rich in bioactive compounds.[3] such as essential amino acids, carotenoids, vitamins, polyunsaturated fatty acids, and protein,[4] and is considered a superfood of the future. Interestingly, spirulina has been reported to exert biological activities and have beneficial properties in the management of cardiovascular diseases.[5] Thus, the identification of novel bioactive peptides from this natural source could be useful in developing alternative therapeutic tools for reducing, or even preventing, cardiovascular diseases. On this point, simulated

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human gastrointestinal digestion (GID) of natural matrices represents the best method to obtain functional, readily absorbed bioactive peptides from larger parental proteins.[6]

Here, we demonstrate for the first time that GID-spirulina evokes dose-dependent vasorelaxation of ex vivo mouse resistance vessels: the mechanism identified was a PI3K/AKT pathway-dependent one that converged on nitric oxide (NO) release. Peptidomic analysis of GID-spirulina identified a decameric peptide, herein denominated SP6 (spirulina peptide 6), whose sequence (GIVAGDVTPI) has never been reported. In vivo administration of SP6 reduced blood pressure, improved endothelial vasorelaxation, and exerted an antihypertensive action in experimental models of hypertension, working through a NO-dependent mechanism. The identification of this novel compound extends the scope of applications of spirulina-derived peptides, laying the foundation for the development of promising nonpharmacological products aimed at containing vascular dysfunction associated with misregulation of NO, such as arterial hypertension.

Methods

The data that support the findings of this study are available from the corresponding authors on reasonable request. For detailed methodologies, please see the online-only Data Supplement. Simulated GID of spirulina was conducted in vitro following Pepe et al.[7] The peptides in the digest were identified by Orbitrap-mass spectrometry. Subsequently, the crude digest was fractionated and the main peptides identified by UHPLC-MS/MS (ultra-high performance liquid chromatography–tandem mass spectrometry) and synthesized through F-moc protocol. Vascular reactivity studies were performed with a myograph-based technique on second-order branches of the mesenteric arterial tree removed from animals. Vascular precontraction was performed administering increasing doses of phenylephrine (10^{-9}–10^{-6} M) to obtain a similar level of precontraction equal to 80% of initial KCl-induced contraction. Blood pressure was measured using noninvasive tail-cuff and invasive in vivo methods. Some vessels obtained for vascular studies were rapidly subjected to protein extraction for molecular studies evaluating total and phosphorylated eNOS (endothelial NO synthase), PI3K, and AKT. Another set of vessels was used to measure in-situ NO levels by the DAF-FM (4-amino-5-methylamino-2',7-difluorofluorescein diacetate) technique. Blood samples were used to measure nitrite levels on a 280i Nitric Oxide Analyzer (Sievers Instruments) and circulating levels of SP6 by UHPLC-MS/MS.

Statistical Analysis

All data are presented as mean±SEM. For continuous variables, we used a *t* test to compare 2 independent groups. When >2 independent groups were compared, we used 1-way ANOVA followed by Bonferroni post hoc test. To analyze the effects of our treatments on endothelium-dependent vasorelaxation in response to increasing doses of acetylcholine or SP6, we performed a 2-way repeated-measures ANOVA with Bonferroni post hoc test for multiple comparisons. A P value of <0.05 was considered statistically significant. All statistical analyses were conducted with Prism statistical software (GraphPad, La Jolla, CA).

Results

GID-Spirulina Evokes Dose-Dependent Vasorelaxation Through NO Release

Bioactive peptides usually arise from enzymatic hydrolysis of their parent proteins. Indeed, in the gastrointestinal tract, enzymes digest food proteins to release hundreds of peptides that can act either locally or after being absorbed by the body.[7] To mimic the physiological digestion process, we performed in vitro GID of spirulina (Figure 1A). Because the resulting digest was extremely complex, we employed Orbitrap-based mass spectrometry to identify peptide sequences (Figure S1 in the online-only Data Supplement). A total of 97 peptides were identified belonging to phycocyanin (α and β chains) and to allophycocyanin (α and β chains; Table S1); molecular weights spanned from 487 Da (IAGID) to 2084 Da (AIVNDPAGITPGDCSALASEIA).

To evaluate whether GID-spirulina exerted any effect on vascular function, we exposed ex vivo phenylephrine-preconstricted mouse mesenteric arteries—a prototype of resistance vessel involved in the modulation of blood pressure—to increasing doses (2–200 µg/mL) of GID-spirulina. GID-spirulina evoked vasorelaxation in a dose-dependent manner, an effect completely abolished by the prior exposure of vessels to N(ω)-nitro-L-arginine methyl ester (L-NAME), a well-characterized inhibitor of eNOS (Figure 1B). Of note, GID-spirulina did not evoke vasorelaxation of vessels that had the endothelium mechanically removed, suggesting that endothelial cells are the cellular target (Figure 1C). To confirm that eNOS was necessary for GID-spirulina's effect, we repeated the experiments with mesenteric arteries from eNOS-knockout mice. Coherently, GID-spirulina failed to induce any dose-dependent vasorelaxation of these vessels (Figure 1D).

GID-Spirulina-Evoked Vasorelaxation is Mediated Through the PI3K/AKT Pathway

To dissect the molecular mechanism involved in GID-spirulina's action, we performed another series of experiments using Compound C, LY294002, or AKT Inhibitor X, pharmacological inhibitors of AMPK, PI3K, and AKT, respectively, 3 important intracellular molecules involved in NO regulation. GID-spirulina was still able to induce dose-dependent vasorelaxation in the presence of Compound C (Figure 2A), but not in the presence of LY294002 or AKT Inhibitor (Figure 2B and 2C), clearly demonstrating that the PI3K/AKT pathway is recruited by spirulina. At the molecular level, GID-spirulina–induced the phosphorylation of PI3K at tyrosine 458 and of AKT at serine 473, 2 clear activation markers of these kinases that drive eNOS activation by phosphorylation of the enzyme at serine 1177. Of note, inhibition of either PI3K or AKT blocked phosphorylation of eNOS at serine 1177; moreover, when AKT was inhibited, PI3K was still activated (Figure 2D), an observation suggesting that PI3K is the upstream modulator.

Fractionation of Crude Spirulina Digest and Vascular Evaluation of the Peptide Fractions

In vitro simulated GID employs endogenous peptidases, such as pepsin and trypsin, to mimic the physiological process. This leads to the hydrolysis of parent proteins into hundreds of peptides, resulting in an extremely complex crude digest. Thus, we fractionated the crude hydrolysate on the basis of hydrophobicity by preparative liquid chromatography. Five different fractions (A–E) were obtained (Figure 3A): fraction A was discarded because of the absence of peptides, leaving fractions B to E to be screened for bioactivity.

Of the 4 fractions studied, only E was capable of evoking vasorelaxation of vessels ex vivo, an activity that occurred in a dose-dependent manner (Figure 3B); this effect was abolished

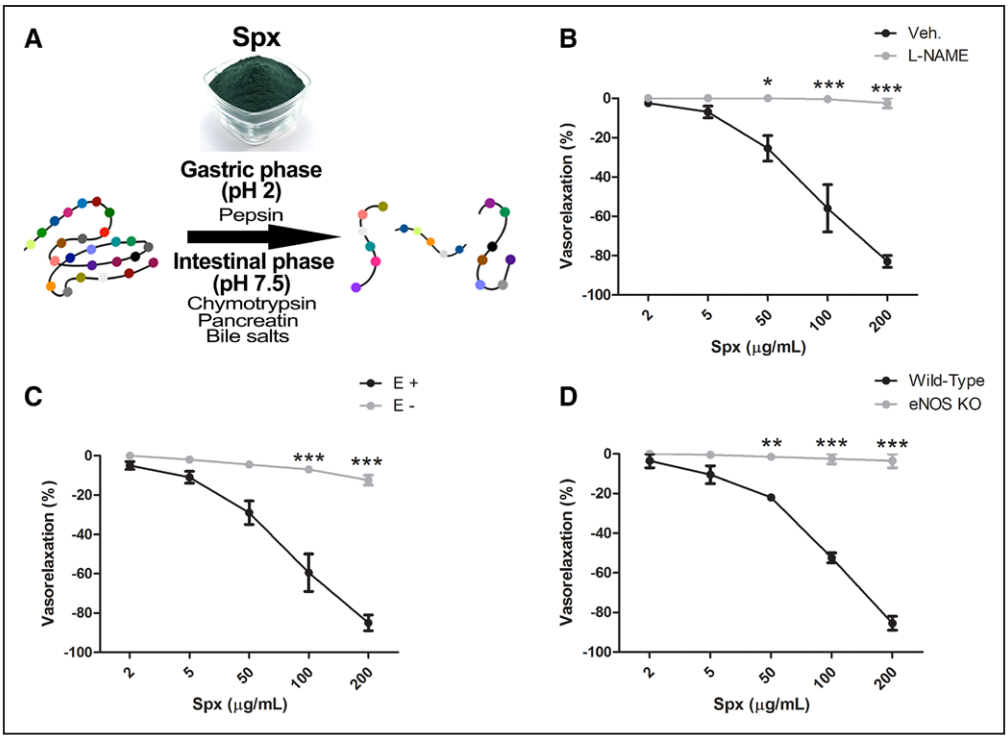

Figure 1. Spirulina evokes a dose-dependent vasorelaxation effect through nitric oxide. **A**, Workflow of in vitro simulated gastrointestinal digestion (GID). **B**, Vascular response of phenylephrine-precontracted mouse mesenteric arteries to increasing doses (2–200 μg/mL) of GID-spirulina (Spx) in the presence (E+) or absence of endothelium (E-). **C**, Inhibition of eNOS (endothelial NO synthase) with N(ω)-nitro-L-arginine methyl ester (L-NAME) abolishes the effect of Spx. **D**, Mesenteric arteries from eNOS-knockout mice (eNOS KO) are refractive to the effects of Spx. Data are mean±SEM of n=7 independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001. Veh indicates vehicle.

by pretreatment with L-NAME (Figure 3C). We then tested for hemodynamic effects in vivo, administrating a single oral dose (10 mg/kg) of each fraction separately to normotensive mice. Interestingly, E reduced systolic blood pressure significantly between 4 and 8 hours postadministration, whereas the other fractions had no effect (Figure 3D, left). Vascular reactivity studies were then performed on mesenteric arteries excised from mice 4 hours after fractions were administered, a time when blood pressure was maximally reduced. Only vessels harvested from mice administered fraction E had a significantly improved vasorelaxation response to acetylcholine, candidating it as the most vascular bioactive fraction (Figure 3D, right). The administration of lower doses of E (5 and 2.5 mg/kg) to mice was not sufficient to modulate blood pressure significantly (Figure 3E, left), although they were still associated with a significantly improved vascular function in harvested mesenteric arteries (Figure 3E, right). In line with all prior findings that the effect seen on the vasculature was dependent on intact NO metabolism, administration of E did not reduce blood pressure in eNOS-knockout mice (Figure 3F).

Bioactive Peptides in Fraction E of Spirulina

To identify the peptide/peptides responsible for the vascular activity of GID-spirulina, fraction E was profiled by UHPLC-UV-MS/MS. We found 4 abundant spirulina peptides—termed SP3, SP4, SP5, SP6—having a percent UV area significantly higher than those of the other peaks (Figure 4A). Sequence analysis identified the source proteins from which they were derived: C-phycocyanin α-chain (YNKFPY, f:60–65, 830.3962) for SP3; allophycocyanin β-chain (FATGEL, f:31–35, 636.3119) for SP4; allophycocyanin β-chain (NSLGVPI, f:117–123, 698.4026) for SP5; and allophycocyanin α-chain (GIVAGDVTPI, f:95–104, 940.5229) for SP6 (Figure 4B). Tandem mass spectra are reported in Figure S3.

Interestingly, SP6 was detectable in the serum of mice treated with 10 mg/kg of fraction E, a finding indicating that the native form of this peptide is resistant to hydrolysis in the circulation, allowing it to remain bioavailable (Figure 4C and Figure S2).

Characterization of the Vascular and Molecular Action of Bioactive Spirulina Peptides

The 4 peptides were then synthesized to obtain sufficient pure compounds for the testing of their vascular effects. When used on ex vivo mouse mesenteric arteries, only SP5 and SP6 induced significant dose-dependent vasorelaxation, with the latter developing the stronger effect (maximal relaxation: SP5, ≈32%; SP6, ≈68%; Figure 4D).

SP5 and SP6 were then selected for in vivo studies in which we orally administered a single dose of either 2.5, 5, or 10 mg/kg of one of the peptides to normotensive mice. Only 10 mg/kg of SP6 reduced systolic blood pressure, an effect that was significant between 2 and 8 hours from administration, with return to the basal level by 24 hours (Figure 4E). Ex vivo evaluation of vascular effects on vessels harvested after 8 hours from in vivo administration revealed that SP5 was ineffective in improving acetylcholine-evoked vasorelaxation (Figure 4F left), whereas SP6 was effective at the 2 highest doses (Figure 4F, right). Western blotting analysis revealed

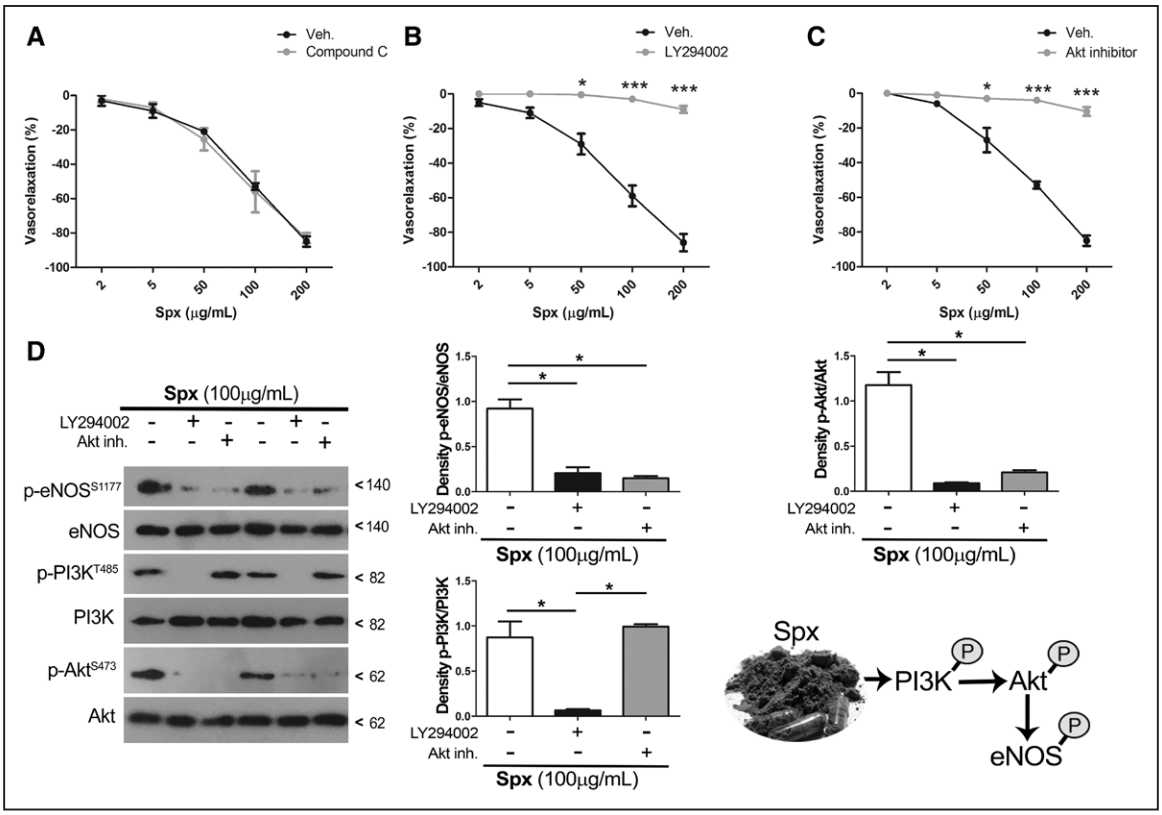

Figure 2. GID (gastrointestinal digestion)-spirulina exerts its vascular effect through the PI3K-AKT-eNOS (endothelial NO synthase) pathway. **A**, Response of phenylephrine-precontracted mouse mesenteric arteries to increasing doses of GID-spirulina (2–200 μg/mL) in the presence of the AMPK inhibitor compound C. **B**, The PI3K inhibitor LY294002 abolishes GID-spirulina–evoked vasorelaxation. **C**, Also Akt Inhibitor X abolishes GID-spirulina–evoked vasorelaxation. Data are mean±SEM of n=7 independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 vs vehicle (Veh). **D**, **left**, Representative immunoblotting conducted on extracts of mouse mesenteric artery exposed to GID-spirulina (100 μg/mL) alone or in the presence of either LY294002 or Akt Inhibitor X; **middle** and **right**, Semiquantitative analyses of phosphorylation levels of phospho-eNOS (endothelial NO synthase) serine 1177; phospho-PI3K (phosphoinositide-3-kinase) tyrosine 485; phospho-Akt (serine/threonine kinase Akt) serine 473 (mean±SEM; n=3 independent experiments) and schematic of the spirulina/PI3K/AKT pathway leading to release of NO. **P*<0.05. SP indicates spirulina peptide.

that the PI3K/AKT/eNOS pathway was activated in mesenteric arteries by administration of SP6 (Figure 4G).

Coherently with previous observations, SP6 failed to evoke dose-dependent vasorelaxation of mesenteric arteries harvested from eNOS-knockout mice or from wild-type mice when pretreating vessels with L-NAME (Figure 5A and Figure S4). Moreover, SP6 failed to reduce blood pressure when administered orally to eNOS-knockout mice (Figure 5B and Figure S5A).

SP6 Enhances Endothelial Function of Vessels Harvested From Hypertensive Animals

We then focused our attention on an experimental model of hypertension, namely the spontaneously hypertensive rat (SHR), in which endothelial dysfunction is associated with high blood pressure. SP6 evoked vasorelaxation of ex vivo SHR mesenteric arteries in a dose-dependent manner, albeit to a reduced extent with respect to vessels harvested from normotensive WKY (Wistar Kyoto) rats (Figure 5C and 5D and Figure S6); analogous findings were obtained with vessels harvested from Ang II (angiotensin II)-infused wild-type mice, a second model of hypertension (Figure S7).

The characterization of the intracellular pathway at functional and molecular levels using pharmacological inhibitors showed that the PI3K/AKT intracellular signaling pathway was recruited by SP6 to phosphorylate eNOS, and this was necessary to release NO (Figure 5E). That inhibition of eNOS abolished SP6-evoked vasodilation in both SHRs and WKY rats demonstrated that NO represents the only determinant of SP6-evoked relaxation (Figure S6A and S6B; see also results in the online-only Data Supplement). An interesting finding emerging from the molecular studies on vessels subjected to functional analyses is that, although we observed reduced vasodilation in vessels from SHRs, SP6 induced an increase of eNOS phosphorylation in this animal model compared with their relative control rats (Figure 5E).

Thus, we decided to incubate dysfunctional vessels from SHRs and evaluate acetylcholine-evoked vasorelaxation. Interestingly, SP6 enhanced acetylcholine-evoked vasorelaxation in both SHRs and WKY rats, an effect that disappeared upon eNOS inhibition (Figure S8). The data so far described show that the vascular action of SP6 is characterized by a direct vasorelaxation effect on constricted vessels and a sensitizing effect on acetylcholine-evoked vasorelaxation by potentiating NO release.

NO release was then assessed visually under the microscope with a reagent that becomes fluorescent in the presence of NO. SP6 increased the green fluorescence signal selectively at the endothelium of wild-type vessels, indicating NO release at this level (Figure S9A); similar observations were made on SHR and Ang II–infused mouse vessels (Figure S9B–S9D).

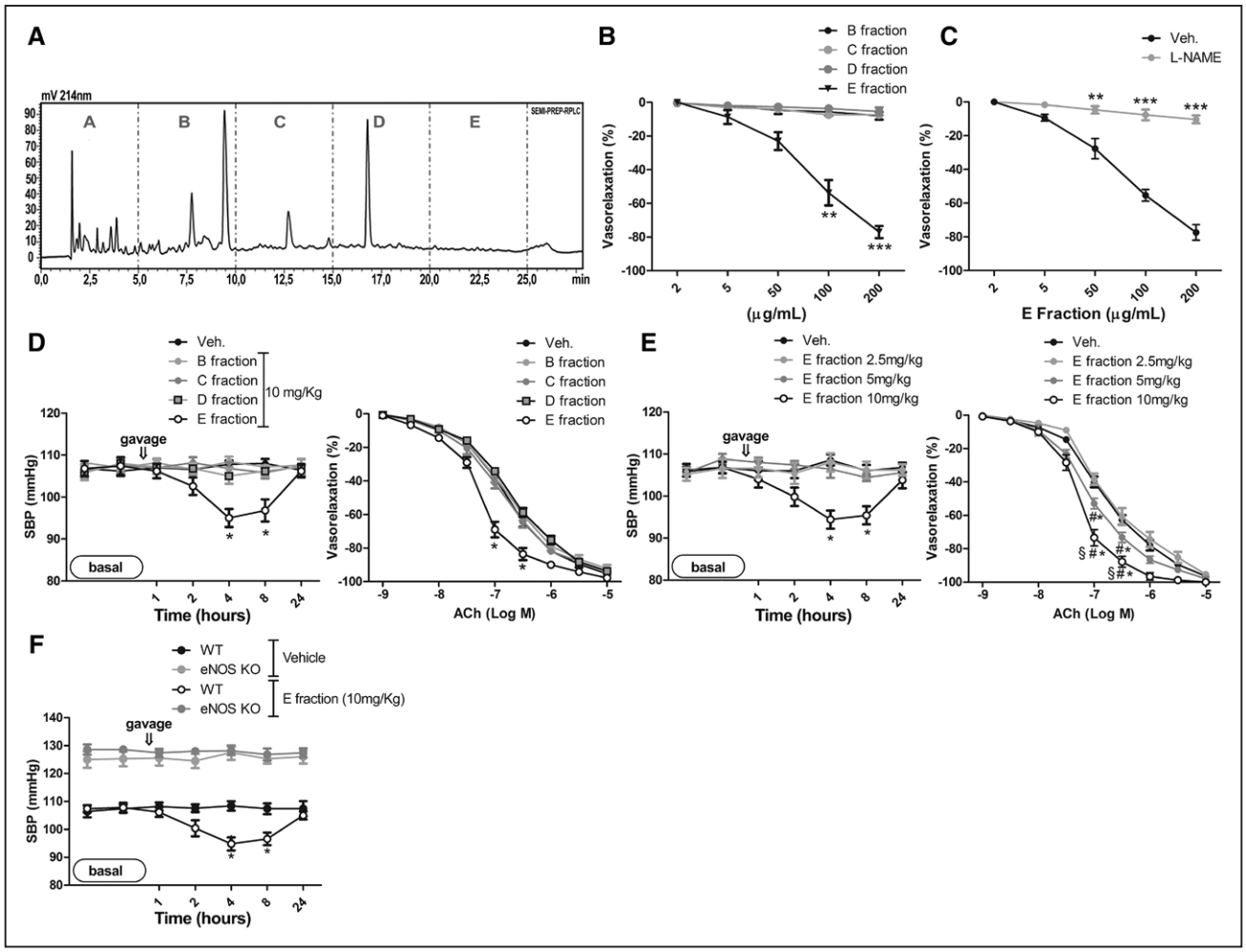

Figure 3. Characterization of the vascular effects of spirulina fractions. **A**, Semi-prep reversed-phase high-performance liquid chromatography UV chromatogram showing fractions A–E. **B**, Response of phenylephrine-precontracted mouse mesenteric arteries to increasing doses (2–200 μg/mL) of GID-spirulina fractions B–E (n=5). **C**, Response of phenylephrine-precontracted mouse mesenteric arteries to increasing doses of E in the presence of an eNOS (endothelial NO synthase) inhibitor (n=5). **D**, **left**, Time course of systolic blood pressure (SBP) in C57BL/6 mice receiving a single oral dose of vehicle (Veh) or the indicated GID-spirulina fraction (n=6/group). Arrow indicates time of administration via gavage. Data are mean±SEM. *P<0.05 vs all; **right**, dose-response curves to acetylcholine (Ach; 10^{-9} M to 10^{-5} M) of mesenteric arteries excised from mice 8 h after a single oral dose of Veh or the indicated fraction. Values are means±SEM of n=6 experiments. *P<0.05 vs all. **E**, **left**, time course of SBP in C57BL/6 mice given differing doses of fraction E; **right**, Dose-response to ACh of mesenteric arteries excised at 4 h (n=6/group). *P<0.05 vs Veh; #P<0.05 vs 2.5 mg/kg; §P<0.05 vs 5 mg/kg. **F**, SBP in wild-type (WT) and eNOS-knockout (KO) mice administered Veh. or fraction E (n=5/group). L-NAME indicates N(ω)-nitro-L-arginine methyl ester.

In agreement with functional and molecular data, the presence of acetylcholine further enhanced SP6-induced endothelial fluorescence, indicating potentiation of NO release (Figure S9A–S9D). As expected, there was no green fluorescence emanating from eNOS-knockout vessels (Figure S9B).

SP6 Exerts an Antihypertensive Action Through a NO-Dependent Mechanism

The results obtained in vitro led us to explore the hemodynamic properties of SP6 in vivo in a hypertensive context. Administration of a single oral dose of 10 mg/kg of SP6 to SHRs induced a significant decrease (≈43 mm Hg) of blood pressure between 2 and 8 hours after gavage (Figure 5F), demonstrating that this peptide is active also in a hypertensive context in vivo. The evaluation of serum nitrite levels—a well-recognized biomarker of the state of NO metabolism—showed that there was a dose-dependent increase of this parameter in SP6-treated SHRs (Figure 5G).

To define the role of NO in the hemodynamic effects of the peptide, SP6 was then administered to SHRs after receiving L-NAME intraperitoneally. As expected, SP6 was unable to hinder the blood pressure increase induced by inhibition of eNOS (Figure 6A and 6B, left). Moreover, SP6 reduced blood pressure in SHRs to a much greater degree than in normotensive rats. Vascular reactivity studies performed on vessels excised from rats at the time of SP6's maximal effect demonstrated that administration of the peptide protected from endothelial dysfunction because acetylcholine-evoked vasorelaxation was significantly enhanced (Figure 6A and 6B, right).

Analyses at the molecular level further strengthened our findings on the mechanisms induced by spirulina because in vessels from rats treated with SP6 the action of acetylcholine on eNOS phosphorylation was significantly potentiated (Figure 6C). Moreover, SP6 enhanced green fluorescence emissions in SHRs and WKY rats, an effect lost in the presence

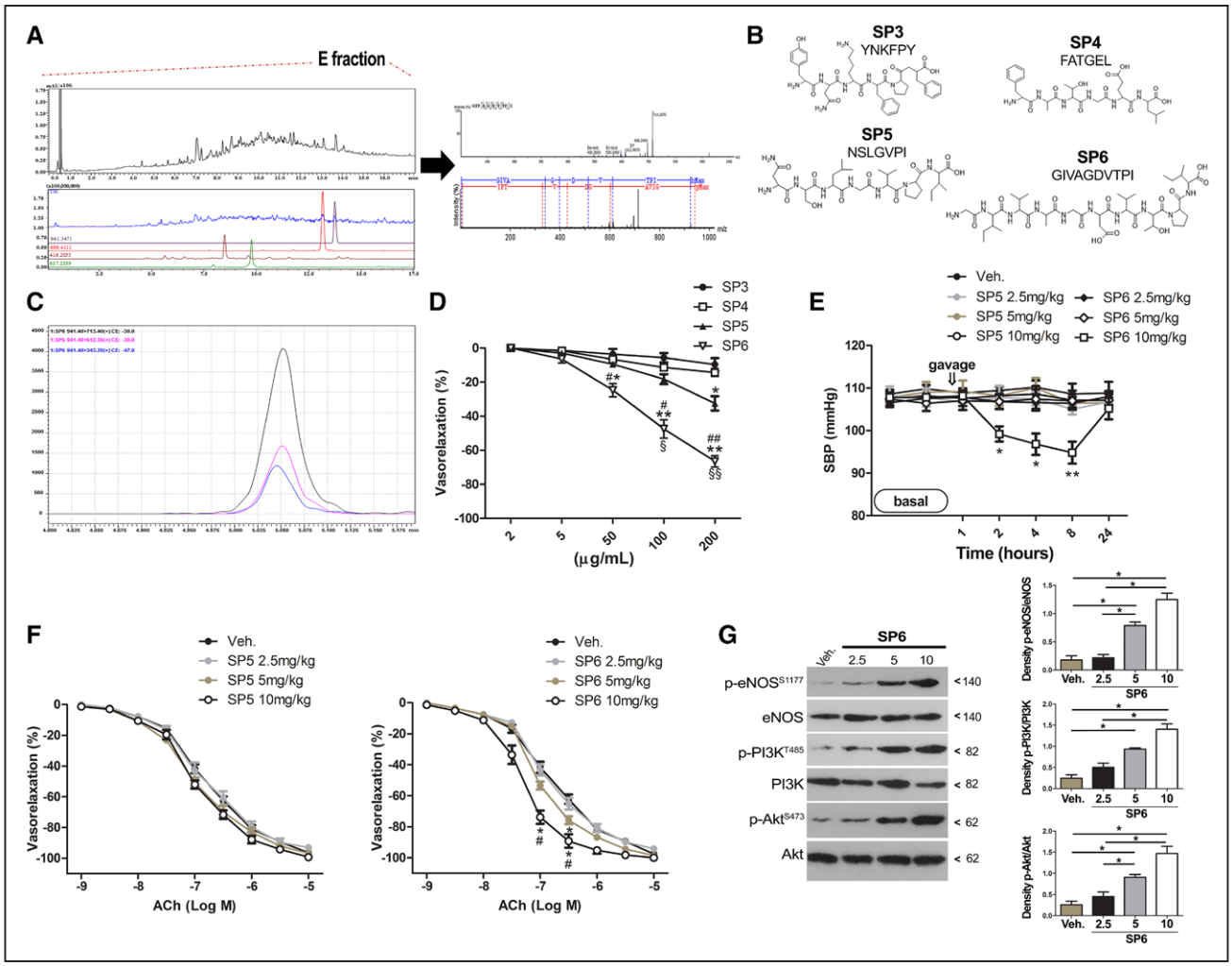

Figure 4. Characterization of single peptides isolated from fraction E of spirulina. **A**, UV and extracted-ion chromatograms of fraction E, revealing the peaks indicating the presence of the 4 spirulina peptides (SP)3, SP4, SP5, and SP6 (**left**), and the MS/MS (tandem mass spectrometry) spectrum of the peptide sequence of SP6**. B**, Chemical structures of the individual SPs present in fraction E. **C**, Selected reaction monitoring of the presence of SP6 in mouse serum after oral administration of 10 mg/kg of fraction E. **D**, Response of phenylephrine-precontracted mouse mesenteric arteries to increasing doses of SP3, SP4, SP5, and SP6 (n=5). **P*<0.05, ***P*<0.01 vs SP3 and SP4; #*P*<0.05, ##*P*<0.01 vs SP5. **E**, Time course of systolic blood pressure (SBP) in C57BL/6 mice administered vehicle (Veh) or different doses of the indicated peptide. Arrow indicates time of administration via gavage. Data are mean±SEM of n=6/group. **F**, **left** and **right**, Dose-response curves to acetylcholine (ACh) of mesenteric arteries excised from mice 8 h after administration of SP5 (**left**) or SP6 (**right**) given at the indicated dosage (n=6/group). **P*<0.05, ***P*<0.01 vs Veh; #*P*<0.05 vs 2.5 mg/kg. **G**, **left**, Representative immunoblottings conducted on extracts of mouse mesenteric arteries harvested 8 h after administration of SP6 at different doses; **right**, Semiquantitative densitometric analyses of phosphorylation levels of phospho-eNOS (endothelial NO synthase) serine 1177; phospho-PI3K (phosphoinositide-3-kinase) tyrosine 485; phospho-Akt (serine/threonine kinase Akt) serine 473. Data are the mean±SEM of 3 independent experiments. **P*<0.05. eNOS indicates endothelial NO synthase.

of L-NAME (Figure 6D). Careful analysis of our data revealed that the acetylcholine-evoked increase in green fluorescence was significantly higher in SHRs versus normotensive rats, in agreement with molecular studies showing enhanced eNOS phosphorylation in the former; in vessels treated with SP6, acetylcholine incremented green fluorescence, indicating higher NO release (Figure 6D).

Discussion

This study identifies for the first time a novel decameric peptide, generated by simulated GID of spirulina, that induces endothelial vasorelaxation in a dose-dependent manner. Activation of the PI3K/AKT/eNOS signaling pathway was responsible for normalizing blood pressure in experimental models of hypertension.

S platensis—a microscopic filamentous blue/green alga rediscovered in the 1960s—has garnered increasing attention over the past 10 years after the publication of reports on the health benefits of its dried biomass, spirulina.[8–10] Because of a high protein content, spirulina may be a useful substance from which bioactive peptides can be obtained. Indeed, small tripeptides have been identified—mainly through hydrolysis with nonendogenous proteases, such as alcalase[11] or papain[12]—that exert an antihypertensive effect by inhibiting Ang II signaling and oxidative stress in experimental models.[12,13] However, differently from these studies, we decided to process spirulina through simulated human GID, using endogenous enzymes that mimic the physiological process. In particular, this method provides enormous advantages: (1) it generates products that are resistant to digestion after

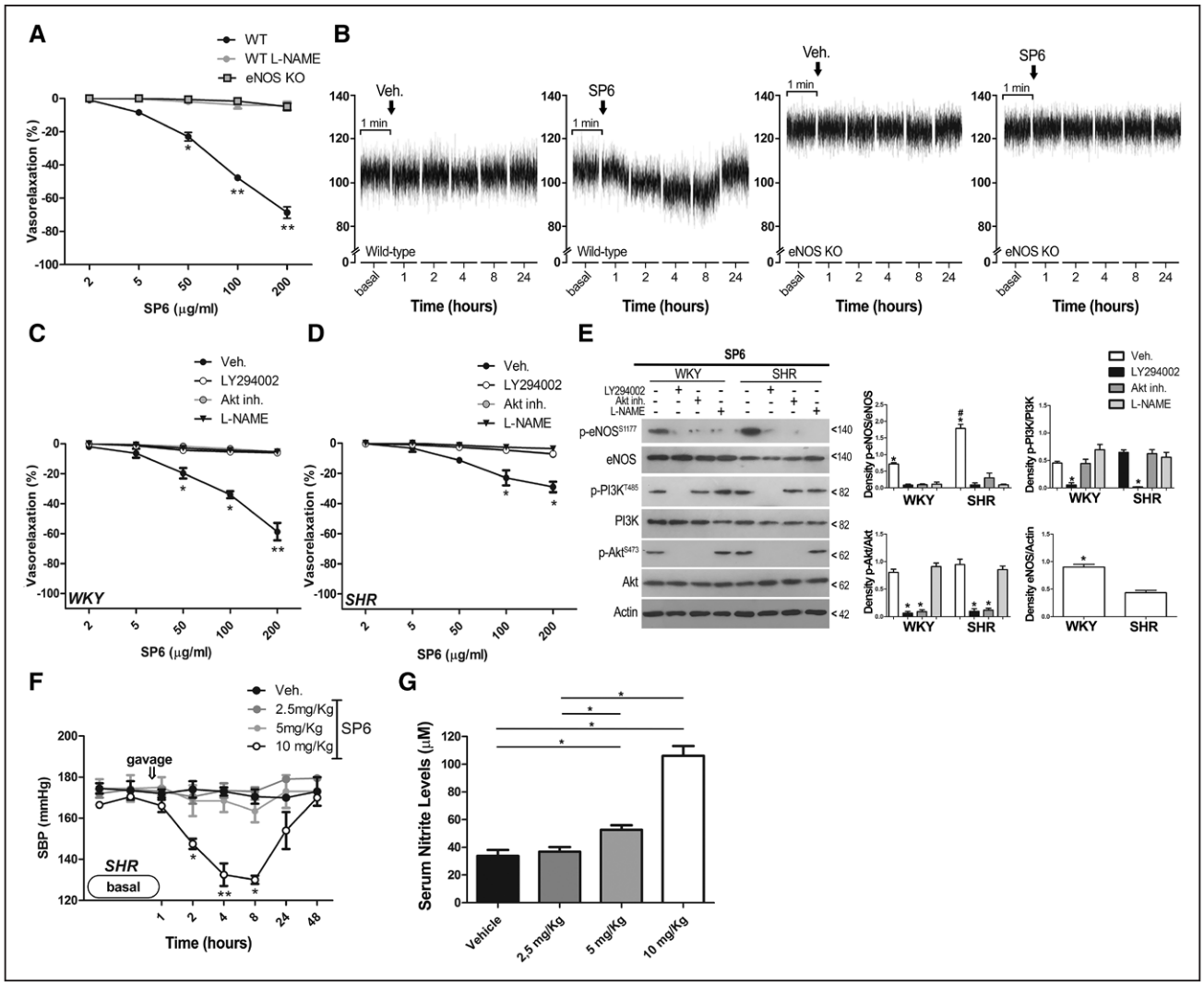

Figure 5. Molecular mechanism and hemodynamic effects evoked by SP6 (spirulina peptide 6). **A**, Response of phenylephrine-precontracted mesenteric arteries harvested from wild-type (WT) and eNOS (endothelial NO synthase)-knockout (KO) mice to increasing doses of SP6, the former also in the presence of the eNOS inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME). **P*<0.05, ***P*<0.01 vs all. **B**, Representative intrafemoral blood pressure (BP) traces (1-min intervals) measured in C57BL/6 (WT) and eNOS KO mice receiving vehicle (Veh) or 10 mg/kg of SP6, at different times after the administration by gavage (indicated by an arrow). **C** and **D**, Response of phenylephrine-precontracted mesenteric arteries harvested from normotensive WKY (Wistar Kyoto) rats (**C**) and spontaneously hypertensive rats (SHR; **D**) to increasing doses of SP6 in the presence of a PI3K inhibitor (LY294002), an AKT inhibitor (Akt inh), or an eNOS inhibitor (L-NAME). (n=7) **P*<0.05, ***P*<0.01, ****P*<0.001 vs vehicle. **E**, **left**, Representative immunoblottings conducted on extracts of mesenteric arteries, harvested from hypertensive and normotensive rats, exposed to SP6 (100 μg/mL) alone or in the presence of LY294002, AKT Inhibitor X, or L-NAME; **right**, Semiquantitative analyses of phosphorylation of phospho-eNOS (endothelial NO synthase) serine 1177; phospho-PI3K (phosphoinositide-3-kinase) tyrosine 485; phospho-Akt (serine/threonine kinase Akt) serine 473. Data are mean±SEM of 3 independent experiments. **P*<0.05 vs all, #*P*<0.05 vs WKY. **F**, Time course of systolic blood pressure (SBP) in SHRs receiving vehicle or different doses of SP6. Values are the means±SEM. (n=5/group). **P*<0.05, ***P*<0.05 vs all. **G**, Serum concentration of stable NO metabolites in SHRs treated with vehicle or different doses of SP6. n=6 per group. **P*<0.05.

oral administration;[7] (2) it provides information on the products' release sites; and (3) more-potent bioactive products are obtained.[14]

In the first part of our study, we demonstrate that GID-spirulina directly induces vasodilation of mouse resistance arteries, an important vascular district involved in blood pressure regulation. This action was dependent upon the activation of eNOS in the endothelium because vascular effects were completely lost in eNOS-knockout mice. However, pharmacological inhibition of AMPK—an important sensor of cellular energy status and a key molecule involved in metabolic control that has been reported to be efficiently activated by several natural compounds[15,16]—did not influence the vasorelaxant properties of GID-spirulina. In contrast, we found that eNOS was activated by phosphorylation on Ser[1177] through a PI3K/AKT pathway.

Using a 2-stage approach, we divided whole GID-spirulina matrix into 5 fractions (A to E) to identify the most bioactive portion. Only fraction E evoked NO-dependent vasorelaxation of ex vivo vessels and reduced blood pressure in mice. Of note, a low dose of fraction E did not influence blood pressure homeostasis but was still able to enhance NO-mediated vasorelaxation, indicating that its administration at lower doses may help contain vascular diseases without influencing blood pressure.

These findings led us to hypothesize that fraction E contained stable peptides that survived GID and that once absorbed exerted a direct vascular effect. Indeed, through a

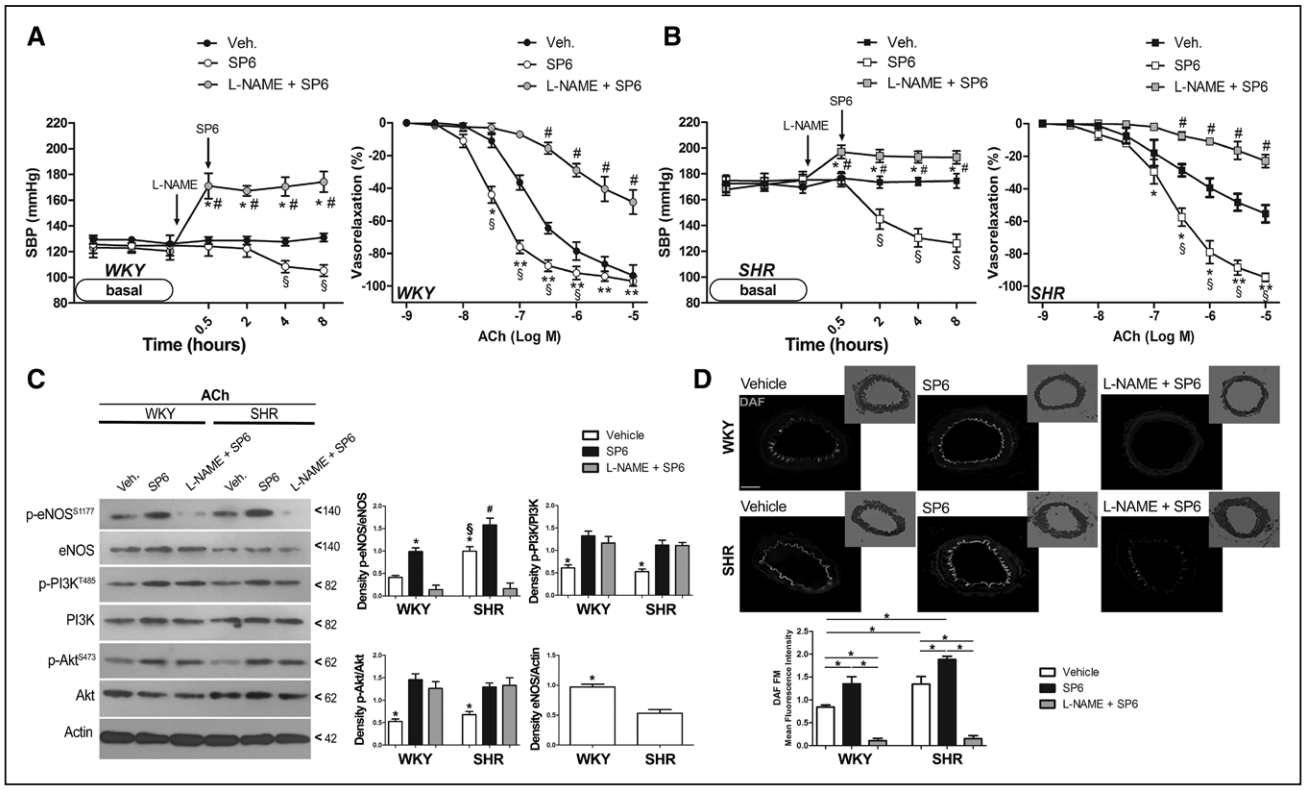

Figure 6. SP6 (spirulina peptide 6) reduces blood pressure in normotensive and hypertensive rats through a NO-dependent mechanism. **A** and **B**, **left**, Systolic blood pressure (SBP) in normotensive WKY (Wistar Kyoto) rats (**A**) and in spontaneously hypertensive rats (SHRs; **B**) administered vehicle (Veh), 10 mg/kg of SP6 alone, or SP6 30 min after pretreatment with L-NAME (75 mg/kg IP). Values are means±SEM (n=6 per group). **P*<0.05 vs all; #*P*<0.05 vs Veh; **right**, Response of ex vivo phenylephrine-precontracted rats mesenteric arteries to increasing doses of acetylcholine, excised 8 h from in vivo treatments. **P*<0.05 vs all; #*P*<0.05 vs Veh. **C**, **left**, Representative immunoblots conducted on extracts of mesenteric arteries excised from rats after 8 h from in vivo treatment; **right**, Semiquantitative analyses of phosphorylation levels of phospho-eNOS (endothelial NO synthase) serine 1177; phospho-PI3K (phosphoinositide-3-kinase) tyrosine 485; phospho-Akt (serine/threonine kinase Akt) serine 473. Data are mean±SEM of 3 independent experiments. **P*<0.05, §*P*<0.05 vs WKY+Veh, #*P*<0.05 vs WKY+SP6. **D**, Representative micrographs of DAF-FM fluorescent signal in mesenteric arteries excised from in vivo-treated rats (Veh, SP6 alone, or N(ω)-nitro-L-arginine methyl ester [L-NAME] then SP6) stimulated with acetylcholine (Ach; 10^{-5} M). Inset: images merged with sections counterstained with hematoxylin-eosin. Scale bar, 100 μm. The graph gives mean fluorescence intensity of fluorescence measurements. Data are mean±SEM of 5 independent experiments. **P*<0.05.

chemical approach, we isolated 4 different spirulina peptides from fraction E, finding that SP6 was unique in its ability to activate the endothelial NO machinery. Of note, the quantification of the circulating level of SP6 after oral administration of fraction E to mice revealed that the native form of this decapeptide was present, clearly indicating that the peptide is bioavailable and resists further hydrolysis in the blood.

It is well known that hypertensive patients have a defect in the endothelium-dependent NO system; this contributes significantly to increased vascular resistance in the basal condition and to impairment of endothelium-dependent vasorelaxation. Moreover, oral administration of SP6 to SHRs—one of the best animal models reproducing the alterations seen in human hypertension—increased serum nitrite levels and exerted an antihypertensive effect. In addition, results obtained in vivo on inhibition of eNOS clearly demonstrated that the hemodynamic action of SP6 is mediated by NO. Thus, the antihypertensive effect observed in hypertensive rats could be ascribed to SP6 directly inducing enhancement of endothelial function and, hence, vasorelaxation.

In conclusion, the health-enhancing potential and safety profile of SP6 designate this stable bioactive peptide—which is obtainable from a complex natural matrix—as an interesting candidate for future studies on the prevention of cardiovascular diseases associated with NO dysregulation.

Study Limitations

The data presented here demonstrate acute, beneficial cardiovascular effects of SP6, but clearly, more studies are needed to clarify whether its effects can also be extended to chronic conditions. Preliminary analysis of serum in animals treated with 10 mg/kg of fraction E revealed the presence of an intact decapeptide, the concentration of which was obviously low, but the analysis did not take into account metabolization and excretion processes. Characterization of the tissue distribution and pharmacokinetics of SP6 will be the aim of future studies necessary to fully understand the effects evoked by the peptide.

Perspectives

The current study provides new information on the potential effects of spirulina on cardiovascular homeostasis. Moreover, the identification of a single bioactive peptide from the plethora of molecules generated from simulated GID of spirulina, and the description of its efficacy in vivo, opens a new scenario in the development of non-pharmacological adjuvants that may be combined with

pharmacological compounds to improve endothelial function and control blood pressure.

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Disclosures

None.

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Novelty and Significance

What Is New?

- We used a peptidomic approach comprising a model of in vitro gastrointestinal digestion, fractionation, and high-resolution mass spectrometry of the dried biomass of *Spirulina platensis* to identify a single biologically active peptide–SP6 (spirulina peptide 6).
- SP6 is a novel, decameric, spirulina-derived peptide that modulates endothelial function to increase the production of NO; of note, it is effective when administered orally in reducing blood pressure in animal models of hypertension.

What Is Relevant?

- The identification of bioactive peptides such as spirulina peptide 6 [GIVAGDVTPI] that modulate endothelial function and blood pressure could lead to the development of novel natural coadjutants to the pharmacological treatment of hypertension.
- The existence of doses of spirulina peptide 6 that do not modulate blood pressure but are still able to enhance endothelial NO-mediated vasorelaxation strongly suggests that the peptide could be considered for novel natural therapeutic approaches to contain vascular diseases associated with NO dysfunction, independently from a hemodynamic effect.

Summary

Food-derived materials are usually extremely complex, containing hundreds of compounds, and, thus, represent a challenging task. This is compounded for proteins because hydrolysis by endoproteases releases large numbers of peptides of differing molecular weights. Here, we used a complex, multistep, mass-spectrometry-assisted platform to isolate the most bioactive peptide (spirulina peptide 6) in a spirulina digest. We identified its exact amino acid sequence and defined its action on endothelial function and blood pressure, finding that it acted through a PI3K/AKT/eNOS (endothelial NO synthase)-dependent pathway. The identification of spirulina peptide 6 may open the door to the development of new natural therapies aimed at containing the incidence of arterial hypertension and vascular diseases.