Pergamon

Biochemical Pharmacology, Vol. 50, No. 7, pp. 991–999, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00227-8

PROTECTION OF HYPOXIA-INDUCED ATP DECREASE IN ENDOTHELIAL CELLS BY GINKGO BILOBA EXTRACT AND BILOBALIDE

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(*Received* 18 *October* 1994; *accepted* 17 *May* 1995)

Abstract—Due to their localization at the interface between blood and tissue, endothelial cells are the first target of any change occurring within the blood, and alterations of their functions can seriously impair organs. During hypoxia, which mimics *in vivo* ischemia, a cascade of events occurs in the endothelial cells, starting with a decrease in ATP content and leading to their activation and release of inflammatory mediators. EGb 761 and one of its constituents, bilobalide, were shown to inhibit the hypoxia-induced decrease in ATP content in endothelial cells *in vitro*. Under these conditions, glycolysis was activated, as evidenced by increased glucose transport, as well as increased lactate production. Bilobalide was found to increase glucose transport under normoxic but not hypoxic conditions. In addition, EGb and bilobalide prevented the increase in total lactate production observed after 60 min of hypoxia. However, after 120 min of hypoxia, the total lactate production was similar under normoxic and hypoxic conditions, and both compounds increased this production. These results indicate that glycolysis slowed down between the 60th and 120th minute of hypoxia, while EGb and bilobalide delayed the onset of glycolysis activation. In another experimental model, both compounds were shown to increase the respiratory control ratio of mitochondria isolated from liver of rats treated orally. Since ischemia is known to uncouple mitochondria, the protection of ATP content and the delay in glycolysis activation observed during hypoxia in the presence of EGb 761 or bilobalide is best explained by a protection of mitochondrial respiratory activity, at least during the first 60 min of hypoxia incubation. Both products retain the ability to form ATP, thereby reducing the cell's need to induce glycolysis, probably by preserving ATP regeneration by mitochondria as long as oxygen is available.

Key words: hypoxia, endothelial cells; mitochondrial respiration; glycolysis; bilobalide; Gingko biloba extract

Ischemia is a common feature of various vascular diseases: impairment of blood circulation is involved in thrombosis, myocardial infarction, cerebral ischemia, or chronic venous insufficiency. A deficit in blood transport leads to a decrease in oxygen and nutriment supply to the tissue. Due to their localization at the interface between blood and tissue, endothelial cells are the first targets of variations in oxygen pressure within the blood [1, 2, 3]. These cells fulfill numerous important functions: They regulate coagulation by the release of antithrombic molecules; they synthesize vasoactive molecules as well as compounds regulating leukocyte, platelet, and smooth muscle cell functions; and they act as a selective barrier and secrete various metabolites [4, 5, 6]. Thus, alteration of the endothelium can lead to important disturbances of vessel functions [7].

Previous results have shown that endothelial cells can be strongly activated by hypoxia, a condition that mimics the *in vivo* decrease in oxygen supply during ischemic events. This activation is due to a decrease in the ATPs§ content of the cells, and leads to an increase in cytosolic calcium concentration [8] and to activation of phospholipase A_2, then responsible for an increase in the synthesis of prostaglandins [9] and of platelet-activating factor. This activation results in an increased adhesiveness of endothelial cells for neutrophils [10] and to their activation [11]. Hypoxic tissues can thus initiate, via the active role of the endothelium, an inflammatory response, which will then induce tissue damage [12, 13]. However, it must be noted that endothelial cells from different blood vessels show different sensitivity to hypoxia. Not all are sensitive to it, heart endothelial cells, for example, being almost completely resistant [14].

A leaf extract of EGb 761 is widely prescribed as therapy for several vascular diseases involving ischemic events, such as cerebrovascular and peripheral vascular and chronic venous insufficiencies. The extract contains 24% ginkgo-flavone glycosides, 6% terpenes (ginkgolides and bilobalide), and approx. 7% proanthocyanidines and other uncharacterized compounds [15]. It has been shown to enhance glucose transport *in vitro* in erythrocytes [16] and in smooth muscle cells [17], as well as *in vivo* in rats with cerebral ischemia [18]. It also protects endothelial cells against hypoxia-induced mortality [19] and prevents hypoxia-induced ATP decrease. This latter effect is due to the terpenic fraction of EGb 761 and, more importantly, to bilobalide [20]. Because of the crucial role played by endothelial cells in initiating ischemia-induced tissue damages, it was interesting to investigate how EGb 761 and bilobalide could protect endothelial cells against the effects of hypoxia, in particular, their influence on the energetic metabolism.

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§ Abbreviations: ATP, adenosine triphosphate; EGb 761, *Ginkgo bilabo* extract; HBSS, Hank's balanced salt solution; HUVEC, human umbilical vein endothelial cells; P/O ratio, number of moles of ATP generated from ADP per atom of oxygen; RCR, respiratory control ratio.

In this report, we show that EGb 761 and—more efficiently—bilobalide inhibit the hypoxia-induced decrease in ATP content in endothelial cells. This effect is not due to an increase in glucose transport nor to an increase in glycolytic activity as measured by lactate production, but probably to an increase in respiration efficiency, as indicated by a better coupling of mitochondria isolated from liver of treated rats.

MATERIAL AND METHODS

Reagents

Modified Hank's balanced salt solution (0.14 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 3 mM $Na_2HPO_4 \cdot 2H_2O$, 0.4 mM KH_2PO_4, 5.5 mM glucose, pH 7.35) containing 1 mM $CaCl_2$ (HBSS) was prepared in our laboratory. EGb 761 and purified bilobalide (BN 52023) were generously donated by the Institut Henri Beaufour, Paris, France. EGb 761 is a standardized extract of leaves of *Ginkgo biloba* by acetone/water. It contains flavonoids (coumarinic esters of quercetin, kaempferol, proantocyanidines, and flavones) and terpenes (ginkgolides and bilobalide) [14]. EGb was directly dissolved in HBSS for the studies on HUVEC and in water for treating the rats. Bilobalide was dissolved in pure ethanol at 10 mg/mL and then serially diluted in HBSS. The final concentration of ethanol in the solution of 100 ng/mL of bilobalide is 0.001%. Chemicals of analytical grade were from Merck (Darmstadt, Germany).

HUVEC isolation and culture

Human umbilical vein endothelial cells were isolated according to Jaffe *et al.* [21]. Cords were stored at 4°C just after birth in stock buffer (4 mM KCl, 140 mM NaCl, 10 mM Hepes, 1 mM glucose, 100 μg/mL streptomycin (Sigma, St. Louis, MO), 100 U/mL penicillin (Sigma), and 0.25 μg/mL fungizone (Gibco, Paisley, Scotland), pH 7.3). Before manipulation, they were rinsed with 20 mL phosphate-buffered saline (PBS) containing antibiotics and fungizone at concentrations aforementioned. Umbilical veins were incubated for 35 min at 37°C with 4 mL collagenase type II (Sigma) 0.05% in PBS. The cells were then harvested in M199 (Gibco) + 20% foetal calf serum (Gibco), centrifuged 10 min at 1000 rpm, and seeded in 0.20% gelatin-coated culture dishes (25 cm^2, Falcon Plastics, Oxnard, CA). The following day, the cells were washed with medium to eliminate blood cell contamination. Only monolayers of primary cultures that were tightly confluent were used for these studies. Confirmation of their identity as endothelial cells was obtained by detecting factor VIII antigen assessed by immunofluorescence staining [22]. For one experiment, several cultures were mixed and seeded in the different dishes so that very low variability was observed from one dish to the next. However, from experiment to experiment using cultures coming from different umbilical cords the variability was higher. One dose-response curve was always obtained on one batch of cells in order to decrease variability.

In vitro *model of hypoxia*

Ischemia was simulated by exposing cells to hypoxia (100% N_2) at 37°C. Cells were seeded in gelatine-coated Petri dishes (ϕ = 35 mm, Falcon Plastics, Oxnard, CA). For incubation, cells were rinsed twice with HBSS and covered with 0.7 mL of HBSS. Medium was reduced to a uniform thin layer to decrease the diffusion distances of atmospheric gases. Hypoxia was produced with an atmosphere of 100% N_2 in an incubator gas chamber. PO_2 was 130 mmHg under normal conditions, decreasing to 10 mmHg after 30 min hypoxia as described here [23]. One hundred twenty minutes of hypoxia was chosen because it is the maximal hypoxia time that endothelial cells can sustain without loss of viability. Corresponding controls were always performed with cells incubated under the same conditions but kept in normoxia (ambient atmosphere).

ATP assay

ATP assay was performed using a bioluminescent ATP assay kit (FL-ASC, Sigma) using luciferase and luciferine. To ensure reproducibility and low background, all the technical precautions described in the corresponding manual sheet were observed. HUVEC were seeded at 50,000 cells/cm^2 in Petri dishes one day before the experiment. After incubation under normoxia or hypoxia, cells were rinsed with PBS, lysed with 0.5 mL of somatic cell ATP releasing reagent (Sigma) for a few seconds, and the supernatant recovered for the assay performed in a luminometer (Biocounter M2010, Lumac, Landgraaf, The Netherlands). The absolute values of ATP yielded from the experiments were expressed in RLU (relative light unit)/mg of proteins. The amount of proteins was assayed for each test to correct the amount of ATP measured in the bioluminescent assay with the number of cells present in each dish.

Glucose transport studies

Measurement of the rates of glucose transport was carried out during the hypoxia or normoxia incubation using 2-deoxy-D-glucose, a non-metabolizable glucose analogue according to Louis [24]. HUVEC seeded at confluence in Petri dishes were rinsed twice with HBSS and incubated for 120 min under normoxia or hypoxia in 0.7 mL of HBSS containing 1 μCi/mL of 2-deoxy-D-[2,6-^{3}H]-glucose (specific activity = 52 Ci/mmol, Amersham Laboratories, Buckinghamshire, U.K.). The reaction was terminated by rapidly removing the media and washing each dish three times with NaCl 0.15 M. The cell layer was allowed to dry and then dissolved in 1 mL of NaOH 0.5 N for one night. The radioactivity of 200 μL aliquots neutralized with 200 μL HCl 0.5 N was measured in 5 mL scintillation liquid (Aqualuma, Lumac, Landgraaf, the Netherlands). Two hundred μL aliquots were also assayed for protein content according to Lowry *et al.* [25], with results expressed in dpm/μg of proteins.

Lactate assay

HUVEC at confluence in Petri dishes were rinsed twice and incubated with 0.7 mL HBSS for 60 or 120 min under normoxia or hypoxia. After incubation, the medium was collected and kept at 4°C. Cells were lyzed in 0.6 mL NaOH 0.5 N for 1 hr at 4°C, and the cell lysate then neutralized with 0.2 mL HCl 1 N. The amount of lactate was measured both in the media and in the cell lysates using lactate dehydrogenase (LDH) [26]. Sixty μL of sample was added to 0.8 mL of reaction buffer (glycine 0.5 M, hydrazine (Sigma) 0.45 M, pH 9) and 60 μL of NAD^+ (Sigma) (30 mg/mL), and the absorbance at 340 nm measured (E1). Six μL of LDH (type XXVII, 5 mg/mL, Sigma) was then added, the samples incubated for 30 min at 37°C, and the absorbance measured (E2).

The lactate concentration was then calculated according to the formula:

$$[(E2 - E1) - (b2 - b1)] \times 2.35,$$

b1 and b2 being the blanks corresponding to E1 and E2.

Isolation of rat liver mitochondria

Female Wistar rats (IFFA CREDO, Brussels, Belgium) were housed in groups of 5 before the experiments, and allowed to acclimatize to their new laboratory conditions for at least 15 days. They were then treated orally for 10 to 14 days with EGb 761 or bilobalide or distilled water (0.6 mL per day). The rats were starved during the last 18 hr and, after killing, their livers were chilled in a medium containing 0.25 M saccharose, 0.1 M EDTA, 2 M HEPES, and 2 g/L bovine serum albumin (BSA). Three g of liver were homogenized by two successive passages in a Teflon homogenizer (Type C, AH Thomas Co., Philadelphia, PA). A nuclear fraction was prepared in an International PR-J Centrifuge (International Equipment Co., Needham Heights, MA) by a 10-min centrifugation at 754 g at 4°C. The supernatant was kept at 4°C. The pellet was centrifuged once more for 10 min at 580 g, and the supernatant added to the previous one and adjusted to a final volume of 45 mL. Two times 8 mL were sampled to isolate mitochondria by a 3-min centrifugation at 10,300 g in a Beckman L5 65B ultracentrifuge (Beckman Instruments Inc., Spinco Div., Palo Alto, CA). Resuspension of the mitochondrial pellet was carried out carefully with a 7-mL Dounce loose homogeneizer (Kontes Glass Co., Vineland, NY) in NaCl 7.05 mM, KCl 70.5 mM, K_2HPO_4 5.45 mM, KH_2PO_4 4.55 mM, BSA 0.15%, pH 7.2 [27].

Respiration determination

The rate of oxygen consumption by the mitochondrial fraction was assayed by an oxypolarographic method using a Clark-type electrode (39065 oxygen sensor, Beckman Instruments Inc.). The suspension was maintained in a thermostatic chamber at 27°C under magnetic agitation. The respiratory control ratio (RCR) was calculated according to Chance & Williams [28]; this is the ratio between the oxygen consumption rate in the presence of both exogenous succinate 5 mM and ADP 0.16 mM and the rate before ADP consumption.

Statistical analyses

Results are presented as means ± 1 SD, and statistical comparisons were made using Student's *t*-tests.

RESULTS

Effect of EGb 761 and bilobalide on ATP content

When HUVEC were exposed to hypoxia for 120 min, their ATP content decreased from approx. 50 to 75% of the value of control cells maintained under normoxic conditions. The variability observed in the effect of hypoxia from experiment to experiment was due to the use of different cultures of HUVEC originating from different cords. The ATP level decreased linearly during the overall incubation under hypoxia [6]. However, when incubated in the presence of EGb 761 or bilobalide during the hypoxic period, the cells showed a strong inhibition of this decrease (Fig. 1). Significant protections of 44% and 42% were obtained for 0.5 and 1 µg/mL EGb 761, respectively, while protections of 36% and 53% were recorded for 100 and 10 ng/mL bilobalide, respectively. The protection at higher concentrations of bilobalide was limited by the cytotoxic effect of this molecule observed in this model.

Effect of EGb 761 and bilobalide on glucose transport

Both compounds were able to inhibit the ATP decrease induced by hypoxia in HUVEC. This decrease was probably the result of a decrease in mitochondrial

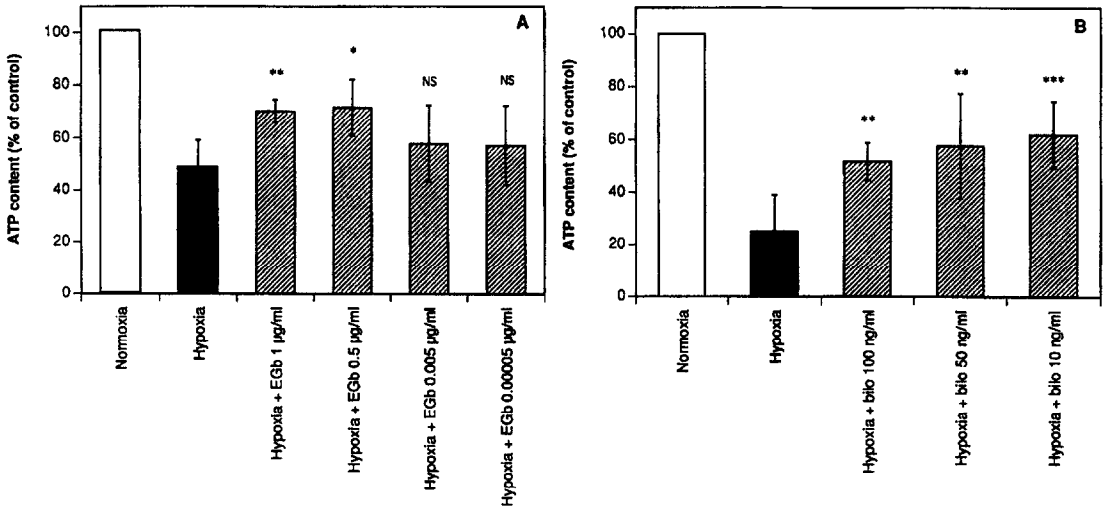

Fig. 1. Effect of EGb 761 and bilobalide on ATP content.
HUVEC were incubated for 120 min under normoxia or hypoxia in the absence or in the presence of different concentrations of (A) EGb 761 or (B) bilobalide and the ATP content was assayed. Results are expressed in percentages of control as means ± 1 SD for $n = 5$.
NS: statistically non-significantly different from cells incubated for 120 min under hypoxia.
*, **, or ***: statistically significantly different from cells incubated for 120 min under hypoxia with $P < 0.05$, $P < 0.01$, or $P < 0.001$.

respiratory chain activity due to the lack of oxygen. To compensate for this energy deficiency, the cells activated the glycolytic pathway. Two mechanisms could be responsible for the beneficial effect of EGb 761 and bilobalide on the preservation of the ATP pool of hypoxia-incubated cells: they either enhanced glycolytic activity or preserved respiratory chain activity. Both hypotheses were investigated.

To determine whether glycolytic activity was enhanced, we first studied glucose transport within the cells by measuring deoxy-D-[^{3}H]-glucose incorporation. Figure 2 (A and B) shows that hypoxia by itself significantly increased glucose transport by between 60% and 95%, which suggests that glycolysis was indeed activated under hypoxic conditions as described here. EGb 761 was not able to increase glucose transport further under hypoxic conditions, whereas a small induction (20%) was observed at 5 µg/ml under normoxic conditions (Fig. 2A). On the other hand, bilobalide significantly increased glucose transport under normoxia by 57% and 70% at 5 and 50 ng/ml, respectively. While hypoxia also increased glucose transport by 60% in this experiment, bilobalide did not further increase glucose transport under hypoxic conditions (Fig. 2B). Two explanations can be proposed for this effect: Either glucose transport was already saturated by the hypoxia-induced activation and thus bilobalide could not further increase it, or bilobalide did not influence glucose transport under hypoxic conditions. Whichever explanation is correct, neither EGb 761 nor bilobalide protection of ATP content under hypoxia can be explained by their effect on glucose transport.

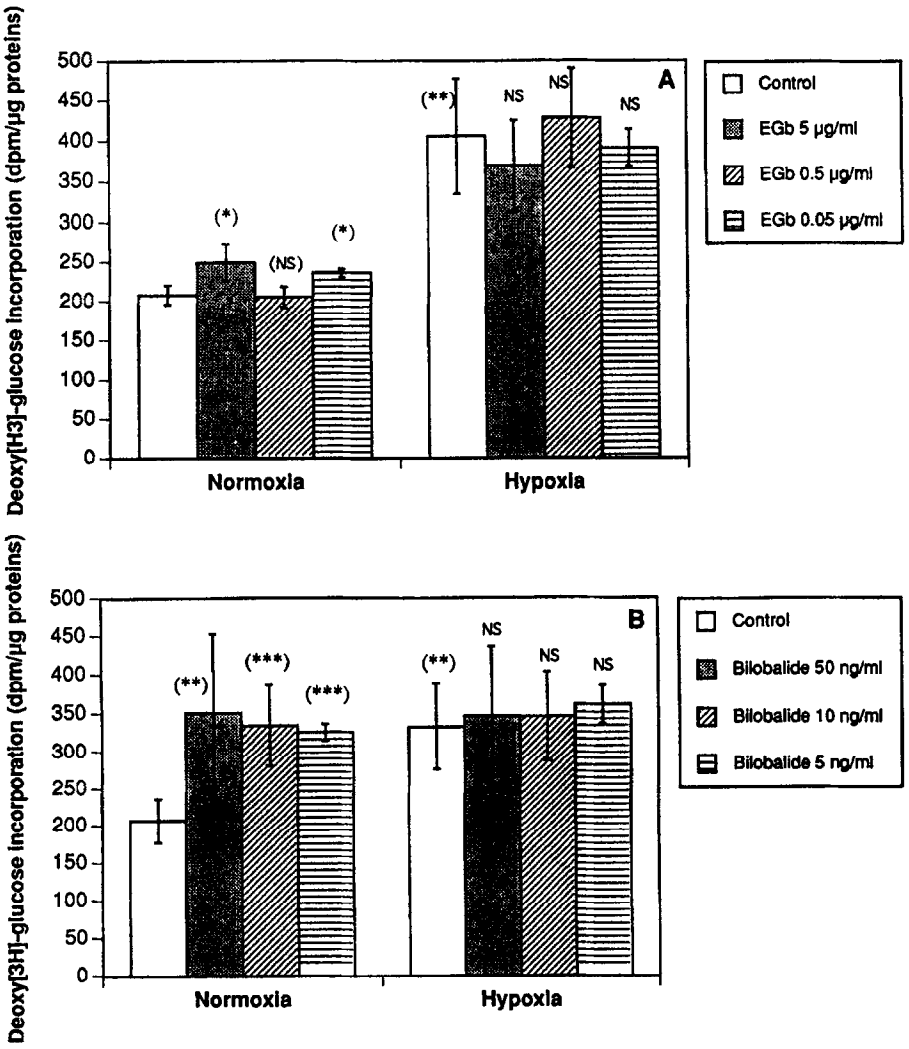

Fig. 2. Effect of EGb 761 and bilobalide on glucose transport.
HUVEC were incubated for 120 min under normoxia or hypoxia in the absence or in the presence of different concentrations of (A) EGb 761 or (B) bilobalide and glucose transport was measured by deoxy-[^{3}H]-glucose incorporation. Results are expressed in dpm/µg of proteins as means ± 1 SD for $n = 3$ for A and $n = 5$ for B. (NS): statistically non-significantly different from cells incubated for 120 min under normoxia.
(*), (**), or (***): statistically significantly different from cells incubated for 120 min under normoxia with $P < 0.05$, $P < 0.01$, or $P < 0.001$. NS: statistically non-significantly different from cells incubated for 120 min under hypoxia.

Table 1. Effect of EGb 761 on lactate production after 60 min hypoxia

	Medium	Cell lysate	Total
Normoxia (n = 3)	0.150 ± 0.008	0.176 ± 0.058	0.326 ± 0.060
Hypoxia (n = 3)	0.249 ± 0.011 (†††)	0.218 ± 0.010 *NS*	0.466 ± 0.013 (††)
Hyp + EGb 0.5 µg/ml (n = 3)	0.163 ± 0.024**	0.263 ± 0.010**	0.426 ± 0.037 NS
Hyp + EGb 0.05 (n = 3)	0.181 ± 0.008***	0.167 ± 0.016**	0.348 ± 0.023***
Hyp + EGb 0.005 (n = 3)	0.159 ± 0.015***	0.220 ± 0.025 NS	0.379 ± 0.032**

HUVEC were incubated for 60 min under normoxia or hypoxia in the absence or in the presence of different concentrations of EGb 761, and lactate content was measured in the medium and in the cell lysate. Results are expressed in µmol/dish as means ± 1 SD.

NS: statistically non-significantly different from cells incubated for 60 min under normoxia.

(††) or (†††): statistically significantly different from cells incubated for 60 min under normoxia with $P < 0.01$ or $P < 0.001$.

NS: statistically non-significantly different from cells incubated for 60 min under hypoxia.

** or *** statistically significantly different from cells incubated for 60 min under hypoxia with $P < 0.01$ or $P < 0.001$.

Effect of EGb 761 and bilobalide on lactate production

Because the glycolytic pathway can also occur through glycogen degradation, we also investigated lactate production as the end product of glycolysis.

HUVEC were first exposed to hypoxia for 60 min, and the total lactate production was found to increase by 44%. This increase was due to an increase in the amount of lactate release in the medium. This result again suggests that hypoxia activated glycolysis. EGb 761 reduced the increase in lactate release by HUVEC during the first 60 min of hypoxia to normoxic values. It also significantly inhibited total lactate production during this period (Table 1). Lactate content was slightly increased in cell lysate at the highest EGb concentration (0.5 µg/mL), but decreased at 0.05 µg EGb/mL. This suggests that lactate could be partially sequestered within the cell at the high EGb concentration. This effect is, however, much smaller than the reduction in total lactate production. Bilobalide also completely prevented the hypoxia-induced increase in lactate release as well as total lactate production (Table 2). The amount of lactate assayed in the cell lysate was not affected by hypoxia or by bilobalide.

Tables 3 and 4 present the lactate production measured after 120 min of hypoxia. An incubation of 120 min under hypoxic conditions led to a complete loss of lactate in the cell lysate and to an increase in lactate in the medium, resulting in a total lactate production similar to that observed under normoxic conditions. This suggests that glycolysis following early activation (observed at 60 min, see Tables 1 and 2) was then arrested, and that there was a leakage of lactate in the medium.

EGb 761 did not change lactate release in the medium after 120 min of hypoxia, whereas it significantly inhibited the loss of lactate in the cell lysate induced by hypoxia. Both effects resulted in a significant increase in total lactate production at concentrations between 0.5 and 0.005 µg/mL (Table 3). Similar effects were observed in the presence of bilobalide; this molecule totally prevented the loss of lactate in cell lysate while it did not affect lactate release, the net result being an increase in total lactate production of 41% and 47% at 5 and 50 ng/mL, respectively (Table 4). These results suggest that between 60 and 120 min of hypoxia, glycolysis was activated in the presence of EGb 761 and bilobalide. Figure 3 presents a time course of the effect of hypoxia on total lactate production in the absence or in the presence of EGb 761 or bilobalide. It shows that in the presence of these two products, the rate of lactate production was similar to that observed under normoxic conditions during the first hour; however, it then increased during the second hour and was identical to the rate obtained during the first hour of hypoxia in the absence of the drugs.

Effect of EGb 761 and bilobalide on mitochondrial respiration

The different results of the studies on the glycolytic pathway suggest that EGb 761 and bilobalide prevented the hypoxia-induced decrease of ATP content in HUVEC, not by activating glycolysis but rather by preserving the energy produced by the respiratory chain. Unfortunately, we were not able to purify enough mitochondria from HUVEC to measure their respiratory activity; the number of endothelial cells obtained was limiting. For this reason and to see whether EGb 761 and bilobalide could affect the respiratory activity of mitochondria, we used another experimental model: Rats were treated orally with EGb 761 or bilobalide for 10 to 14 days, and the mitochondria purified from liver and the respiratory control ratio (RCR) measured. RCR is a direct measure of the coupling of mitochondria and of the efficiency of ATP regeneration from the mitochondrial electron transport chain.

Figure 4A shows that EGb 761 treatment dose-dependently increased the RCR of the mitochondria; a significant increase from 8.43 for control mitochondria to 10.80 for mitochondria from rats treated with 100 mg/kg

Table 2. Effect of bilobalide on lactate production after 60 min hypoxia

	Medium	Cell lysate	Total
Normoxia	0.160	0.215	0.375
Hypoxia	0.335	0.209	0.571
Hyp + bilo 5 ng/ml	0.175	0.235	0.410
Hyp + bilo 10 ng/ml	0.172	0.210	0.382
Hyp + bilo 50 ng/ml	0.187	0.206	0.393

HUVEC were incubated for 60 min under normoxia or hypoxia in the absence or in the presence of different concentrations of bilobalide, and lactate content was measured in the medium and in the cell lysate. Results are expressed in µmol/dish as means for $n = 2$.

Table 3. Effect of EGb 761 on lactate production after 120 min hypoxia

	Medium	Cell lysate	Total
Normoxia ($n = 6$)	0.435 ± 0.049	0.186 ± 0.102	0.622 ± 0.116
Hypoxia ($n = 5$)	0.596 ± 0.083 (††)	0.018 ± 0.017 (††)	0.614 ± 0.098 *NS*
Hyp + EGb 5 μg/ml ($n = 2$)	0.441 NS	0.134**	0.575 NS
Hyp + EGb 0.5 ($n = 6$)	0.591 ± 0.095 NS	0.250 ± 0.113***	0.841 ± 0.153*
Hyp + EGb 0.05 ($n = 6$)	0.651 ± 0.085 NS	0.194 ± 0.104**	0.844 ± 0.147*
Hyp + EGb 0.005 ($n = 3$)	0.649 ± 0.077 NS	0.201 ± 0.060***	0.850 ± 0.127*

HUVEC were incubated for 120 min under normoxia or hypoxia in the absence or in the presence of different concentrations of EGb 761, and lactate content was measured in the medium and in the cell lysate. Results are expressed in μmol/dish as means ± 1 SD.

NS: statistically non-significantly different from cells incubated for 120 min under normoxia.

(††) statistically significantly different from cells incubated for 120 min under normoxia with $P < 0.01$.

NS: statistically non-significantly different from cells incubated for 120 min under hypoxia.

*, **, or *** statistically significantly different from cells incubated for 120 min under hypoxia with $P < 0.05$, $P < 0.01$, or $P < 0.001$.

was observed. On the other hand, the P/O ratio, which gives the number of moles of ATP generated per atom of oxygen consumed in the respiration did not change; the values were 2.65 for the control mitochondria and 2.61, 2.66, and 2.60 for rats treated with 50, 75, and 100 mg/kg, respectively.

The length of the treatment was important: The increase in RCR was not significant (9%) after 9 days of treatment with 100 mg/kg, slightly significant (18%) after 10 days, and highly significant after 12 days (36%) (Fig. 4B). In addition, the P/O ratio was not different for treated rats compared to control rats (data not shown).

Bilobalide treatment also dose-dependently increased RCR: A slightly significant increase (12%) was already observed at 1 mg/kg, and a highly significant increase was obtained at 2, 4, and 8 mg/kg; the effect was maximal at 8 mg/kg, a 72% increase (Fig. 5). This increase was much higher than that observed with EGb 761. P/O ratios did not change: A value of 2.92 was observed for the control rats and 2.97 for rats treated with 8 mg/kg. One hundred mg of EGb 761 contains 3 mg of bilobalide, and its effect on the RCR could probably be explained by the presence of this molecule. These different results suggest that EGb 761 and—more efficiently—bilobalide treatments were able to increase the respiratory efficiency of mitochondria.

We then investigated whether bilobalide could also increase the RCR of mitochondria isolated from untreated rats when these mitochondria were incubated directly in the presence of this molecule. Figure 6 shows that this was indeed the case; the RCR of naive mitochondria incubated with 0.8 and 8 μg/mL bilobalide increased by 62% and 27%, respectively, after 2 hours of incubation, while their P/O ratios remained unchanged (data not shown). The lower effect of 8 μg/mL bilobalide could probably be explained by the cytotoxic effect of this molecule (see Fig. 1). This result indicates a direct effect of bilobalide on the efficiency of respiratory activity.

Table 4. Effect of bilobalide on lactate production after 120 min hypoxia

	Medium	Cell lysate	Total
Normoxia	0.351	0.265	0.616
Hypoxia	0.592	0.021	0.613
Hyp + bilo 5 ng/ml	0.575	0.291	0.866
Hyp + bilo 10 ng/ml	0.573	0.296	0.869
Hyp + bilo 50 ng/ml	0.594	0.310	0.904

HUVEC were incubated for 120 min under normoxia or hypoxia in the absence or in the presence of different concentrations of bilobalide, and lactate content was measured in the medium and in the cell lysate. Results are expressed in μmol/dish as means for $n = 2$.

DISCUSSION

During ischemia, oxygen supply to the tissue is impaired and endothelial cells, which line all the blood vessels, are the first to undergo changes in PO_2. Glycolysis is an important pathway for energy supply in endothelial cells [29, 30], but oxidative phosphorylations occurring during mitochondrial respiration are essential to

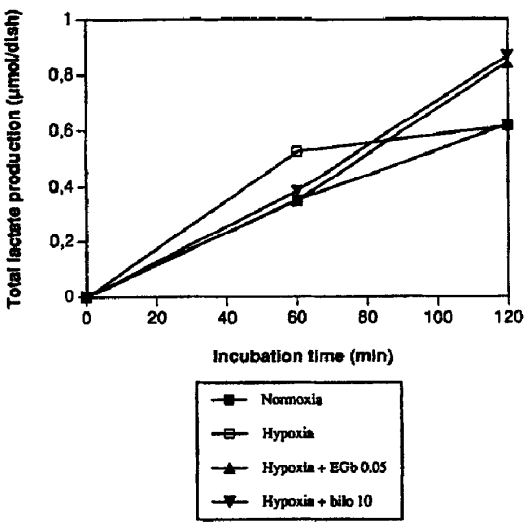

Fig. 3. Effect of EGb 761 and bilobalide on total lactate production.

HUVEC were incubated for 60 or 120 min under normoxia or hypoxia in the absence or in the presence of EGb 761 at 0.05 μg/ml or bilobalide at 10 ng/mL and lactate content in the medium and in the cell lysates was measured. Results are expressed in μmol/dish as means from data from Tables 1 to 4.

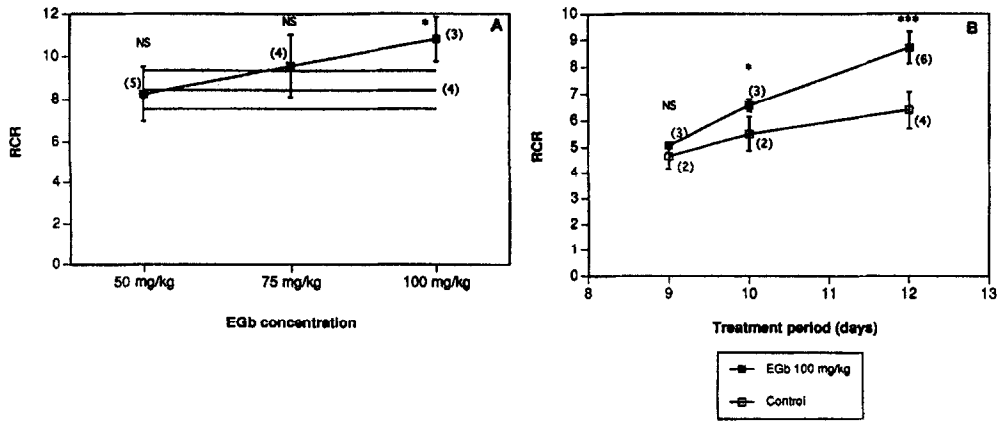

Fig. 4. (A) Effect of EGb 761 treatment on mitochondrial respiration.
Rats were treated for 12 days with different EGb 761 concentrations or with distilled water. The liver mitochondria were then purified, and the respiratory control ratio (RCR) measured. Results are presented as means ± 1 SD with the number of rats indicated between parentheses. The three horizontal lines represent the mean ± 1 SD of RCR of the control rats.
NS: statistically non-significantly different from rats treated with distilled water.
*: statistically significantly different from rats treated with distilled water with $P < 0.05$.
(B) Effect of the length of EGb 761 treatment on mitochondrial respiration. Rats were treated for 9, 10, or 12 days with EGb 761 at 100 mg/kg (■) or with distilled water (□). The liver mitochondria were then purified and the respiratory control ratio (RCR) measured. Results are presented as means ± 1 SD with the number of rats indicated between parentheses.
NS: statistically non-significantly different from rats treated with distilled water.
* or ***: statistically significantly different from rats treated with distilled water with $P < 0.05$ or $P < 0.001$.

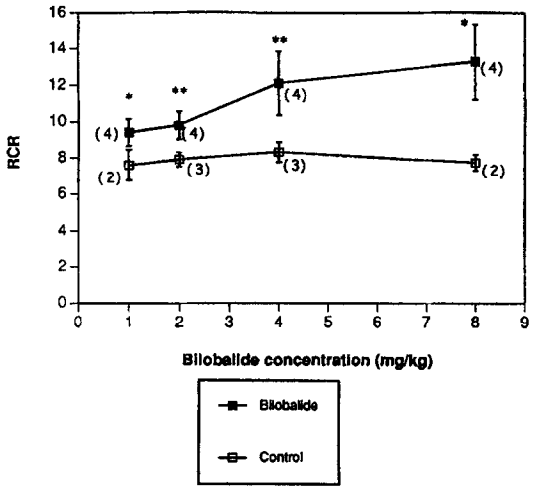

Fig. 5. Effect of bilobalide treatment on mitochondrial respiration. Rats were treated for 12 days with different bilobalide concentrations (■) or with distilled water (□). The liver mitochondria were then purified and the respiratory control ratio (RCR) measured. Results are presented as means ± 1 SD with the number of rats indicated between parentheses.
NS: statistically non-significantly different from rats treated with distilled water.
* or **: statistically significantly different from rats treated with distilled water with $P < 0.05$ or $P < 0.01$.

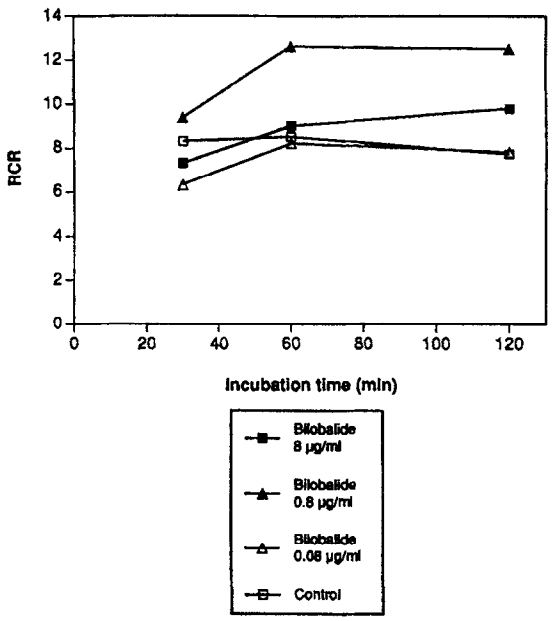

Fig. 6. Effect of bilobalide on mitochondrial respiration of naive mitochondria. Mitochondria of non-treated rats were purified and incubated with different concentrations of bilobalide and the RCR was measured after different incubation times.

maintaining a high level of ATP. The ATP level is a steady state of synthesis and degradation. It is generally accepted that cells adapt under hypoxic conditions to decrease their ATP utilization to housekeeping functions [31]. For example, protein synthesis in hypoxic endothelial cells is partially arrested to save ATP [32]. Unfortunately, the adaptative mechanism of the reduction of ATP demand is not powerful enough to compensate for reduced ATP production. Under these conditions, a decrease in ATP level during hypoxia is observed, mainly due to a decrease in production. It is this aspect that we investigated in this work.

When oxygen supply is impaired, as occurs during

ischemia or hypoxia, mitochondrial respiration decreases and glycolytic metabolism is enhanced to compensate for the decrease in ATP regeneration. This is evidenced by increased lactate production and increased activities of pyruvate kinase and phosphofructokinase [14]. However, glycolysis is not sufficient to fully regenerate the ATP needed by the cells, and a series of modifications occur in cellular functions: lowering or arrest of the Na^+ and Ca^{++} ATPase pumps, alteration of the plasma membrane and of the regulation of cellular volume, dissociation of the cytoskeleton elements, inhibition of protein synthesis, mitochondria swelling, lysosome dilatation and rupture, etc. All these events can lead to cell death [33, 34].

When endothelial cells are exposed *in vitro* to hypoxia, an important decrease in their ATP content is observed within 2 hours of incubation. In parallel, glycolysis is activated, as evidenced by an increase in glucose transport and in lactate production. This latter effect was observed after 1 hour of hypoxia incubation, but was not detected after 2 hours, indicating that after early activation, glycolysis was then arrested. Due to the decrease in oxidative phosphorylations, there was an increase in $NADH/NAD^+$ ratio [35]. Increased NADH concentration inhibited glyceraldehyde-3-phosphate dehydrogenase, and this can explain why after a transient activation, glycolysis strongly decreased in cells incubated under hypoxia in the presence of glucose [36], as in the experiments described here.

EGb 761 was shown to protect the ATP content in HUVEC during hypoxia incubation. A previous study demonstrated that this protective effect of EGb 761 was due to terpenes present in this plant extract, with the most active compound being bilobalide [20]. Bilobalide alone also protects against ATP decrease: a 36% protection was observed at 10 ng/mL, which can account for the 44% protection observed with 0.5 ng/mL EGb 761, since bilobalide represents 3% of EGb 761.

The mechanism by which EGb 761 and bilobalide preserved the ATP content of hypoxia-incubated HUVEC was investigated. We first checked a possible effect on glycolysis. Bilobalide, and to a much lesser extent EGb 761, increased glucose transport within HUVEC under normoxic conditions. Bruel *et al.* [17] have already shown that EGb 761 preincubation of smooth muscle cells also increased glucose transport *in vitro*, and Rapin *et al.* [16] observed a similar effect in erythrocytes of rats treated *in vivo* with this compound. However, neither compound affected glucose transport of HUVEC under hypoxic conditions, suggesting that they do not act as activators of glycolysis. This hypothesis was confirmed by the studies on lactate production. Lactate production increased after 1 hour hypoxia, indicating that hypoxia activated glycolysis. However, this activation did not occur in the presence of EGb 761 and bilobalide during this first hour of hypoxia incubation. These results suggest that glycolysis is not induced probably because it is not needed to produce ATP when EGb 761 or bilobalide is present, suggesting that they could act by preserving ATP production by the mitochondria. This hypothesis was reinforced by the results obtained after 2 hours of hypoxia: glycolysis seemed to be arrested in HUVEC, but was then activated in the presence of EGb 761 or bilobalide. In conclusion, EGb 761 and bilobalide preserve the ability to form ATP, thereby reducing the need of the cell to induce glycolysis, probably by preserving ATP regeneration by mitochondria as long as a small amount of oxygen is available. When this oxygen is consumed or when mitochondrial respiratory activity is inhibited, then glycolysis is activated. This effect indicates that the protection of the hypoxia-induced ATP decrease observed in Fig. 1 represents a delay in the decrease in ATP content in the presence of EGb 761 or bilobalide.

Mitochondria have been shown to be altered in ischemic organs both morphologically and functionally [37–38]; their alterations seriously impair the functions of the ischemic organ [31], so that a protection of their functions by EGb 761 or bilobalide would be very beneficial. The results presented in this report show that EGb 761 or bilobalide treatment of rats markedly ameliorates the coupling of the liver mitochondria, and thus their respiratory efficiency. This effect was dose-dependent, and led to a very high RCR of 13.3 at 8 mg/kg bilobalide. On the other hand, the P/O ratio was not changed.

It was previously shown that ischemia decreases the coupling of mitochondria [39]. This effect may be due to a change in mitochondrial membrane fluidity, as observed by Shin *et al.* [37], or to a loss or denaturation of the compounds of the respiratory chain, as described by Piper *et al.* [31]. Among them, NADH reductase (complex I) is impaired early in ischemic myocardium [40, 41]. This uncoupling of mitochondria results from a collapse of the transmembraneous proton gradient, thereby impairing ATP regeneration as well as electron transfer; electrons are also found to leak at the ubisemiquinone transporter [42]. From all the results presented in this manuscript, a mechanism of the protective effect of EGb 761 and bilobalide on hypoxia-induced ATP decrease may be proposed. The protection of the coupling of mitochondria in hypoxic cells by these drugs will preserve their capacity to regenerate ATP as long as oxygen is available, thereby delaying the toxic effects of hypoxia. The protection of mitochondria by EGb would explain the reduced need of the cell to induce glycolysis. This hypothesis, of course, needs to be confirmed, and the precise site where bilobalide acts is currently under investigation.

The decrease in the ATP content of endothelial cells due to hypoxia is the first event in a cascade of reactions that leads to the release of inflammatory mediators, resulting in adherence and activation of neutrophils and stimulation of the growth of smooth muscle cells [43, 12]. By protecting alterations in endothelial cell metabolism induced by energy deficiency during ischemia, EGb 761 and bilobalide could prevent the subsequent inflammatory response and further damages to the ischemic tissue, accounting for their beneficial pharmacological effects.

Acknowledgements—We thank the doctors and nurses of the Clinique Sainte Elisabeth for providing the umbilical cords. This work was supported by a grant from the Fonds de la Recherche Fondamentale Collective. C. Michiels is a Senior Research Assistant at the Fonds National de la Recherche Scientifique (Brussels, Belgium). This text presents results of the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming. Scientific responsibility is assumed by its authors. The support of the Institut Henri Beaufour is also gratefully acknowledged.

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