British Journal of Pharmacology (2000) 130, 1513–1524

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Effect of venotropic drugs on the respiratory activity of isolated mitochondria and in endothelial cells

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1 Several drugs used in the treatment of chronic peripheral ischaemic and venous diseases, i.e. aescine, Cyclo 3, Ginkor Fort, hydroxyethylrutosides, naftidrofuryl, naphthoquinone and procyanidolic oligomers, were tested on the mitochondrial respiratory activity.

2 The results show that all these drugs protected human endothelial cells against the hypoxia-induced decrease in ATP content. In addition, they all induced a concentration-dependent increase in respiratory control ratio (RCR) of liver mitochondria pre-incubated with the drugs for 60 min.

3 The drugs were divided into two groups according to their effects. The first group (A), comprising aescine, Ginkor Fort, naftidrofuryl and naphthoquinone, increased RCR by decreasing state 4 respiration rate. The second group of drugs (B), comprising hydroxyethylrutosides, procyanidolic oligomers and Cyclo 3, increased RCR by increasing state 3 respiration rate. The drugs of group A were able to prevent the inhibition of complexes I and III respectively by amytal and antimycin A while the first two drugs of group B increased adenine nucleotide translocase activity. Cyclo 3 inhibited the carbonylcyanide m-chlorophenyl hydrazone (mCCP)-induced uncoupling of mitochondrial respiration. None of these seven drugs could protect complexes IV and V, respectively, from inhibition by cyanide and oligomycin.

4 When tested on endothelial cells the drugs of group A, in contrast to group B, prevented the decrease in ATP content induced by amytal or antimycin A.

5 The present results suggest that the protective effects on mitochondrial respiration activity by these venotropic drugs may explain their protective effect on the cellular ATP content in ischaemic conditions and some of their beneficial therapeutic effect in chronic vascular diseases.

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Keywords: Venotropic drugs; hypoxia; endothelial cells; mitochondria; respiration; cellular; phosphorylation; oxidative; adenine nucleotide translocase

Abbreviations: FAD, flavin adenine dinucleotide; HBSS, Hanks balanced salt solution; HUVEC, human umbilical vein endothelial cells; mCCP, carbonylcyanide m-chlorophenyl hydrazone; NAD, nicotinamide adenine dinucleotide; PBS, phosphate-buffered saline; RCR, respiratory control ratio

Introduction

Vascular diseases affect a high proportion of the population in developed countries. They vary in nature, including such conditions as arteritis, thrombosis, myocardial infarction, cerebral ischaemia and chronic venous insufficiency. All of these diseases are associated with more or less pronounced ischaemic conditions. Chronic venous insufficiency is one of these vascular diseases. A possible mechanism for the development of varicose veins was recently described to explain the irreversible changes occurring in the vessel wall (Michiels *et al*., 1997).

The pathological activation of endothelial cells by ischaemic conditions developing during blood stasis has been proposed to play a key role in this process (Michiels *et al*., 1994). The initial event of the pathological activation of endothelial cells by hypoxia *in vitro* is a decrease in intracellular ATP content which leads to an increase in cytosolic calcium concentration (Arnould *et al*., 1992). This decrease is due to a decrease in mitochondrial respiration. Calcium can then induce the activation of phospholipase A_2, which is responsible for an increase in the synthesis of prostaglandins and of platelet-activating factor (Michiels *et al*., 1993a). This process results in an increase in endothelial cell adhesiveness for neutrophils (Arnould *et al*., 1993) and to the subsequent activation of these blood cells (Arnould *et al*., 1993; 1994). Hypoxia is thus able to initiate, *via* an active role of the endothelium, an inflammatory response which then initiates and promotes tissue damage. According to this hypothesis, preservation of ATP regeneration by drugs should prevent the hypoxia-induced endothelial activation and, hence, would protect tissue from subsequent alteration.

The critical role played by mitochondria in the maintenance of cellular energy metabolism has long been recognized (Jennings & Ganote, 1976; Trump *et al*., 1976). Electron transport from the oxidation of reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$) to O_2 is tightly coupled to the synthesis of ATP. The electron transport occurs through protein-bound redox centres, from complex I (NADH-Coenzyme Q reductase) or II (succinate-Coenzyme Q reductase) to III (Coenzyme Q-cytochrome c reductase) and then to IV (cytochrome c oxidase). The free energy released by this transport is conserved by pumping out protons in order to create an electrochemical H^+ gradient across the inner mitochondrial membrane. The electrochemical potential of this gradient is then harnessed in the synthesis of ATP by complex V (ATP synthase): this process is known as oxidative phosphorylation.

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In ischaemic conditions it has been established that, in a large number of organs, there is a relationship between mitochondrial dysfunction and the irreversibility of pathological damage (Veitch *et al*., 1992; Allen *et al*., 1995). Indeed, the transition from reversible to irreversible ischaemia is probably dependent on the functional state of mitochondria (Jennings, 1969; Taegtmeyer *et al*., 1985). Mitochondria have been shown to be altered both morphologically and functionally in ischaemic organs (Yanagiya, 1994), while ischaemia induces a decrease in the respiratory activity of mitochondria in several species, mainly at the level of NAD^+-dependent substrate (Almeida *et al*., 1995). Damage occurring in mitochondria during ischaemia is associated with a loss of respiratory activity of complex I in the electron transport chain; the activity of complex III is also altered but this occurs at later time points. A decrease in NADH-ubiquinone reductase and adenine nucleotide translocase activities and in cytochromes a and a_3 and cytochromes c and c_1 then leads to complete loss of respiratory activity (Duan & Karmazyn, 1989; Veitch *et al*., 1992). These alterations finally result in mitochondrial swelling and ultrastructural alterations such as convolutions of the mitochondrial inner membrane and appearance of amorphous dense bodies in the matrix (Yanagiya, 1994).

In addition to these processes, an increase in mitochondrial calcium content is observed during and after hypoxia (Silverman, 1993). This phenomenon may be responsible for cytochrome c release (Borutaite *et al*., 1999), matrix swelling through opening of the permeability transition pore (Saris *et al*., 1998), respiratory uncoupling (Lemasters *et al*., 1997) or calcium cycling and collapse of the proton-motive force (Richter & Frei, 1988). However, the significance of this increase for the ischaemic impairment of respiratory activity remains controversial.

The understanding of the mechanism through which many of the drugs claimed to be beneficial in chronic venous insufficiency and arteriopathy work is currently not known; this is particularly true for drugs derived from plant extracts. In this work, some of these drugs were tested in order to investigate whether they could protect the cellular ATP content in hypoxic conditions and whether they have a direct effect on mitochondrial respiration. In a previous study we showed that bilobalide, a molecule present in one of these drugs, Ginkor Fort, could prevent the hypoxia-induced decrease in ATP content in endothelial cells. This effect was explained by an increase in the respiratory activity of isolated mitochondria (Janssens *et al*., 1995). Other studies also showed that three other drugs (aescine, hydroxyethylrutosides and naftidrofuryl) used in the treatment of chronic venous insufficiency or arteriopathy could also prevent the ATP decrease induced by hypoxia in cultured endothelial cells (Arnould *et al*., 1996; Janssens *et al*., 1996; Michiels *et al*., 1993a). Since the mechanism of this protection is not known, we investigated here the effect of these drugs, as well as some others also used in the treatment of chronic venous insufficiency or of arteriopathy, on the respiratory activity of isolated mitochondria. In addition, their effects on the different complexes of the respiratory chain were studied.

Methods

Materials

Modified Hanks balanced salt solution (HBSS: (mM): NaCl 140, KCl 5, $MgSO_4$ 0.4, $MgCl_2$ 0.5, Na_2HPO_4 3, KH_2PO_4 0.4, glucose 5.5, pH 7.35) was prepared in our laboratory. Medium 199 and foetal calf serum were obtained from Gibco Life Technologies (Paisley, U.K.). ADP, amytal, antimycin A, atractyloside, bovine serum albumin (BSA, essentially fatty acid- and γ-globulin-free), collagenase type II, ferrocytochrome c, β-hydroxybutyrate, oligomycin and succinic acid were from Sigma Chemical Co. (St Louis, MO, U.S.A.). [^{14}C]-ADP (specific activity 55 mCi $mmol^{-1}$) was purchased from Dupont-NEN (Boston, MA, U.S.A.). Dinitrophenol, KCN, $Fe(CN)_3$ and all other chemicals of analytical grade were from Merck KG (Darmstadt, Germany).

Drugs were obtained from the following sources: Aescine (Madaus AG, Cologne, Germany); Cyclo 3 (Laboratoires Fabré, Castre, France); Ginkor Fort (Laboratoires Beaufour, Paris, France); hydroxyethylrutosides (Novartis SA, Basel, Switzerland); melilot extract (Boots plc, Nottingham, U.K.); naftidrofuryl (Lipha Santé SA, Lyon, France); naphthoquinone (Aldrich SA, Beerse, Belgium); procyanidolic oligomers (Sanofi Synthelabo, Paris, France).

HUVEC isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated according to Jaffe *et al*. (1973b). Cords were stored at 4°C immediately after birth in stock buffer ((mM): KCl 4, NaCl 140, HEPES 10, glucose 1, streptomycin 100 μg ml^{-1}, penicillin 100 u ml^{-1}, fungizone 0.25 μg ml^{-1}). Before manipulation, cords were rinsed with 20 ml phosphate-buffer saline (PBS) containing antibiotics and fungizone at the above concentrations. Umbilical veins were incubated for 35 min at 37°C with 4 ml type II collagenase 0.05% in PBS. The cells were then harvested in Medium 199 containing 20% foetal calf serum, centrifuged for 10 min at 1000 r.p.m. and seeded in 0.20% gelatine-coated 25-cm^2 culture dishes (Falcon Plastics, Oxnard, CA, U.S.A.). The following day, the cells were washed with medium to eliminate blood cell contamination. Only primary cultures were used for these studies. Confirmation of their identity as endothelial cells was provided by detection of factor VIII antigen, assessed by immunofluorescence staining (Jaffe *et al*., 1973a).

In vitro *model of hypoxia*

Hypoxic incubation was performed by exposing cells to 100% N_2 at 37°C. Cells were seeded in gelatine-coated 35-mm Petri dishes (Falcon). 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. Partial pressure of O_2 was 130 mmHg under normal conditions, decreasing to 10 mmHg after 30 min hypoxia (Michiels *et al*., 1992). Two hours of hypoxia was chosen because it is the maximal 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 normoxic conditions (ambient atmosphere).

ATP assay

ATP assay was performed using a bioluminescence ATP assay kit (FL-ASC, Sigma) using luciferase and luciferin. To ensure reproducibility and low background, all technical precautions described in the corresponding manual sheet were observed. HUVEC were seeded at 50,000 cells $dish^{-1}$ in Petri dishes 1 day before the experiment. After incubation under normoxic

or hypoxic conditions, cells were rinsed with PBS and lysed with 0.5 ml of somatic cell ATP releasing reagent (Sigma) for a few seconds; the supernatant was recovered for the assay performed in a luminometer (Biocounter M2010, Lumac, Landgraaf, The Netherlands). The values of ATP yielded from the experiments were expressed in RLU (relative light unit) μg protein^{-1}. The quantity of protein was assayed for each test to correct the amount of ATP measured in the bioluminescence assay with the number of cells present in each dish. Results for each drug are expressed as percentage protection: (hypoxia + drug − hypoxia)/(normoxia − hypoxia) × 100.

The effects of amytal and of antimycin on the ATP content were also investigated. HUVEC were pre-incubated for 60 min with the drugs and in the presence of one of these metabolic inhibitors. The concentrations of the drugs in these experiments were 0.75 μg ml^{-1} aescine, 75 μg ml^{-1} diosmin, 0.3 mg ml^{-1} Ginkor Fort, 0.3 mg ml^{-1} melilot extract, 47 ng ml^{-1} naftidrofuryl, 0.16 μg ml^{-1} naphthoquinone, 22.5 μg ml^{-1} Cyclo 3, 1 mg ml^{-1} hydroxyethylrutosides and 5 μg ml^{-1} procyanidolic oligomers. Results are expressed in RLU μg protein^{-1}.

Isolation of rat liver mitochondria

Female Wistar rats (IFFA Credo, Brussels, Belgium) were housed before the experiments in groups of four and allowed to acclimatize to their new laboratory conditions for at least 14 days. Rats were fasted for at least 18 h and following sacrifice the liver was chilled in a medium containing sucrose 0.25 M, EDTA 1 mM, HEPES 1 mM and bovine serum albumin 0.2% w v^{-1}. Liver (3 g) was homogenized by two successive passages in a Teflon homogenizer (Type C, AH Thomas Co, Philadelphia, PA, U.S.A.). A nuclear fraction was prepared by a 10-min centrifugation at 754 × *g* at 4°C. The supernatant was stored at 4°C. The pellet was centrifuged for a further 10 min at 580 × *g* and the supernatant added to the previous one and adjusted to a final volume of 45 ml. Two 8-ml portions were sampled to isolate mitochondria by a 3-min centrifugation at 10,300 × *g*. Resuspension of the mitochondrial pellet was carried out carefully with a 7-ml Dounce loose glass homogenizer (Kontes Glass Co., Vineland, NY, U.S.A.) in mitochondria buffer ((mM): NaCl 7.05, KCl 70.5, K_2HPO_4 5.45, KH_2PO_4 4.55, BSA 0.15% w v^{-1}, pH 7.2).

Mitochondrial incubation with drugs

Mitochondria isolated from rat liver were suspended in mitochondria buffer at a final protein concentration of approximately 40 mg ml^{-1}. The mitochondria were then incubated for 60 min in the presence of the different drugs at 4°C. At the end of the incubation, different parameters were assayed. For each experiment – i.e. to study the effect of one drug at different concentrations on one parameter or to study the effect of different drugs at one concentration – two separate experiments were performed using one liver preparation. It should be noted that state 3 and state 4 respiration rates are usually very similar from one preparation to another.

Incubations were conducted in triplicate for 60 min and then state 3 and state 4 were measured for each sample. Incubations were performed sequentially – control followed by first drug concentration, second drug concentration and third drug concentration – and then the same sequence was repeated twice. This same order was maintained for measurement, so that the effects observed are not due to differences related to time after isolation or order of measurement (in this design, the data for the different controls were stable over time).

Respiration determination

The rate of oxygen consumption by the mitochondrial fraction was assayed by an oxypolarographic method using a Clark type electrode. The respiratory control ratio (RCR) was calculated according to Chance & Williams (1953): it is the ratio between the oxygen consumption rate in the presence of both exogenous succinate 5 mM or glutamate/malate 10 mM and ADP 0.16 mM (state 3) and the rate after ADP consumption (state 4). The values for states 2 and 4 were similar under our experimental conditions.

Inhibition of mitochondrial electron transfer

Oxygen consumption was followed by a Clark electrode. Reactions were performed at 25°C in a 2.5-ml chamber containing 2 mg of mitochondrial suspension in the incubation buffer. Respiration rates were measured using 10 mM D-β-hydroxybutyrate (for complex I), 5 mM of succinate (for complex III and complex V) or 6 mM $Fe(CN)_3$ (for complex IV) as substrates. Inhibition studies were performed by the addition of different concentrations of amytal (for complex I), antimycin A (for complex III), KCN (for complex IV) or oligomycin (for complex V) to a reaction containing non-limiting amounts of substrate.

The experiments were performed as follows: O_2 consumption was measured for mitochondria in the presence of the substrate and then the inhibitor was added into the chamber and O_2 consumption measured again. The inhibition was thus measured on state 4 respiration rate. This was done both for control mitochondria and for mitochondria pre-incubated with bilobalide or isolated from bilobalide-treated rats. The percentage inhibition was calculated as (O_2 consumption without inhibitor $-O_2$ consumption with inhibitor)/O_2 consumption without inhibitor × 100.

The concentrations of drugs used in these experiments were 75 ng ml^{-1} aescine, 0.3 mg ml^{-1} Ginkor Fort, 47 ng ml^{-1} naftidrofuryl, 15.8 ng ml^{-1} naphthoquinone, 4.5 μg ml^{-1} Cyclo 3, 0.1 mg ml^{-1} hydroxyethylrutosides and 1.5 μg ml^{-1} procyanidolic oligomers.

Adenine nucleotide translocase

Adenine nucleotide translocase activity was determined in terms of atractyloside-sensitive ADP uptake using the procedure of Duée & Vignais (1969), adapted by Duan & Karmazyn (1989). Briefly, 50 nmol [^{14}C]-ADP was added to the reaction medium containing 110 mM KCl, 20 mM Tris and 1 mM EDTA (pH 7.4) and 500 μg mitochondrial proteins. The reaction was carried out on ice and stopped after 40 s by the addition of 100 μM atractyloside. This reaction time was selected because preliminary results showed that, by this time, ADP was at 50% of the maximum transport in the mitochondria. The reaction mixture was centrifuged at 25,000 × *g* for 5 min and the supernatant was discarded. The pellet was washed twice with ice-cold reaction medium, dissolved in 0.5 ml NaOH 0.5 M and counted for radioactivity in 5 ml scintillation fluid (Aqualuma, Lumac, Landgraaf, The Netherlands) in a liquid scintillation counter.

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Statistical analysis

Results are presented as means ± 1 s.d. Statistical analyses were performed using Student's *t*-tests, one-way analysis of variance (ANOVA 1) with Scheffé's contrasts for multiple comparison within one experiment, or two-way ANOVA (crossed ANOVA 2 with one fixed factor, drug, and one randomized factor, mitochondrial preparation or cell culture) with Scheffé's contrasts for multiple comparison of data from more than one experiment.

Results

ATP content of HUVEC under hypoxia

The effects of nine venotropic drugs commonly used in the treatment of chronic venous insufficiency or arteriopathy were first investigated on HUVEC exposed to hypoxic conditions: aescine, Cyclo 3, diosmin, Ginkor Fort, hydroxyethylrutosides, melilot extract, naftidrofuryl, naphthoquinone and procyanidolic oligomers. The respective efficiency of the different drugs was compared for the protection of ATP content. When HUVEC are exposed to hypoxia for 120 min, the ATP content decreases by 30–40% of its normoxic value. Since each experiment was performed on primary cultures isolated from different umbilical cords, there is some variability in the absolute values. Therefore, we expressed the results in percentage protection with, for each experiment, hypoxic control cells (0% protection) and normoxic cells (100% protection).

All of the drugs demonstrated a protective effect which increased with concentration (Figure 1). Some of the drugs presented a bell-shaped concentration-response curve. However, the protective concentration range differed from one drug to another. The maximal protection obtained was 95% for aescine at 0.75 μg ml^{-1}, 79% for Cyclo 3 at 12 μg ml^{-1}, 32% for diosmin at 30 μg ml^{-1}, 103% for Ginkor Fort at 75 μg ml^{-1}, 99% for hydroxyethylrutosides at 1 mg ml^{-1}, 70% for naftidrofuryl at 0.47 μg ml^{-1}, 92% for naphthoquinone at 0.158 μg ml^{-1}, 79% for melilot extract at 0.5 mg ml^{-1} and 97% for procyanidolic oligomers at 7.5 μg ml^{-1}. Four of the drugs were active at concentrations below 1 μg ml^{-1} while the others needed much higher concentrations.

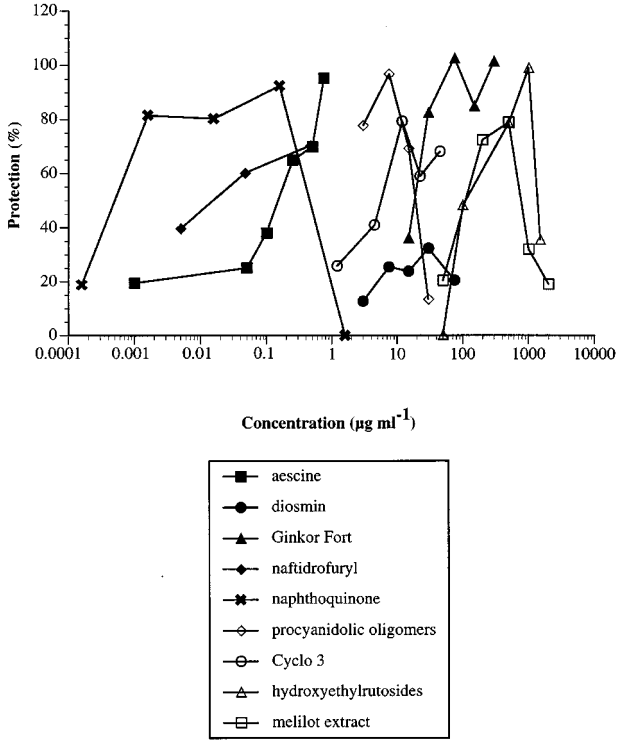

Figure 1 The effects of nine venotropic drugs used in the treatment of chronic venous insufficiency or arteriopathy on hypoxia-induced decrease in ATP content in HUVEC. HUVEC were incubated 120 min in normoxia or hypoxia in the presence or absence of different concentrations of aescine, Cyclo 3, diosmin, Ginkor Fort, hydroxyethylrutosides, melilot extract, naftidrofuryl, naphthoquinone or procyanidolic oligomers. Results are expressed as percentage of protection, with 0 and 100% protection being the values for non-protected hypoxic cells and normoxic cells, respectively. Data are mean of two experiments conducted in triplicate as described in Methods.

Respiratory control ratio

Previous studies showed that bilobalide, one component of *Ginkgo biloba* extract, demonstrated similar properties on the ATP content of hypoxic endothelial cells and that this protection was attributable to an increase in the mitochondrial respiration control ratio (Janssens *et al*., 1995). The effect of these nine drugs was investigated on mitochondrial respiration: isolated rat liver mitochondria were incubated 60 min in the presence of different concentrations of each of the drugs and the RCR was measured. The mean RCR value for control mitochondria was 5.95 ± 0.51 (mean value for 10 different mitochondria preparations). Whenever the initial RCR value for freshly prepared mitochondria was lower than 5, or if there was a drop in control RCR values greater than 10% during the experiment, the experimental data were discarded.

Figure 2 shows that all the nine drugs tested were able to increase RCR, with an optimal effect of 18% at 75 ng ml^{-1} aescine, 14% at 4.5 μg ml^{-1} Cyclo 3, 12% at 0.608 μg ml^{-1} diosmin, 28% at 0.3 mg ml^{-1} Ginkor Fort, 23% at 0.1 mg ml^{-1} hydroxyethylrutosides, 18% at 47 ng ml^{-1} naftidrofuryl, 19% at 15.8 ng ml^{-1} naphthoquinone, 16% at 1 mg ml^{-1} melilot extract and 15% at 1.5 μg ml^{-1} procyanidolic oligomers. Each drug presented a bell-shaped concentration-response curve, indicating that the effect was limited at high concentrations for all of them. Again, there was very large variation between the effective concentrations of the various drugs.

RCR is calculated as the ratio between state 3, which is the rate of phosphorylating respiration in the presence of exogenous ADP, and state 4, which is the rate of resting respiration without ADP. We analysed the effect of each drug on state 3 and state 4 (Table 1). Interestingly, the increase in RCR induced by aescine, diosmin, Ginkor Fort, naftidrofuryl and naphthoquinone was due to a larger decrease in state 4 than in state 3. The decrease in state 4 was respectively by 42% at 75 ng ml^{-1} aescine, 26% at 0.608 μg ml^{-1} diosmin, 21% at 0.3 mg ml^{-1} Ginkor Fort, 32% at 47 ng ml^{-1} naftidrofuryl and 24% at 15.8 ng ml^{-1} naphthoquinone. On the other hand, the increase in RCR induced by Cyclo 3, hydroxyethylrutosides, melilot extract and procyanidolic oligomers was due to a larger increase in state 3 than in state 4, by 43% at 4.5 μg ml^{-1} Cyclo 3, 19% at 0.1 mg ml^{-1} hydroxyethylrutosides, 41% at 1 mg ml^{-1} melilot extract and 33% at 1.5 μg ml^{-1} procyanidolic oligomers. It must be noted that several of the drugs also affected the other respiratory state but to a lesser extent so that the overall effect was an increase in RCR. None of the drugs at the concentrations tested here was able to increase or decrease the ADP/O ratio (data not shown).

Increase in state 4 respiration rate is often linked to calcium cycling, which increases proton leak (Richter & Frei, 1988). To exclude any effect of the drugs on calcium cycling or calcium uptake, 0.4 mM EGTA was added to the incubation buffer of mitochondria pre-incubated for 60 min in the presence of the optimal concentration of each drug. While each of the drugs tested did significantly increase the RCR, this increase was not significantly affected by EGTA except for the hydroxyethylrutosides (25% increase in the presence of EGTA compared to 39% increase without; Table 2).

These results indicate that, while all drugs increased the mitochondrial respiratory control ratio, they seem to do so by at least two different mechanisms: decrease in state 4 or stimulation of state 3 respiration rate. These mechanisms do not seem to involve inhibition of calcium uptake and/or cycling.

Inhibition of mitochondrial respiration

The identity of the target of these two groups of drugs was then investigated by the study of various factors which modulate the RCR, i.e. the efficiency of the electron transport chain, the ATPase activity, the adenine nucleotide translocase activity and the proton leak. Their possible effect was first tested on the electron transport chain complexes by studying their protection of the mitochondrial respiratory activity when the various complexes were blocked by specific inhibitors.

The specific inhibitors of the different complexes of the electron transport chain were amytal, which blocks the complex I activity, antimycin A for complex III and cyanide for complex IV. For each inhibitor, we first performed a concentration-inhibition curve for the oxygen consumption (Figures 3A, 4A and 5A). We then selected the concentration which gave around 50% inhibition of the oxygen consumption. These concentrations of inhibitors were, respectively, 0.5 mM, 0.75 μM and 0.3 mM for amytal, antimycin A and cyanide. The effect of the drugs was then studied on state 4 respiration rate in the presence of each of the different inhibitors.

Mitochondria were pre-incubated for 60 min in the presence of each drug, at the concentration which was the most active on RCR. After this pre-incubation, the oxygen consumption was measured in the absence and then in the presence of the inhibitor. Protections of 26, 32, 33 and 27% for the amytal-induced inhibition of oxygen consumption were observed in the presence of aescine, Ginkor Fort, naftidrofuryl

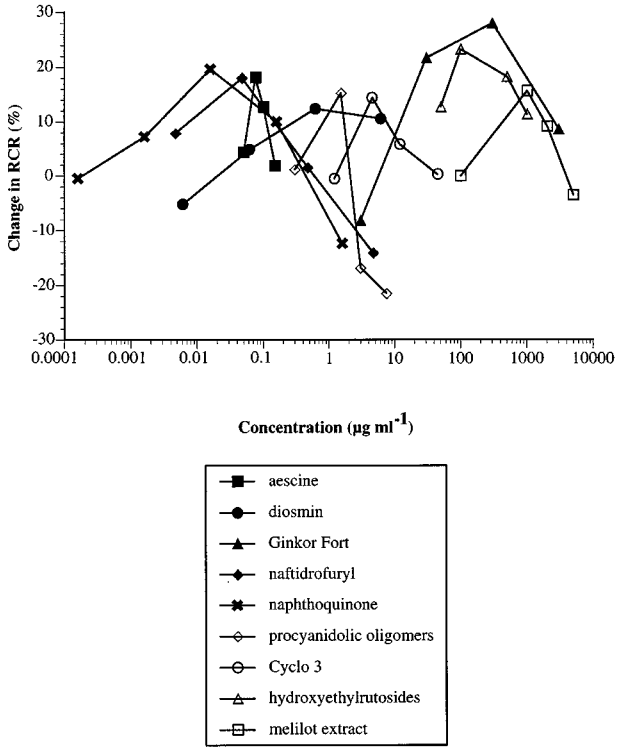

Figure 2 The effects of venotropic drugs on mitochondrial respiration. Mitochondria of non-treated rats were incubated 60 min with different concentrations of aescine, Cyclo 3, diosmin, Ginkor Fort, hydroxyethylrutosides, melilot extract, naftidrofuryl, naphthoquinone and procyanidolic oligomers, and the RCR was measured. Results are expressed as percentage of RCR of control mitochondria incubated for 60 min with the incubation medium alone. Data are mean of three independent samples of one mitochondrial preparation.

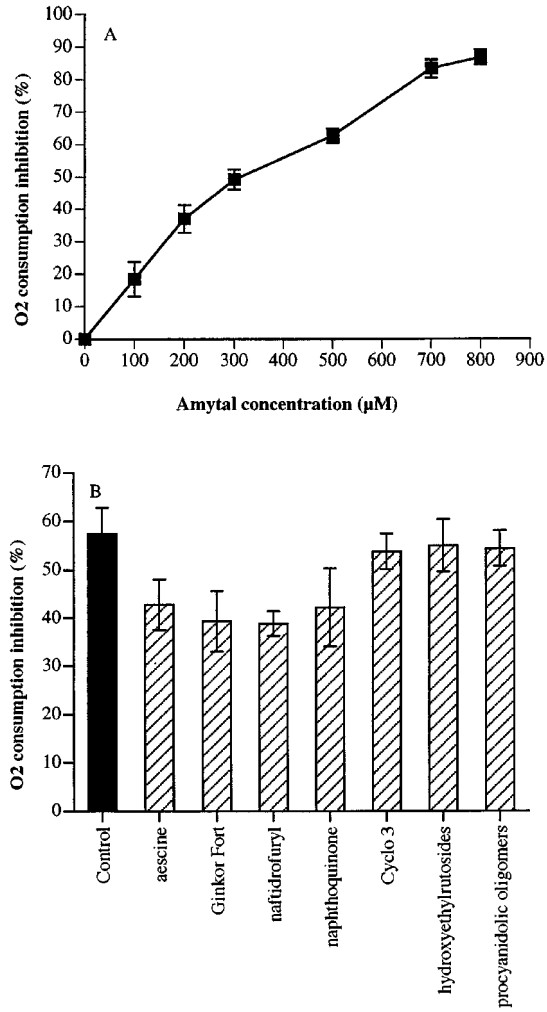

Figure 3 (A) Effect of complex I inhibition by amytal on O_2 consumption of isolated mitochondria. Oxygen consumption was measured in presence of 10 mM D-β-hydroxybutyrate. Results are expressed as percentage O_2 consumption inhibition and presented as mean $\pm$ s.d. for three independent samples of one mitochondrial preparation. (B) Effect of venotropic drugs on the inhibition of O_2 consumption induced by 0.5 mM amytal. Mitochondria were incubated for 60 min in the presence of 75 ng ml^{-1} aescine, 0.3 mg ml^{-1}1 Ginkor Fort, 47 ng ml^{-1} naftidrofuryl, 15.8 ng ml^{-1} naphthoquinone, 4.5 mg ml^{-1} Cyclo 3, 0.1 mg ml^{-1} hydroxyethylrutosides or 1.5 μg ml^{-1} procyanidolic oligomers and thereafter the inhibition of O_2 consumption by amytal was measured. Results are expressed as percentage inhibition of O_2 consumption. Data are mean of two experiments conducted in triplicate, as described in Methods.

Table 1 Effect of nine drugs used in the treatment of chronic venous insufficiency or arteriopathy on state 3 and state 4 respiration rates

Drugs	*Concentration* (mg ml^{-1})	*RCR*	*State 3* (ng-atoms O.min^{-1}.mg proteins^{-1})	*State 4* (ng-atoms O.min^{-1}.mg proteins^{-1})
Aescine	0.150	5.9±0.2	53±9.9	9±1.4
	0.100	6.5±0.2**	41.5±2.1**	6.4±0.6**
	0.075	6.8±0.1***	38±6.4**	5.5±0.7**
	0.050	6.0±0.1*	38.3±10.3*	6.4±1.6*
	Control	**5.8±0.2**	**54.7±3.8**	**9.5±0.7**
Cyclo 3	45	6.5±0.5	44.3±1.1	6.6±0.6
	12	6.9±0.06***	42.8±0.4	6.2±0.5
	4.5	7.5±0.3**	61.3±5.3**	8.3±1.1*
	1.2	6.5±0.1	42.8±3.2	6.6±0.6
	Control	**6.5±0.05**	**42.8±1.4**	**6.6±0.2**
Diosmin	6080	5.3±0.6	59.5±2.3*	11.3±1
	608	5.8±0.8	62.5±2.6	10±0.5
	60	6.3±0.4*	59.1±6.5	9.5±1.5
	6	6.2±0.4	59±4.8*	11.3±1.4
	Control	**5.6±0.3**	**70.3±7.0**	**12.8±2.6**
Ginkor Fort	3000	5.5±0.2*	50.4±2.6	9.2±0.5
	300	6.5±0.2***	51.2±4.1	7.9±0.7*
	30	6.2±0.1***	48±5.6	7.8±0.9*
	3	5.5±0.2*	40.7±7.2	7.5±1.3*
	Control	**5.1±0.1**	**50.3±6.3**	**10.0±1.0**
Hydroxyethyl-rutosides	1000	6.0±0.4	34.3±0.4	5.8±0.9
	500	6.3±0.2**	59.5±7.8**	9.4±0.9**
	100	6.6±0.2**	43.0±1.4*	6.5±0.3
	50	6.0±0.3*	34.8±0.4	5.8±0.4*
	Control	**5.4±0.3**	**35.0±5.7**	**6.5±0.7**
Melilot extract	5000	6.3±0.2	65.5±7.9	10.4±0.5
	2000	7.1±0.3	78.2±6.3*	11.0±0.9*
	1000	7.5±0.3**	84.7±9.5*	11.2±0.6*
	100	6.3±0.2	68.2±6.5	10.8±1.0
	Control	**6.5±0.1**	**60.2±5.8**	**9.3±0.8**
Naftidrofuryl	4.7	6.6±0.4	31.2±4.6	4.8±0.4*
	0.47	7.2±0.1***	30.5±2.1	4.3±0.4*
	0.047	6.2±0.1	40.2±3.9	6.5±0.7
	0.005	5.2±0.71	31.8±8.1	6.3±0.7
	Control	**6.1±0.1**	**38±6.4**	**6.3±1.1**
Naphthoquinone	1.58	6.1±0.1	39.5±7.7	6.5±1.4
	0.158	6.6±0.3*	32.0±7.0	4.9±1.3
	0.016	7.3±0.3**	34.5±1.3	4.7±1.7
	0.0016	6.7±0.1***	31.0±4.2	4.6±0.5*
	0.0002	5.4±1.1	36.0±5.6	6.7±0.3
	Control	**6.1±0.03**	**38.2±6.7**	**6.2±1**
Procyanidolic oligomers	7.5	4.8±0.01***	58.5±12	12.8±1.8
	3	5.1±0.3**	61.5±3.5	12.5±0.7*
	1.5	7.0±1.2	101.5±12*	14.5±0.7**
	0.3	6.2±0.1	88±2.8*	14.8±0.4***
	Control	**6.1±0.1**	**67.8±11**	**11.2±1.9**

Mitochondria of non-treated rats were incubated 60 min with different concentrations of the drugs and state 3 and state 4 respiration rates were measured and RCR was calculated. Results for state 3 and state 4 respiration rates are expressed in ng-atoms O.min^{-1}.mg proteins^{-1}. Results are presented as means±1 s.d. *,**or***: statistically different from the corresponding control with $P<0.05$, 0.01 or 0.001 using Student's *t*-test.

and naphthoquinone, respectively. These protections did not reach statistical significance. No effect of Cyclo 3, hydroxyethylrutosides or procyanidolic oligomers was observed. The same four drugs also protected O_2 consumption when antimycin A was used to inhibit the complex III activity (Figure 4B). Protection of 32, 36, 42 and 43% was obtained for aescine, Ginkor Fort, naftidrofuryl and naphthoquinone, respectively. These protections were statistically significant. These effects were more pronounced than for complex I. Again, no protection of the antimycin A-induced complex III inhibition was observed in the presence of Cyclo 3, hydroxyethylrutosides and procyanidolic oligomers. In contrast, there was no effect of any of these seven drugs on complex IV activity when partially inhibited by cyanide (Figure 5B).

The rate of mitochondrial respiration is also coupled to the activity of ATP synthase. The possible effect of the drugs on this activity was tested in the presence of 10 ng ml^{-1} oligomycin, a concentration which gave about 50% inhibition of RCR (Figure 6A). Results obtained in the presence of the drugs showed no significant protection on the oligomycin-induced inhibition of oxygen consumption for any of the drugs except for Ginkor Fort (Figure 6B).

Since the availability of ADP inside the mitochondrial matrix can also affect the rate of oxidative phosphorylation, the effects of the drugs were also tested on the adenine nucleotide translocase activity. The activity of adenine translocase was measured as atractyloside-sensitive [^{14}C]-ADP uptake into mitochondria. When mitochondria were incubated in the presence of hydroxyethylrutosides or procyanidolic oligomers, a highly significant increase in the ADP uptake was observed. Aescine, Cyclo 3, Ginkor Fort and naphthoquinone did not affect this activity (Figure 7A). The effect of hydroxyethylrutosides and procyanidolic oligomers on adenine nucleotide translocase activity was then further investigated. Both drugs increased the rate of [^{14}C]-ADP incorporation when compared to the control (Figure 7B). A marked increase was observed at 30, 45 and 60 s.

Table 2 Effect of nine drugs used in the treatment of chronic venous insufficiency or arteriopathy on respiratory activity in the presence of EGTA

Drugs	*RCR without EGTA*	*RCR with EGTA*
Control	5.46±0.22	5.54±0.50#
Aescine	7.43±0.52**	7.75±0.35#
Hydroxyethylrutosides	7.60±0.24***	6.93±0.27§
Ginkor Fort	7.67±0.41***	7.72±0.24#
Control	7.72±0.39	7.76±0.68#
Diosmin	9.49±0.41**	9.69±0.30#
Melilot extract	8.62±0.51*	8.95±0.14#
Procyanidolic oligomers	9.17±0.53**	8.66±0.56#
Control	6.18±0.31	5.91±0.36#
Cyclo 3	7.38±0.66*	7.31±0.11#
Naftidrofuryl	7.46±0.47**	7.40±0.80#
Naphthoquinone	7.78±0.92*	8.07±0.38#

Mitochondria were incubated 60 min in the presence of 75 ng ml^{-1} aescine, 0.3 mg ml^{-1} Ginkor Fort, 0.047 μg ml^{-1} naftidrofuryl, 0.0158 μg ml^{-1} naphthoquinone, 4.5 μg ml^{-1} Cyclo 3, 0.1 mg ml^{-1} hydroxyethylrutosides and 1.5 μg ml^{-1} procyanidolic oligomers and in the presence or in the absence of 0.4 mM EGTA. Thereafter, state 3 and state 4 respiration rates were measured and RCR calculated. Results are presented as means±1 s.d. for three different samples of one mitochondria preparation. *, ** or ***: significantly different from the control with $P<0.05$, 0.01 or 0.001; #: non significantly different from the corresponding test without EGTA; §: significantly different from the corresponding test without EGTA with $P<0.05$ using Student's *t*-test.

Drugs can act on enzymes located in the inner mitochondrial membrane as shown above but they can also interact with membrane in a less specific way and affect the leakage of protons or the function of the electron transporters. In order to test this hypothesis, their influence on the partial uncoupling induced by mCCP was investigated. mCCP (carbonyl cyanide m-chlorophenyl hydrazone) is a proton-transporting ionophore, hence dissipating the proton electrochemical gradient.

Addition of mCCP to mitochondria led to a decrease in RCR due to an increase in state 4 respiration. This decrease was not affected by the presence of the drugs except for Cyclo 3 (Figure 8A). To confirm this result, we performed a complete mCCP-induced uncoupling curve in the presence and absence of Cyclo 3. Figure 8B shows a significant protective effect of Cyclo 3 on the inhibition of oxygen consumption at 0.5 and 1 μM mCCP. This result indicates that Cyclo 3 may indeed affect the fluidity of the inner mitochondrial membrane.

ATP content of HUVEC incubated with amytal or antimycin A

In order to link the findings observed both in HUVEC exposed to hypoxia and on isolated mitochondria, the effect of the drugs on complex I and complex III inhibition in whole cells was investigated. When HUVEC were exposed to amytal, a decrease in ATP content of the cells was observed which was concentration-dependent (Figure 9A). From these results, an incubation of HUVEC in the presence of 0.25 mM amytal for 30 min was chosen for the next experiments because these conditions induced a decrease in ATP content similar to that observed after 2 h hypoxia. The effect of the drugs was then investigated on the amytal-induced decrease in ATP content. The cells were pre-incubated for 60 min in the presence of the different drugs before addition of amytal. Optimal concentrations from Figure 1 were chosen for these experiments. The drugs which increased RCR by decreasing state 4 and which were able to prevent amytal- or antimycin A-induced decrease in O_2 consumption on isolated mitochondria – i.e. aescine, Ginkor Fort, naftidrofuryl and naphthoquinone – were also able to prevent the amytal-induced decrease in ATP content in whole HUVEC (Figure 9B). Protections of 85, 66, 82, 59 and 77% were observed for aescine, diosmin, Ginkor Fort, naftidrofuryl and naphthoquinone, respectively. These protections were statistically significant for aescine and Ginkor Fort. No protection was obtained for the other drugs which increase RCR by increasing state 3.

Similar results were observed for antimycin A. Antimycin A decreased in a time- and concentration-dependent manner the ATP content of HUVEC (Figure 10A). Aescine, diosmin, Ginkor Fort, naftidrofuryl and naphthoquinone were able, in contrast to Cyclo 3, hydroxyethylrutosides and procyanidolic oligomers, to prevent this decrease, with significant protections of 81, 67, 93, 93 and 88%, respectively (Figure 10B).

Discussion

Ischaemic conditions are often associated with vascular diseases and may contribute to their development. Due to their localization at the blood-tissue interface, endothelial cells are the first target of any change occurring within the blood, such as O_2 tension decrease, and alteration of their functions will seriously impair organs. Hypoxic conditions which develop during haemostasis can activate the endothelium and lead to a cascade of events eventually resulting in the pathophysiological changes observed in varicose veins (Michiels *et al*., 1997). The first step of the endothelial cell pathological activation by hypoxia *in vitro* is the decrease in intracellular ATP (Arnould *et al*., 1992). The drugs tested in this work, commonly used in the treatment of chronic venous insufficiency or arteriopathy, were able to maintain the ATP content of endothelial cells under hypoxic conditions. These nine drugs are aescine, Cyclo 3, diosmin, Ginkor Fort, hydroxyethylrutosides, melilot extract, naftidrofuryl, naphthoquinone, and procyanidolic oligomers. By preventing the hypoxia-induced ATP decrease, we hypothesize that they will protect cells from the pathological activation process which follows, and hence could protect the vein from the alterations resulting from this activation.

Similar effects have been described for *Ginkgo biloba* extract and for its most active molecule, bilobalide (Janssens *et al*., 1995). Previous results have shown that this anti-ischaemic effect was due to an increase in RCR. *Ginkgo biloba* extract and bilobalide are able to increase the RCR of rat liver mitochondria when rats are treated for 14 days or when naïve mitochondria are pre-incubated *in vitro* in the presence of one of the drugs. These results suggest that

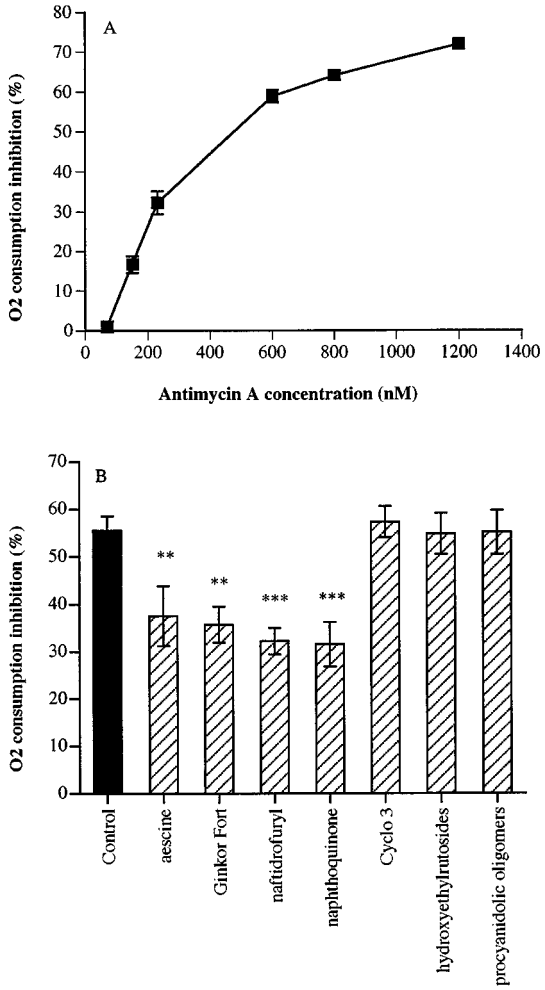

Figure 4 (A) Effect of complex III inhibition by antimycin A on the O_2 consumption of isolated mitochondria. Oxygen consumption was measured in the presence of 5 mM succinate. Results are expressed as percentage inhibition of O_2 consumption and presented as mean$\pm$1 s.d. for three independent samples of one mitochondria preparation. (B) Effect of venotropic drugs on the inhibition of O_2 consumption induced by 0.75 μM antimycin A. Mitochondria were incubated for 60 min in the presence of 75 ng ml^{-1} aescine, 0.3 mg ml^{-1} Ginkor Fort, 47 ng ml^{-1} naftidrofuryl, 15.8 ng ml^{-1} naphthoquinone, 4.5 μg ml^{-1} Cyclo 3, 0.1 mg ml^{-1} hydroxyethylrutosides or 1.5 μg ml^{-1} procyanidolic oligomers and thereafter the inhibition of O_2 consumption by 0.75 mM antimycin A was measured. Results are expressed in percentage inhibition of O_2 consumption. Data are mean of two experiments conducted in triplicate, as described in Methods. $^{**}P<0.01$, $^{***}P<0.001$ compared to control using ANOVA 2 and Scheffé's contrasts.

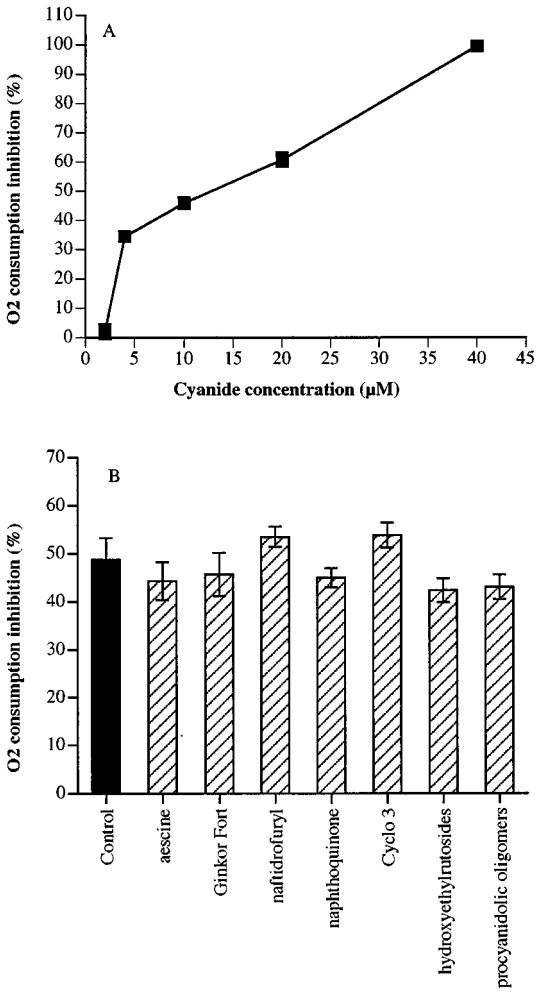

Figure 5 (A) Effect of complex IV inhibition by cyanide on the O_2 consumption of isolated mitochondria. Oxygen consumption was measured in the presence of 6 mM $Fe(CN)_3$. Results are expressed as percentage inhibition of O_2 consumption and presented as mean$\pm$s.d. for three independent samples of one mitochondria preparation. (B) Effect of venotropic drugs on the inhibition of O_2 consumption induced by 0.3 mM cyanide. Mitochondria were incubated 60 min in the presence of 75 ng ml^{-1} aescine, 0.3 mg ml^{-1} Ginkor Fort, 47 ng ml^{-1} naftidrofuryl, 15.8 ng ml^{-1} naphthoquinone, 4.5 μg ml^{-1} Cyclo 3, 0.1 mg ml^{-1} hydroxyethylrutosides or 1.5 μg ml^{-1} procyanidolic oligomers and thereafter the inhibition of O_2 consumption by cyanide was measured. Results are expressed in percentage inhibition of O_2 consumption. Data are mean$\pm$s.d. for three independent samples of one mitochondria preparation.

other drugs which protect cells from the hypoxia-induced ATP decrease may also act on mitochondrial respiration.

Further studies indeed showed that each of these nine drugs increased RCR in a concentration-dependent fashion. All nine drugs increased the mitochondrial respiration but interestingly they did not increase RCR by the same mechanism. One group (A) of drugs (aescine, diosmin, Ginkor Fort, naftidrofuryl and naphthoquinone) increased the RCR by decreasing state 4 respiration. State 4 reflects the dissipation of the proton gradient and is in direct relationship with the coupling and the phosphorylation process. The second group (B) of drugs (Cyclo 3, hydroxyethylrutosides, melilot extract and procyanidolic oligomers) increased RCR by increasing state 3 respiration. State 3 is especially dependent on the electron transport chain, on the import of substrates into the mitochondrial matrix and on the activities of ATP synthase and of adenine nucleotide translocase (Duan & Karmazyn, 1989). One possible explanation for the bell-shaped concentration-response curves observed for some of the drugs is that these molecules probably insert themselves in the inner mitochondrial membrane (IMM) where they could interact with the different enzymatic complexes of the respiratory chain. The higher the concentration, the higher the effect on the RCR, until an excess of the molecule is inserted so that IMM fluidity is impaired or that the organization of the different subunits within the complexes or in relation to other complexes is disturbed. Further investigation is still needed to allow full understanding of these results.

The effects of the drugs on different enzymes of the respiratory chain were then further investigated. The drugs of group A were able to markedly protect complex III from inhibition by antimycin A and, to a lesser extent, complex I

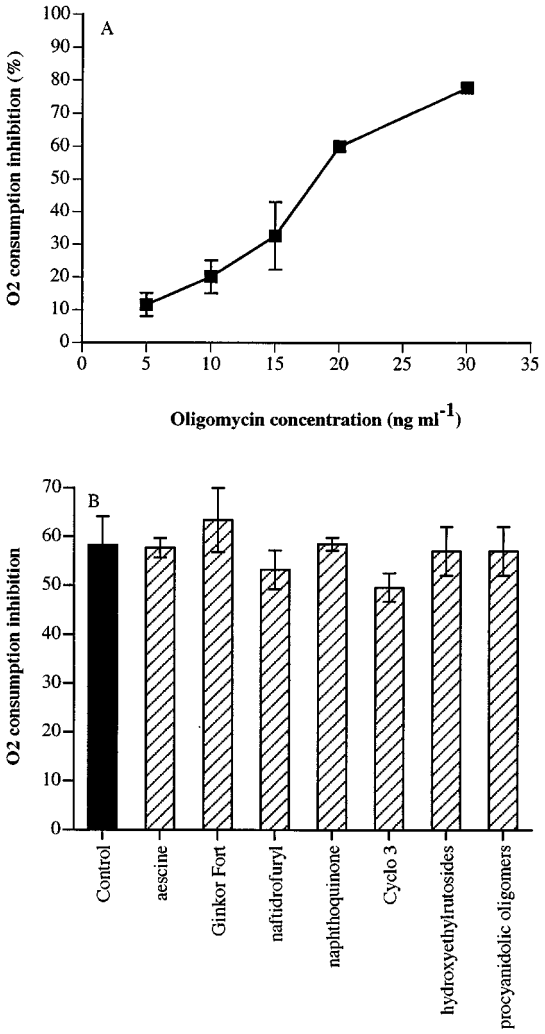

Figure 6 (A) Effect of ATP synthase inhibition by oligomycin on the O_2 consumption of isolated mitochondria. Oxygen consumption was measured in presence of 5 mM succinate. Results are expressed as percentage inhibition of O_2 consumption and presented as mean ± s.d. for three independent samples of one mitochondrial preparation. (B) Effect of venotropic drugs on the inhibition of O_2 consumption induced by 10 ng ml^{-1} oligomycin. Mitochondria were incubated for 60 min in the presence of 75 ng ml^{-1} aescine, 0.3 mg ml^{-1} Ginkor Fort, 47 ng ml^{-1} naftidrofuryl, 15.8 ng ml^{-1} naphthoquinone, 4.5 μg ml^{-1} Cyclo 3, 0.1 mg ml^{-1} hydroxyethylrutosides or 1.5 μg ml^{-1} procyanidolic oligomers and thereafter the inhibition of O_2 consumption by oligomycin was measured. Results are expressed in percentage inhibition of O_2 consumption. Data are mean ± s.d. for three independent samples of one mitochondrial preparation.

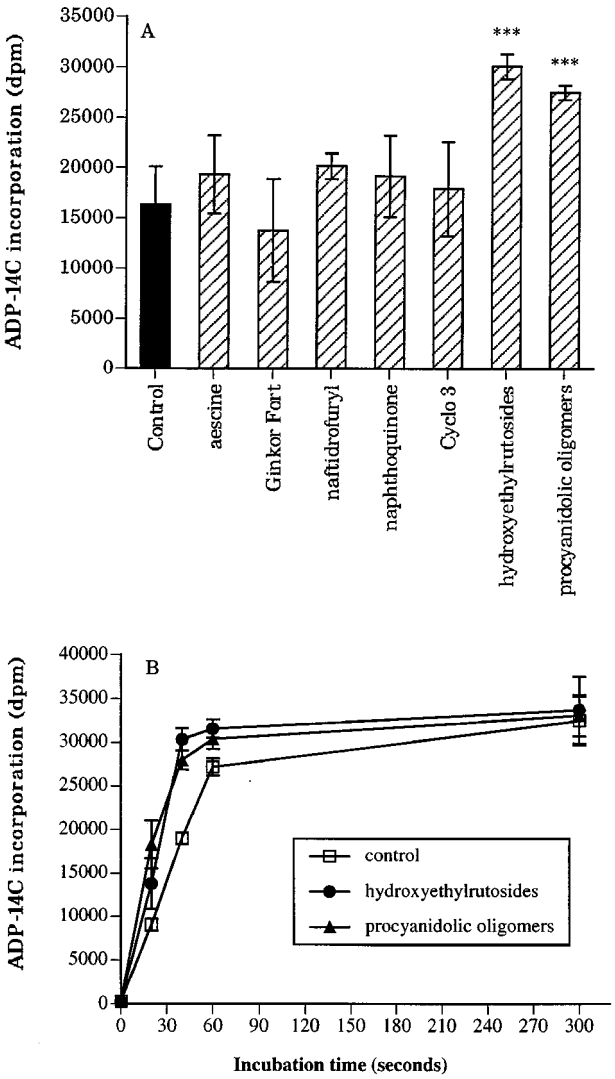

Figure 7 (A) Effect of venotropic drugs on the atractyloside-sensitive [^{14}C]-ADP incorporation. Mitochondria were incubated for 60 min in the presence of 75 ng ml^{-1} aescine, 0.3 mg ml^{-1} Ginkor Fort, 47 ng ml^{-1} naftidrofuryl, 15.8 ng ml^{-1} naphthoquinone, 4.5 μg ml^{-1} Cyclo 3, 0.1 mg ml^{-1} hydroxyethylrutosides or 1.5 μg ml^{-1} procyanidolic oligomers. At the end of this incubation, mitochondria were incubated at 4°C with 50 nmol [^{14}C]-ADP. Incorporation of [^{14}C]-ADP was stopped by 100 μM atractyloside after 40 s. Results are expressed in d.p.m. as mean ± s.d. Data are mean of three independent samples of one mitochondrial preparation except for controls, for which two experiments were included. ***$P<0.001$, compared to control, using ANOVA 1 and Scheffé's contrasts. (B) Effect of hydroxyethylrutosides and procyanidolic oligomers on [^{14}C]-ADP incorporation over time. Mitochondria were incubated for 60 min in the presence of incubation medium (control), 0.1 mg ml^{-1} hydroxyethylrutosides or 1.5 μg ml^{-1} procyanidolic oligomers. Results are expressed in d.p.m. as mean ± s.d. for three independent samples of one mitochondrial preparation.

from inhibition by amytal. No significant protection was observed for the drugs of group B. Two drugs of group B, hydroxyethylrutosides and procyanidolic oligomers, increased the incorporation of [^{14}C]-ADP into the mitochondrial matrix while Cyclo 3 demonstrated a protection of the coupling of the mitochondrial respiration in the presence of mCCP. None of the drugs could influence the inhibition of complex IV. There was also no effect of the drugs on ATP synthase activity when partly inhibited by oligomycin. A schematic classification of the drugs according to the mechanism whereby they work on mitochondria is presented in Figure 11.

If targets within the inner mitochondrial membrane have been identified for these drugs, the exact mechanism by which they operate on these targets is not known. State 4 reflects the proton leak across the inner mitochondrial membrane. At least two processes can influence the level of such a leak. First, state 4 mainly depends on the optimal activity and integrity of the respiratory chain, i.e. of the different electron transporters (complexes I, III and IV) which extrude protons from the mitochondrial matrix toward the intermembrane space, thus generating the proton gradient (Nicholls, 1974). By increasing complex I and/or complex III activity, the drugs of group A would favour the establishment of the proton gradient and thus decrease state 4 respiration rate. Secondly, when cytosolic calcium concentration rises and/or when mitochondria are slightly damaged, calcium cycling takes place, dissipating the

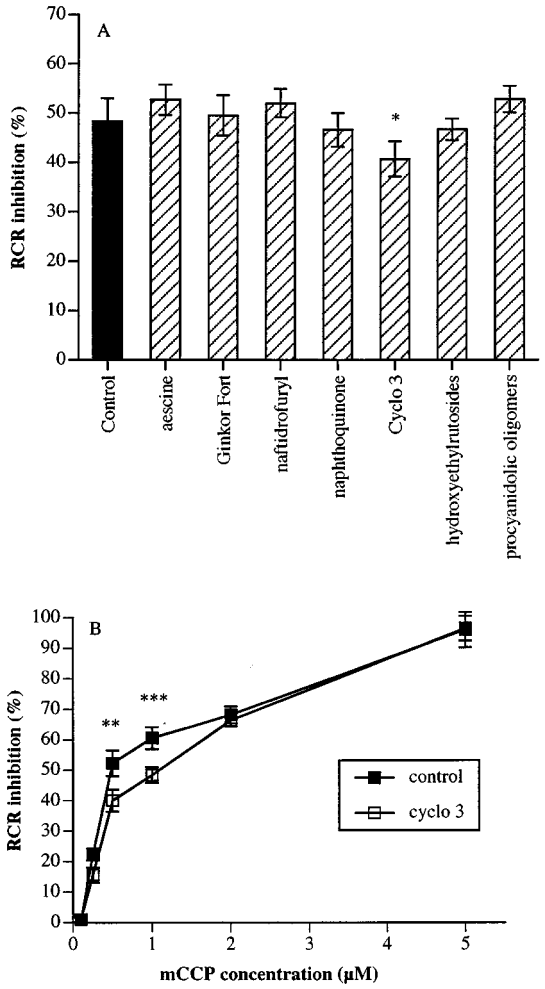

Figure 8 (A) Effect of venotropic drugs on mCCP-induced decoupling of mitochondrial respiration. Mitochondria were incubated for 60 min in the presence of 75 ng ml^{-1} aescine, 0.3 mg ml^{-1} Ginkor Fort, 47 ng ml^{-1} naftidrofuryl, 15.8 ng ml^{-1} naphthoquinone, 4.5 μg ml^{-1} Cyclo 3, 0.1 mg ml^{-1} hydroxyethylrutosides or 1.5 μg ml^{-1} procyanidolic oligomers and thereafter the RCR was measured in the absence then in the presence of 1 μM mCCP. Results are expressed in percentage inhibition of O_2 consumption. Data are mean$\pm$s.d. for three different samples of one mitochondrial preparation. $^*P<0.05$, compared to control, using ANOVA 1 and Scheffé's contrasts. (B) Effect of Cyclo 3 on the mCCP-induced uncoupling of mitochondrial respiration. Mitochondria were incubated for 60 min in the presence of incubation medium (control) or 4.5 μg ml^{-1} Cyclo 3 and thereafter the RCR was determined in the absence then in the presence of different concentrations of mCCP. Results are expressed in percentage inhibition of RCR. Data are mean$\pm$s.d. for three independent samples of one mitochondrial preparation. $^{**}P<0.01$, $^{***}P<0.001$, compared to control, using Student's *t*-test.

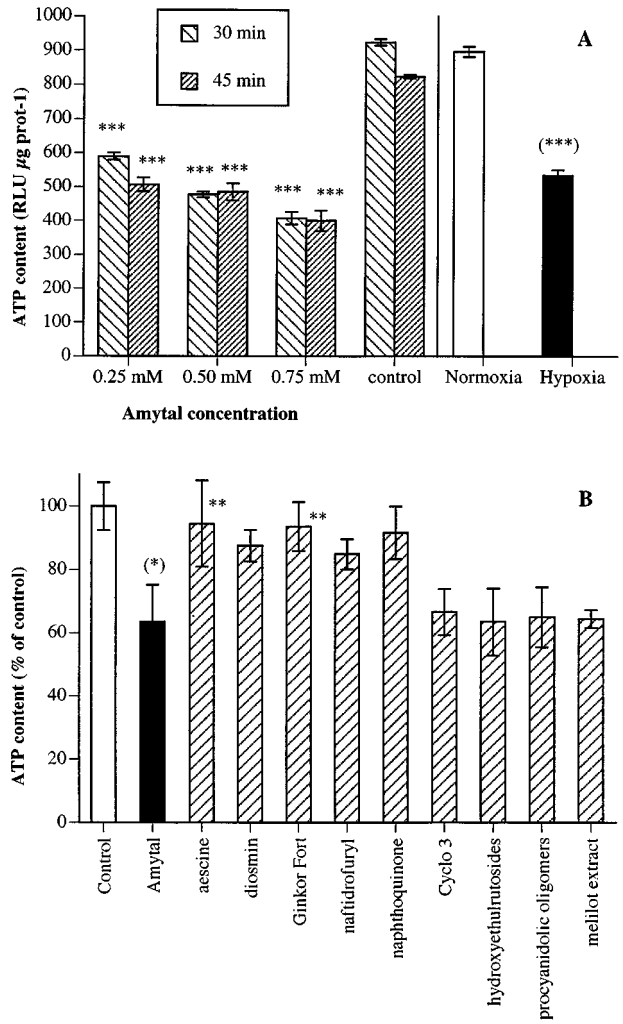

Figure 9 (A) Effect of complex I inhibition by amytal on ATP content in HUVEC. HUVEC were incubated either for 30 min or 45 min in the presence or absence of different amytal concentrations or for 2 h in normoxic or hypoxic conditions. Results are expressed in relative light units (RLU) μg $protein^{-1}$ and presented as mean$\pm$s.d. for one experiment conducted in triplicate. $^{***}P<0.001$ compared to the corresponding control, $(^{***})P<0.001$ compared to normoxia, using ANOVA 1 and Scheffé's contrasts. (B) Effect of venotropic drugs on the amytal-induced decrease in ATP content in HUVEC. HUVEC were incubated for 30 min in the presence or absence of 0.25 mM amytal and in the presence of 750 ng ml^{-1} aescine, 75 μg ml^{-1} diosmin, 0.3 mg ml^{-1} Ginkor Fort, 0.3 mg ml^{-1} melilot extract, 47 ng ml^{-1} naftidrofuryl, 0.16 μg ml^{-1} naphthoquinone, 22.5 μg ml^{-1} Cyclo 3, 1 mg ml^{-1} hydroxyethylrutosides or 5 μg ml^{-1} procyanidolic oligomers. Results are expressed in RLU μg $protein^{-1}$ and presented as mean$\pm$s.d. for two experiments conducted in triplicate. $(^*)P<0.05$ compared to control cells, $^{**}P<0.01$ compared to amytal-incubated cells, using ANOVA 2 and Scheffé's contrasts.

proton gradient and increasing state 4. This could be prevented by calcium chelators such as EGTA (Richter & Frei, 1988). However, the results presented in Table 2 show that the effect of the drugs in the presence of EGTA was the same as that in the absence of EGTA, thus excluding a role as a calcium chelator or as an inhibitor of calcium cycling. Similar results were already observed for bilobalide, another molecule which works as the group A drugs (Janssens *et al*., 1999). Finally, the possibility that these molecules could act as electron transporters by themselves shunting complexes cannot be excluded. Concerning hydroxyethylrutosides and procyanidolic oligomers, their interaction with adenine nucleotide translocase is not understood but leads to an increase in the velocity of the ADP exchange without any effect on the maximal transporter capacity.

It has been established that ischaemic conditions impair the coupling of mitochondria (Duan & Karmazyn, 1989). This effect may be due to a change in mitochondrial membrane fluidity (Shin *et al*., 1989; Sun & Gilboe, 1994) or to a loss or denaturation of compounds of the respiratory chain (Kotaka *et al*., 1982; Rouslin, 1983b). Among these, NADH-ubiquinone reductase (complex I) is rapidly impaired in ischaemic myocardium (Veitch *et al*., 1992; Allen *et al*., 1995). This uncoupling of mitochondria leads to a reduction or a collapse of the transmembrane proton gradient, thereby impairing ATP regeneration. A proton leak has also been

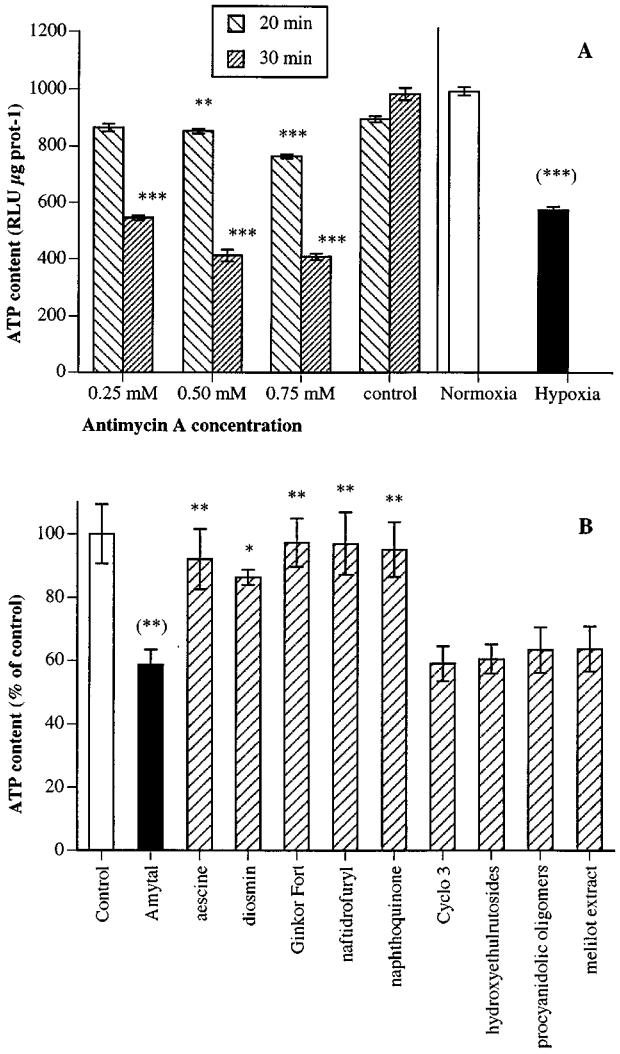

Figure 10 (A) Effect of complex III inhibition by antimycin A on ATP content in HUVEC. HUVEC were incubated either for 20 min or 30 min in the presence or absence of different antimycin A concentrations or for 2 h in normoxic or hypoxic conditions. Results are expressed in RLU μg protein^{-1} and presented as mean$\pm$s.d. for one experiment conducted in triplicate. $^{**}P<0.01$, $^{***}P<0.001$, compared to the corresponding control; $(^{***})P<0.001$ compared to normoxia, using ANOVA 1 and Scheffé's contrasts. (B) Effect of venotropic drugs on the antimycin A-induced decrease in ATP content in HUVEC. HUVEC were incubated for 30 min in the presence or absence of 0.5 mM amytal and in the presence of 0.75 μg ml^{-1} aescine, 75 μg ml^{-1} diosmin, 0.3 mg ml^{-1} Ginkor Fort, 0.3 mg ml^{-1} melilot extract, 47 ng ml^{-1} naftidrofuryl, 0.16 μg ml^{-1} naphthoquinone, 22.5 μg ml^{-1} Cyclo 3, 1 mg ml^{-1} hydroxyethylrutosides or 5 μg ml^{-1} procyanidolic oligomers. Results are expressed in RLU μg protein^{-1} and presented as mean$\pm$s.d. for two experiments conducted in triplicate. $(^{**})P<0.01$ compared to control cells; $^{*}P<0.05$, $^{**}P<0.01$ compared to amytal-incubated cells, using ANOVA 2 and Scheffé's contrasts.

observed (Borutaite *et al*., 1995) as well as an electron leak at the ubisemiquinone transporter (Nohl *et al*., 1993). Finally, alteration of the adenine nucleotide translocase activity has also been observed in ischaemic tissue (Duan & Karmazyn, 1989) and this decrease is often linked to a decrease in the oxidative phosphorylations, especially affecting state 3 (Kobayashi *et al*., 1991; Dagani *et al*., 1988).

Mitochondrial dysfunction in ischaemic conditions can thus be attributed mainly to inhibition of adenine nucleotide translocase (Duan & Karmazyn, 1989), decrease in complex I and III activity (Veitch *et al*., 1992; Allen *et al*., 1995),

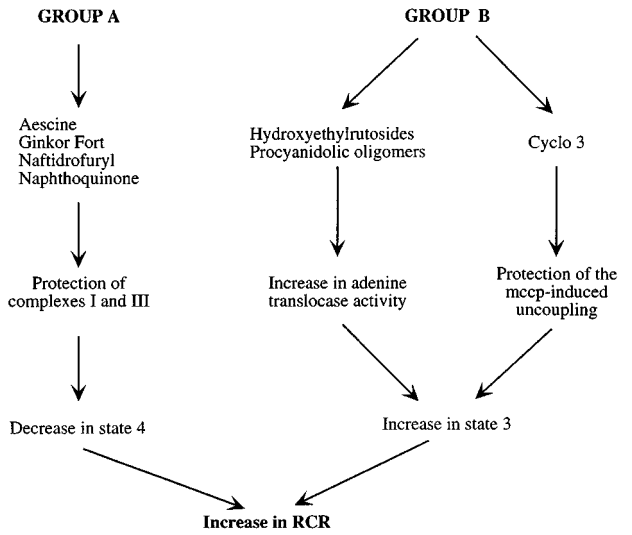

Figure 11 Schematic representation of the effects of the drugs investigated in this work.

alteration of membrane fluidity (Sun & Gilboe, 1994; Nakahara *et al*., 1991) or calcium overload (Silverman, 1993). Very strikingly, the nine drugs tested in this work all protected mitochondria by acting on one of these processes.

Most interestingly, the drugs of group A were also able to prevent the decrease in ATP content induced by complex I or complex III inhibition on whole cells. These results suggest that these drugs are able to reach the inner mitochondrial membrane on whole cells where they could operate in the same way as observed on isolated mitochondria. They thus provide a biochemical basis for their protective effect on the decrease in ATP content induced by hypoxia in these cells.

These results thus suggest that at least part of the therapeutic benefit of these drugs would be to protect mitochondria in ischaemic conditions, thus presenting the endothelial cell decrease in ATP content induced by hypoxia and hence delaying the pathological activation cascade which then occurs in these cells. Indeed, such a protective effect on this cascade has been well demonstrated for Ginkor Fort (Arnould *et al*., 1998), hydroxyethylrutosides (Janssens *et al*., 1996), aescine (Arnould *et al*., 1996) and naftidrofuryl (Michiels *et al*., 1993b). Protection or delay of cell death in ischaemic conditions would also be predicted if the decrease in ATP content is delayed. This proposal gives a general rational explanation for a long term beneficial preventive effect of these nine drugs which is independent of the antioxidant activity of some of them. These protections have now to be confirmed in *in vivo* pathological situations.

C. Michiels is Research Associate of Fonds National de la Recherche Scientifique, Brussels, Belgium). This work was partly supported by FRRC and SSTC. 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. The scientific responsibility is assumed by its authors. The financial support of Région Wallonne is greatly acknowledged.

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(Received February 24, 2000
Revised April 25, 2000
Accepted May 5, 2000)