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Inhibitory effect of the neuroprotective agent idebenone on arachidonic acid metabolism in astrocytes

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Abstract

Idebenone, a compound with protective efficacy against neurotoxicity both in in vitro and in in vivo models, exists in two different oxidative states: the ubiquinol-derivative (reduced idebenone) and the ubiquinone-derivative (oxidised idebenone). In the present study, we have observed that both the redox forms of idebenone have a dose-dependent inhibitory effect on the enzymatic metabolism of arachidonic acid in astroglial homogenates (IC_{50} reduced idebenone: 1.76 ± 0.86 μM; IC_{50} oxidised idebenone: 16.65 ± 3.48 μM), while in platelets, they are apparently less effective (IC_{50} reduced idebenone: 18.28 ± 4.70 μM; IC_{50} oxidised idebenone: > 1 mM). We have also observed that the oxidised form preferentially inhibited cyclooxygenase vs. lipoxygenase metabolism (IC_{50} ratio lipoxygenase/cyclooxygenase: 3.22), while the reduced form did not discriminate between the two pathways (IC_{50} ratio lipoxygenase/cyclooxygenase: 1.38). In this respect, the inhibitory action of reduced idebenone resembled that of the antioxidant nordihydroguaiaretic acid, while oxidised idebenone behaved similarly as indomethacin and piroxicam—two typical anti-inflammatory agents. Our results suggest the existence of two distinct mechanisms of action for the two redox forms of idebenone and a preferential action of the drug on arachidonic acid metabolism in the central nervous system.

Keywords: Idebenone; Neuroprotection; Arachidonic acid; Cyclooxygenase; Lipoxygenase; Astrocyte

1. Introduction

Idebenone, 6-(10-Hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone (oxidised form), is a compound with protective efficacy against cerebrovascular disorders in vivo, including cerebral ischemia (Yamazaki et al., 1984) and hypertension-induced vascular lesions (Nakaoga et al., 1984). In addition, idebenone blocks glutamate neurotoxicity in both in vitro (Miyamoto et al., 1989; Bruno et al., 1994; Ratan et al., 1994) and in vivo models (Miyamoto and Coyle, 1990). It has been suggested that idebenone exerts its pharmacological action as electron carrier after conversion to its ubiquinol form (reduced form) by complex I in the mitochondrial respiratory chain (Sugiyama et al., 1985). Furthermore, idebenone (reduced form) possibly acts as an antioxidant by blocking lipid peroxidation and other oxidative processes (Suno et al., 1989). For instance, Miyamoto et al. (1989) have shown that idebenone protected against oxidative stress toxicity secondary to depletion of glutathione induced by glutamate administration in neuroblastoma cell lines.

The neurotoxic action of glutamate, particularly via activation of the NMDA receptors, is mediated by an elevated influx of Ca^{2+}, which triggers several intracellular Ca^{2+}-dependent events, including phospholipase A_2-mediated arachidonic acid release (Dumuis et al., 1988; Sanfeliu et al., 1990; Rordorf et al., 1991), and oxygen free radical formation (Lafon-Cazal et al., 1993), which are thought to play a major role in the cascade leading to neuronal damage. Arachidonic acid can undergo oxidative metabolism to eicosanoids via the cyclooxygenase, lipoxygenase (Murphy et al., 1988), and epoxygenase pathways (Amruthesh et al., 1993). Metabolic conversion of arachidonic acid in central nervous system cultured cells has been shown to occur preferentially in astrocytes with respect to neurons (Oomagari et al., 1991).

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The aim of the present study was to evaluate the action of idebenone, in both its redox forms, on the enzymatic metabolism of arachidonic acid in homogenates of hippocampal astrocyte cultures and to compare this with their effects on platelet homogenates.

2. Materials and methods

2.1. Materials

The two redox forms of idebenone were a gift from Takeda Chemical Ind. (Japan and Italy). Piroxicam, indomethacin, and ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA) were purchased from Sigma (USA). Nordihydroguaiaretic acid was from Aldrich Chemical (USA). Cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) was a gift from Prof. S. Feinmark. All salts were from Fluka, Switzerland. [^{3}H]-Arachidonic acid, [^{14}C]-oleic acid, 6-keto-prostaglandin$F_{1\alpha}$, TromboxaneB_2, prostaglandinD_2, prostaglandinE_2, prostaglandin$F_{2\alpha}$, 12-hydroxyheptadecatrienoic acid, 5-, 12- and 15-hydroxyeicosatetraenoic acid were purchased from NEN du Pont de Nemours (Germany).

2.2. Homogenates from primary hippocampal astrocyte cultures

Primary astroglial cell cultures obtained from embryotic (ED18) rat hippocampi, were grown in minimal essential medium (MEM, Gibco) to which was added 20% fetal calf serum (ICN Flow). They were plated on poly-ornithine-treated 35 mm Petri dishes and utilised after reaching monolayer confluence (10–12 days in vitro), when about 95% of the cells showed glial fibrillary acidic protein-positive immunoreactivity. Cells were scraped from dishes

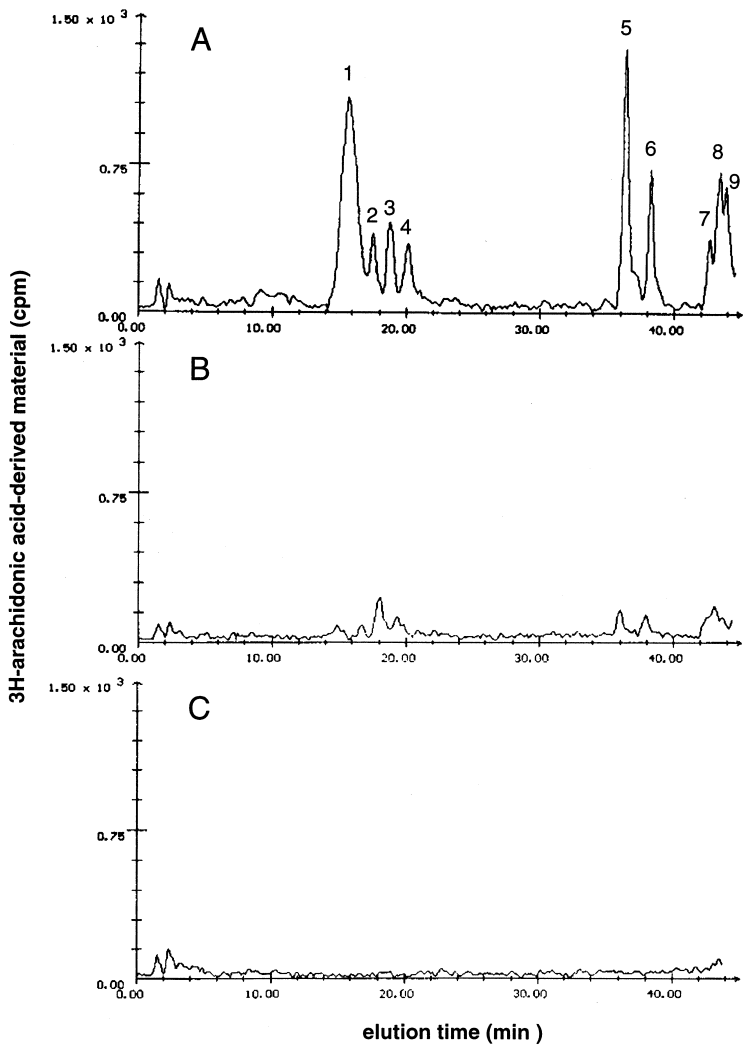

Fig. 1. Representative chromatograms of [^{3}H]-arachidonic acid metabolic products obtained from control astrocyte homogenates (A) and homogenates treated with pharmacological agents: 100 μM idebenone (oxidised form) (B) or 100 μM idebenone (reduced form) (C). Peaks of unmetabolised [^{3}H]-arachidonic acid and [^{14}C] oleic acid internal standard are not shown because largely out of scale. Protein content was: (A) 605 μg; (B) 555 μg; (C) 636 μg.

with 3 ml Dulbecco's buffer containing (in mM): 2.68 KCl, 1.47 KH_2PO_4, 137 NaCl, 8.06 $NaH_2PO_4 \cdot 2H_2O$, 2 glucose, pH 7.4, and immediately sonicated for 30 s at 110 Watt (Model XL2020, Heat Systems, USA).

2.3. Platelet homogenates

Blood was obtained by cardiac puncture from rats (Sprague–Dawley, male, 250–300 g, CD-COBS;Charles River Breeding Laboratories) under ether anaesthesia and diluted with a 3.8% solution of citric acid (trisodium salt dihydrate) (9:1). Platelets were collected upon different centrifugation steps: whole blood was centrifuged for 18 min at 1300 rpm ($287 \times g$) at 15°C and the platelet-rich plasma was collected as supernatant of the pelleted erythrocytes. Then, the platelet-rich plasma was centrifuged at 2500 rpm ($1061 \times g$) for 15 min at 158°C. The resulting platelet pellet was resuspended in Tyrode–HEPES buffer containing (in mM): 140 NaCl, 3 KCl, 1 $MgCl_2 \cdot 6H_2O$, 1.5 $CaCl_2$, 10 glucose HEPES/Na, pH 7.4. Finally, this suspension was centrifuged at 12 000 rpm ($1061 \times g$) for 2 min at 15°C and the pellet stored at −20°C. To perform the experiments, the platelets were resuspended in Dulbecco's buffer devoid of Ca^{2+} and added of 1 mM EGTA (Ca^{2+}-free) and sonicated for 30 s at 110 Watt.

2.4. [^{3}H]-Arachidonic acid metabolism in homogenates

Homogenates of platelets (about 50 μg/sample) or astrocytes (about 500 μg/sample) were preincubated for 5 min at 37°C in 3 ml Dulbecco's buffer without or with pharmacological agents added in ethanol to a final concentration of 0.3% v/v. This procedure was performed in the dark when using the reduced form of the idebenone. Then, 1 μCi [^{3}H]-arachidonic acid (100 mCi/mmol, NEN) was added to the suspension, and the medium was incubated for 30 min at 37°C. The reaction was stopped by adding 100 μl formic acid 5 N. [^{14}C]-Oleic acid (0.1 mCi, 60 Ci/mmol, NEN) was added as an internal standard. In all experiments, a control in the absence of homogenates was run to correct for nonspecific autooxidation of arachidonic acid. Then, the reaction mixture was extracted three times with an equal volume of ethyl acetate. The organic phase was evaporated under vacuum and nitrogen. To perform high-pressure liquid chromatography (HPLC) analysis, each sample was resuspended in 100 μl of buffer B (see Section 2.5).

2.5. HPLC analysis

100 μl samples were injected into a Jasco 880 P HPLC coupled to a Flo-one/β series A100 Radiomatic (Canberra-Packard) radiodetector linked to a personal computer. Reversed-phase chromatography was performed following the method of Powell (1985) with slight modifications. Flow rate through a C-18 RP column (Bio Rad, 250×4.6 mm^2, particle size: 5 μm) was 1.4 ml/min with the following elution gradient: 0 min, 100% buffer A (75% H_2O, 25% CH_3CN, 0.001% CF_3COOH); 40 min, 20% buffer A and 80% buffer B; 60–70 min, 100% buffer B (7.5% H_2O, 54% CH_3OH, 38.5% CH_3CN, 0.001% CF_3COOH).

Retention times of the biological peaks were compared with those of [^{3}H]-eicosanoids standards: 6-keto-prostaglandin$F_{1\alpha}$, TromboxaneB_2, prostaglandinD_2, prostaglandinE_2, prostaglandin$F_{2\alpha}$, 12-hydroxyheptadecatrienoic acid, 5-, 12-, 15-hydroxyeicosatetraenoic acid (60–150 Ci/mmol, NEN).

2.6. Protein assay

The aqueous phase of the extraction mixture containing the cell homogenate was lyophilised and the protein content was determined by the method of Lowry et al. (1951).

2.7. Statistical analysis

The peaks were evaluated as area under the curve and expressed as percentage of total arachidonic acid metabolism. Samples were corrected according to their recovery coefficient (r. coefficient = [^{14}C] radioactivity recovered/[^{14}C] radioactivity added) and normalised for the protein content. Finally, the data were expressed for the same amount of protein (50 μg).

The IC_{50s}, representing the concentration at which the drugs exert 50% of their maximal inhibitory effect, were calculated using the program MacAllFit based on the method of De Lean et al. (1978) using six to eight

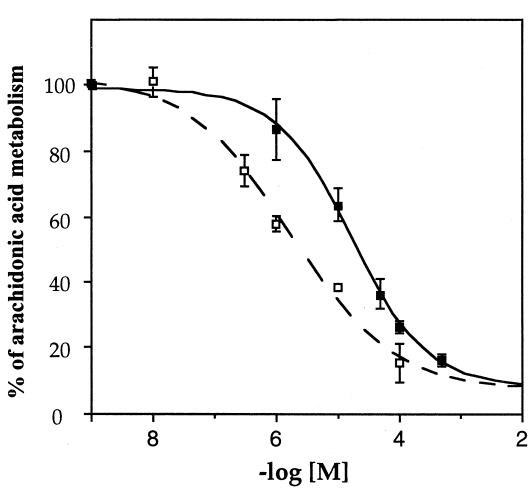

Fig. 2. Dose–response curve for oxidised idebenone (filled square and solid line) and reduced idebenone (open square and dotted line) on the basal metabolism of arachidonic acid in astrocytes. The curve of oxidised idebenone was significantly shifted to the right as compared to that of reduced idebenone. The maximal inhibitions for the two forms were not significantly different.

Table 1
Inhibitory potencies of the two redox forms of idebenone on arachidonic acid metabolism in astrocyte homogenates IC_{50} [μM] (mean ± S.E.M.)

	Total arachidonic acid metabolism	Cyclooxygenase metabolism	Lipoxygenase metabolism	Lipoxygenase/ cyclooxygenase
Oxidised idebenone	16.65 ± 3.48	14.44 ± 2.99	46.51 ± 7.20	3.22
Reduced idebenone	1.76 ± 0.86	1.20 ± 0.50	1.66 ± 0.46	1.38
Nordihydroguaiaretic acid	0.84 ± 0.29	0.78 ± 0.26	0.92 ± 0.30	1.18
Piroxicam	0.15 ± 0.03	0.15 ± 0.02	0.57 ± 0.05	3.80
Indomethacin	0.09 ± 0.05	0.09 ± 0.04	0.67 ± 0.10	7.44
CDC	11.70 ± 1.07	11.59 ± 1.29	11.03 ± 0.81	0.95

This table shows the IC_{50} values for all drugs on astroglial arachidonic acid metabolism. They are reported for total arachidonic acid metabolism and metabolism through the cyclooxygenase or lipoxygenase pathway. In the last column, the ratios between the IC_{50s} on lipoxygenase and cyclooxygenase metabolism are listed as an index of selectivity. Three experiments were carried out in duplicate for oxidised idebenone and reduced idebenone. One experiment was carried out in duplicate for other compounds. Each curve was constructed on six to eight data points (i.e., drug concentrations). CDC (Cinnamyl-3,4-dihydroxy-α-cyanocinnamate).

concentration points. The ratio between the IC_{50s} represents the relative selectivity for the two enzymatic pathways. Statistical significance was evaluated by using the *t*-test (paired, two-tails).

3. Results

Fig. 1A represents the basal pattern of [^{3}H]-arachidonic acid metabolites observed in astroglial homogenates, already characterised in a previous work (Volterra et al., 1995). Briefly, the origin of the peaks was established by using both retention time and selective pharmacological inhibition criteria. Retention times of the endogenous peaks were compared to those of standards for the major cyclooxygenase and lipoxygenase derivatives. In addition, homogenates were incubated in the presence of either indomethacin, a cyclooxygenase inhibitor (Shen and Winter, 1977; Salari et al., 1984), or nordihydroguaiaretic acid, reported as a lipoxygenase inhibitor (Hope et al., 1983) and the resulting metabolic patterns evaluated. In agreement between retention time and pharmacology, peaks 1 (TromboxaneB_2), 2 (prostaglandin$F_{2\alpha}$), 3 (prostaglandinE_2), 4 (prostaglandinD_2), and 5 (12-hydroxyheptadecatrienoic acid) were assigned to cyclooxygenase derivates and peak 9 (12-hydroxyeicosatetraenoic acid) to lipoxygenase derivates. Peak 6 was considered a lipoxygenase derivate given its indomethacin-insensitivity and nordihydroguaiaretic acid-sensitivity. Peaks 7–8 were indomethacin-sensitive and therefore considered cyclooxygenase products, as observed also by Oomagari et al. (1991). In summary, peaks 1, 2, 3, 4, 5 and 7, 8 were assigned to cyclooxygenase and peaks 6 and 9 to lipoxygenase (Volterra et al., 1995). The oxidised (B) and reduced (C) forms of idebenone were both added at 100 μM and the experiments were carried out with about 500 μg of astrocytes which were able to metabolise 8% of the added [^{3}H]-arachidonic acid (1 μCi). The reduced form is more potent than the oxidised in inhibiting arachidonic acid metabolism.

Fig. 2 shows the complete dose–response curves of the inhibitory effects of both the oxidised and reduced form of idebenone. The curve of the oxidised form was signifi-

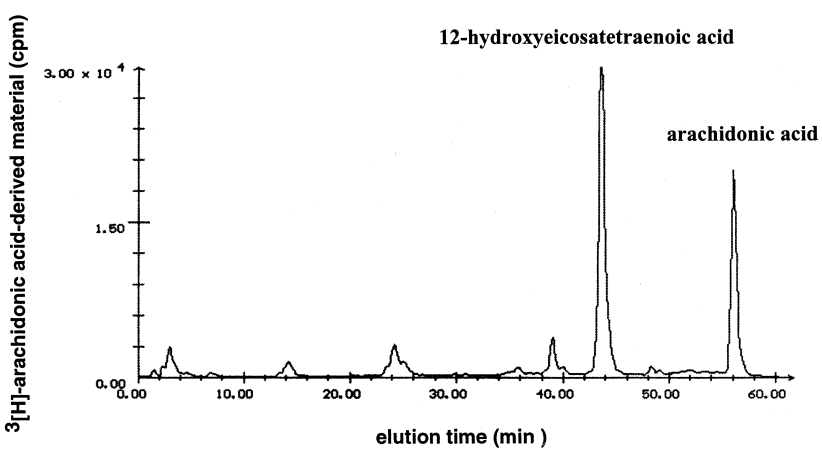

Fig. 3. Chromatogram of basal arachidonic acid metabolism in platelet homogenates, with 12-hydroxyeicosatetraenoic acid as the main metabolite (peak with retention time 44′). Protein content was about 50 μg.

Table 2
Inhibitory potencies of the two redox forms of idebenone on arachidonic acid metabolism in platelet homogenates IC_{50} [μM](mean ± S.E.M.)

	Total arachidonic acid metabolism	12-hydroxyeicosatetraenoic acid
Oxidised idebenone	> 1 mM	> 1 mM
Reduced idebenone	18.28 ± 4.70	17.78 ± 5.60
Nordihydroguaiaretic acid	3.34 ± 0.76	2.19 ± 0.74
Indomethacin	no effect	no effect
CDC	1.70 ± 0.13	1.70 ± 0.13

This table shows the IC_{50} values for all drugs on arachidonic acid platelet metabolism. They are reported for total arachidonic acid metabolism and/or 12-hydroxyeicosatetraenoic acid, the major platelet metabolite in our conditions. For each pharmacological agent, three experiments were carried out in duplicate. Each curve constructed on six to eight data points (i.e., drug concentrations). CDC (Cinnamyl-3,4-dihydroxy-α-cyanocinnamate).

cantly ($p < 0.05$) shifted to the right as compared to that of the reduced form, while the maximal effects of the two forms were not different. In Table 1, the IC_{50s} of either redox form of idebenone in inhibiting arachidonic acid astroglial metabolism are indicated: IC_{50} oxidised idebenone was 16.65 ± 3.48 μM; while IC_{50} reduced idebenone was 1.76 ± 0.86 μM; IC_{50} ratio: 9.46. When we compared the inhibitory potency of idebenone (oxidised form) on cyclooxygenase vs. lipoxygenase metabolism, we found that idebenone (oxidised form) preferentially inhibited cyclooxygenase (IC_{50} cyclooxygenase: 14.44 ± 2.99 μM; IC_{50} lipoxygenase: 46.51 ± 7.20 μM, $p < 0.05$; IC_{50} ratio: 3.22). In contrast, idebenone (reduced form) did not show selectivity. In fact, the two pathways were inhibited by the same doses of idebenone (reduced form) (IC_{50} cyclooxygenase: 1.20 ± 0.50 μM; IC_{50} lipoxygenase: 1.66 ± 0.46μM; IC_{50} ratio: 1.38).

The effects of idebenone in astrocytes were compared with those of compounds with a well-established inhibitory effect on the arachidonic acid metabolism: nordihydroguaiaretic acid and CDC were selected as lipoxygenase-selective inhibitors (Hope et al., 1983; Cho et al., 1991), while piroxicam and indomethacin as cyclooxygenase inhibitors (Shen and Winter, 1977; Carty et al., 1980; Salari et al., 1984).

As expected, both piroxicam and indomethacin preferentially inhibited cyclooxygenase metabolism. In contrast, nordihydroguaiaretic acid and CDC appeared rather nonselective and inhibited cyclooxygenase and lipoxygenase at similar concentrations (Table 1).

The pattern of basal arachidonic acid metabolism in platelet homogenates under our conditions is shown in Fig. 3. The main metabolite was 12-hydroxyeicosatetraenoic acid, which by itself accounted for more than 60% of the total metabolism.

Arachidonic acid metabolism in astroglia and platelet homogenates was different both qualitatively and quantitatively (compare Fig. 1A and Fig. 3).When normalised for protein content, the metabolism of arachidonic acid in platelet homogenates was about 80 times higher than that of astroglial homogenates. In addition, under our experimental conditions, platelets metabolised arachidonic acid to a major peak, 12-hydroxyeicosatetraenoic acid, and other minor more polar peaks which probably represent lipoxygenase products since they were blocked by nordihydroguaiaretic acid and CDC, but not by indomethacin. As shown, astrocyte homogenates form lipoxygenase and cyclooxygenase metabolites with cyclooxygenase products present in higher amounts.

The IC_{50s} of all pharmacological agents were evaluated either on total arachidonic acid metabolism in platelets or on the production of the main metabolite, 12-hydroxyeicosatetraenoic acid, with similar results (Table 2). Both the redox forms of idebenone inhibited platelet arachidonic acid metabolism less effectively than astroglial metabolism. Idebenone (oxidised form) was practically inactive at the concentration of 100 μM, while it elicited a 39% inhibition of arachidonic acid metabolism at 1 mM. Idebenone (reduced form) had an IC_{50} of 18.28 ± 4.70 μM in platelet homogenates, i.e., about 11 times higher than in astrocytes.

4. Discussion

In this work, we have investigated the actions of the two redox forms of the neuroprotective agent idebenone on arachidonic acid metabolism in rat astrocyte and platelet homogenates. Interestingly, both forms of idebenone proved to be effective in inhibiting arachidonic acid metabolism in astrocytes. The IC_{50s} for both the oxidised and the reduced compound were in the low micromolar range, similar to the reported levels of the drug in the central nervous system (Nagai et al., 1989).

In platelets, idebenone was active only at high concentrations (= 100 μM). A possible explanation is that the basal capacity of platelets to metabolise arachidonic acid is about 80-fold higher than that of astrocytes, therefore requiring higher idebenone concentrations to obtain the same extent of inhibition observed in astrocytes. A previous work studying the in vivo distribution of the drug (Torii et al., 1985) had shown that idebenone preferentially concentrates in central nervous system. This observation is probably important as the marked inhibitory effect on arachidonic acid metabolism in astrocytes, and the lower

effectiveness in platelets suggest that, in a specific range of concentrations, idebenone might act preferentially on central arachidonic acid metabolism. Therefore, the compound seems particularly suited for therapeutic application in central neurological diseases such as ischemia, hypoxia and in other disorders in which dysregulation of the arachidonic acid metabolism is thought to play a major pathophysiological role.

Another relevant observation of this study was that in astrocytes, idebenone (oxidised form), contrary to idebenone (reduced form), preferentially inhibited cyclooxygenase vs. lipoxygenase metabolism, while the reduced form potently inhibited both pathways. Idebenone (reduced form) behaves like nordihydroguaiaretic acid, a compound which has been proposed to act as a lipoxygenase inhibitor, but has in addition antioxidant properties, while idebenone (oxidised form) behaved similar to indomethacin and piroxicam—two typical anti-inflammatory agents. In platelets, idebenone (oxidised form) and indomethacin were basically not able to inhibit arachidonic acid metabolism, while idebenone (reduced form) and nordihydroguaiaretic acid, though less potent than in astrocytes, were active. The lack of effect of idebenone (oxidised form) and indomethacin in inhibiting arachidonic acid metabolism in platelet homogenates could be explained by the fact that under our specific experimental conditions, most of the arachidonic acid metabolism seemed to be through the 12-lipoxygenase pathway.

5. Conclusion

These results suggest that the two redox forms of idebenone exert an inhibiting action on arachidonic acid metabolism via different mechanisms. This implies that idebenone (oxidised form) could act by itself without prior being transformed to the reduced form, as generally thought. The type of action of idebenone (reduced form) resembles that of a potent anti-oxidant as proposed, while the oxidised form inhibits quite selectively cyclooxygenase metabolism and resembles typical anti-inflammatory agents, although its potency is significantly lower. Idebenone has effects on arachidonic acid metabolism similar to both nordihydroguaiaretic acid and piroxicam, which are known to be neuroprotective in vitro (Rothman et al., 1993) as well as in vivo (Patel et al., 1993).

The preferential effect of idebenone on central arachidonic acid metabolism may represent an important aspect of its pharmacological profile as a potential neuroprotective agent.

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