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ORIGINAL CONTRIBUTION

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Complementary cardioprotective effects of flavonoid metabolites and terpenoid constituents of Ginkgo biloba extract (EGb 761) during ischemia and reperfusion

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Abstract Hemodynamic and electron spin resonance (ESR) analyses were performed on isolated ischemic and reperfused rat hearts to assess the cardioprotective and antioxidant effects of therapeutically relevant concentrations of *Ginkgo biloba* extract (EGb 761; 5, 50 or 200 µg/ml), its terpenoid constituents (ginkgolide A; 0.05 µg/ml and ginkgolide B; 0.05, 0.25 or 0.50 µg/ml), and a terpene-free fraction of EGb 761 (CP 205; 5 or 50 µg/ml). Hearts underwent 10 min of low-flow ischemia, 30 min of no-flow global ischemia, and 60 min of reperfusion. Test substances were added to the perfusion fluid during the last 10 min of control perfusion, low-flow ischemia and the first 10 min of reperfusion. A separate group of rats was treated with CP 205 (60 mg/kg/day; p.o.) for 15 days, after which the hearts were perfused with plain buffer. In ESR experiments, the spin-trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was added to the perfusate to determine the effects of treatments on post-ischemic myocardial free radical generation. Results showed that in vitro exposure of hearts to EGb 761 (5 or 50 µg/ml) or to ginkgolides A and B (both at 0.05 µg/ml), or in vivo pretreatment of the rats with CP 205 delayed the onset of contracture during ischemia. The strong reperfusion-induced elevation of left ventricular end-diastolic pressure observed in untreated hearts was significantly reduced by in vitro exposure to the lowest concentrations of EGb 761, by ginkgolide A, and to a lesser extent by ginkgolide B, or by prior oral treatment with CP 205. Post-ischemic functional recovery was significantly improved by in vivo administration of CP 205, by perfusion with 5 µg/ml of EGb 761 or with both terpenoids as compared to untreated group but in vitro CP 205 was not effective. ESR analyses revealed that DMPO-OH (the DMPO / hydroxyl radical spin-adduct) concentrations in coronary effluents were markedly decreased by all treatments, except for the lowest concentration of ginkgolide B. Perfusing 5 µg/ml EGb 761 resulted in a better inhibition of baseline DMPO-OH concentration than 5 µg/ml CP 205 (–70 % and –48 % vs. control, respectively), indicating that both terpenoid and flavonoid constituents of EGb 761 are required to produce this effect. CP 205 was significantly more efficient in reducing DMPO-OH concentration when administered in vivo than when applied in vitro, indicating that the antioxidant effect of flavonoid metabolites (formed in vivo) is superior to that of intact flavonol glycosides (present in vitro). Collectively, these findings provide the first evidence that part of the cardioprotection afforded by EGb 761 is due to a specific action of its terpenoid constituents and that this effect involves a mechanism independent of direct free radical-scavenging. Thus, the terpenoid constituents of EGb 761 and the flavonoid metabolites that are formed after in vivo administration of the extract act in a complementary manner to protect against myocardial ischemia-reperfusion injury.

Key words *Ginkgo biloba* extract (EGb 761) – Langendorff heart preparation – myocardial reperfusion injury – flavonoids – ginkgolides – spin-trapping

Abbreviations used: ESR, electron spin resonance; EGb 761, *Ginkgo biloba* extract; CP 205, fraction of EGb 761 devoid of terpenes; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; TEMPO, 2,2,6,6-tetramethylpiperidin-1-yloxyl; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; dP/dt, first derivative of left ventricular developed pressure with time; RPP, rate pressure product; PAF, platelet-activating factor.

Introduction

Among the extracts of dried leaves of *Ginkgo biloba* that are used therapeutically, the standardized extract termed "EGb 761" is one of the most widely employed medicinal plant products in Europe. Numerous clinical studies have delineated a wide array of indications for EGb 761, including conditions associated with age-related physical and mental deterioration, cerebral vascular insufficiency and peripheral arterial occlusive disease (reviewed in 4, 5). The results of recent clinical trials support new indications for EGb 761 in the treatment of cardiovascular disease, particularly in the prevention of ischemic heart syndromes (5, 23). Thus, pretreatment with EGb 761 improved the clinical outcome following cardiopulmonary bypass surgery (23), supporting the hypothesis that an antioxidant can inhibit free radical-mediated processes that are involved in myocardial ischemia-reperfusion injury (reviewed in 11, 21). In this regard, the effects of EGb 761 of scavenging hydrogen peroxide and free radical species of biological importance such as superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical (HO•), nitric oxide (NO) and peroxyl radicals have been assessed in several chemical and cellular systems (3, 8, 9, 13, 16, 19, 20, 28, 29, 37). In contrast, only a few studies have addressed the effects that in vivo administration of EGb 761 might exert on myocardial reperfusion injury in experimental animals (8, 31, 34, 35), and in such studies only concentrations of EGb 761 in excess of those that would be achieved with a therapeutic dose of the extract were tested and found to confer significant protection, as assessed by hemodynamic, biochemical, or electron spin resonance (ESR) indices of reperfusion injury (8, 31, 34).

Recent advances concerning the pharmacokinetics and bioavailability of the flavonoid and terpenoid constituents of EGb 761 following its oral administration to experimental animals or humans (1, 6, 26, 27, 36) have allowed us to examine the effects of therapeutically relevant in vivo doses (or in vitro concentrations) of EGb 761 and three of its terpenoid constituents, ginkgolides A and B and bilobalide, on hemodynamic and ESR indices of myocardial reperfusion injury (24). It was discovered that a significant part of the hemodynamic and antioxidant protection afforded by in vitro perfusion or in vivo administration of EGb 761 in ischemic-reperfused rat hearts was related to an action of highly bioavailable terpene trilactones (especially ginkgolide A), and that such protection involved their inhibition of free radical formation rather than direct free radical-scavenging (24). These data prompted us to reexamine the general assumption that the cardioprotective effects of EGb 761 are related mainly to the direct free radical-scavenging properties of its flavonoid constituents.

In the present study, rat isolated hearts were examined using experimental perfusion protocols, hemodynamic monitoring and ESR spin-trapping techniques that were identical to those described previously (24), except that the ischemia-reperfusion sequence was slightly modified (including more severe ischemia). The effects of CP 205 (a terpene-free fraction of EGb 761) on post-ischemic functional recovery and free radical generation were determined with the aim of establishing whether a link may exist between antioxidant and cardioprotective effects. The same indices were also measured in in vitro perfusion experiments conducted with increasing concentrations of EGb 761 and ginkgolide B, and the results were compared with those obtained with ginkgolide A which was taken as the reference terpene trilactone.

Materials and methods

Drugs and chemicals

EGb 761, ginkgolide A (BN 52020), ginkgolide B (BN 52021), and CP 205 (an extract corresponding to EGb 761 that is devoid of terpene trilactone constituents) were kindly provided by the Institut Henri Beaufour (Paris, France). All of these test substances were used in their water-soluble forms. The nitrone 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and the free radical standard 2,2,6,6-tetramethylpiperidin-1-yloxyl (TEMPO) were obtained from Aldrich-Sigma (Saint Quentin Fallavier, France). Aqueous DMPO stock solutions were purified and checked for diamagnetism as previously described (22). All other reagents were of analytical grade.

Animals and conditions for isolated heart perfusion

Male Wistar rats, weighing 300–350 g, which were randomly assigned to one control group and seven treatment groups, were used. Anesthesia and surgical procedures for Langendorff perfusion and monitoring of hemodynamic parameters have been previously described in detail (24). The perfusion fluid, a modified Krebs-Henseleit bicarbonate-buffered medium, contained: 25.0 mM $NaHCO_3$, 119.0 mM NaCl, 4.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.5 mM EDTA, and 10 mM glucose, and it was continuously gassed with 95 % O_2 / 5 % CO_2 to maintain its pH at 7.4. Prior to use, the perfusion medium was filtered through a 0.22 µm Millipore filter. Myocardial contractions were monitored by means of a noncompressible balloon introduced into the left ventricle and coupled to a Gould Statham P23 pressure transducer. The balloon was inflated until left ventricular end-diastolic pressure (LVEDP) was in the range of 8–12 mmHg and the volume (60–70 µl) was held constant throughout the experiment. The output signal, adapted in impedance and amplified with a home-made adaptor-amplifier, was digitalized using a data acquisition and control card (Advantech, PCL-711S PC-Multilab, USA). The hemodynamic data were processed in real time on a personal computer using a software developed in our laboratory and stored in a standard ASCII format. LVEDP, left ventricular developed pressure (LVDP) and its first derivative (LV dP/dt), and heart rate were computed at 5-min intervals by signal averaging 10 s-blocks. Rate pressure product (RPP), an index of overall cardiac mechanical recovery, was calculated as the product of heart rate and LVDP. Coronary flow was measured by collecting the coronary perfusate over a known period of time.

Experimental protocol and groups

To assess the effects of in vitro administration of test substances on hemodynamic parameters, hearts were allowed to equilibrate for 30 min at 37 °C and were then subjected to 10 min of low-flow ischemia (30–35 % of control coronary flow), followed by 30 min of no-flow normothermic global ischemia and 60 min of reperfusion. In six in vitro groups of hearts (n = 12 / group) each test substance was added to the perfusion medium during the last 10 min of control perfusion, the entire period of low-flow ischemia and the first 10 min of reperfusion. Concentrations used in in vitro experiments were 5, 50 or 200 µg/ml for EGb 761, 0.05 µg/ml for ginkgolides A and B, and 5 µg/ml for CP 205. Hearts from the untreated group (n = 13) were perfused with plain buffer. The lowest EGb 761 concentration (i.e., 5 µg/ml) and the concentrations of ginkgolides and CP 205 that were tested were considered to represent their actual circulating concentrations following in vivo treatment of rats with a therapeutically relevant dose of EGb 761, estimated to be 60 mg/kg/day (1, 6, 24).

In experiments involving in vivo administration, a group of 12 rats received CP 205 (60 mg/kg/day) orally for 15 days. On the last day of treatment, the animals were anesthetized and their hearts were perfused with plain buffer according to the ischemia-reperfusion protocol described above.

The investigation conformed to the *Guide for the care and use of laboratory animals*, published by the US National Institutes of Health (N.I.H. publication 85–23, revised 1985).

Spin-trapping experiments

The effects of the test substances on post-ischemic myocardial free radical generation were examined using ESR spin-trapping. Eight experimental groups of hearts that had been randomly treated in the same manner as the eight experimental groups described above and three additional in vitro groups of hearts that were perfused with CP 205 at 50 µg/ml or with ginkgolide B at 0.25 µg/ml or 0.50 µg/ml were studied (n = 6 in each group). The perfusion conditions and the ischemia-reperfusion sequence were identical to those described for hemodynamic experiments, except that the Krebs-Henseleit buffer did not contain EDTA. The spin-trap DMPO (final concentration, 28 mM) was added to the perfusion fluid during the last 3 min of the control period and the first 5 min of reperfusion using a special device that prevented the formation of radical artifacts (22). After 4 min of reperfusion, coronary effluents were collected for 15 s, immediately frozen in liquid N_2, and subsequently thawed under identical conditions for ESR analysis at 22°C. ESR spectra were acquired at 9.81 GHz in 50-µl calibrated glass capillaries (Corning Inc., New York, USA) using a Bruker ESP 300 spectrometer (Karlsruhe, Germany) equipped with a 100 kHz field modulation frequency. The instrumental settings were: microwave power 10 mW, gain 3.2 x 10^5, modulation amplitude 0.05 mT, time constant 20.48 ms, and sweep rate, 0.36 mT/s for a sweep width of 7.5 mT. Hyperfine coupling constants and g-factors were measured using a Bruker ER 035M gaussmeter and a Hewlett-Packard 5350B frequency counter, respectively. Spin-adduct concentrations in coronary effluents were estimated by double integration of the ESR signals and comparison with that of a 0.6 µM solution of TEMPO in 10 mM phosphate buffer.

Statistical analyses

Hemodynamic data (absolute values, or percent recovery of pre-ischemic values) are presented as means ± SEM. Statistical analyses were performed using a two-way analysis of variance (ANOVA) with repeated measures. If a difference

was found ($p < 0.05$), experimental groups were compared further using the Newman-Keuls test. A one-way ANOVA was then carried out to test for differences among the mean values of all groups at every time point, and this was followed by a Duncan test. ESR data are expressed as means ± SEM. Intergroup differences were evaluated using a one-way ANOVA, followed by a Duncan test. Differences having values of $p < 0.05$ were considered to be significant.

Results

Hemodynamic experiments

Among the perfused hearts, less than 5 % remained in irreversible fibrillation upon reperfusion and did not achieve a steady state level of functional recovery (i.e., RPP < 3 % of preischemic value); these hearts were excluded from the study. During the initial 30 min of normoxic perfusion, the experimental groups showed no significant intergroup differences in baseline hemodynamic performance, except for coronary flow which was improved following in vitro perfusion with EGb 761 (50 µg/ml) or after oral administration of CP 205 ($p < 0.02$ vs. untreated group in both cases; see Table 1). After 10 min of low-flow ischemia, a general decrease in ventricular function was observed in all groups, RPP values ranging from 34.3 to 42.7 % of pre-ischemic values with no significant intergroup differences. No concomitant increase in LVEDP was found in any group (data not shown). After global ischemia, the contractile activity of the hearts was markedly decreased in all groups. Systolic oscillations of less than 2 mmHg in amplitude affected only 3 % of the hearts of each group during the first 15 min of ischemia, but thereafter all hearts remained asystolic. Diastolic contracture, as assessed by LVEDP measurements, gradually increased in all groups during ischemia, culminating at 53.3–59.3 mmHg after 18–20 min in the untreated group and in groups of hearts perfused with either CP 205 or with the high EGb 761 concentration of 200 µg/ml. In vitro exposure of hearts to either EGb 761 at lower concentrations (5 and 50 µg/ml) or to ginkgolides, or in vivo pretreatment of the rats with CP 205 delayed the onset of contracture by at least 5 min ($p < 0.02$), the maximum LVEDP values being significantly decreased to 44.4–47.5 mmHg ($p < 0.02$). As shown in Table 1, this pattern remained unchanged upon terminating ischemia.

Table 2 displays hemodynamic variables after 10 min (taken as early reperfusion) and at the end of reflow (60 min of reperfusion). Due to the severity of the ischemic periods, values for LVEDP in the untreated group, which peaked within the first 5 min of reperfusion, declined slowly, and the untreated hearts remained in intense ventricular contracture after 60 min of reflow (Table 2; Fig. 1A). This reperfusion-induced elevation of LVEDP was significantly limited over the entire reperfusion by in vitro application of the two low concentrations of EGb 761, by ginkgolide A, and to a lesser extent by ginkgolide B, or by oral (in vivo) treatment with CP 205,

Table 1 Effect of treatments on global hemodynamic variables after 30 min of normoxia and on LVEDP at the end of ischemic periods (10 min low-flow ischemia + 30 min global ischemia) in rat hearts

Group	LVEDP (mmHg)	LVDP (mmHg)	RPP (mmHg.beats/min)	LV dP/dt (mmHg/s)	CF (ml/min/g wet wt)
Untreated	11.7 ± 1.2 (43.8 ± 3.1)	122.6 ± 6.0	29163 ± 1461	3943 ± 100	14.5 ± 0.8
EGb 761 (5 µg/ml)	9.6 ± 1.9 (38.9 ± 1.7)[abc]	113.1 ± 3.5	28207 ± 704	3851 ± 88	14.7 ± 0.8
EGb 761 (50 µg/ml)	11.2 ± 1.2 (34.0 ± 1.7)[abc]	124.7 ± 6.8	30218 ± 2501	4157 ± 134	16.9 ± 0.8[a]
EGb 761 (200 µg/ml)	8.9 ± 1.7 (44.8 ± 2.1)	107.6 ± 3.9	28318 ± 1307	3820 ± 130	15.5 ± 1.0
Ginkgolide A (0.05 µg/ml)	9.4 ± 1.7 (36.2 ± 2.0)[abc]	117.4 ± 5.1	28531 ± 1079	3930 ± 67	15.3 ± 0.4
Ginkgolide B (0.05 µg/ml)	10.0 ± 1.4 (39.1± 2.1)[bc]	117.0 ± 3.5	28050 ± 1027	3854 ± 67	15.7 ± 0.9
CP 205 (5 µg/ml)	10.9 ± 1.6 (43.8 ± 1.2)	119.1 ± 4.0	28626 ± 1185	3851 ± 71	15.9 ± 0.6
CP 205 (60 mg/kg orally)	10.6 ± 1.6 (35.5 ± 2.4)[abc]	130.2 ± 5.6	29830 ± 1503	3927 ± 79	18.3 ± 0.4[a]

n = 13 in untreated group and n = 12 for all other groups, means ± SEM. Data in parentheses correspond to values at the end of ischemia. LVEDP = left ventricular end-diastolic pressure; LVDP = left ventricular developed pressure; RPP = rate pressure product; LV dP/dt = first derivative of LVDP with time; CF = coronary flow. One-way ANOVA ($p < 0.05$) followed by Duncan test: [a]$p < 0.02$ vs. untreated; [b]$p < 0.02$ vs. EGb 761 (200 µg/ml); [c]$p < 0.02$ vs. oral CP 205

Table 2 Effect of treatments on hemodynamic variables after early (10 min) and late (60 min) reperfusion of rat hearts

Group	LVEDP (mmHg)	LVDP (% of pre-ischemic value)	RPP	LV dP/dt	CF
	Reperfusion 10 min				
Untreated	101.5 ± 5.5	7.1 ± 2.7	4.5 ± 1.9	6.4 ± 2.1	35.5 ± 3.1
EGb 761 (5 µg/ml)	85.1 ± 4.5[a]	27.4 ± 3.5[a]	14.4 ± 2.4[a]	20.5 ± 2.9[a]	41.2 ± 3.7[ab]
EGb 761 (50 µg/ml)	76.3 ± 4.1[a]	17.7 ± 3.6	9.8 ± 1.7	16.4 ± 3.4[a]	32.9 ± 4.4[b]
EGb 761 (200 µg/ml)	90.0 ± 4.0	18.6 ± 5.2	6.7 ± 3.3	13.9 ± 4.5	23.1 ± 3.0[a]
Ginkgolide A (0.05 µg/ml)	83.0 ± 4.2[a]	30.8 ± 3.2[a]	13.9 ± 2.3[a]	23.8 ± 3.7[a]	42.1 ± 3.9[ab]
Ginkgolide B (0.05 µg/ml)	87.2 ± 3.8	23.6 ± 3.5[a]	12.0 ± 2.2[a]	18.6 ± 2.3[a]	37.9 ± 3.1
CP 205 (5 µg/ml)	88.7 ± 3.1	7.6 ± 2.3	5.6 ± 2.2	7.6 ± 2.3	43.4 ± 4.5[ab]
CP 205 (60 mg/kg orally)	85.8 ± 3.4[a]	10.9 ± 3.5	11.2 ± 1.6[a]	9.2 ± 3.2	51.2 ± 3.6[ab]
	Reperfusion 60 min				
Untreated	77.6 ± 4.5	25.9 ± 3.6	17.8 ± 2.3	25.5 ± 3.3	34.8 ± 3.5
EGb 761 (5 µg/ml)	58.2 ± 5.4[a]	43.7 ± 3.3[a]	30.1 ± 2.4[a]	41.0 ± 4.8[a]	43.7 ± 3.4[ab]
EGb 761 (50 µg/ml)	59.4 ± 4.5[a]	27.5 ± 4.2	18.8 ± 2.5	25.8 ± 7.6	31.0 ± 2.8[b]
EGb 761 (200 µg/ml)	66.7 ± 4.5	30.3 ± 6.8	21.1 ± 2.3	33.9 ± 5.6	23.8 ± 2.4[a]
Ginkgolide A (0.05 µg/ml)	55.3 ± 3.6[a]	46.4 ± 3.1[a]	34.0 ± 2.8[a]	41.1 ± 4.0[a]	43.7 ± 3.1[ab]
Ginkgolide B (0.05 µg/ml)	68.5 ± 3.5[a]	36.3 ± 3.9[a]	24.0 ± 2.9[a]	34.1 ± 2.9[a]	36.6 ± 3.0[b]
CP 205 (5 µg/ml)	74.0 ± 2.7	30.3 ± 5.6[d]	22.7 ± 3.4[d]	30.9 ± 5.3[d]	33.1 ± 3.4[bd]
CP 205 (60 mg/kg orally)	63.8 ± 2.5[a]	32.7 ± 4.4[a]	28.9 ± 2.4[a]	34.1 ± 4.3[a]	52.0 ± 3.5[ab]

n = 13 in untreated group and n = 12 for all other groups, means ± SEM. LVEDP = left ventricular end-diastolic pressure; LVDP = left ventricular developed pressure; RPP = rate pressure product; LV dP/dt = first derivative of LVDP with time; CF = coronary flow. One-way ANOVA ($p < 0.05$) followed by Duncan test: [a]$p < 0.02$ vs. untreated; [b]$p < 0.02$ vs. EGb 761 (200 µg/ml); [d]$p < 0.02$ vs. ginkgolide A

as compared to the untreated group (Fig. 1; Table 2). In marked contrast, in vitro reperfusion of hearts in the presence of CP 205 (5 µg/ml) did not significantly improve post-ischemic cardiac function, and subjecting the hearts to the highest concentration of EGb 761 (200 µg/ml) was only slightly effective (not significant) in limiting the increase in reperfusion-induced LVEDP (Fig. 1; Table 2).

As expected, hearts from the untreated group showed an increase in coronary vascular resistance, as assessed by the time course of coronary flow recovery which followed a pattern parallel to that of LVEDP. In these untreated hearts, coronary flow reached only 34.8 % of the pre-ischemic value after 60 min of reperfusion (Table 2). Of the treatments tested, only in vitro perfusion with the lowest concentration of EGb 761 or with ginkgolide A or in vivo administration of CP 205 significantly improved the recovery of coronary flow, as compared to the untreated group (Table 2). This beneficial effect on post-ischemic coronary flow did not occur when the concentration of flavonoid compounds in the perfusion fluid was increased; i.e., with 5 µg/ml of CP 205 or 50 µg/ml of EGb 761. Perfusion with the highest concentration of EGb 761 (200 µg/ml) caused a significant worsening of post-ischemic coronary flow (Table 2).

In untreated hearts, values for RPP, LVDP, and dP/dt were markedly decreased by reperfusion, as compared to respective pre-ischemic values (see Fig. 2; Table 2). These hemodynamic variables were significantly preserved by in vivo administration of CP 205 and by in vitro perfusion with 5 µg/ml of EGb 761 or with both terpene trilactones. In vitro perfusion with 50 µg/ml or 200 µg/ml of EGb 761 or with 5 µg/ml of CP 205 provided only slight and non-significant effects on functional hemodynamic parameters at reflow (Table 2; Fig. 2).

Measurements of free radical generation

For ESR analyses, 15-s samples of coronary effluents were collected after 4 min of reperfusion since strong spin-adduct formation has been demonstrated at this time in untreated rat hearts that were subjected to a similar ischemia-reperfusion sequence (24). In all groups of hearts, no ESR signal was observed when coronary effluent samples were collected before inducing ischemia, whereas all samples collected at reflow exhibited the characteristic 1:2:2:1 quartet of DMPO-OH nitroxide ($a_N = a_{H\beta} = 1.49$ mT; $g = 2.0056$), the DMPO / HO• spin adduct (Fig. 3, insert). In matched control experiments with aerobically perfused hearts from all groups, no signals were seen (data not shown).

The mean concentration of DMPO-OH in the coronary effluents of untreated hearts (0.66 ± 0.03 µM, n = 8; Fig. 3) was significantly higher than the value that we recently reported for DMPO spin-trapping studies using a similar perfusion protocol, a difference that was probably due to the fact that hearts were subjected to ischemia for only 20 min in the previous

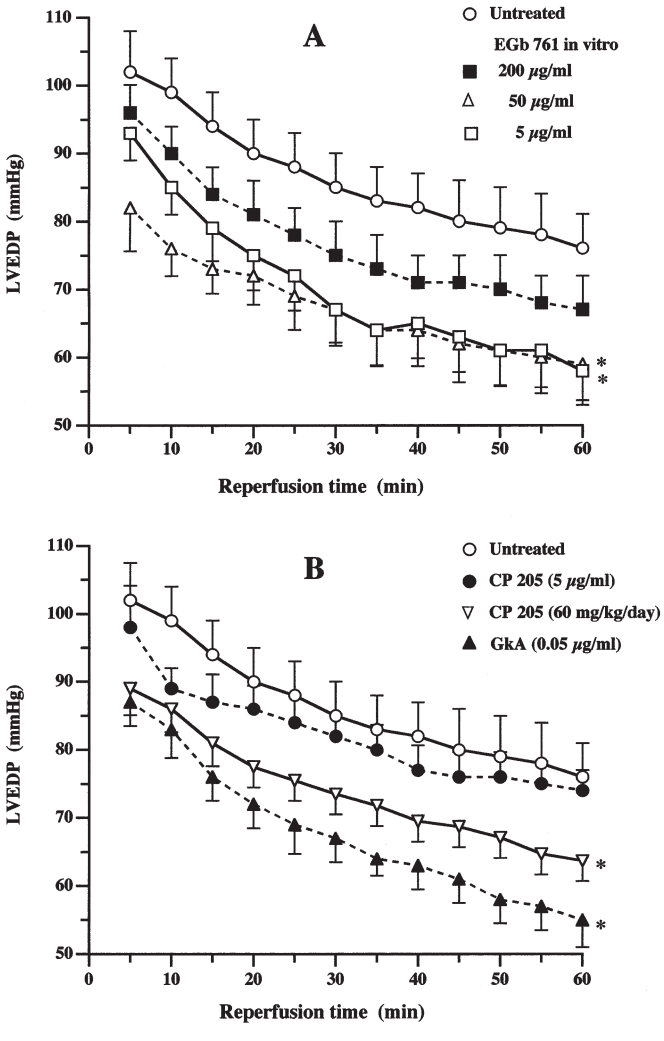

Fig. 1 Effect of treatments on the time course of recovery of post-ischemic left ventricular end-diastolic pressure (LVEDP) of isolated Langendorff perfused rat hearts undergoing 10 min of low-flow ischemia followed by 30 min of no-flow ischemia. The total extract (EGb 761; panel A), the fraction devoid of terpenes (CP 205) and ginkgolide A (GkA) were added to the perfusion medium at indicated concentrations during ischemic periods and reflow. In one group, CP 205 was given orally at 60 mg/kg/day for 15 days before drug-free perfusion (panel B). Values represent means ± SEM from 12 or 13 experiments per group. *$p < 0.05$ vs. untreated group by two-way ANOVA followed by a Newman-Keuls test ($p < 0.05$), over the entire period of reperfusion.

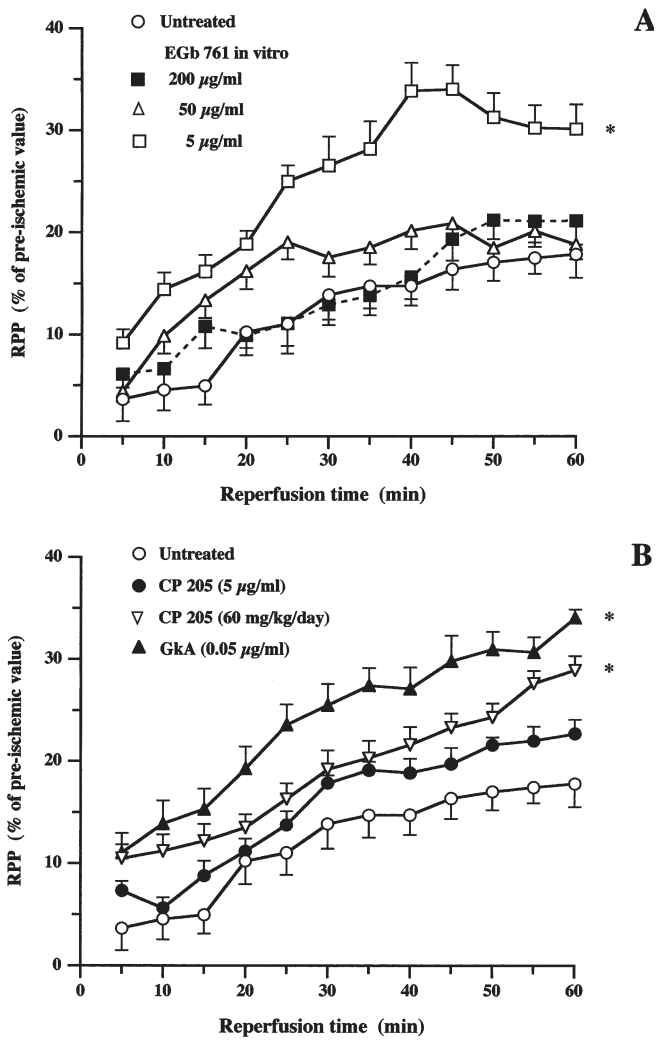

Fig. 2 Effect of treatments on the time course of recovery of rate pressure product (RPP). The perfusion protocol and experimental groups are described in the caption to Fig. 1. *$p < 0.05$ vs. untreated group by two-way ANOVA followed by a Newman-Keuls test ($p < 0.05$), over the entire period of reperfusion.

study (24). This finding corroborates earlier low-temperature (38) and spin-trapping (14) ESR studies which showed that post-ischemic levels of free radicals peaked when the antecedent duration of ischemia was 30 min.

Results provided in Fig. 3 indicate further that post-ischemic DMPO-OH concentrations in coronary effluents were markedly decreased in all groups with respect to the untreated group ($p < 0.01$), except for the group that was exposed to the lowest concentration of ginkgolide B. The arrows in Fig. 3 indicate the estimated concentrations of EGb 761 and its flavonoid and terpenoid constituents that may reach the general circulation following oral intake of 60 mg/kg of EGb 761 in the rat. The data clearly show that the extent of inhibition of baseline DMPO-OH concentration that followed in vitro application of the total extract (–70 % vs. untreated group at 5 µg/ml) could not be obtained by perfusing only its flavonoid fraction CP 205 (–48 % vs. untreated group at 5 µg/ml), indicating that the presence of both terpenoid and flavonoid constituents of EGb 761 was required to produce this effect. In this regard, 0.05 µg/ml of ginkgolide A (–47 % vs. untreated group) was as effective as 5 µg/ml of CP 205, whereas the decrease in post-ischemic DMPO-OH concentration observed with 0.05 µg/ml of ginkgolide B (–26 % vs. untreated group) was not statistically significant.

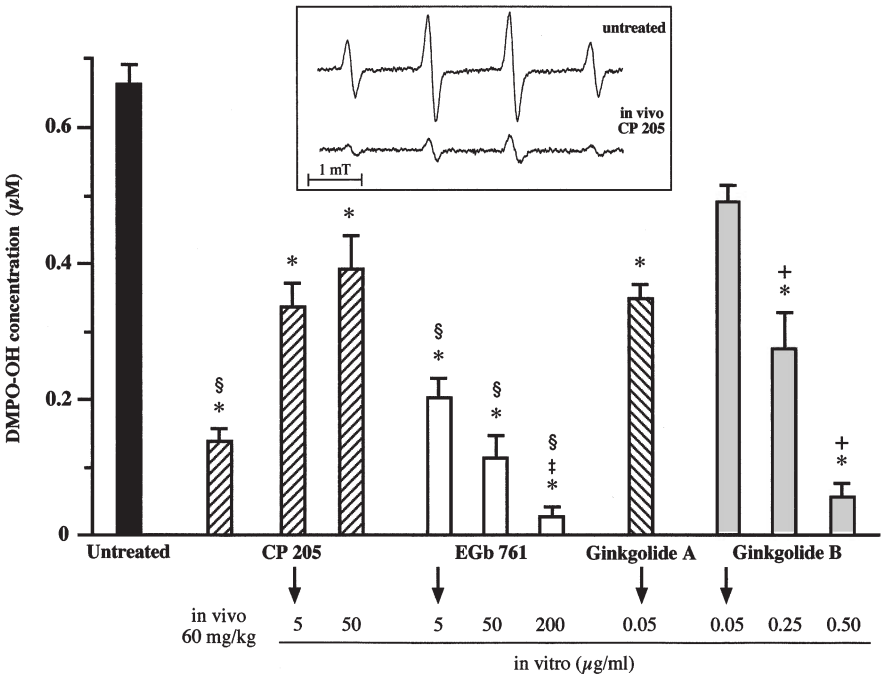

Fig. 3 Effects of in vivo and in vitro treatments on DMPO-OH concentration (means ± SEM; n = 6 / group) in the coronary effluents collected for 15 s after 4 min of post-ischemic reperfusion. DMPO (28 mM) was added to the perfusion fluid during the last 3 min of aerobic control perfusion and the first 5 min of reperfusion. The arrows indicate the estimated circulating concentrations in humans corresponding to a therapeutic dose in rats of EGb 761 (i.e., 60 mg/kg/day). One-way ANOVA ($p < 0.0001$) followed by a Duncan test: *$p < 0.01$ vs. untreated group; §$p < 0.01$ vs. CP 205 (5 µg/ml and 50 µg/ml); ‡$p < 0.01$ vs. EGb 761 (5 µg/ml); +$p < 0.01$ vs. ginkgolide B (0.05 µg/ml). Insert: typical ESR spectra of the DMPO-OH spin adduct in the coronary effluents of hearts from the untreated and in vivo CP 205 groups that underwent ischemia/reperfusion in the presence of the spin trap DMPO, showing the beneficial effect of treatment on free radical generation.

Figure 3 also shows that the decreases in baseline DMPO-OH concentration that occurred with EGb 761 and ginkgolide B exhibited a concentration-dependency. Considering the lowest concentrations tested, EGb 761 (5 µg/ml) was significantly more effective than ginkgolide B (0.05 µg/ml), but a two-fold higher increase in EGb 761 concentration than in ginkgolide B concentration was required to further decrease post-ischemic DMPO-OH concentration to the same extent. At the two tested concentrations of 5 and 50 µg/ml, CP 205 was found less efficient than EGb 761 in reducing post-ischemic DMPO-OH concentration ($p < 0.01$). Moreover, increasing the 10-fold higher CP 205 concentration of 50 µg/ml had an adverse effect (but not significant) on DMPO-OH levels with respect to 5 µg/ml. Considering in vivo studies (6), the in vitro concentration of 5 µg/ml for CP 205 was selected as the maximal concentration of non-terpene constituents of EGb 761 that could reach the general circulation following oral administration of CP 205 at a dose 60 mg/kg. Results provided in Fig. 3 show that this in vivo dose of CP 205 was significantly more efficient in reducing post-ischemic DMPO-OH concentration than in vitro application of 5 µg/ml of CP 205 ($p < 0.01$).

Discussion

Until now, the leading hypothesis for explaining the cardioprotective effects of EGb 761 on reperfusion injury has involved direct scavenging of the free radicals that are formed upon reperfusion of the ischemic heart (8, 34). Among the pharmacologically active substances identified in EGb 761, the flavonoid compounds [which account for about 31 % of EGb 761, mainly as flavonol glycosides (about 24 %) and proanthocyanidins (about 7 %) (5) initially seemed to be ideal candidates for oxyradical-scavenging due to their well-known in vitro antioxidant properties. This view has been reinforced by studies which showed that none of the terpene trilactone constituents of EGb 761 (which represent 6 % of EGb 761 and which are unique to the extract) that have been tested displayed significant in vitro inhibitory actions on $O_2^{\bullet -}$ or HO• (3, 20, 24, 32). Therefore, it was expected that the terpene-free preparation of EGb 761 (CP 205) would afford a similar degree of cardioprotection as the total extract upon in vitro perfusion to ischemic-reperfused hearts.

However, the results of the present study have shown that when CP 205 is added to the perfusion fluid at the same concentration that occurs in EGb 761, it is significantly less effective than the total extract in protecting hemodynamic function

and post-ischemic free radical release into coronary effluents. This finding, taken together with the results that ginkgolides A and B both decreased DMPO-OH release into coronary effluents, demonstrates for the first time that part of the cardioprotection that occurs following in vitro perfusion with EGb 761 is due to a specific action of its terpenoid constituents which involves a mechanism independent of direct free radical-scavenging. From the hemodynamic and ESR data presented herein, it can be estimated that a large part of the beneficial effects of perfusing ischemic-reperfused hearts with a therapeutically relevant concentration of EGb 761 (5 µg/ml) is due to the action of its terpene trilactone constituents.

With further regard to this cardioprotective role of the terpenoid constituents of EGb 761, a series of studies conducted with hypoxic cultured endothelial cells treated with bilobalide (9), with ischemic-reperfused isolated hearts (24), or with heart mitochondrial preparations (10) from rats that had been pretreated orally with ginkgolide A or bilobalide have recently indicated they might act at the membrane and/or at the mitochondrial levels during the pre-ischemic period. This hypothesis is in accord with the present observation that the ventricular contracture that occurred during ischemia was significantly suppressed in hearts that were exposed to ginkgolides or to a low concentration of EGb 761, as compared to untreated hearts (Table 1). Although an in vitro $O_2^{\bullet -}$-scavenging activity of the terpenoid constituents of EGb 761 (except for ginkgolide A) in a lipophilic environment has been recently reported (32), the published second-order rate constants are relatively modest. Hence, direct $O_2^{\bullet -}$-trapping within the membrane of the ischemic-reperfused heart does not seem to be a relevant mechanism for explaining their cardioprotective action.

In our recent study (24), we have reported that since the DMPO-OH ESR signal detected in the coronary effluents of rat hearts reperfused in the presence of DMPO could be abolished by co-administration of superoxide dismutase, it can be taken as an indirect indicator of $O_2^{\bullet -}$ formation. In the present investigation, the post-ischemic DMPO-OH concentration of the coronary perfusate was dose-dependently decreased by EGb 761 and ginkgolide B, but based on estimated dose-related effects (Fig. 3) the latter substance appeared to be significantly more effective. Thus, it seems possible that some constituents of EGb 761, when occurring at high concentrations in the myocardium, may have relatively toxic effects able to counteract the antioxidant effects of the flavonoidic and terpenoic substances during ischemia and/or reperfusion. In this respect, hemodynamic measurements performed during the early phase of reperfusion clearly showed that although addition of EGb 761 to the perfusion fluid had some cardioprotective effects at the lower concentrations tested, increasing its concentration to 200 µg/ml resulted in a worsening of functional recovery, as compared with the low therapeutic concentration of 5 µg/ml (Table 2; Figs. 1A, 2A). These data provide the first evidence that the relationship between EGb 761 concentration in vitro and its protection of post-ischemic cardiac function in experimental ischemia-reperfusion injury is best characterized by a bell-shaped curve. From the relative loss of the inhibitory effect of CP 205 towards DMPO-OH levels occurring when its concentration in the myocardium was increased (Fig. 3), it can be suggested that some non-terpenoid constituents of EGb 761 at high concentrations may actually stimulate free radical formation in the post-ischemic heart. A bell-shaped response has been recently demonstrated for EGb 761 and ginkgolide B for the increase in viability and inhibition of apoptosis of primary cultures of rat hippocampal neurons exposed to a peroxyl radical-generator (30). In these studies (30), the maximal beneficial effects were obtained at 10-20 µg/ml for EGb 761 and 0.2–0.4 µg/ml for ginkgolide B.

We have previously proposed (24) that proanthocyanidins could be involved in the relative toxicity of high concentrations of EGb 761 since these phenolic polymers were reported to bind specifically to proteins and inactivate enzymes such as catalase, lactate dehydrogenase and glutathione peroxidase in vitro (7). However, EGb 761 contains many other classes of substances that could be incriminated in the toxicity observed at high in vitro concentrations, the nature of which remaining to be determined. The finding that the in vitro effect of ginkgolide B on the concentration of DMPO-OH in the post-ischemic coronary effluent was maximal at the high concentration of 0.5 µg/ml (Fig. 3) could indicate that mechanisms related to inhibition of platelet-activating factor (PAF) could be, at least in part, involved in the observed antioxidant effect. Indeed, ginkgolide B, which is a potent PAF antagonist (4, 5), has been reported to inhibit ischemia-induced ventricular fibrillations and tachycardia in isolated rat hearts when perfused at concentrations that were more than 10 times the maximal concentration used in the present study (12). Since a direct link between the occurrence of ventricular fibrillations and post-ischemic DMPO-OH signals has been established for ginkgolide B (33), a possible interpretation of the present results is that this terpenoid may have limited free radical production to some extent by reducing PAF-induced myocardial dysrhythmias. However, from the finding that ginkgolide A, which is a poor PAF antagonist (4, 5), acts as a better cardioprotector and free radical inhibitor than ginkgolide B (Table 2, Fig. 3), it can be reasonably assumed that PAF inhibition is not a main mechanism involved in the global effect.

In recent years, the in vivo fate of the flavonoid and terpenoid constituents of EGb 761 following oral intake of the total extract has been precisely determined in rats (1, 26) and humans (6, 27, 36). Although the bioavailabilities of ginkgolides A and B and bilobalide were found to be practically complete after oral ingestion of EGb 761 in rats (1), it was not possible to demonstrate any significant bioavailability for flavonoid glycosides or their aglycones (26). In this lat-

ter study (26), the main metabolites that were detected in blood were propionic and phenolic acids resulting from the degradation of proanthocyanidins and flavonols (such as quercetin), respectively. Since the phenolic acids possess antioxidant properties (17, 18), they could conceivably participate in the protective effect afforded by in vivo administration of CP 205 during ischemia and reperfusion, an effect that would be revealed in hemodynamic and ESR indices (see Tables 1 and 2; Figs. 1B, 2B, 3). Hence, the significantly better cardioprotective effect that was found after in vivo administration of CP 205 than with in vitro perfusion of this non-terpenoid extract in the present study may have been due to the combination of a superior antioxidant effect of flavonoid metabolites (formed in vivo) vs. flavonoid glycosides and aglycones (present in vitro) and a greater ability for phenolic acids (found after in vivo administration) to reach the cardiac cell. Particularly, some metabolized proanthocyanidins may be involved in the cardioprotective effects of CP 205 and EGb 761 following in vivo treatment. Recent studies have reported a significant cardioprotection in post-ischemic rat hearts that were fed with procyanidins-enriched fractions from natural standardized mixtures such as Crataegus extracts or Vitis vinifera seeds (2, 15). However, the relevance of these results (2, 15) to the exact cardioprotective role of the proanthocyanidins of EGb 761 administered in vivo should be carefully considered since some of the effects reported (2, 15) may have been due to the antioxidant action of some constituents that are not present in EGb 761 (e.g., catechins) (4).

In conclusion, our results have demonstrated that the terpenoid constituents of EGb 761 and the flavonoid metabolites that are formed after its in vivo administration are both required for explaining the protection that this extract affords against myocardial ischemia-reperfusion injury. Although many such complementary interactions, and possibly even some synergistic interactions, may occur among the various biologically active constituents of EGb 761, it is believed that the particular findings presented herein may provide a basis for improving the beneficial effects of the extract as a treatment for cardiovascular disease. This might be accomplished by increasing its content of terpenoid compounds and/or by modulating the affinity of these compounds for cellular membranes. Efforts in this latter direction, which are currently in progress in our laboratory (25), have already revealed that a synthetic lipophilic analogue of ginkgolide C has cardioprotective properties that may be due to an increased affinity for the myocardium.

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