Biochemical Pharmacology Vol 35, No 13, pp 2119–2122, 1986
Printed in Great Britain

0006–2952/86 $3.00 + 0.00

(+)-CYANIDANOL-3 PREVENTS THE FUNCTIONAL DETERIORATION OF RAT LIVER MITOCHONDRIA INDUCED BY Fe^{2+} IONS

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(*Received* 19 *September* 1985; *accepted* 11 *December* 1985)

Abstract—The clinically effective hepatoprotective flavonoid, (+)-Cyanidanol-3, prevented the Fe^{2+}-induced functional deterioration of rat liver mitochondria Fe^{2+} treatment of mitochondria resulted in increased lipid peroxidation (MDA-formation), decreased mitochondrial membrane potential, impaired Ca^{2+} uptake capacity and caused large amplitude swelling of mitochondria All of the consequences of Fe^{2+} treatment were inhibited by (+)-Cyanidanol-3 in a concentration dependent manner The mitochondrial protective action of the drug is comparable with its free radical scavenging property

Many hepatotoxic agents (CCl_4, ethanol, Fe^{2+} ions, etc.) induce the peroxidation of various biological membranes (see reviews [1–3]). The involvement of mitochondrial damage in drug-induced hepatotoxicity has been demonstrated both *in vivo* and *in vitro* [4–10].

Fe^{2+} ions are capable of inducing enhanced peroxidation of the mitochondrial membranes both *in vivo* [11–13] and *in vitro* [14–16] This can be used to study the peroxidation-associated damage of these cell organelles Mitochondria prepared from Fe^{2+}-treated rats built up a significantly lower membrane potential and contained less K^+ than the controls [17]. Addition of Fe^{2+} ions in micromolar concentration to isolated mitochondria caused the release of certain enzymes and K^+ from the matrix space [15, 18], deterioration of ADP/O ratio [18] and loss of membrane potential [19].

Cyanidanol,† a clinically effective flavonoid, inhibited the CCl_4 and Fe^{2+}-induced lipid peroxidation of microsomes, both *in vivo* and *in vitro* [20] It also prevented the morphologic abnormalities of mitochondria and endoplasmic reticulum in hepatocytes [5]. In addition Cyanidanol was shown to counteract the depletion of cellular ATP and reduced glutathion content brought about by peroxidative agents [21, 22] and to decrease the extra oxygen consumption of hepatocytes induced by tert-butylhydroperoxide [23] However, it remains questionable whether the protective effect at the mitochondrial level contributes to the action of the drug.

The aim of the present study was to investigate whether Cyanidanol when added to isolated mitochondria was able to prevent the lipid peroxidation-induced functional damage in these organelles. This presumed effect was tested for by parallel investigations of malondialdehyde (MDA) formation, membrane potential, Ca^{2+} uptake capability and permeability changes of the mitochondrial inner membrane.

MATERIALS AND METHODS

Rat liver mitochondria were isolated by the method of Johnson and Lardy [24] Mitochondrial protein content was determined by the biuret method using bovine serum albumin as standard.

Mitochondrial membrane potential ($\Delta\psi$) was measured by a tetraphenyl-phosphonium selective electrode prepared according to [25] The experimental set-up and the calculation of the $\Delta\psi$ value was described in detail in [26]

Mitochondrial swelling was measured by following the changes of apparent absorbance at 546 nm with a Beckman DK-2A recording spectrophotometer

Malondialdehyde concentration was determined by thiobarbituric acid reaction [27]

Ca^{2+} ion movements were monitored by a Ca^{2+} selective electrode (produced by Redelkis, Hungary) connected to an OP-205 pH meter and an OH-814 potentiometric recorder (Radelkis, Hungary) The Ca^{2+} electrode was calibrated with a multiple point calibration technique combined with an iterative determination of the initial Ca^{2+} concentration of the mitochondrial suspension as in [28] The electrode selectivity coefficient for Fe^{2+} ions was 0 1 determined by the fixed interference method [29] The gradients of the voltage–metal-ion concentration of the Ca^{2+} electrode for Ca^{2+} and Fe^{2+} were 23 mV and 1 mV, respectively. When following changes of the free Fe^{2+} concentration by the Ca^{2+} electrode, the amplification of the apparatus was augmented accordingly.

The experiments were carried out in a basic medium containing 130 mM KCl, 20 mM Tris–HCl, 1 5 mM $MgCl_2$ at pH 7 4. Further additions are indicated in the figure captions (Figs 1–4).

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† Abbreviations Cyanidanol, (+)-Cyanidanol-3, MDA, malondialdehyde (thiobarbituric acid reactive substance), TPP^+, tetraphenylphosphonium ion, Tris, Tris-(hydroxymethyl)-aminomethan, $\Delta\psi$, mitochondrial transmembrane electric potential difference

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All the reagents were of highest purity commercially available Cyanidanol was a product of Zyma-Biogal (Debrecen, Hungary), commercialized under the name of Catergen. Desferal (Desferroxamine mesylate) was a product of Ciba-Geigy (Basle, Switzerland).

RESULTS

The magnitude of mitochondrial membrane potential ($\Delta\psi$) could display abnormalities in mitochondria of iron-treated rats which were not revealed by the respiratory parameters [30]. Thus we measured the changes of $\Delta\psi$ during Fe^{2+}-induced lipid peroxidation and the effect of Cyanidanol on this parameter

The original TPP^+ electrode recording shows that mitochondria suspended in the incubation medium built up only a transient $\Delta\psi$ by the oxidation of the endogenous substrates (Fig 1A). After the addition of 3-OH-butyrate a stable $\Delta\psi$ value was maintained as high as 180 mV. If the mitochondria were pre-incubated with 17 6 μM Fe^{2+} for 5 min, even in the presence of respiratory substrate a $\Delta\psi$ value less than 120 mV was developed (Fig 1B) In contrast, if the mitochondria were pre-incubated in the presence of 23 nmol Cyanidanol/mg protein (34.5 μM) before Fe^{2+} treatment, the addition of respiratory substrate raised the value of $\Delta\psi$ to the control level (Fig 1C)

The effect of Cyanidanol on the $\Delta\psi$ value was concentration dependent Using succinate or 3-OH-butyrate as respiratory substrate the fall of $\Delta\psi$ was prevented by 50% in the presence of 5 nmol Cyanidanol/mg (6 μM) and 8 nmol Cyanidanol/mg (12 μM), respectively (Figs 2A and B)

The Fe^{2+}-induced decrease of $\Delta\psi$ was probably due to the enhanced lipid peroxidation of the mitochondrial inner membrane To analyze the relation between the changes of MDA level and of $\Delta\psi$, these parameters were measured in parallel, varying the time of incubation with Fe^{2+} (Fig 3). After 5 min incubation with Fe^{2+} the addition of respiratory substrate resulted in a $\Delta\psi$ value of only 115 mV. During the same incubation period the MDA level increased

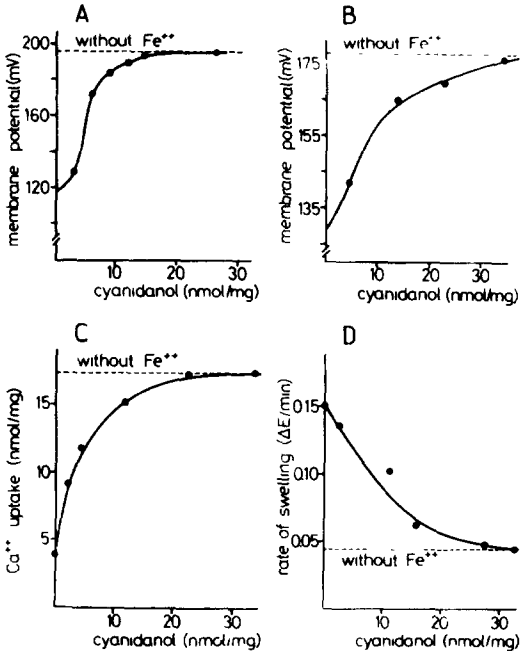

Fig 2 The prevention of Fe^{2+}-induced deterioration of various mitochondrial parameters by Cyanidanol (A) and (B) The effect of various amounts of Cyanidanol on the $\Delta\psi$ value after Fe^{2+} addition Sequence of additions 0 min, mitochondria plus the indicated amount of Cyanidanol, 2 min, 17 6 μM $FeSO_4$, 7 min, respiratory substrate, (A) 1 2 mg mitochondrial protein per ml, 5 mM Tris-succinate (B) 1 5 mg mitochondrial protein/ml, 5 mM 3-OH-butyrate (C) The effect of Cyanidanol on the Fe^{2+}-induced impairment of mitochondrial Ca^{2+} uptake Mitochondria (1 5 mg/ml) were pre-incubated in the basic medium which contained 32 μM Ca^{2+} (21 1 nmol Ca^{2+}/mg protein) Further additions were the same as in (B) The ordinate shows the maximal amount of Ca^{2+} uptake after the addition of 3-OH-butyrate (D) The effect of Cyanidanol on the Fe^{2+}-induced mitochondrial swelling Mitochondria (1 5 mg/ml) were pre-incubated in the presence of the indicated amount of Cyanidanol for 2 min Subsequently 17 6 μM $FeSO_4$ was added The rate of swelling was measured in the first minute after Fe^{2+} addition

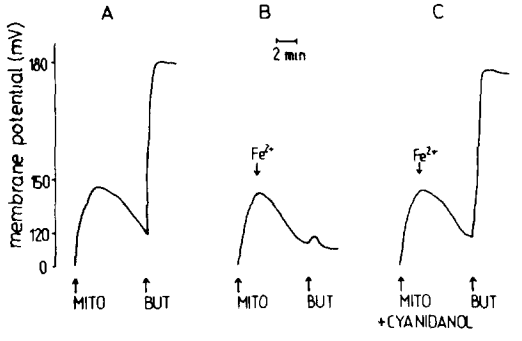

Fig 1 The effect of Fe^{2+} and Cyanidanol on the mitochondrial membrane potential Original trace of the TPP^+ electrode The basic medium was supplemented by 3 μM TPP^+ Further additions mito, 1 5 mg mitochondrial protein/ml, BUT, 5 mM 3-OH-butyrate, Fe^{2+}, 17 6 μM $FeSO_4$, Cyanidanol, 23 nmol/mg protein (34 5 μM)

by 1.7 nmol/mg. In contrast, in the presence of 23 nmol Cyanidanol/mg (34.5 μM) the MDA concentration failed to increase and $\Delta\psi$ reached the control value (Fig. 3). The MDA formation and $\Delta\psi$ decrease ran parallel in time and both processes were prevented by Cyanidanol The collapse of $\Delta\psi$ induced by Fe^{2+} ions was also prevented by the well-known free radical scavanger butylated hydroxianisol (data not shown)

Many peroxidative agents perturb the Ca^{2+} homeostasis of liver cells by decreasing mitochondrial and other intracellular Ca^{2+} pools [31–33] We investigated the effect of Cyanidanol on the Ca^{2+} uptake capability of Fe^{2+}-treated mitochondria Incubation of mitochondria in the presence of 17 6 μM Fe^{2+} for 5 min decreased the extent of maximal Ca^{2+} uptake from the control value of 17 3 nmol/mg to 4 nmol/mg When mitochondria were treated with increasing amounts of Cyanidanol before the addition of Fe^{2+} the extent of Ca^{2+} uptake gradually increased and

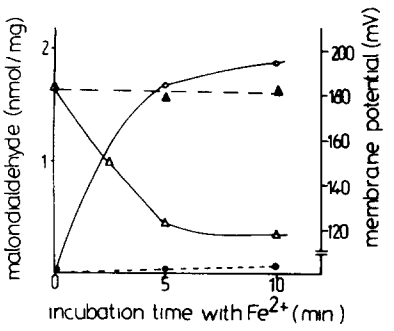

Fig 3 The effect of incubation time with Fe^{2+} on the MDA formation and $\Delta\psi$ changes in the presence and absence of Cyanidanol After the time indicated on the abscissa samples were removed for MDA determination (○, ●) or 5 mM Tris–succinate was added to measure $\Delta\psi$ in the presence of 3 μM TPP^+ (△, ▲) Lipid peroxidation was initiated with 17 6 μM $FeSO_4$ Mitochondrial protein was 1 2 mg/ml Open symbols, in the absence of Cyanidanol; full symbols, in the presence of 23 nmol Cyanidanol/mg mitochondrial protein

finally reached the control value (Fig. 2C). Half maximal effect was obtained with 4.2 nmol Cyanidanol/mg protein (6.3 μM). Although the decrease of $\Delta\psi$ value might explain the impairment of the Ca^{2+} uptake, an additional effect on the Ca^{2+} uniporter and/or on the Ca^{2+} efflux pathway can not be ruled out.

The intact inner membrane has low permeability for K^+ and Cl^- ions. Thus volume changes are not observed if mitochondria are suspended in an iso-osmotic KCl solution [34] Addition of Fe^{2+} to mitochondria suspended in the basic medium resulted immediately in a rapid fall of apparent absorbance indicating an increase of the mitochondrial volume. This high rate of the mitochondrial swelling points to the increased K^+ and Cl^- permeability of the mitochondrial inner membrane, probably due to enhanced lipid peroxidation. The Fe^{2+}-induced swelling was prevented by Cyanidanol in a concentration dependent manner (Fig. 2D). Above 20 nmol/mg (30 μM) concentration the swelling rate decreased to the control value, i.e. to the swelling rate measured in the presence of Cyanidanol alone (Fig 2D)

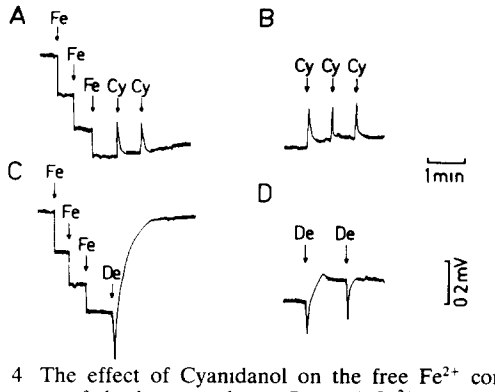

Fig 4 The effect of Cyanidanol on the free Fe^{2+} concentration of the basic medium Original Ca^{2+} electrode recording Additions Fe^{2+}, 8 8 μM $FeSO_4$; Cy, 17.2 μM Cyanidanol, De, 50 μM Desferal

It was shown previously that under certain conditions flavonoid compounds are able to chelate metal ions [35] Therefore we investigated the effect of Cyanidanol on the free Fe^{2+} concentration in the absence of mitochondria with Ca^{2+} electrode (see Methods). Addition of Cyanidanol in a final concentration of 34 5 μM did not decrease the Fe^{2+} (26.4 μM) concentration of the medium (Fig. 4A). The upward deflection of the recorder after the first addition of Cyanidanol was observed also in the absence of Fe^{2+} ions (Fig. 4B). In contrast to Cyanidanol, addition of the iron-chelator Desferal decreased the medium Fe^{2+} concentration (Fig. 4C). The artefactual effect of Desferal on the electrode is significantly smaller than the effect of Desferal on the level of Fe^{2+} concentration (Figs. 4D and 4B). These observations suggest that under our conditions Fe^{2+} chelation by Cyanidanol must have been negligible. This fact is in accordance with the results of Bindoli *et al* [36], who found that the protection of mitochondria by the oxoflavonoid Sylimarin during Fe^{2+}-induced lipid peroxidation is not a consequence of Fe^{2+} chelation.

DISCUSSION

The results reported in the present paper indicate that Cyanidanol prevents simultaneously both the lipid peroxidation and the functional deterioration of mitochondria brought about by Fe^{2+} ions. Cyanidanol in itself did not alter the mitochondrial membrane potential and the Ca^{2+} uptake and retention capability. It was found earlier that Cyanidanol did not alter the rate of O_2 consumption in state 3 respiration [24]

Fe^{2+} ions exert their action through the catalysis of free radical generation [3]. Cyanidanol being a potent free radical scavenger [37,38] most probably prevents the radical-induced injury (e g. lipid peroxidation) of the mitochondrial inner membrane Similar observations were made on microsomal membrane vesicles [20].

The hepatic conjugation of Cyanidanol proves that the drug can penetrate the cell membranes *in vivo* [39]. This fact supports our suggestion that the above described mitochondrion-protective effect may have pharmacological relevance, too. Under conditions when enhanced free radical generation occurs in the mitochondrial environment, Cyanidanol may enable the cells to preserve their bioenergetic intactness, i e. full capacity of ATP synthesis and the capability to keep the cytoplasmic Ca^{2+} concentration at the physiologically low level

Acknowledgements—The authors are indebted to Professor Janos Fehèr for initiating the work and kindly providing Cyanidanol, to Professor Attila Fonyò and Dr Erzsèbet Ligeti for valuable discussions and help during the preparation of the manuscript and to Jeno Kunovics for providing a Ca^{2+}-selective membrane We also thank Miss Edit Fedina and Mrs Erzsèbet Seres-Horvàth for their expert and devoted technical assistance Experimental work was financially supported by grant 17/1-06/439 of the Hungarian Ministry of Health and by a grant of the Orszàgos Muszaki Fejlesztèsi Bizottsàg (National Committee for Technical Development) of Hungary

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