The Effect of *Ginkgo Biloba* in Isolated Ischemic/Reperfused Rat Heart

A Link between Vitamin E Preservation and Prostaglandin Biosynthesis

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Abstract: The effect of *Ginkgo biloba* extract (EGb 761) was studied in rat hearts submitted to ischemia/reperfusion. Isolated hearts perfused in Langendorff mode were subjected to 60 minutes of global ischemia and 15 minutes of reperfusion. EGb 761 was administered by chronic or acute treatment: intra-peritoneal injections of 5 mg/Kg extract for 5 days, or 100 mg /L extract addition to the perfusion buffer, respectively. In hearts not treated with EGb 761, ischemia induced a 20% decrease in the concentration of membrane α-tocopherol. This effect was not worsened by reperfusion. α-tocopherol consumption was accompanied by about 650% increase in 6-ketoPGF1α release within 3 minutes of reperfusion. Moreover, ischemia induced activation of transcription factor NF-κB, as compared with the untreated group. In both chronic and acute treatment with EGb 761, heart concentration of α-tocopherol was completely spared during ischemia as much as after reperfusion, and a significant decrease of 6-ketoPGF1α release was observed at 3 minutes of reperfusion. Nuclear translocation of NF-κB was lowered during ischemia. EGb 761 might act as direct free radical scavenger or as tocopheryl radical recycler; in both cases sparing membrane vitamin E should affect phospholipase A2 activity. Finally, EGb 761, by lowering ROS produced during ischemia, challenges nuclear translocation of NF-κB.

Key Words: rat heart, EGb 761, ischemia, reperfusion, antioxidants, transcription factor NF-κB

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Extracts from the leaves of *Ginkgo biloba* trees have long been used for the management of vascular and neurologic disorders.[1] Many molecular mechanisms have been proposed to explain the beneficial effects of the most-used *Ginkgo biloba* extract, EGb 761. In particular, its antioxidant action has been unmasked by several models of oxidative stress experimentally performed in different tissues and organs.[2–5] Specifically, EGb 761 revealed cardioprotective effects against oxidative damages induced by ischemia/reperfusion in isolated hearts. In fact, Tosaki et al[6] have shown that adding EGb 761 to the perfusion buffer of isolated rat hearts significantly reduces the incidence of reperfusion-induced ventricular fibrillation and tachycardia. The same method of administration of EGb 761 showed to improve all post-ischemic hemodynamic parameters,[7] reduce LDH leakage in post-ischemic coronary effluent, and preserve ascorbic acid content of myocardial tissue.[8] Evidences of the beneficial effects of *Ginkgo biloba* were confirmed by studies performed on isolated heart of rats chronically treated with oral doses of EGb 761.[7,9]

Cardioprotective effects of *Ginkgo biloba* extracts are complex and multifactorial but seem to rely mainly on its properties of free radical scavenger,[7,10,11] which are due to the complementary action of both its terpenoid and flavonoid constituents.[12] Moreover, both the constituents of EGb 761 decrease the expression of inducible nitric oxide synthase (iNOS), responsible for the formation of large and sustained amounts of NO during oxidative stress. An excessive formation of NO may be potentially deleterious as it may damage the surrounding tissues, mainly by the formation of peroxynitrite. EGb 761 extract acts as potent inhibitor of NO production at both myocardial and microvascular levels under the condition of ischemia/reperfusion.[8,13]

Ischemic/reperfused hearts represent a suitable and well-characterized model for studying free radical induced oxidative stress and inflammatory response, which can contribute significantly to tissue injury and functional damage of myocardium.[14,15] Previous studies demonstrated that ischemia/reperfusion leads to a decrease of α-tocopherol content in myocardial membranes,[16] and an increase of prostaglandin biosynthesis.[17] In addition, several lines of evidence indicate that oxidative reactions and the altered cellular redox state of ischemic myocardium control early expression of genes codifying for proteins involved in inflammatory and stress responses (eg, inducible nitric oxide synthase and cyclooxygenase).[18–20] The induction of pro-inflammatory genes usually results from the increased activity of a subset of transcription factors, which have thus become attractive targets in the development of novel drugs. Among the inducible transcription

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factors that control gene expression, nuclear factor NF-κB plays a central role in modulating the expression of various pro-inflammatory mediators (eg, cytokines, chemokines, and prostaglandins).[21–23] The activation of NF-κB is itself sensitive to the cellular redox state[24] and, in the isolated heart model, is induced during ischemia.[25]

In the past decade many studies have addressed the in vitro and in vivo effects of tocopherols on eicosanoid synthesis, and α-tocopherol has proved to act as inhibitor of cyclooxygenase, phospholipase, and lipooxygenase,[26–28] thus modifying cellular arachidonic acid metabolism for the synthesis of biologically active prostaglandins and related compounds.

Moreover, because oxidative stress has been implicated in the signaling pathway, there has been greater attention on the use of antioxidants in inhibiting NF-κ B activation.

The present study aimed to assess the effect of EGb 761 on oxidative damage in isolated rat hearts undergoing ischemia/reperfusion. The drug was administered according to two different protocols of treatment: chronic or acute administration. We monitored prostaglandin synthesis, membrane vitamin E content, the nuclear translocation of the transcription factor NF-κB, and LDH release. Our results further confirmed the benefits of administering EGb 761 extracts reported in literature. Moreover, we sought possible interactions among these different parameters, to provide further input on the overview of the effects of *Ginkgo biloba* on oxidative stress.

METHODS

Animal Model

All studies employed Wistar rats weighing 250 to 300 g. Animals were used in accordance to Italian law (DL 116, 1992) and conformed to the "Guiding Principles for Research Involving Animals and Human Beings" approved by the Council of the American Physiological Society.

Rats were anesthetized with diethyl ether, and anticoagulated by injection of 500 IU/Kg heparin in the femoral artery. The hearts were immediately removed and placed in cold Krebs-Henseleit buffer. The aorta was then cannulated and the hearts perfused according to the non-recirculating Langendorff configuration. Krebs-Heinseleit buffer gassed with 95% O_2 / 5% CO_2 and containing 10 mmol/L glucose was perfused at a constant pressure of 80 cm H_2O. Myocardial and buffer temperature were kept at 37°C with water-jacket heart chamber and buffer reservoirs.

The hearts were allowed to beat spontaneously. The volume of coronary effluent was measured at different time collection during the experiment and stored at −20°C.

Three protocols were designed. The number of hearts studied in each protocol is plotted in Table 1.

Protocol 1: Hearts were subjected to 90 minutes of aerobic perfusion (control condition).

Protocol 2: In ischemia experiments, after 15 minutes of aerobic perfusion, the hearts underwent 60 minutes of no-flow global ischemia.

Protocol 3: In ischemia/reperfusion experiments, the 60 minutes of ischemia were followed by 15 minutes of reperfusion.

At the end of the experiments, all hearts were freeze-clamped in liquid nitrogen and stored at −80°C.

Two different modalities of EGb 761 (Tanakan, Beaufour Ipsen Pharma, Paris) administration were used (Table 1): chronic and acute treatment. Chronic treatment consisted of daily intra-peritoneal doses of EGb 761 (5 mg/kg dissolved in saline solution) for 5 days (chronic group). A comparable amount of animals received daily injections with the same volume of saline solution (no drug group).

In studies involving acute administration of the drug, EGb 761 (100 mg/L) was added to the perfusion buffer (acute group).

Vitamin E Assay

Five mg of lyophilized powder of myocardial tissue was added with 50 μL of 45 mmol/L butylated hydroxytoluene; 950 μL of ice-cold bi-distilled water was added, and immediately homogenized. Vitamin E was extracted according to the procedure by Lang et al.[29] Briefly, homogenates were mixed with 1 mL of 100 mmol/L sodium dodecylsulfate solution in water, 2 mL of ethanol/isopropanol (95:5 vol/vol), 2 mL of *n*-hexane, and mixed with a vortex mixer for 2 minutes. The hexane phase was separated by centrifugation; 1.5 mL aliquots were evaporated under nitrogen flux and re-suspended in 800 μL of methanol. Determinations by high performance liquid chro-

TABLE 1. Number of Experiments Performed in Each Experimental Condition

	Protocol		
Drug	**Control 90 min Aerobic Perfusion**	**Ischemia 60 min No-Flow Ischemia**	**Ischemia/Reperfusion 60 min Ischemia/15 min Reperfusion**
No drug	10	10	10
Chronic EGb 761 treatment	10	10	10
Acute EGb 761 treatment	7	7	7

matography (HPLC) were performed utilizing a 5 μm ODS C-18 reverse phase column using methanol as eluent at 1 mL/min and fluorometric detection (excitation 286 nm, emission 330 nm). Data from HPLC were collected and analyzed using SYSTEM GOLD program (Beckman Instruments, Palo Alto, CA).

6-ketoPGF1α Measurements

The biosynthesis of prostaglandins was tested by measuring the stable metabolite of prostacyclin, 6-ketoPGF1α, in the coronary effluent by radioimmunoassay.

Lactate Dehydrogenase Release

Lactate dehydrogenase (LDH) activity was spectrophotometrically measured as previously described[30] at λ = 340 nm, in samples collected from the coronary effluent before ischemia, at 3 and 13 minutes of reperfusion. Each value was obtained as IU/min/g wet weight, and reported in the results as percentage of the pre-ischemia value.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins of at least 2 hearts for each condition and each group were isolated using a method previously described[25,31] and adapted for our samples. The nuclear extracts were stored at −70°C. Protein concentration was estimated by using BCA method (Pierce Chemical, Rockford, IL).

The oligonucleotide 5′-CCTGGGTTTCCCCTTGA-AGGGATTTCCCTCC-3′, encompassing 2 consensus sequences of NF-κB binding sites (underlined sequences represent site 1 and 2, respectively) was used to assess NF-κB activation. The oligonucleotides were end-labeled by T4 polynucleotide kinase and [γ $^{-32}$P]ATP (3000 Ci/mmol). Nuclear extract (15 μg) was added to ^{32}P-labeled oligonucleotide in 20 μL reaction buffer containing 50 μg/mL of poly(dI-dC), 10 mmol/L Tris. HCl (pH 7.5), 50 mmol/L NaCl, 0.5 mmol/L DTT, and 10% glycerol. After 15 minutes incubation at room temperature, DNA-protein complexes were resolved in 5% native polyacrylamide gel electrophoresis in low-ionic-strength buffer (0.25 × Tris-borate-EDTA). The gels were dried and exposed for autoradiography. Self competitions were carried out under the same conditions using a 160-fold molar excess of unlabeled NF-κB oligonucleotide probe in the binding reaction of nuclear extracts from ischemic myocardium of untreated rats. Quantification of densities of autoradiographic bands for EMSA was performed with NIH Image 1.6 software.

Statistical Analysis

Results were expressed as mean ± standard error (S.E.). Student's *t* test was used for comparison between two means. A *P* value of $P < 0.05$ was considered as significant against the control.

RESULTS

Effect of EGb 761 on Myocardial Vitamin E Content

The α-tocopherol content of non-ischemic (control condition) hearts of untreated rats was 214.6 ± 4.1 ng/mg of dry weight. This value was comparable with that obtained with both chronic and acute EGb 761 administration (213.3 ± 5.7 and 214.8 ± 11.3 ng/mg, respectively, Fig. 1).

In the untreated group, hearts were exposed to 60 minutes ischemia and showed a significant decrease of tissue α-tocopherol content (172.9 ± 6.7 ng/mg; $P < 0.005$), corresponding to about 20% reduction of the related control content. Conversely, EGb 761 treatment spared tissue vitamin E during ischemia, independently of the modality of drug administration. The myocardial α-tocopherol content was 215.7 ± 5.6 and 221.5 ± 15.1 ng/mg, for chronic and acute drug supply, respectively (Fig. 1).

A comparable pattern was reproduced after 15 minutes of reperfusion. Untreated hearts showed a significant decrease: tissue α-tocopherol content was 179.1 ± 5.3 ng/mg ($P <$ 0.005), corresponding to about 17% of the related control value. Chronic and acute EGb 761 treatment maintained vitamin E content at the control values (211.3 ± 8.5, and 216.2 ± 10.7 ng/mg respectively, Fig. 1).

Release of 6-ketoPGF1α in the Effluent

A marked inter-subject variability in 6-ketoPGF1α release was observed during the initial 15 minutes of aerobic perfusion, independently of the animal group. Figure 2 shows the release of 6-ketoPGF1α measured in the myocardial effluent in the first 3 minutes of reperfusion according to the ischemia/reperfusion protocol in the 3 experimental conditions.

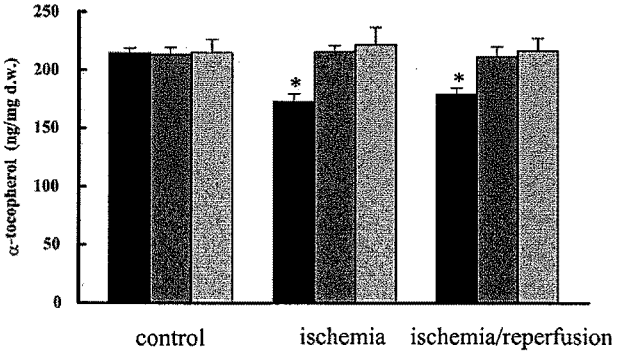

FIGURE 1. α-tocopherol content of rat hearts under the 3 conditions: control, ischemia, and ischemia/reperfusion. Black columns: untreated animals (n = 10 for each condition). Dark gray columns: chronic treatment with EGb 761 (n = 10 for each condition). Pale gray columns: acute treatment with EGb 761 (n = 7 for each condition). Vitamin E is expressed as ng for mg of tissue dry weight. Results shown are means ± SE. *$P <$ 0.005 versus control condition of untreated group.

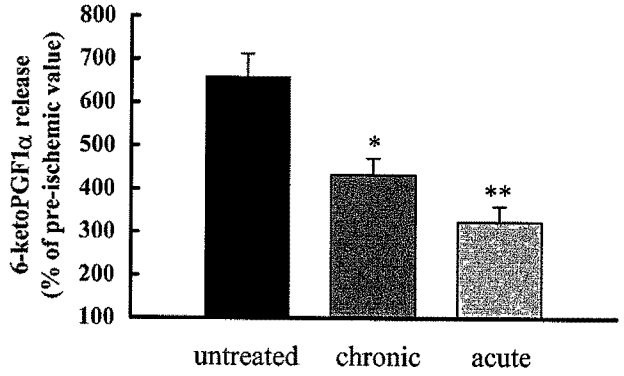

FIGURE 2. 6keto-PGF1α release in the coronary effluent collected in the first 3 minutes of reperfusion. Black column: untreated animals (n = 10 for each condition). Dark gray column: chronic treatment with EGb 761 (n = 10 for each condition). Pale gray column: acute treatment with EGb 761 (n = 7 for each condition). 6keto-PGF1α release is expressed as percentage of its own value measured before ischemia. Results shown are means ± SE. $P < 0.005$ (*) and $P < 0.001$ (**) versus untreated group, respectively.

While the untreated group showed more than a 6-fold increase in 6-ketoPGF1α release in comparison to the basal level (659% ± 54), chronic and acute EGb 761 treatment yielded a significant reduction of 6-ketoPGF1α release (434% ± 38, $P < 0.005$ and 325% ± 36, $P < 0.001$) compared with the untreated group.

The pre-ischemia value of prostaglandin release (100%) was restored within 6 minutes in the untreated group and within 4 minutes in both treated groups.

Lactate Dehydrogenase Release

In untreated hearts subjected to ischemia/reperfusion protocol, LDH leakage at 3 minutes after the onset of reperfusion was significantly higher than the pre-ischemia value (367% ± 101), as shown in Figure 3. However, chronic EGb 761 treatment significantly reduced LDH release at 3 minutes of reperfusion compared with the untreated group (111% ± 23, $P < 0.05$).

At 13 minutes from the onset of reperfusion, LDH release decayed to 175% ± 21 and 142% ± 9 of pre-ischemia value in untreated and chronic treated rats respectively.

No data are available for LDH release in the acute group because of the marked interference of EGb 761 extract with the optical absorbance at λ = 340 nm.

Nuclear Translocation of Transcription Factor NF-κB

Nuclear translocation of transcription factor NF-κB was determined in untreated and EGb 761 treated hearts. For this purpose the ischemia period was shortened to 30 minutes to obtain reliable signal/noise ratio conditions in consideration of the time course of the nuclear translocation of NF-κB and the cytosolic restoration of IκBα as reported by Li et al.[25]

Electrophoretic mobility shift assay (EMSA) demonstrated increased NF-κB binding activity in nuclear extracts of untreated hearts perfused for 15 minutes (control). When nuclear extracts were prepared from hearts subjected to 30 minutes of ischemia the gel-shift band was further increased. The gel-shift band increment persisted after 15 minutes of reperfusion (Figs. 4 and 5). EMSA of nuclear extracts prepared from hearts submitted to chronic (Fig. 4) or acute (Fig. 5) treatment with *Ginkgo biloba* demonstrated a decrease in the intensity of the shifted band induced by both ischemia and reperfusion, independently of the modality of drug administration.

DISCUSSION

As previously reported, the present results show that 60 minutes of "warm" ischemia followed by reperfusion can induce a decrease in membrane concentration of the lipophilic antioxidant vitamin E, and an increase of prostaglandin release[16,17] as well as an increase of enzyme leakage providing evidence of ischemia-reperfusion cellular damage.[8,32] These results confirm that during heart ischemia reactive oxygen species are involved in the genesis of tissue injury and inflammatory response. This effect is in part mediated by the activation of the nuclear factor NF-κB, a cytoplasmatically sequestered transcription factor that up-regulates the expression of many inflammatory genes. The biosynthesis of prostaglandins relies

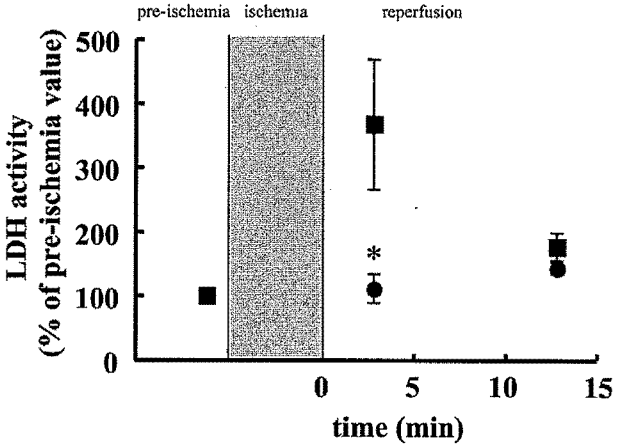

FIGURE 3. Lactate dehydrogenase leakage during reperfusion following 60 minutes of ischemia in untreated (squares, n = 5) and in chronic EGb 761 treated (circles, n = 5) hearts. LDH activities are expressed as percentage of the pre-ischemia value. Background gray area indicates the ischemia period. Time 0 corresponds to the onset of reperfusion. Each value is expressed as mean ± S.E. *$P < 0.05$ versus untreated group.

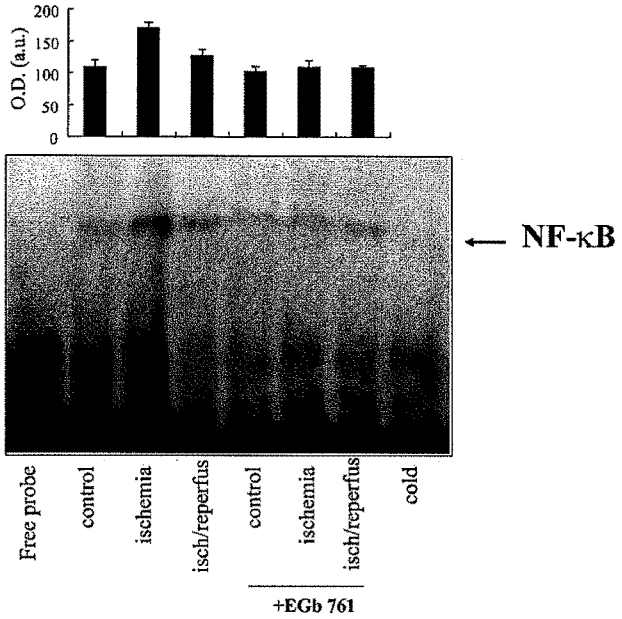

FIGURE 4. Electrophoretic mobility shift assay of NF-κB nuclear activation in hearts of untreated and treated rats with chronic EGb 761 administration. Top: bar histograms of densitometric scanning (mean ± S.E.) for 2 different nuclear heart extracts at each condition. The optical density (O.D.) of autoradiographic bands is expressed as arbitrary units (a.u.). Representative EMSA gel is shown at the bottom of panel. Lane 1: Free probe. Lane 2,3,4: nuclear extracts prepared from untreated groups. Lane 5,6,7: nuclear extracts prepared from hearts of chronic treatment groups. Lane 8: nuclear extracts from ischemic heart plus unlabeled NF-κB oligonucleotide (cold). NF-κB band is labeled at right.

on the activity of phospholipase A2 (PLA_2), a rate-limiting enzyme in the arachidonic acid cascade. PLA_2 activity depends on free radicals, lipid peroxidation, or free calcium levels. The increase in PLA_2 activity, in turn, accelerates the production of free arachidonic acid, the substrate for non-enzymatic- and enzymatic-eicosanoid production and platelet-activating factors.[33] The eicosanoids have multifaceted physiological effects at extremely low concentrations. However, an increased production of eicosanoids leads to the state of inflammation. Since PLA_2 is one of the enzymes involved in the synthesis of eicosanoids, its inhibition and the consequent constraint of arachidonic acid cascade would reduce both the oxidative damage and the inflammatory response induced by ischemia and reperfusion. In tissues vitamin E acts as chain-breaking antioxidant that mainly prevents peroxidation of polyunsaturated fatty acids in cell membranes, thus challenging the trigger for increasing PLA_2 activity. In addition, vitamin E may attenuate cyclooxygenase activity by scavenging the oxidant hydroperoxide necessary for cyclooxygenase activation.[34–36] However, although the antioxidant ability of vitamin E could be useful for the oxidative events triggered by ischemia-reperfusion these antioxidant properties can only partly account for the extensive protective effects of vitamin E. In the past decade cellular functions of α-tocopherol, some of which are independent of antioxidant capacity, have been documented. Activation of protein phosphatase 2A, and inhibition of protein kinase C, cyclooxygenase, lipoxygenase, PLA_2, and cytokine release are examples of non-antioxidant action of α-tocopherol at cellular level.[37–40] The great majority of these activities affects enzymes or factors that modulate eicosanoid synthesis and inflammatory response.

The present results suggest that, in our model, a modulation of PLA_2 activity could be related to the variation of vitamin E concentration in myocardial membranes. It could be reasonable, in fact, that both the activation of the nuclear factor NF-κB and the increased prostaglandin biosynthesis at heart reperfusion observed in the present study could be secondary to the reduced membrane concentration of α-tocopherol induced by ischemia. Moreover, the present observations strongly suggest that the beneficial effects of EGb 761 might

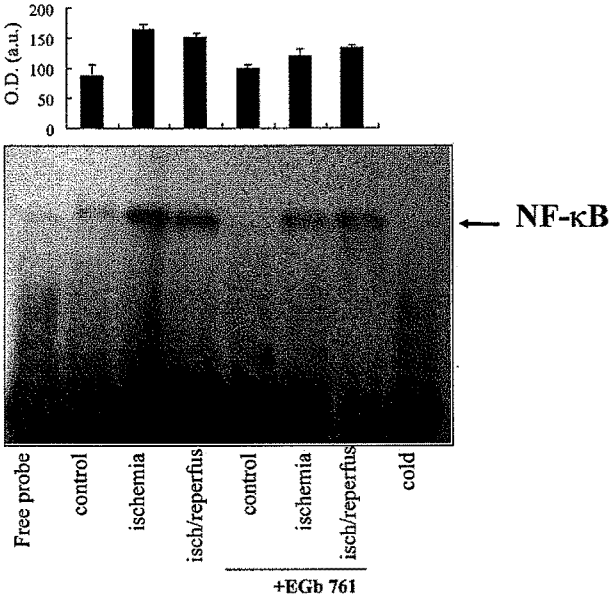

FIGURE 5. Electrophoretic mobility shift assay of NF-κB nuclear activation in hearts of untreated and acutely EGb 761 treated groups. Top: bar histograms of densitometric scanning (mean ± S.E.) for 2 different nuclear heart extracts at each condition. The optical density (O.D.) of autoradiographic bands is expressed as arbitrary units (a.u.). Representative EMSA gel is shown at the bottom of panel. Lane 1: Free probe. Lane 2,3,4: nuclear extracts prepared from untreated groups. Lane 5,6,7: nuclear extracts prepared from hearts of acute treatment groups. Lane 8: nuclear extracts from ischemic heart plus unlabeled NF-κB oligonucleotide (cold). NF-κB band is labeled at right.

depend upon its primary preventive radical-quenching properties. The mechanism by which EGb 761 exerts its protective effect in a cardiac ischemia-reperfusion model could be linked mainly to its free radical scavenging properties, thus preventing the consumption of the endogenous scavengers[8] and challenging ROS production. Moreover, EGb 761 also exerts its protective effect on the nuclear translocation of NF-κB and against the ischemia-reperfusion cellular damage induced by the experimental conditions. Despite the inability of EGb 761 constituents (terpenoids and flavonoids) to penetrate the cytosol, the antioxidant effect of the extract could be the consequence of the reduction of radical species forming at the membrane sites during ischemia and reperfusion. The reduction of cytosolic ROS provides the mean of facing the activation of nuclear factor NF-κB, the increase of PLA_2 activity induced by ischemia, and as a consequence, the increased release of eicosanoid products.

It is notable that, in our model, the modality of drug administration does not affect the beneficial properties of *Ginkgo biloba*. In fact, we obtained similar results both when treating animals for 5 consecutive days and then testing their isolated hearts in the absence of EGb 761, and when perfusing isolated hearts of untreated animals with EGb 761 acutely added in the perfusion solution. In our assays 15 minutes of perfusion with EGb 761 was a sufficient time for the drug to be optimally incorporated into the membranes. In addition, the protective effect observed in the hearts of pre-treated rats suggests that the very compounds of EGb 761 are actually incorporated into the membrane to challenge oxidative stress.

CONCLUSION

Ginkgo biloba has a documented effect in vascular and neurologic disorders. The mechanisms of this powerful protective effect are however still elusive and not fully understood.

According to the "radical theory", ischemic tissue injury associated with ischemia-reperfusion determines an increased oxidative stress, which surely oxidizes vitamin E, and thus limits the cytoprotective effect of it provided through both antioxidant and non-antioxidant mechanism. In this view, the antioxidant properties of EGb 761 might be helpful by preventing the oxidation of vitamin E in cell membrane, thus, providing the cell with a potent support to challenge the ischemia/reperfusion damage.

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