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Antioxidant effect of dipyridamole and its derivative RA-25 in mitochondria: correlation of activity and location in the membrane

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Abstract

Dipyridamole (DIP), a coronary vasodilator, presents coactivator activity for a number of antitumor drugs as well as antioxidant activity in membrane systems. DIP and derivatives interact with membrane systems such as micelles, phospholipid monolayers and vesicles. The antioxidant effect of DIP and several derivatives upon iron-induced lipoperoxidation on mitochondria has been reported and a good correlation between the hydrophobicity and their protective effect was found (M.F. Nepomuceno et al., Free Radic. Biol. Med., 23 (1997) 1046–1054). In the present work an effort is made to better understand the role of DIP as inhibitor of Fe^{2+}-induced lipid peroxidation in mitochondria. At low concentration, no significant effect on either state IV or state III respiration was found, discarding a possible direct interaction of DIP or RA-25 with the peripheral benzodiazepine receptor. The association constants for DIP and RA-25 in mitochondria were estimated, being 0.7 $(mg/ml)^{-1}$ for DIP and 0.2 $(mg/ml)^{-1}$ for RA-25. Oxygen consumption studies in the presence of $FeSO_4$ showed that the antioxidant effect of DIP or RA-25 did not involved the initial step of Fe^{2+} oxidation. Our data strongly support the hypothesis that the antioxidant effect of both DIP and RA-25 is related to their partition in the lipid phase of the mitochondrial membrane and not to a specific interaction with membrane proteins. This protection may be due either to a direct inhibition of the propagation steps or a scavenger effect on the radicular species that would trigger the peroxidative process.

Keywords: Dipyridamole; Oxidative stress; Mitochondrial membrane; Binding constant; Lipid peroxidation

1. Introduction

Dipyridamole (DIP) is a compound that has been used in coronary heart diseases for its antiplatelet and vasodilating activities [1,2]. This drug also showed an antioxidant action as superoxide and hydroxyl radical scavenger [3,4], and its chain-breaking activity places it as an efficient and powerful biological antioxidant, as potent as vitamin E [5]. Our data demonstrated that DIP was a potent antioxidant in mitochondrial membrane mainly against Fe^{2+} effects and that its antioxidant action may be associated to its partition in the lipid bilayer, where lipid peroxidation takes place [6].

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Lipid peroxidation seems to play a major role in many pathological situations such as ischemia–reperfusion [7]. The alterations caused by the lipid peroxidation may affect the mitochondrial energy transduction related processes [8]. Moreover, the damage to the membrane may also increase the Fe^{2+} free concentration [9]. The requirement for Fe^{2+} to induce lipid peroxidation in biological membranes has long been recognized, although the mechanism of this process, particularly the identity of the initiating species, remains controversial [10–15]. Besides $^{\bullet}OH$ produced through the Fenton reaction, as the species responsible for the attack to the membrane [12,14], there is also experimental evidence in the literature that lipid peroxidation requires as inducers both Fe^{2+} and Fe^{3+}, probably as a dioxygen–iron complex [14–17], perferryl radical, ferryl radical [15], or even membrane-bound Fe^{2+} [18].

The interaction of DIP in model membrane systems has been described by Borissevitch et al. [19], demonstrating different affinities of DIP and its derivatives (RA14, RA47 and RA-25) in micelles. According to these studies the hydrophobicity of the compound correlates with its location in the membrane [19].

On the other hand, the binding of DIP to proteins such as the benzodiazepine receptor of kidney mitochondrial membranes was also described in the literature [20]. The subunit composition of the mitochondrial purified benzodiazepine receptor complex apparently includes the voltage-dependent anion channel (VDAC), the adenine nucleotide carrier (ADC) and another protein of 18 kDa [21]. It has been proposed that all three proteins comprise the activity receptor, with a high affinity for a number of drugs leading to an increase in the state IV and a decrease in the state III respiration rates, resulting in a significant decrease in the respiratory control ratio (RC) [22]. The benzodiazepine receptor is also suspected to play some role during hypoosmotic stress in astrocytes [23], and superoxide radical generation by phagocytes was found to be markedly sensitive to benzodiazepine receptor ligands [24]. It is possible that these compounds would be modulating the oxidative response capacity of these cells [25].

The purpose of the present work is to verify whether the DIP antioxidant action is correlated with its partition in the mitochondrial membrane, dependent of a specific protein binding site in the membrane or simply due to iron chelation. O_2 consumption of isolated mitochondria submitted or not to Fe^{2+} in the presence or in the absence of DIP and its derivative RA-25 (structural formulae are presented in Fig. 1) were monitored. To study their location in the membrane we estimated the association constant of DIP and RA-25 to the mitochondrial membrane.

Our results strongly support that the partition of DIP and RA-25 in the mitochondrial membrane according to their lipophilicity order is responsible for the inhibitory effect of DIP on the lipid peroxidation induced by Fe^{2+}. Apparently, the binding to the mitochondrial benzodiazepine receptor, in liver mitochondria and under our experimental conditions, does not occur to a significant extent. Moreover, a possible chelation of Fe^{2+} or Fe^{3+} by DIP in our reaction medium has been discarded, although a direct scavenger effect of DIP on the radicular species initiating the peroxidative process could also be an explanation for our data.

2. Materials and methods

2.1. Isolation of mitochondria

Liver mitochondria were isolated from overnight fasted adult female Wistar rats, according to the procedure described in the literature [26]. The mitochondrial pellet was resuspended in isosmotic buffer containing 120 mM KCl and 20 mM Hepes (pH 7.2) to give a final protein concentration of 80–100 mg, determined by the biuret method modified with cholate addition [27].

2.2. Oxygen uptake measurements

Oxygen consumption was measured using a Clark-type electrode in a 1.8-ml glass chamber equipped with a magnetic stirrer and without O_2 limitation.

2.3. Standard incubation conditions

The fluorescence experiments were performed in basic medium containing 120 mM KCl plus 20 mM Hepes, pH 7.2. Previous incubations were done at

temperature of 30°C and for 50 min. Oxygen consumption experiments were performed in basic medium containing 125 mM saccharose, 65 mM KCl, 10 mM Hepes (pH 7.2), 3 mM phosphate, 5 mM succinate and 5 μM rotenone. $FeSO_4$ was added at concentrations shown in the figure legends and tables.

2.4. Determination of association constants

Determination of association constants of the drugs to the mitochondrial membranes was performed with titration of the drug solution at fixed concentration with variable mitochondrial membrane protein concentration (from 0.8 to 4.0 mg protein/ml). Either DIP or RA-25, at a fixed concentration, were added to the medium immediately before the mitochondrial suspension. Samples were exposed to a previous incubation or not, followed by a centrifugation at $4000 \times g$ for 15 min. The fluorescence emission spectra of the supernatant were measured in the range 425–600 nm with excitation at 405 nm for DIP and in the range 400–600 nm with excitation at 370 nm for RA-25. Data from the titrations were analyzed as double reciprocal plots of fluorescence intensity change (ΔF) at 480 nm for DIP and 430 nm for RA-25 and membrane concentration (determined as mg of protein/ml) or by direct fitting of the law of mass action, which gives ΔF versus membrane concentration as previously described [19,28–30].

2.5. DIP and RA-25 binding to mitochondria

Measurement of DIP or RA-25 binding to mitochondria was made by titration as described for determination of the association constants. The fluorescence intensity of the supernatants decreased with the increase in the membrane protein concentration. In order to estimate the amount of drug left in the pellet (bound to the membrane) the following procedure was performed. The pellets were immediately placed in liquid nitrogen [31] and then dissolved in 1 ml of 1% Triton X-100, 10% NaCl and centrifuged at $4000 \times g$ in a Beckman microfuge for 25 min. The fluorescence of the drug in the supernatants obtained from this solubilization in the detergent was determined as already described in the previous section for the supernatants from the initial titration. The amount of drug in the pellet was estimated with standard solutions of drug in the same media in the absence of mitochondria.

2.6. Estimation of lipid peroxidation

Mitochondria (1 mg protein) were added to 1 ml of medium immediately followed by DIP or its derivative RA-25 and oxidant addition at different concentrations as indicated in the figures, with subsequent incubation for 20 min. After this incubation period, 0.1 ml of 50 μM butylhydroxytoluene was added to prevent further lipoperoxidation, and the samples were treated with 3 ml of 0.04 M H_2SO_4 and 2 ml of 0.8% thiobarbituric acid (TBA) in 0.1 M NaOH. The samples were boiled for 45 min at 100°C, and after cooling, 4.0 ml of *n*-butanol was added. The mixture was thoroughly mixed and centrifuged at $900 \times g$ for 10 min. The TBA-reactive substances (TBARS) were determined in the organic layer with a spectrophotometer at 535 nm. The content of TBARS was calculated from a standard curve of 1,1,3,3-tetraetoxypropane as described by Yagi et al. [32].

3. Results

3.1. DIP and RA-25 do not significantly alter mitochondrial respiration

A decrease in the respiratory control (RC) due to an increase on state IV respiration and a decrease on state III respiration in mitochondria incubated with peripheric benzodiazepine receptor ligands [22], including DIP [20,22], have previously been reported. To verify if DIP presented an effect per se on mitochondrial respiration, we measured the rate of oxygen consumption by mitochondria energized with succinate during states IV and III and the RC was calculated for different DIP or RA-25 concentrations. The data in Table 1 show that under our experimental conditions both compounds exhibit a very slight effect on these parameters, even when used at low concentrations. Since higher concentrations of RA-25 are necessary to produce its antioxidant effect, this derivative caused a higher decrease in RC as compared to DIP.

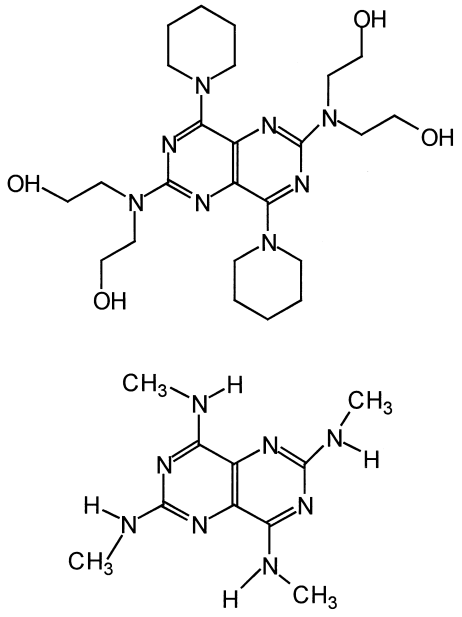

Fig. 1. Structural formulae of dipyridamole and its derivative RA-25.

Table 1
Effect of DIP and RA-25 on oxygen consumption by mitochondria

	V_4	V_3	$RC = V_3/V_4$
	(natom of O/min per mg protein)		
[DIP] μM			
0	58 ± 4	212 ± 25	4.0 ± 0.4
2.5	59 ± 5	207 ± 20	3.5 ± 0.4
5.0	62 ± 6	200 ± 22	3.5 ± 0.2
7.5	65 ± 4	198 ± 18	3.2 ± 0.2
10.0	65 ± 4	201 ± 19	3.2 ± 0.2
50.0	101 ± 15	250 ± 20	2.4 ± 0.2
100.0	118 ± 18	262 ± 21	2.1 ± 0.2
[RA-25] μM			
0	58 ± 4	212 ± 25	4.0 ± 0.4
50	75 ± 7	233 ± 25	3.0 ± 0.4
100	80 ± 3	235 ± 22	2.7 ± 0.2
250	84 ± 6	233 ± 22	2.7 ± 0.2
500	88 ± 5	218 ± 25	2.5 ± 0.2

Oxygen consumption was measured in the presence of DIP or RA-25 increasing concentrations. Mitochondria were added in the basic medium in the presence of succinate. Values are mean ± S.D. from four experiments.

3.2. DIP and RA-25 effect on oxygen consumption by mitochondria in the presence of $FeSO_4$

When Fe^{2+} is added to respiring mitochondria we observe bursts of extra oxygen consumption, separated by an increasing lag time (Δt) as shown in Fig. 2. Unpublished results from our laboratory indicate that the first burst of extra O_2 consumption (V_1) is related with Fe^{2+} oxidation and that the peroxidative damage occurs during the second burst of extra O_2 consumption (V_2). The existence of a latent period, Δt, before the lipid peroxidation induced by iron could start was already described in the literature [15,18] and is explained as the time needed for the formation of the initiating species, which depends on a critical ratio Fe^{2+}:Fe^{3+}. Data from Table 2 show that DIP was able to inhibit the second burst at very low concentration, as already observed when the peroxidation was followed through TBARS formation [6], without changing V_1. An increase in V_1 was observed only when very high DIP concentrations (100 μM) and low Fe^{2+} concentrations were used. It is important to stress that DIP inhibits V_2 even when added just before it starts (not shown).

Table 2
Effect of DIP upon the extra oxygen consumption induced by FeII

[DIP] μM	V_1 [Fe^{2+}] μM				V_2 [Fe^{2+}] μM				Δt_{min} [Fe^{2+}] μM			
	50	100	200	500	50	100	200	500	50	100	200	500
0	50 ± 6	74 ± 8	111 ± 2	218 ± 10	37 ± 5	47 ± 2	81 ± 8	150 ± 6	6 ± 1	7 ± 1	9 ± 1	11 ± 1
2.5	60 ± 2	72 ± 7	108 ± 5	219 ± 6	–	–	–	–	–	–	–	–
5.0	65 ± 5	73 ± 7	110 ± 2	219 ± 8	–	–	–	–	–	–	–	–
7.5	62 ± 4	86 ± 4	111 ± 2	223 ± 9	–	–	–	–	–	–	–	–
10.0	64 ± 7	91 ± 8	111 ± 5	225 ± 7	–	–	–	–	–	–	–	–
50.0	67 ± 7	101 ± 1	141 ± 8	228 ± 3	–	–	–	–	–	–	–	–
100.0	104 ± 5	140 ± 11	165 ± 7	240 ± 9	–	–	–	–	–	–	–	–

Mitochondrial suspensions (1 mg/ml) were incubated in the presence of increasing DIP concentrations for 1 min before Fe^{2+} addition. The oxygen consumption was measured as shown in Fig. 2.

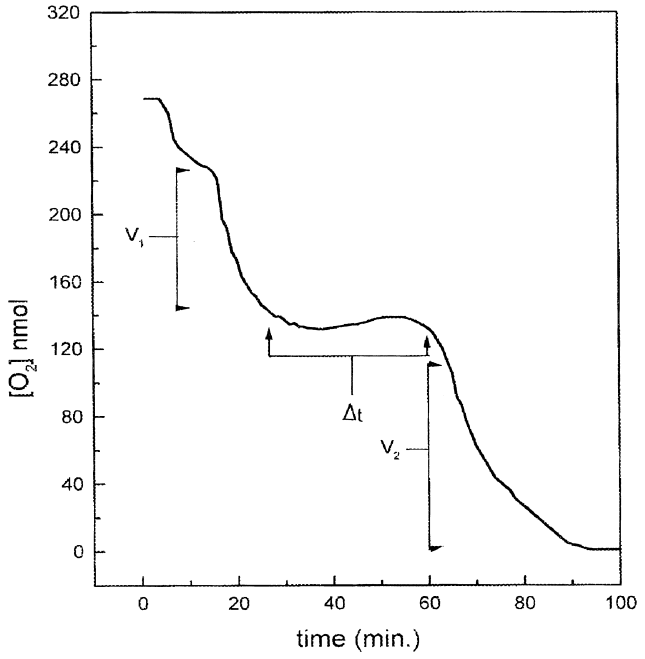

Fig. 2. Effect of $FeSO_4$ on mitochondrial oxygen consumption measured with a Clark-type polarographic oxygen electrode. Mitochondrial suspensions (1 mg/ml) were incubated in basic medium for 1 min and 0.2 mM $FeSO_4$ was added. The figure shows the extra oxygen consumption in respiring mitochondria. The first peak (V_1) was attributed to Fe^{2+} oxidation and the second one (V_2) to lipid peroxidation. Δt is the lag time between the two peaks.

This result suggests that the antioxidant effect of DIP can not be attributed to inhibition of Fe^{2+} oxidation but also does not discard the possibility that it prevents either the initiator formation or the initiator attack, since DIP also eliminates Δt.

Similar results were obtained with RA-25, a less lipophilic derivative than DIP. The protective effect against the peroxidative damage also occurred but at much lower extent and at higher concentrations than DIP (Table 3), in agreement with our previous data [6].

3.3. Previous incubation of DIP with mitochondria results in inhibition of CuOOH-induced lipid peroxidation

DIP was shown to be a powerful antioxidant when the lipid peroxidation was induced by $FeSO_4$ ($IC_{50} = 1$ μM) but was ineffective when CuOOH was the oxidant inductor [6]. In the present work, the mitochondrial suspensions were previously incubated for 50 min in the presence of increasing concentrations of DIP before the CuOOH addition. CuOOH is a lipophilic compound and generates alcoxyl radicals in the membrane when cleaved by mitochondrial cytochromes [33], leading to lipid peroxidation as other organic hydroperoxides [7]. Under these conditions a partial inhibition ($IC_{50} = 50$ μM) can be observed as shown in Fig. 3. However this concentration is much higher when compared to the IC_{50} obtained when peroxidation was induced by Fe^{2+}.

3.4. Binding constants of DIP and RA-25 to mitochondria

Titration of a 2 μM solution of DIP with variable concentrations of mitochondria gave a series of emission spectra obtained for the supernatants after centrifugation and shown in Fig. 4. It is seen that for higher membrane concentrations the intensity of fluorescence emission decreases, consistent with more binding of the drug to the membrane and less drug remaining in the supernatant. These changes in emis-

Table 3
Effect of RA-25 upon the extra oxygen consumption induced by Fe^{2+}

[RA-25] μM	V_1 [Fe^{2+}] μM				V_2 [Fe^{2+}] μM				Δt_{min} [Fe^{2+}] μM			
	50	100	250	500	50	100	250	500	50	100	250	500
0	50 ± 6	74 ± 8	111 ± 2	218 ± 10	37 ± 7	47 ± 2	80 ± 8	149 ± 7	6 ± 1	7 ± 1	9 ± 1	11 ± 1
50	71 ± 12	92 ± 13	130 ± 12	227 ± 13	31 ± 6	34 ± 3	62 ± 8	83 ± 2	7 ± 1	12 ± 2	10 ± 2	11 ± 1
100	79 ± 12	105 ± 13	146 ± 9	241 ± 2	25 ± 4	29 ± 2	48 ± 1	74 ± 2	8 ± 1	15 ± 2	14 ± 2	13 ± 1
250	86 ± 13	121 ± 13	156 ± 15	243 ± 14	–	–	–	–	–	–	–	–
500	92 ± 7	151 ± 13	171 ± 16	246 ± 11	–	–	–	–	–	–	–	–

Mitochondrial suspensions (1 mg/ml) were incubated in the presence of increasing RA-25 concentrations for 1 min before $FeSO_4$ addition. The oxygen consumption was measured as shown in Fig. 2.

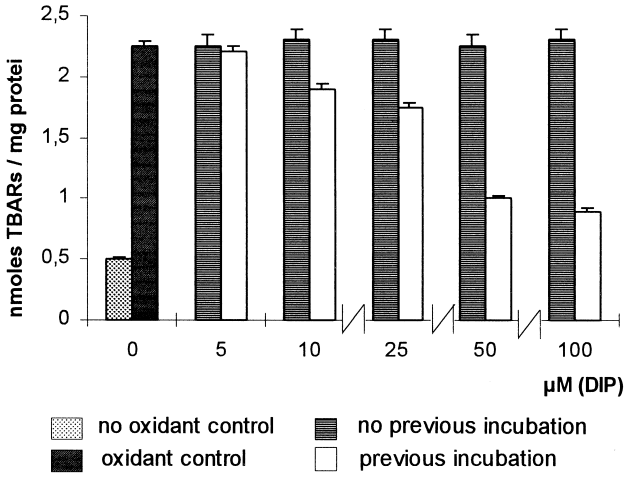

Fig. 3. DIP action upon lipid peroxidation induced by CuOOH with or without previous incubation. Mitochondria (1 mg/ml) were incubated in basic medium with increasing DIP concentration for 50 min before CuOOH addition. When no previous incubation was performed, both DIP and CuOOH were added simultaneously. The first column shows the control experiment.

sion spectra were used to estimate the association constant of DIP to the membrane.

Considering the equilibrium:

$$D + M \leftrightarrow D{-}M \quad (1)$$

where D represents the drug and M the mitochondria, the total fluorescence observed is due to the free and bound species of D. ΔF represents the difference between the fluorescence emission intensity of the drug at a certain concentration of the mitochondria (F) and the initial fluorescence intensity (F^0), in the absence of the mitochondrial membrane. This difference (ΔF) is related to the quantity of the drug associated with the mitochondria. In order to obtain quantitatively the association constants for the binding of DIP to mitochondria, data for titrations of the drug with mitochondrial suspension were treated using the mass-action law and the method of the double reciprocal plot [29,30]. This latter treatment is based on the following equation:

$$1/\Delta F = 1/\Delta F_{max} + 1/\Delta F_{max} \bullet 1/K_b \bullet 1/[M] \quad (2)$$

A plot of $1/\Delta F$ as a function of 1/[M] may be used to find K_b, the binding constant. The results obtained for both treatments are listed on Table 4. Some typical results of these titrations are presented in Fig. 5. In this figure the binding of DIP to mitochondria at 37°C and pH 7.0 is shown, while the insert corresponds to the same data presented as a double reciprocal plot.

In Table 4 data for binding of DIP to phospholipid vesicles of pure dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were also included for comparison [30,34]. The data for binding of the drugs to mitochondria are presented in $(mg/ml)^{-1}$ since the protein concentration in mg/ml was used in the plots and in the calculations. In order to transform these data to a molar basis and compare them to the binding to pure lipids and micelles, the following procedure was performed. For every lipid component of mitochondrial membrane an average molecular mass was calculated assuming a mixture of steroyl and palmitoyl alkyl chains as well as the unsaturated oleyl, so that for phosphatidylcholine as an example an average molecular mass of 760 g/mol was obtained (this is the average of dipalmitoyl phosphatidylcholine (DPPC), palmitoyl oleylphosphatidylcholine (POPC), distearoyl phosphatidylcholine (DSPC) and steroyloleylcholine (SOPC) taken with identical proportions). After this calculation, an average molecular mass for the

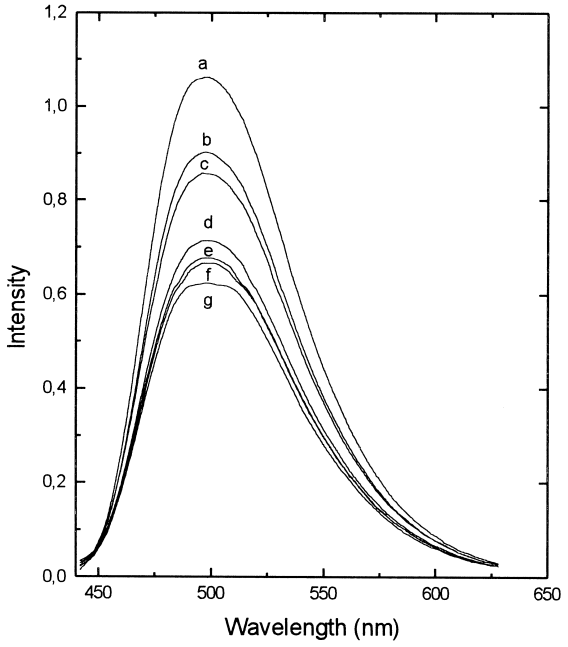

Fig. 4. Fluorescence emission spectra of DIP in supernatants from mitochondria at different protein concentrations. Concentration of DIP 2×10^{-6} M. Excitation wavelength 405 nm. Protein concentration in the range 0.8–4.0 mg/ml.

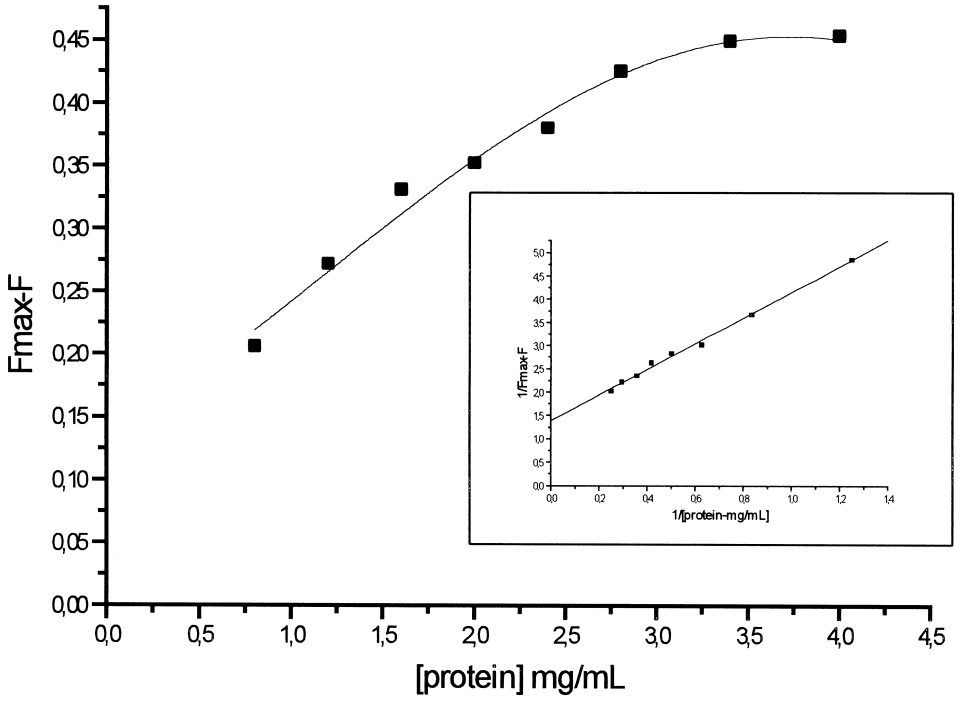

Fig. 5. Fitting of fluorescence data for the binding of DIP to mitochondrial membrane using the mass-action law as the change in emission intensity ΔF versus mitochondrial suspensions protein concentrations. Concentration of DIP 2×10^{-6} M. Protein concentrations the same as in Fig. 4. Excitation at 405 nm and emission at 480 nm. Inset corresponds to the double reciprocal plot for the same data.

lipids in the outer membrane was obtained using the known composition and the percentage of lipids relative to proteins [35].

For the outer membrane the total composition is 52% protein and 48% lipid. So, for 1 mg/ml of total protein in the sample 0.48/0.52 = 0.92 mg/ml is lipid. Our calculation gave an average lipid molecular mass of 756 g/mol for the outer and 861 g/mol for the inner membrane. Assuming that the binding of DIP takes place at the outer membrane, 1 mg/ml of protein corresponds to 1.22 mM of lipid and this can be used to transform the estimated binding constant to the outer membrane from 0.5 $(mg/ml)^{-1}$ to 410 M^{-1}. Since in the inner mitochondrial membrane the proportions of protein and lipid are quite different, 75% and 25%, respectively, the binding of the drug to the inner membrane even to a small extent would lead to a considerable increase of the molar binding constant to lipids. It is interesting that the binding constants to mitochondrial membranes estimated above (Table 4) are smaller as compared to pure phospholipids and micelles [19,28,30,34]. In the case of RA-25 it

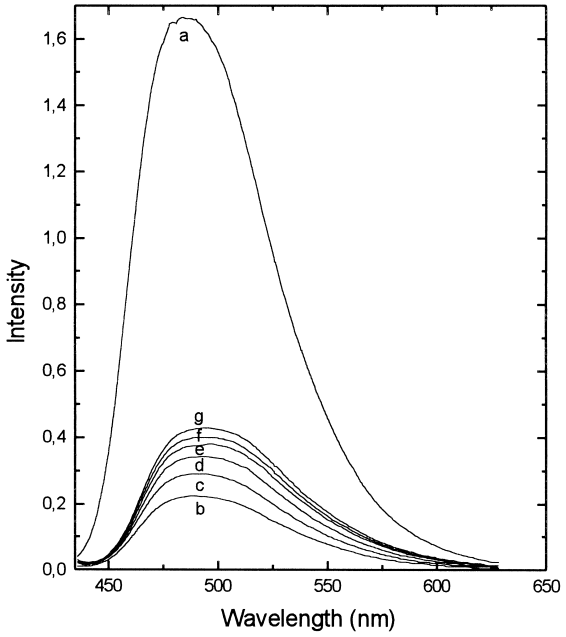

Fig. 6. Fluorescence emission spectra of DIP in pellets from the same preparations treated with detergent as described in Section 2. Concentration of DIP 2×10^{-6} M. Excitation wavelength 405 nm. Protein concentration in the range 0.8–4.0 mg/ml.

Table 4
DIP and RA-25 binding constants in mitochondrial membranes and phospholipid vesicles

	pH	Temp. (°C)	K_b (M^{-1})[a]	K_d (10^{-3} M)	K_b (M^{-1})[b]
DIP					
DPPC	7	30	633 ± 22	1.52 ± 0.08	658 ± 35
		50	1149 ± 16	0.85 ± 0.02	1176 ± 28
DPPG	7	30	727 ± 81	1.39 ± 0.09	719 ± 46
		50	1500 ± 60	0.70 ± 0.02	1428 ± 41
MITO[c]	7.2	37	0.54 ± 0.04 (443 ± 33)		
MITO[d]	7.2	37	0.42 ± 0.04 (344 ± 33)		
MITO[e]	7.2	37	0.7 ± 0.1 (574 ± 82)		
MITO[f]	7.2	37	0.58 ± 0.05 (475 ± 41)		
RA 25					
DPPC	7	50	295 ± 70	4.3 ± 1.1	232 ± 59
MITO[g]	7.2	37	0.25 ± 0.07 (205 ± 57)		–

Binding constants of dipyridamole (DIP) and RA-25 to phospholipids (from [30,34]) calculated by the method of the [a]double reciprocal and by [b]the mass-action law. In mitochondria the concentrations of DIP are [c,d]2 μM, [d]5 μM and [g]that of RA-25 is 2 μM. Data were obtained [f]without incubation and [e]with 50 min incubation. Binding constants to mitochondria are in $(mg/ml)^{-1}$ based on total protein concentration. Values in parentheses correspond to data in M^{-1} calculated as described in the text. K_d is a dissociation constant and K_b an association constant for DPPC and DPPG [34].

is also seen that the binding constant is reduced by a factor of two as compared to DIP while the concentration to achieve 50% inhibition of lipid peroxidation is two orders of magnitude different, higher for the more polar derivative RA-25. Our data also show that, when the mitochondrial suspensions were preincubated with DIP, the binding constant increased and saturation occurred at lower membrane concentrations. In fact, these results showed that DIP permeates the membrane and this process is time dependent.

All of the above results show the binding as the decrease of drug concentration in the supernatant. In order to assess the amount of drug bound to the membrane in a more direct way, the same pellet of the samples used to measure the emission in the supernatants were dissolved in Triton X-100 detergent in the presence of NaCl, centrifuged, and the new supernatants were examined for their fluorescence emission. The obtained emission spectra are presented in Fig. 6, and show the increase in fluorescence with the increase in membrane protein concentration. In Fig. 7 a plot of the fraction of DIP in the pellet as a function of membrane protein concentration is presented. This plot was fitted by the mass-action law, resulting in an association constant of 0.8 ± 0.1 $(mg/ml)^{-1}$ and maximum saturation at 47% of DIP. This means that at 2 micromolar DIP and an excess of mitochondria, around 47% of the drug is bound. The value of binding constant is quite close to the value obtained from the change of fluorescence of the supernatants after incubation of the drug (Table 4).

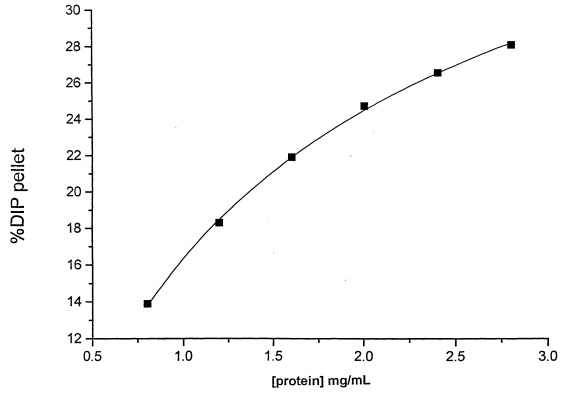

Fig. 7. Fitting of fluorescence data for the binding of DIP to mitochondrial membrane obtained from measurement of fluorescence spectra of pellet treated with detergent as described in Section 2. Same data as in Fig. 6.

4. Discussion

DIP is a potent antioxidant against the Fe^{2+}-induced lipid peroxidation in mitochondria [6] and this effect seems to be related to its high hydrophobicity,

since less hydrophobic derivatives [19,30] are also less effective in the protection against this peroxidation in mitochondria [6]. However, it has also been reported that DIP can bind to the peripheral benzodiazepine receptor located at the mitochondria outer membrane [20,22], although its affinity for the benzodiazepine receptor was found to be five times lower (IC_{50} 170 nM) than those reported for other benzodiazepine receptor ligands such as R_O5-4864 and PK 11195 [22]. The mitochondrial respiratory control is decreased due to this binding since DIP changes both rates of oxygen consumption, increasing state IV and decreasing state III respiration [22]. However, these data refer to experiments performed with isolated rat kidney mitochondria, which contain significantly higher amounts of benzodiazepine receptors as compared to liver [36]. Under our experimental conditions, working with liver mitochondria, to observe such alterations much higher concentrations of both DIP and RA-25 were necessary. The data presented in Table 1 show that although DIP per se presents a small effect on the respiratory chain even at low concentrations, a significant decrease in RC values occurs only at DIP or RA-25 concentrations much higher than those required for the inhibition of lipid peroxidation ($IC_{50} = 1$ μM for DIP protection [6]). The decrease in RC values at concentrations above 10 μM could be due to cytotoxic effects resulting from the high drug concentration. The concentrations of DIP and RA-25 required to cause 50% inhibition of iron-induced lipid peroxidation differ by two orders of magnitude, being 1 μM and 100 μM, respectively [6]. These results are corroborated by the data shown in Table 2, where 2.5 μM DIP completely abolishes the second peak of extra oxygen consumption (v_2), attributed to the peroxidative process, whereas an RA-25 concentration 100 times higher is needed to observe the same effect (Table 3). The binding constants (K_b) of both compounds, DIP and RA-25, to the mitochondrial membrane were calculated (Table 4). A two- to threefold difference in K_b values was obtained, certainly related to the higher hydrophobicity of DIP as compared to RA-25 [19,30]. Interestingly, the binding constants of DIP and RA-25 measured from the fluorescence spectra in the membrane pellet are 0.8 ± 0.1 and 0.17 ± 0.03 $(mg/ml)^{-1}$, which correspond to a ratio similar to that obtained in DPPC vesicles and equal to 4. However, this ratio is much smaller than the observed IC_{50} ratio [6] and cannot explain by itself the two orders of magnitude difference in antioxidant activity.

The results showing that DIP is able to permeate the membrane in a time dependent way with only a partial inhibitory effect against CuOOH-induced lipid peroxidation indicate that the binding to the membrane is not sufficient either to avoid the radical species formation by CuOOH or the propagation of this process.

One general observation on iron-induced peroxidation is the existence of a latent period (Δt) until the peroxidation reaction effectively starts. This lag time was explained as the period necessary for the radical initiator formation prior to the membrane attack [18]. Recently it was shown that the removal of either Fe^{2+} or Fe^{3+} by adding a free iron chelator at the end of the lag time resulted in inhibition of the lipid peroxidation, supporting the hypothesis that an Fe^{2+}–Fe^{3+} complex is responsible for the iron-initiated lipid peroxidation [9,37]. Indeed, our results also have shown the effectiveness of DIP, since independently of its addition during the observed Δt or just before it, and even after the beginning of V_2, it was able to inhibit the peroxidation process. So it is more likely that DIP prevents both the radical formation and the propagation reaction.

The lack of effect of low concentrations of DIP on the first peak of extra oxygen consumption (V_1) was a first indication that there was no direct interaction of DIP with Fe^{2+}. Since DIP is a highly fluorescent compound, its complexation with iron would result in quenching of the fluorescence due to either heavy metal or paramagnetic quenching. The possibility of a direct interaction between DIP and Fe^{2+} or Fe^{3+} was investigated through the monitoring of the fluorescence quenching by iron of a solution of DIP in the reaction medium in the absence of mitochondria. The direct experiment with ferrous sulfate quenching was not quantitative due to the rapid oxidation of iron (Fe^{2+} to Fe^{3+}), which leads to a strong time-dependent optical absorption of the solution preventing the analysis. Besides the iron oxidation, the solution becomes turbid, probably, due to precipitation of iron hydroxide. We therefore made a control experiment by incubating the solution of DIP with $FeSO_4$ and $FeCl_3$ for 3 h followed by centrifugation.

All of the DIP initially present in the solution remained in the supernatant as judged by both optical absorbance and fluorescence of the drug. This is also an indication against the possibility of direct complex formation of DIP with either Fe^{2+} or Fe^{3+}, excluding a chelation effect.

Although this complex mechanism has not been fully elucidated, our data indicate that binding to the peripheral benzodiazepine receptor is not likely to be important at the low concentrations under which DIP completely inhibits the peroxidative process. The partition of the drugs into the mitochondrial membrane gave a better explanation to the effects of DIP on the propagation reactions of the lipid peroxidation induced by Fe^{2+}. Moreover, the results presented in this work also confirm the radical scavenger ability of DIP on Fe^{2+}-induced lipid peroxidation in isolated mitochondria. It is possible that simplifying the system, using mitoplasts instead of mitochondria, will allow further experimental evidence to be obtained, giving a more detailed understanding of the mechanisms involved in the protection of peroxidation by DIP. Such experiments are under way in our laboratory.

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