Wine polyphenols improve cardiovascular remodeling and vascular function in NO-deficient hypertension

IVETA BERNÁTOVÁ,1 OLGA PECHÁNOVÁ,1 PAVEL BABÁL,2 SONA KYSELÁ,1 SVETOSLAV STVRTINA,2 AND RAMAROSON ANDRIANTSITOHAINA3

1Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, 813 71 Bratislava; 2Department of Pathology, Medical Faculty, Comenius University, 81108 Bratislava, Slovak Republic; and 3Pharmacologie et Physico-Chimie des Interactions Cellulaires et Moléculaires, Université Louis Pasteur de Strasbourg, Unité Mixte de Recherche Centre National de la Recherche Scientifique 7034, Faculté de Pharmacie, Illkirch, France

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Very recently, we provided evidence for in vivo cardiovascular effects of red wine polyphenolic compounds (RWPCs) (10). Oral administration of RWPCs including Provinol is able to produce a decrease in blood pressure in normotensive rats. This hemodynamic effect was associated with enhanced endothelium-dependent relaxation and induction of inducible nitric oxide (NO) synthase and cyclooxygenase 2 gene expression within the arterial wall. This effect probably involved an NO pathway inasmuch RWPCs are able to produce in vivo endothelium-dependent relaxation as a result of enhanced NO synthesis (2).

Endothelial abnormalities with reduced activity of NO synthase are often observed in several vascular diseases leading to structural and functional changes of the cardiovascular system. In a model of chronic inhibition of NO synthesis by administration of the inhibitor NG-nitro-L-arginine methyl ester (L-NAME), development of arterial hypertension occurs and is associated with left ventricular (LV) hypertrophy and cardiac and vascular remodeling (3, 5, 12). Taking into account the therapeutically relevant effect of polyphenols on the pathway leading to NO generation in the cardiovascular system, it was suggested that Provinol might protect against the development of pathological changes in a rat model of L-NAME-induced hypertension. Therefore, the effect of oral administration of polyphenols, they were also reported to affect the activity of enzymes critically involved in pathways that regulate cell proliferation and cell activation (18). In addition to the latter effect of polyphenols...
Provinol on blood pressure recovery as well as LV hypertrophy, myocardial fibrosis, and vascular remodeling was investigated in a model of hypertension produced by chronic L-NAME treatment. The hypothesis that an increased NO pathway contributes to the probable antiatherosclerotic effect of Provinol was tested by measuring NO synthase activity within cardiovascular tissue.

**MATERIALS AND METHODS**

All procedures were performed in accordance with institutional guidelines. Male Wistar rats, 12–13 wk old, were divided into groups as follows. Forty-two rats received L-NAME at a dose of 40 mg·kg⁻¹·day⁻¹ in tap water for 4 wk. After this period, the rats were randomly divided into three groups: the L-NAME group, the recovery group, and the Provinol group. In the L-NAME group (n = 6), the rats were killed immediately at the end of L-NAME treatment. In the recovery group, the L-NAME treatment period was followed by a 1-, 2-, or 3-wk drug-free recovery period (n = 6 in each subgroup). In the Provinol group, the period of L-NAME treatment was followed by 1, 2, or 3 wk of Provinol treatment (40 mg·kg⁻¹·day⁻¹ in tap water; n = 6 in each subgroup). A further six animals served as a control group without any treatment. All animals were housed at a temperature of 22–24°C in individual cages and were fed with a regular pellet diet ad libitum.

Systolic blood pressure (SBP) was measured by noninvasive tail cuff plethysmography twice weekly. Rats were killed by decapitation, body weight (BW), heart weight (HW), LV weight (LVW), and right ventricle weight (RVW) were determined, and the LVW-BW and RVW-BW ratios were calculated. Samples of the anterior wall of the LV and thoracic aorta were used for measurement of NO synthase activity, [¹⁴C]leucine incorporation, and protein concentration. The polyphenol contents of Provinol were described as RWPC2 in our previous work (2), and they were (in mg/g of dry powder) 480 proanthocyanidins, 61 total anthocyanins, 19 free anthocyanins, 38 catechin, 18 hydroxycinnamic acid, and 14 flavonols.

**Ex vivo vascular reactivity.** The thoracic aorta was removed and mounted as previously described (24). Endothelium-dependent relaxation was studied in thoracic aorta cut into rings (3- to 3.5-mm length) with functional endothelium and precontracted to maximal response obtained with phenylephrine (10 μmol/l). Acetylcholine was applied in a cumulative fashion, and relaxation was expressed as a percentage of phenylephrine-induced contraction. A concentration-response curve for norepinephrine was constructed in a cumulative manner in aortas with functional endothelium. Contractile responses were reported as active stress (mN/mm²).

NO synthase activity. NO synthase activity was determined in crude homogenates of the LV and aorta by measuring the formation of L-[¹⁴C]citrulline (L-Cit) from L-[¹⁴C]arginine (Amersham) as previously described by Breidt and Snyder (6) with minor modifications. Briefly, 50 μl of crude homogenate of the LV (7.5 mg of wet tissue) or aorta (5 mg of wet tissue) were incubated in the presence of 50 mmol/l Tris-HCl, pH 7.4, containing 1 μmol/l L-[¹⁴C]arginine (specific activity 5 GBq/mmol., ~100,000 dpm), 0.5 mg/ml calmodulin, 0.5 mmol/l β-NADPH, 250 μmol/l tetrahydrobiopterin, 4 μmol/l FAD, 4 μmol/l flavin mononucleotide, and 1 mmol/l Ca²⁺, in a total volume of 100 μl. After 10-min incubation at 37°C, the reaction was stopped, the samples were centrifuged, and supernatants were applied to 1-ml Dowex 50WX-8 columns (Na⁺ form). L-Cit was eluted by 2 ml of water and determined by liquid scintillation counting. NO synthase activity was expressed as picokatals (pkat) per gram of protein.

**Determination of [¹⁴C]leucine incorporation.** Protein synthesis in both the LV and aorta was evaluated by measuring [¹⁴C]leucine incorporation into proteins, as previously described by Bernátová et al. (5). The protein concentration was determined by Lowry’s method.

**Histology and morphometry.** Samples of either LV tissue or the aorta were fixed for 24 h in 10% phosphate-buffered formalin. They were then processed routinely in paraffin, and serial 5-μm-thick sections were stained with hematoxylin and eosin and by Van Gieson staining.

Morphometric evaluation was performed on a Olympus light microscope equipped with a two-dimensional image analyzer (Alfa Inotech) as described elsewhere (3). Van Gieson stain was applied to enhance the red color contrast of collagen. Myocardial fibrosis was expressed as percentage of the whole muscle area on three serial sections of each heart ventricle specimen. The aortic cross-sectional area was determined in square micrometers.

**Statistical analysis.** All results are expressed as means ± SE. One-way analysis of variance and Bonferroni test were used for statistical analysis. P < 0.05 was considered significant.

**RESULTS**

SBP was not significantly different in rats in the four groups before the beginning of the treatment. In the control group, SBP did not change significantly for 8 wk (Fig. 1). Treatment of the rats for 4 wk with L-NAME (40 mg·kg⁻¹·day⁻¹) produced a progressive increase in SBP (34% after 4 wk of treatment compared with SBP of control rats; P < 0.01). After cessation of L-NAME treatment, SBP decreased progressively during the period of recovery and returned to the control value after 3 wk (Fig. 1). After cessation of L-NAME treatment, administration of Provinol (40 mg·kg⁻¹·day⁻¹) also produced a progressive decrease of SBP. However, the decrease in SBP was significantly accelerated in the Provinol group such that the SBP value was not significantly different from that of the control group already after the first week of treatment. After

![Fig. 1. Effects of Provinol treatment (PR) on systolic blood pressure. Changes in systolic blood pressure in control (●) and N⁵-nitro-L-arginine methyl ester (L-NAME)-treated (★) rats followed by spontaneous recovery (SR; ▲) or by PR (●) are shown. *P < 0.05 vs. control; **P < 0.05 vs. spontaneous recovery. Values are means ± SE.](http://ajpheart.physiology.org/2002/03/01/ajpheart.htm)
3-wk cessation of L-NAME treatment, the decrease in SBP produced by treatment with Provinol was significantly greater than that of the recovery group (P < 0.05).

BW, HW, LVW/BW, and RVW/BW are given in Table 1. BW and HW were not significantly different among the four groups (i.e., control, l-NAME, recovery, and Provinol groups). Chronic administration of l-NAME induced an increase of LVW/BW without a significant change in RVW/BW. Both in the recovery group and in the Provinol group, the elevation of LVW/BW persisted 3 wk after cessation of l-NAME treatment regardless of decreased blood pressure.

Vasoreactivity of thoracic aorta. Norepinephrine produced concentration-dependent contraction of aortas with functional endothelium in all tested groups (Fig. 2A). In the l-NAME-treated group, the contractile response to norepinephrine was significantly greater compared with that in control rats. The maximal response to norepinephrine was significantly enhanced from 6.07 ± 1.34 to 8.37 ± 0.33 mN/mm² (P < 0.05) in aortas taken from control and l-NAME-treated rats, respectively. Three weeks of spontaneous recovery did not modify the increased response to norepinephrine produced by l-NAME treatment. Thus the maximal response to norepinephrine was significantly different between the l-NAME-treated group and the recovery group, being 8.37 ± 0.33 and 8.18 ± 0.33 mN/mm², respectively. However, Provinol treatment not only restored the increased reactivity of the aortas to norepinephrine induced by the l-NAME treatment but also reduced the norepinephrine response compared with control rats. The maximal contraction to norepinephrine was significantly reduced after Provinol treatment to 3.71 ± 0.47 mN/mm² (P < 0.05 vs. l-NAME group and control group).

Acetylcholine produced concentration-dependent relaxation in aortas from control, l-NAME, recovery, and Provinol groups (Fig. 2B). In the l-NAME-treated group, acetylcholine-induced relaxation was significantly reduced compared with the response obtained in aortas from control rats (P < 0.05). The response to acetylcholine obtained in the recovery group was not significantly different from that of the l-NAME-treated group, but it was significantly reduced compared with the relaxation of aortas from control (P < 0.05). In contrast to the recovery group, the response to acetylcholine was significantly potentiated in aortic rings from Provinol-treated rats (P < 0.05) such that the observed relaxation was not significantly different from that of aortas from control rats. Thus Provinol improved the reduced endothelium-dependent relax-

Table 1. Basic parameters, myocardial fibrosis, and cross-sectional area of control, l-NAME, spontaneous recovery, and Provinol-affected recovery groups

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<tr>
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<th>Control</th>
<th>l-NAME</th>
<th>Recovery</th>
<th>Provinol</th>
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<tr>
<td>BW, g</td>
<td>458 ± 12</td>
<td>450 ± 10</td>
<td>445 ± 15</td>
<td>420 ± 16</td>
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<tr>
<td>HW, g</td>
<td>1.09 ± 0.04</td>
<td>1.12 ± 0.05</td>
<td>1.11 ± 0.05</td>
<td>1.11 ± 0.04</td>
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<tr>
<td>LV/BW, mg/g</td>
<td>1.20 ± 0.04</td>
<td>1.47 ± 0.04</td>
<td>1.36 ± 0.07</td>
<td>1.37 ± 0.05</td>
</tr>
<tr>
<td>RV/BW, mg/g</td>
<td>0.45 ± 0.03</td>
<td>0.39 ± 0.04</td>
<td>0.43 ± 0.03</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>Fibrosis, %</td>
<td>0.07 ± 0.01</td>
<td>4.12 ± 0.92</td>
<td>3.52 ± 0.98</td>
<td>0.91 ± 0.08*</td>
</tr>
<tr>
<td>CSA, μm²</td>
<td>431 ± 48</td>
<td>703 ± 60*</td>
<td>598 ± 71*</td>
<td>513 ± 33</td>
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| Values are means ± SE; n = 6 rats/group. l-NAME, N⁵-nitro-L-arginine methyl ester; Recovery, 3-wk spontaneous recovery; Provinol, 3-wk Provinol-affected recovery; BW, body weight; HW, heart weight; LV, left ventricle; CSA, cross-sectional area. *P < 0.05 vs. control; †P < 0.05 vs. l-NAME group; ‡P < 0.05 vs. Recovery group.
formation to acetylcholine produced by l-NAME treatment toward that of aortic rings taken from control rats.

NO synthase activity. In tissues taken from the control group, NO synthase activities were 3.5 ± 0.22 and 3.33 ± 0.21 pkat/g protein in the LV and the aorta, respectively (Fig. 3). NO synthase activities were significantly reduced in the LV and aorta taken from l-NAME-treated rats (P < 0.05). In the recovery group, NO synthase activities increased slowly to reach a level of 50% and 90% greater than that of the control group in the LV and the aorta, respectively. Interestingly, Provinol treatment accelerated the increase in NO activity in both tissues. In addition, Provinol induced a greater increase of NO synthase activity in both the LV (P < 0.05) and the aorta (P < 0.05) compared with that obtained in the two tissues taken from the recovery group.

[^14C]leucine incorporation. The incorporation of[^14C]leucine into LV and aortic proteins was 75.0 ± 6 and 120 ± 9 dpm/mg protein, respectively, in the control group (Fig. 4). In the l-NAME group,[^14C]leucine incorporation increased significantly by 46% and 41%, respectively, in the LV (P < 0.05) and the aorta (P < 0.05) compared with [^14C]leucine incorporation obtained in tissues taken from control. In the recovery group, the incorporation of[^14C]leucine was not significantly different from that of control, both in the LV and the aorta. Interestingly, Provinol treatment significantly decreased [^14C]leucine incorporation in the LV and the aorta compared with both the l-NAME and control groups. Thus Provinol reduced the protein synthesis in the LV and the aorta.

Histology and morphometric analysis. Hematoxylin and eosin-stained sections revealed large areas of fibrosis in the myocardium of rats treated with l-NAME. These changes were accentuated with Van Gieson staining of collagen. Fibrotic changes were observed in the LV as well as in the right ventricle. The percentage of fibrosis in the heart muscle was significantly greater in the l-NAME-treated and spontaneously recovering groups vs. the control group. Provinol treatment significantly reduced cardiac fibrosis by ~74% compared with that observed in heart muscle taken from the recovery group (Table 1; Fig. 5).

L-NAME treatment induced a significant increase of aortic cross-sectional area. This increase in aortic wall thickness was not significantly reduced after 3 wk of cessation of l-NAME treatment, but it was reduced toward the control value after treatment of the rat with Provinol (Table 1; Fig. 6).

**DISCUSSION**

The present study provides evidence that oral administration of Provinol accelerated the decrease in blood pressure and improved structural and functional cardiovascular changes produced by chronic inhibition of NO synthesis. Indeed, Provinol treatment enhanced the regression of aortic wall thickness and improved the decreased endothelium-dependent relaxation in response to acetylcholine and the increased reactivity of the aorta to norepinephrine in hypertension induced by l-NAME. In addition, Provinol markedly accelerated
the decrease of myocardial fibrosis, even though it did not affect LV hypertrophy under the experimental conditions used. All of the effects of Provinol were associated with a decrease in protein synthesis and an increase of NO synthase activity both in the LV and in the aorta of NO-deficient rats. Data from our previous work and that of other authors show that the model of chronic inhibition of NO synthesis induced by long-term L-NAME treatment in rats results in hypertension associated with hypertrophy of the LV and cardiac fibrosis as well as subsequent thickening of the arterial media and alteration of vascular functions (3, 21).

With regard to the reduction of blood pressure, the SBP of Provinol-treated rats was not different from that of control rats already after the first week of treatment, whereas 2 wk were needed for the recovery group to reach the control value after cessation of L-NAME treatment. Moreover, after 3 wk of treatment, Provinol induced a more pronounced decrease in blood pressure than that in the recovery group. These data suggest that in vivo administration of 40 mg/kg Provinol produced a sufficient circulating concentration of compounds to induce cardiovascular effects. In congruence with our recent work (10), the data showed that oral administration of Provinol leads to a decrease in blood pressure in hypertensive as well as normotensive rats. The molecular identity of the polyphenolic compounds responsible for this effect of Provinol probably included oligomeric condensed tannins and anthocyanins, both of which were reported to be the compounds of RWPCs that mediate the ex vivo endothelial NO-induced relaxation of aortic rings (1).

The mechanism implicated in the antihypertensive effect of in vivo treatment of Provinol and other natural dietary polyphenol compounds is unknown. It has been suggested that the improvement of flow-mediated endothelium-dependent vasodilatation in patients with coronary diseases by ingestion of red grape juice (whose constituents include anthocyanins and tannins; Ref. 22) and the antithrombotic properties (26) of red wine in vivo in the rat involve NO. However, both the origins of NO production and the mechanism by which it might be enhanced in vivo have not been established. The present study documented that the accelerated blood pressure decrease in Provinol-treated rats was associated with an increase of NO synthase activity in both the LV and the aorta compared with that of the spontaneous recovery group. In addition, the increase of NO synthase activity occurred sooner in tissues taken from Provinol-treated rats than in those from the spontaneous recovery group. In light of these findings, the most likely hypothesis is that the effect of Provinol on hypertension produced by chronic L-NAME treatment is the result of in vivo NO production subsequent to increased NO synthase activity both in cardiac and vascular tissues of the rat.

Fig. 5. Myocardium of the left ventricle. There was marked increase of fibrosis (arrow) after chronic L-NAME treatment (LN), without significant change after spontaneous recovery period (LNS) and with reduction of fibrosis after treatment with Provinol (LNP). C, control myocardium. Van Gieson stain, ×50.

Fig. 6. Changes in the aorta wall thickness. Chronic administration of L-NAME (LN) resulted in thickening of the aortic wall. LNS led to moderate decrease and LNP to significant decrease in the wall thickness. C, control aorta. Van Gieson stain, ×100.
Turning to the consequences of hypertension, pressure overload leads to cardiovascular remodeling comprising myocardial and vascular hypertrophy linked to changes of the extracellular matrix compartment. An increase in collagen deposition frequently occurs, resulting in fibrosis that is associated with increased myocardial and vascular stiffness and subsequent abnormalities of cardiac and vascular functions (7, 11). It has been suggested that a major factor determining the progression of LV hypertrophy to failure is the presence of myocardial fibrosis. As reported in our previous studies, chronic inhibition of NO synthesis resulted in hypertrophy of the LV associated with enlargement of myocardial fibrosis and increased aortic wall thickness (3). Increase of protein synthesis both in the LV and the aorta was also observed after L-NAME treatment as previously reported (3, 21). In the present study, we found that, 3 wk after cessation of L-NAME treatment, the reduction of SBP toward the control value was associated with normalization of protein synthesis both in the cardiac and aortic tissue without any improvement of cardiac fibrosis, LV hypertrophy, or aortic wall thickness. Thus 3 wk of recovery was not sufficient to observe regression of cardiovascular remodeling even though blood pressure returned to normal value. It is noteworthy that Provinol treatment reversed cardiovascular remodeling including myocardial fibrosis and protein synthesis and aortic wall thickness produced by chronic inhibition of NO synthesis. Provinol administered simultaneously with L-NAME partially prevents L-NAME-induced hypertension and the associated cardiovascular remodeling and vascular dysfunction (Pechánová et al., unpublished observation). The development of vascular remodeling with medial thickening observed in this model of hypertension has been reported to be the consequence of blockade of the anti-inflammatory and antiarteriosclerotic properties of vascular endothelial NO in vivo (3, 16). The latter effect occurs via the inhibition of the activity of nuclear factor-κB by NO. It is plausible that after Provinol treatment, the inhibition of protein synthesis in the LV and the aorta and the decrease in aortic wall thickness result from the increased NO synthesis in these tissues. In addition, it cannot be excluded that the inhibition of vascular smooth muscle cell proliferation through the reduction of transcription factor expression might participate in the protective effect of Provinol. Downregulation of cyclin A gene expression was reported to contribute to the antiproliferative effect of RWPC (14).

Despite the correction of increased blood pressure and the increase of NO synthase activity in the LV of the spontaneous recovery group, no regression of myocardial fibrosis was observed. In contrast, Provinol treatment produced a marked reduction of myocardial fibrosis. Thus it is possible that other unknown mechanisms not regulated by blood pressure or by in vivo production of NO contribute to the reduction of myocardial fibrosis by Provinol. Such mechanisms might include inhibition of matrix metalloproteinases (8), which are involved in maladaptive extracellular protein matrix remodeling leading to myocardial fibrosis.

The reduction of blood pressure in Provinol-treated rats failed to induce regression of the LV hypertrophy despite the reduction of myocardial fibrosis and the inhibition of protein synthesis within the LV. It was recently shown that prolonged arterial hypertension may be associated not only with fibrosis but also with an increase in interstitial fluid (17). Similarly, L-NAME-induced hypertension is associated with increased permeability of the heart capillaries followed by extracellular microedema as well as mitochondrial edema (25). Under experimental conditions similar to those in the present work, we reported (4) that the decrease in blood pressure induced by an inhibitor of angiotensin-converting enzyme, captopril, after cessation of L-NAME treatment is associated with total regression of LV hypertrophy. It was suggested that the regression of LV hypertrophy in the captopril-induced recovery is associated mainly with a large reduction in fluid volume due to diuretic and natriuretic properties of the inhibitor of angiotensin-converting enzyme. Thus the lack of effect of Provinol on LV hypertrophy might be related to its inability to affect the fluid volume in the LV. In addition, it cannot be excluded that a longer period of recovery is needed to achieve reduction of LV hypertrophy inasmuch as a decrease of protein synthesis in the LV by Provinol, as shown by the reduced [14C]leucine incorporation, might be the first step for the reduction of total LV mass.

The reduced endothelium-dependent vasodilatation seen in response to acetylcholine in aortas from L-NAME-treated rats was potentiated in the Provinol-treated group but not in the spontaneous recovery group. These data are in accordance with our previous work showing that the decrease in blood pressure in normotensive rats by short-term oral administration of Provinol was associated with enhanced endothelium-dependent relaxation (10). This effect of Provinol results from enhanced NO generation but not from reduced NO breakdown by superoxide anions, subsequent to amplification of the pathway leading to second messenger production in the endothelium (10). The present study extends the effect of Provinol treatment in a model of hypertension in which a faster and greater increase of NO synthase activity is also found in the aorta from the Provinol-treated group compared with NO synthase activity in aorta from the spontaneous recovery group. Improved endothelium-dependent vasodilatation is a potential mechanism by which ingestion of Provinol and other red wine polyphenol compounds may reduce cardiovascular risk.

Finally, Provinol treatment not only reversed the increased reactivity of the aortas to norepinephrine-induced contraction by chronic inhibition of NO synthesis but reduced the norepinephrine response compared with that obtained in control rats. We found that RWPC treatment induced expression of inducible NO synthase and cyclooxygenase 2 genes within the arterial wall from normotensive rats, which together main-
tain agonist-induced contractility unchanged (10). A similar mechanism might occur in the present study in vessels from hypertensive animals. The reason why norepinephrine contractility was reduced in Provinol-treated rats after cessation of L-NAME treatment is unknown. It might result from reduced desensitization of smooth muscle guanylyl cyclase to basal release of endothelial NO, from reduced release of vasoconstrictor products from cyclooxygenase, or from the release of other vasodilator factors. We cannot distinguish among these possibilities. Nevertheless, Provinol treatment prevented the increased reactivity of the aorta to norepinephrine after L-NAME treatment.

In conclusion, the present study provided in vivo evidence that oral administration of Provinol induces a faster and more profound decrease of blood pressure in hypertension produced by chronic inhibition of NO synthesis. This effect of Provinol was associated with a regression in myocardial fibrosis even though it did not reduce L-NAME-induced LV hypertrophy. Most interestingly, Provinol treatment reversed the development of aortic wall hyperplasia, improved endothelium-dependent relaxation, and reduced the increased vascular reactivity to vasoconstrictor agonist. The increased NO synthesize activity and reduced tissue protein synthesis may be the key factors for the improvement of structural and functional cardiovascular parameters by Provinol in L-NAME-induced hypertension. All of the above cardiovascular effects of Provinol may contribute to the anti hypertensive and antianteriosclerotic properties of plant-derived polyphenols in vivo.

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