Physiol. Res. 52: 425-432, 2003

Red Wine Polyphenols Induce Vasorelaxation by Increased Nitric Oxide Bioactivity

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Received May 16, 2002
Accepted September 23, 2002

Summary

The aim of the present study was to investigate the mechanism of vasorelaxant responses induced by red wine polyphenolic compounds (Provinol). Rings of rat femoral artery with or without functional endothelium were set up in a myograph for isometric recording and precontracted with phenylephrine (10^{-5} M). Provinol in cumulative doses (10^{-9} to 10^{-3} mg/ml) elicited endothelium- and dose-dependent relaxation of the artery with maximal relaxation of 56 % at the concentration of 10^{-5} mg/ml. The relaxant responses to Provinol correlated well with the increase of NO synthase activity in the vascular tissue after administration of cumulative doses of Provinol (10^{-9} to 10^{-3} mg/ml). N^G-nitro-L-arginine methylester (L-NAME, $3x10^{-4}$ M) significantly attenuated the endothelium-dependent relaxation produced by Provinol. Administration of L-arginine ($3x10^{-5}$ M) restored the relaxation inhibited by L-NAME. The relaxant responses of Provinol were abolished in the presence of Ca^{2+}-entry blocker, verapamil (10^{-6} M). Administration of hydrogen peroxide (H_2O_2) abolished acetylcholine (10^{-5} M)-induced relaxation of the rat femoral artery, while administration of Provinol (10^{-5} mg/ml) together with H_2O_2 helped to maintain the acetylcholine-induced relaxation. Provinol only partially affected the concentration-response curve for the NO donor sodium nitroprusside-induced relaxation in rings without endothelium. In conclusion, Provinol elicited endothelium-dependent relaxation of rat femoral artery by the Ca^{2+}-induced increase of NO synthase activity and by protecting NO from degradation.

Key words

Red wine polyphenolic compounds • Nitric oxide • Free oxygen radicals • Endothelium • Femoral artery

Introduction

The presence of polyphenolic compounds is widespread among plants and plant products (Formica and Regelson 1995, Zenebe and Pecháňová 2002). Several epidemiological studies have shown that consumption of foods rich in polyphenolic compounds is associated with lower incidence of cardiovascular disease. It was hypothesized that the cardioprotective effect of polyphenols results from their ability to protect low-density lipoprotein from oxidation, to prevent platelet aggregation and leukocyte adhesion, and to promote relaxation of vascular smooth muscle (Keli *et al.* 1996, Hertog *et al.* 1997). Polyphenols also act on other targets involved in the metabolism of mammalian cells, including nitric oxide (NO), which by itself regulates hemostasis (Palmer *et al.* 1987), thrombus development (Radomski *et al.* 1987) and vascular tone (Moncada *et al.*

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1991). The beneficial properties of NO may therefore explain, at least in part, the beneficial effects of plant polyphenols.

Recently, several authors have reported that extracts from grapes and wine induce endothelium-dependent relaxation *via* enhanced generation and/or increased biological activity of NO leading to the elevation of cGMP levels (Fitzpatrick *et al.* 1993, Flesch *et al.* 1998). The critical step for the activation of NO synthase in endothelial cells is the increase in Ca^{2+} concentration leading to the production of NO and the subsequent endothelium-dependent vasorelaxation (Andriambeloson *et al.* 1999). The biological activity of NO can be effectively increased by the scavengers of oxygen-free radicals (Bouloumié *et al.* 1997). However, the mechanism leading to the wine extract-induced increase in the concentration of biologically active NO needs further study. Therefore, the aim of the present study was to characterize the mechanism of vasorelaxant activity of polyphenolic compounds isolated from red wine (Provinol). The involvement of endothelial NO in the Provinol-induced relaxation was investigated using NO synthase inhibitor N^G-nitro-L-arginine methylester (L-NAME) and NO synthase substrate L-arginine. NO synthase activity in vascular tissue homogenates was determined after Provinol administration in the cumulative doses. The Ca^{2+}-entry blocker, verapamil, was used to investigate the role of extracellular Ca^{2+} in relaxant responses induced by Provinol. The ability of Provinol to protect NO from degradation was studied using free hydroxyl radicals production system. Finally, sodium nitroprusside was used to analyze the ability of Provinol to prolong the half life of NO produced by NO donor.

Material and Methods

Chemicals and drugs

Dry powder of red wine polyphenolic compounds (Provinol) was provided by D. Ageron (Société Francaise de Distillerie, Vallont Pont d´Arc, France). The composition of Provinol was (in mg/g of dry powder): proanthocyanidins 480, total anthocyanins 61, free anthocyanins 19, catechin 38, hydroxycinnamic acid 18, flavonols 14.

All the chemicals were purchased from Sigma (Germany) except for [^{3}H]-L-arginine (Amersham, United Kingdom).

Preparation of the femoral artery

Male Wistar rats, 12-15 weeks old, were killed by cervical dislocation. The femoral artery was removed and carefully cleaned of adhering fat and connective tissue and then cut into rings (1.5-2 mm long). Two stainless-steel wires were passed through the lumen (taking care not to damage the vessel) and mounted in a Mulvany-Halpern myograph chamber for measuring the isometric wall tension as described elsewhere (Sihm *et al.* 1995). During the experiments, the internal diameter of the vessels was 300-350 μm. The chamber was filled with physiological salt solution (in mM: NaCl 118, KCl 5, $NaHCO_3$ 25, $MgSO_4.H_2O$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, EDTA 1, ascorbic acid 1.1, glucose 11) maintained at 37 °C and continuously bubbled with a 95 % O_2 and 5 % CO_2 mixture. After an equilibration period of 45 min, the rings were precontracted with phenylephrine (10^{-5} M) and tested for the presence of functional endothelium by determining the ability of acetylcholine (10^{-5} M) to induce relaxation greater than 30 %. In some experiments, the endothelium was carefully removed as described elsewhere (Babál *et al.* 2000). The extent of relaxation was expressed as the percentage of phenylephrine-induced contraction.

Analysis of Provinol-induced relaxation

Rings of the rat femoral artery were precontracted with phenylephrine (10^{-5}M). Provinol in concentrations 10^{-9}-10^{-3} mg/ml were added when contraction reached the steady-state.

In order to characterize the role of endothelium-derived NO in the Provinol-induced relaxation, arteries were exposed to the NO synthase inhibitor, L-NAME ($3x10^{-4}$ M) added to the chamber 20 min before phenylephrine. To test the ability of L-arginine to restore relaxation responses blocked by L-NAME, femoral arteries were incubated with L-arginine ($3x10^{-5}$ M) for 10 min before administration of L-NAME into the bath. The source of Ca^{2+} involved in the relaxation induced by Provinol, was analyzed by testing its effect in the presence of Ca^{2+}-entry blocker, verapamil (10^{-5} M).

To examine the hydroxyl radical scavenging activity of Provinol, arteries were incubated with 10 mM H_2O_2 for 15 min before phenylephrine administration, and then relaxation responses to acetylcholine (10^{-5} M) were tested. The same concentration of hydrogen peroxide was added to the chamber in which arteries were primarily exposed to Provinol (10^{-5} mg/ml) for 20 min and then relaxation responses to acetylcholine were assessed. At the end the relaxations were compared.

To test the hypothesis that Provinol prolongs the half life of NO, the concentration response curve to sodium nitroprusside (10^{-9}-10^{-5}M) was assessed in arteries without endothelium, in the absence and presence of Provinol (10^{-5} mg/ml).

Determination of NO synthase activity

NO synthase activity was determined in crude homogenates of vascular tissue by measuring the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine (Amersham, UK) as previously described by Bredt and Snyder (1990) with minor modifications. Briefly, 50 µl of crude homogenate (30 mg of wet tissue) was incubated in the presence of 50 mM Tris-HCl, at pH 7.4, containing 1 µM L-[^{3}H]arginine (specific activity 5 GBq/mmol, about 100 000 DPM), 0.5 mg/ml calmodulin, 0.5 mM β-NADPH, 250 µM tetrahydrobiopterin, 4 µM FAD, 4 µM FMN and 1 mM $CaCl_2$ in a total volume of 100 µl. In some samples Provinol was added to the incubation medium in the concentration range 10^{-9} to 10^{-3} mg/ml. After 10-min incubation at 37 °C, the reaction was stopped, the samples were centrifuged and supernatants were applied onto 1 ml Dowex 50WX-8 columns (Na^+ form). [^{3}H]-L-citrulline was eluted by 2 ml of water and determined by liquid scintilation counting. NO synthase activity was expressed as pkat/g of proteins.

Statistical analysis

The results were expressed as mean ± S.E.M.. Values were considered to differ significantly if the P value was less than 0.05. For analysis one-way ANOVA and Bonferroni test were used.

Results

Relaxant effect of Provinol

Provinol (10^{-9} to 10^{-3} mg/ml) elicited dose-dependent relaxation of rat femoral artery with intact endothelium the maximal relaxation being reached at the concentration of 10^{-5} mg/ml (56 %). Provinol did not relax denuded rings of the femoral artery. Inhibition of endothelial NO synthesis with L-NAME abolished relaxation induced by Provinol. Addition of L-arginine along with L-NAME restored relaxation responses of the femoral artery to control levels (Fig. 1).

Relaxation of femoral artery induced by Provinol was abolished in the presence of the Ca^{2+}-entry blocker, verapamil (Fig. 2).

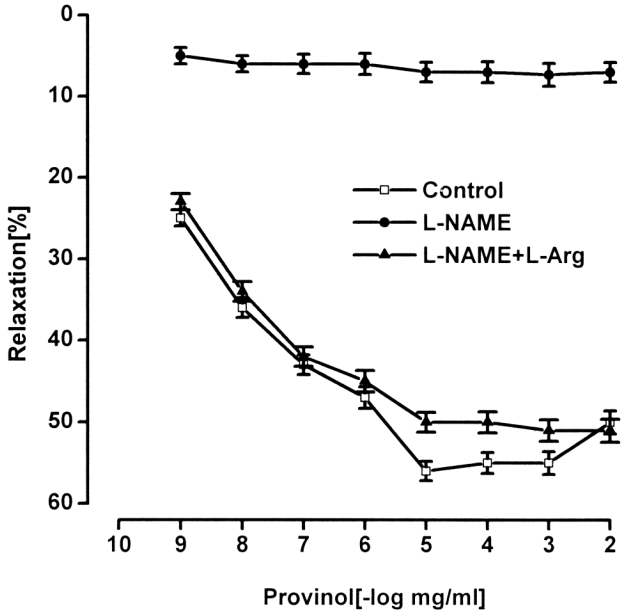

Fig. 1. *Relaxation responses of femoral artery to Provinol administration. Relaxation responses to Provinol (open squares), to Provinol with L-NAME (full circles) and to Provinol with L-arginine and L-NAME (full triangles).*

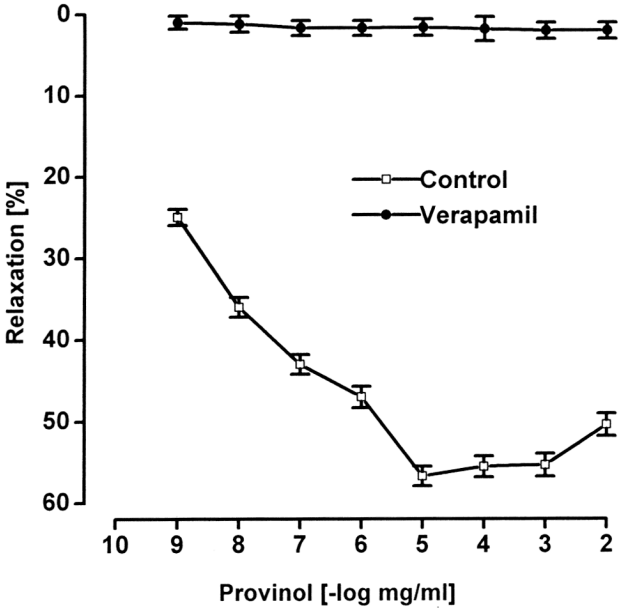

Fig. 2. *Influence of the Ca^{2+} entry blocker verapamil on the relaxation responses of rat femoral artery to Provinol administration. Responses before (open squares) and after (full circles) verapamil administration.*

Administration of H_2O_2 abolished relaxation responses to acetylcholine. Pretreatment of the femoral artery with Provinol (10^{-5} mg/ml) prior to H_2O_2 administration improved the relaxation response of the femoral artery to acetylcholine (Fig. 3).

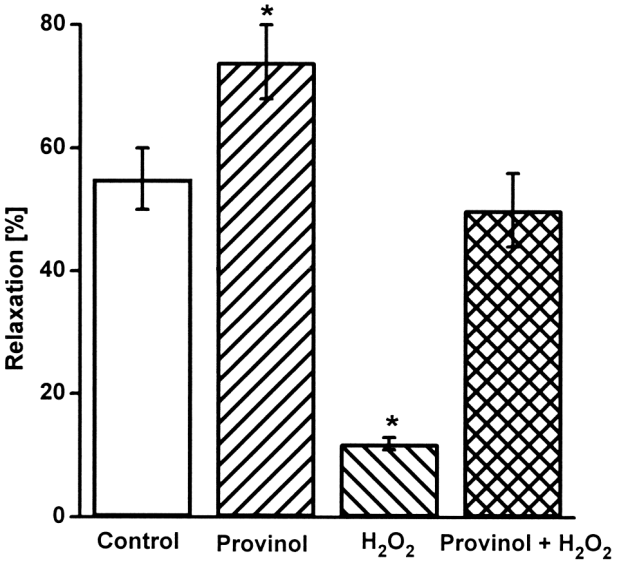

Fig. 3. *Relaxation responses of rat femoral artery to acetylcholine. Control relaxation responses (Control), after exposure of the artery to Provinol (Provinol), after the incubation of the artery with H_2O_2 (H_2O_2) and after administration of Provinol followed by H_2O_2 (Provinol + H_2O_2). * $p<0.05$ as compared to control.*

Provinol at the concentration causing maximal relaxation (10^{-5} mg/ml) partially affected the concentration-response curve for the NO donor sodium nitroprusside-induced relaxation in rings without the endothelium. Although Provinol was not able to affect the maximal relaxation produced by sodium nitroprusside, it significantly shifted the concentration-response curve to the left (Fig. 4).

Nitric oxide synthase activity

In vascular tissue homogenates, NO synthase activity was 5.17±0.31 pkat.g^{-1} protein before the administration of Provinol. Administration of Provinol (10^{-9}-10^{-4} mg/ml) increased NO synthase activity in a dose-dependent manner. The maximal activity of NO synthase was recorded with Provinol at the concentration of 10^{-4} mg/ml and represented 11.20±0.46 pkat.g^{-1} protein (Fig. 5).

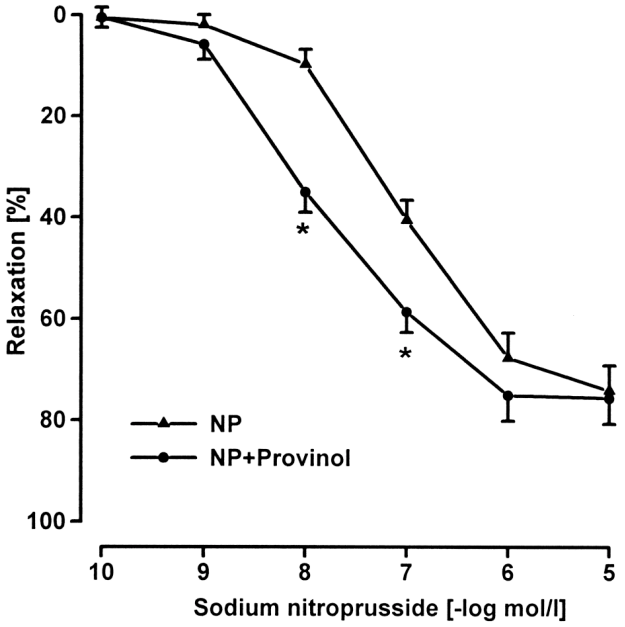

Fig. 4. *Concentration-response curve to NO donor sodium nitroprusside before Provinol administration ((full triangles) and after Provinol administration (full circles).* $p<0.05$ as compared to the relaxation before Provinol administration.*

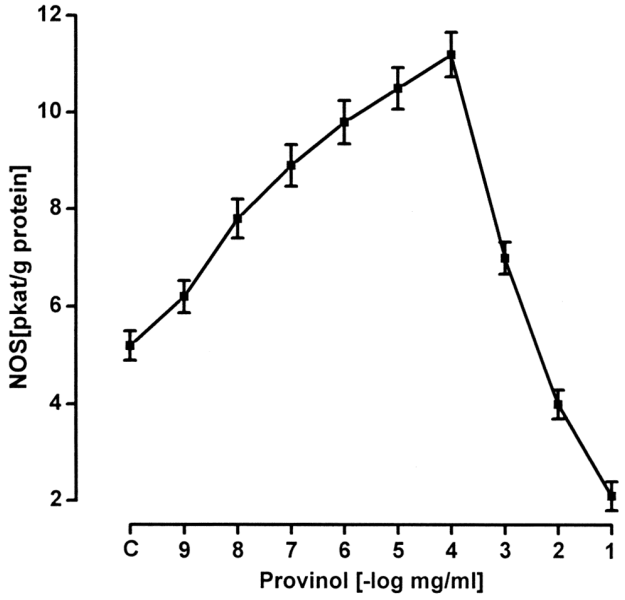

Fig. 5. *NO synthase activity in vascular tissue. NO synthase activity after administration of cumulative doses of Provinol.*

Discussion

The present study provided evidence that Provinol elicits endothelium-dependent relaxation in the

rat femoral artery. The fact that the relaxation abolished by L-NAME was restored by L-arginine confirmed the involvement of NO in the endothelium-dependent vasorelaxation induced by Provinol. Determination of NO synthase activity in the vascular tissue demonstrated that administration of Provinol at the concentration of 10^{-4} to 10^{-9} mg/ml increased the activity dose-dependently. The maximal activation of NO synthase was reached at the concentration of 10^{-4} mg/ml which correlated well with the maximal relaxation of the femoral artery induced by Provinol at the concentration of 10^{-5} mg/ml. The presence of verapamil abolished Provinol-induced relaxation, suggesting that Ca^{2+} entry into the endothelial cells was the crucial step in the relaxant responses induced by Provinol. We assumed that increased intracellular concentration of Ca^{2+} within the endothelial cells after Provinol administration was able to activate NO synthase and to relax the artery.

Moreover, Provinol at a concentration producing the maximal endothelium-dependent relaxation, restored the relaxation of the femoral artery to acetylcholine abolished by H_2O_2 and enhanced partially the relaxant responses of sodium nitroprusside suggesting the ability of Provinol to preserve NO from degradation.

In agreement with our observations, Fitzpatrick *et al.* (1993) reported the relaxation of intact rat aortic rings induced by a red grape skin extract. Similarly to our experiments, relaxation was abolished by L-NAME and reversed by L-arginine indicating the involvement of NO in the relaxant responses. Similarly to the grape skin extract, red wine polyphenolic compounds also caused a dose-dependent relaxation in the rabbit aorta with intact endothelium (Cishek *et al.* 1997). The authors documented that relaxation responses were associated with an increase in cGMP content and were abolished by L-NAME. Flesch *et al.* (1998) also documented increased concentration of the cGMP content in rat aortic rings after exposure to phenolic grape ingredients and red barrique wines. Since guanylate cyclase operates as an intracellular receptor for NO (Lancaster 1992), it is possible that increased concentration of NO was responsible for the enhancement of cGMP levels, which is in agreement with our finding of increased NO synthase activity after Provinol administration. Adriambeloson e*t al.* (1997) also demonstrated that some polyphenols induce endothelium-dependent relaxations *via* enhancement of endothelial NO synthesis. The increase in intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) represents the critical step for the activation of NO synthase in the endothelial cells leading to the production of NO and the subsequent endothelium-dependent vasorelaxation (Lückhoff *et al.* 1988). This increase in $[Ca^{2+}]_i$ can be due to either an influx of extracellular Ca^{2+} or a release of Ca^{2+} from intracellular stores. In our study, the relaxation produced by Provinol was completely prevented in the presence of the Ca^{2+} entry blocker, verapamil, suggesting that the Ca^{2+} influx was crucial for the relaxation ability of the red wine polyphenolic compounds. Similarly, Andriambeloson *et al.* (1999) reported that red wine polyphenolic compounds produced NO-dependent vasorelaxation of rat aortic rings by an extracellular Ca^{2+}-dependent mechanism. However, it cannot be excluded that a release of Ca^{2+} from intracellular stores might play a role in the endothelial NO-dependent relaxation produced by polyphenolic compounds. Indeed, after administration of red wine polyphenolic compounds to the endothelial cell culture, Martin *et al.* (2002) documented an increase of $[Ca^{2+}]_i$ from the intracellular stores, that was sensitive to the phospholipase C inhibitor.

Since Provinol used in our study was a mixture of different polyphenolic compounds, it is not certain which type of the phenolic components is responsible for the vasorelaxant responses. The molecular identity of the polyphenolic compounds responsible for this effect of Provinol probably belong to oligomeric condensed tannins and anthocyanins that were showed to mediate the *ex vivo* endothelial NO-induced relaxation of aortic rings (Andriambeloson *et al.* 1999). The combination of different phenolic compounds might also be the reason for the vasorelaxant effect of Provinol, similarly to the reported antioxidant activity of a mixture of two flavonoids citrin and hesperdin (Scarborough and Bacharach 1949, Kühnau 1976). Fitzpatrick *et al*. (1993) showed that monomers malvidine and resveratrol at concentrations of up to 0.1 mM did not relax aortic rings while tannic acid produced endothelium-dependent L-nitro-arginine inhibitable relaxations. Quercetin only produced slowly developing relaxation in intact aortic rings, but this relaxation was not affected by NO synthase inhibitor. Analogically, Duarte *et al.* (1993) reported that the monomers catechin, epicatechin and quercetin did not exhibit endothelium-dependent relaxation. Although Huang *et al.* (1999) demonstrated epicatechin-induced endothelium-dependent vasorelaxation in rat mesenteric arteries, it seems that polymeric rather than monomeric phenols are responsible for NO-dependent relaxation.

There are at least two mechanisms by which polyphenolic compounds could influence NO release: the described stimulation of NO synthase activity and

preservation or stabilization of NO release under basal conditions. The latter mechanism includes protection of NO from destruction by superoxides and other free radicals (Schuldt *et al.* 2000, Zavillová *et al.* 2001). The antioxidant activity of polyphenols in red wine, grape, green and black tea had been reported by different authors, who showed their inhibitory effect on human low-density lipoprotein oxidation (Frankel *et al.* 1993, Fuhrman *et al.* 1997, Serafini *et al.* 1998). Duthie and Crozier (2000) also reported that most flavonoids are effective antioxidants in a wide range of chemical oxidation systems being capable of scavenging peroxyl radicals, hydroxyl radicals and peroxynitrite. In contrast to these reports, Caccetta *et al.* (2000) did not observe an effect on *ex vivo* lipoprotein oxidation despite an increased plasma phenolic concentration after red wine consumption. In our work Provinol restored relaxation of the femoral artery to acetylcholine which was abolished by H_2O_2. This finding clearly demonstrated that Provinol had an *in vitro* antioxidative effect. Moreover, Provinol partially affected the concentration response curve for the NO donor sodium nitroprusside-induced relaxation in rings without endothelium. Both effects were associated with decreased degradation of NO resulting in the improvement of vasorelaxant responses. In agreement with our results, Diebolt *et al.* (2001) showed that the relaxant effect of red wine polyphenolic compound involved a mechanism sensitive to superoxide anion scavengers and Schuldt *et al.* (2000) reported that polyphenols were able to scavenge free oxygen radicals to a limited extent resulting in an increased NO level. However, Andriambeloson *et al.* (1997) showed that red wine polyphenolic compounds at the concentration producing maximal endothelium-relaxation caused no modification of the 3-morpholino-sydnonimine concentration-effect curve. The use of different NO-donors and different polyphenolic substances may explain the observed difference in vasorelaxant effects. Although several authors reported that the phenolic compounds with the antioxidant effect and those with vasorelaxant effect differ in structure, Provinol possesses both antioxidative and vasorelaxant effects.

In conclusion, our findings indicate that Provinol elicited endothelium-dependent relaxation by stimulation of NO synthase activity concomitantly with scavenging of oxygen free radicals, which together leads to the enhancement of NO concentration.

Acknowledgements

This work was supported by The Slovak Grant Agency for Science (grants No. 2/7165/20, 2/7240/20 and 2/3185/23) and by Société Française de Distilleries.

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