Improved Antioxidant Effect of Idebenone-Loaded Polyethyl-2-Cyanoacrylate Nanocapsules Tested on Human Fibroblasts

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Received July 12, 2001; accepted October 8, 2001

Purpose. The protective antioxidant role of idebenone both as free drug and drug-loaded Tween 80-coated polyethyl-2-cyanoacrylate (PECA) nanocapsules is reported. The relationship between oxidative damage and apoptotic or nonapoptotic cell death is evaluated *in vitro*.

Methods. Idebenone-loaded nanocapsules were prepared with the interfacial polymerization method in the presence of Tween 80. Human nonimmortalized fibroblasts, under different stress conditions, either 0.5 mM diethylmaleate (DEM) for 60 min or 0.1 mM H_2O_2 for 30 min, were used as the experimental *in vitro* model. The production of reactive oxygen species, the cell viability, and the nuclear DNA damage were evaluated. The presence of apoptotic damage was evaluated both by the determination of caspase-3-like protein activity and by Promega's fluorescent apoptotic detection system.

Results. DEM and H_2O_2 affected the cultured cells in different ways. DEM induced a moderate cellular insult, which was efficaciously antagonized by idebenone-loaded PECA nanocapsules. H_2O_2 elicited severe damage to nuclear DNA, which was reduced by idebenone-loaded PECA nanocapsules. The free drug was less effective than idebenone-loaded nanocapsules.

Conclusions. The findings reported here demonstrate that an improved antioxidant effect was obtained with a low idebenone concentration (0.5 μM) when the drug was entrapped within Tween 80-coated PECA nanocapsules.

KEY WORDS: antiapoptotic effect; COMET assay; free radical-induced damage; idebenone; PECA nanocapsules; oxidative stress.

INTRODUCTION

Different environmental, physical, and chemical stresses on cells may cause an oxidative wave, inducing either an overproduction of reactive oxygen species (ROS) or a deficiency in antioxidant enzymes (1). Oxidative stress has been implicated in many physiologic and pathologic conditions occurring at the level of different tissues, i.e., the central nervous system, and the heart as well as in aging (2,3). The determination of massive cellular damage by oxidative stresses, due to necrotic cell death in the presence of lipid peroxidation and alterations of proteins and nucleic acids, has been extensively documented (4,5). An emerging concept is also the involvement of ROS in physiologic death, such as programmed cell death or apoptotic cell death (6).

The regular intake of antioxidant agents seems to prevent or limit the dangerous effects of chronic or transient-drastic oxidative stresses (7). A number of investigations have shown the effectiveness of various molecules such as free radical scavengers to reduce or to prevent oxidative cell damage (8,9).

Our attention has been focused, in particular, on idebenone [6-(10-hydroxydecyl)-2,3-dimetoxy-5-methyl-1,4-benzoquinone], a lipophilic benzoquinone electron carrier, which behaves as an antioxidant free radical scavenging molecule. Idebenone has been reported to be active in central nervous system disorders elicited by both vascular lesions and neurodegenerative processes (10–12). The mechanism of action of idebenone is based on cerebral metabolism improvement (13), activating the mitochondrial electron-transfer system and, hence, reducing the consumption of nonrespiratory oxygen (14).

Unfortunately, this drug is not very water soluble, and the only marketed pharmaceutical formulation is an oral dosage form. Following oral administration in humans, the hematic peak level of idebenone is observed after 3 h and is eliminated showing no accumulation (15). This drug shows an oral bioavailability of ~60% with respect to the administered dose (15). It is well known that the improvement of the water solubility and/or dispersibility of drugs can lead to a more rapid and greater adsorption after oral administration. To improve drug-water solubility idebenone-cyclodextrin complexes were prepared (16). Another possibility for dispersing a poorly water-soluble drug, such as idebenone, in water is represented by encapsulation in polymeric shell nanocapsules (17).

The aim of this work was both to define the effectiveness of nanocapsule-entrapped idebenone with respect to free idebenone and to clarify the protective antioxidant mechanism of action of the drug in our experimental conditions. For this we studied *in vitro* some biochemical parameters that showed evidence of the radical scavenging role exerted by idebenone. As an experimental model we choose human nonimmortalized fibroblasts stressed either by diethylmaleate (DEM) or by H_2O_2.

MATERIALS AND METHODS

Chemicals

DEM and ethyl-2-cyanoacrylate were purchased from Sigma (St. Louis, MO, USA). Ethyl-2-cyanoacrylate was used as the monomer in the preparation of polyethyl-2-cyanoacrylate (PECA) nanocapsules. Miglyol 812 (Eigenmann & Veronelli, Milan, Italy), a neutral oil constituted by

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ABBREVIATIONS: AMC, 7-amino-4-methyl-coumarin; ANOVA, analysis of variance; CPP, caspase-3; DEM, diethylmaleate; HPLC, high-performance liquid chromatography; LDH, lactic dehydrogenase; MTT, tetrazolium salt test; PBS, phosphate-buffered saline; PECA, polyethyl-2-cyanoacrylate; ROS, reactive oxygen species.

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triglycerides with mixed acyl-chains of fractionated coconut fatty acids C_8–C_{10}, was used to achieve the oily nanocapsule core. Tween 80 (Sigma), a nonionic surfactant, was used to stabilize the colloidal nanocapsule dispersion. Apoptosis was tested with a kit purchased from Promega (Madison, WI, USA). Dulbecco's modified eagle's medium and fetal calf serum are products from Life Technologies (Milan, Italy). Inorganic salts (purity >99.5%) NaCl, KH_2PO_4, and NaOH are products of BDH Laboratory Supplies (Poole, UK). Idebenone was kindly provided by Takeda Italia Farmaceutici S.p.A. (Rome, Italy) and was used without further purification (Fig. 1), although its purity was >99.5% as assayed by high-performance liquid chromatography (HPLC) analysis. Double-distilled pyrogen-free water was used. All other reagents and solvents were of analytical grade (Carlo Erba, Milan, Italy).

PECA Nanocapsule Preparation

Lipophilic components, i.e., Miglyol 812 (1 ml), idebenone (150 mg), and the monomer (300 μl), were dissolved in 50 ml acetone. This organic solution was added to 100 ml aqueous phase (sterile double-distilled water, pH 7) containing Tween 80 (at different concentrations) at a flow rate of 0.5 ml/min. The presence of the nonionic surfactant allowed the polymerization of ethyl-cyanoacrylate at the oil/water interface, thus encapsulating Miglyol 812 droplets (18,19). The immediate polymerization triggered the formation of drug-loaded nanocapsule colloidal suspensions. PECA nanocapsule colloidal suspensions were concentrated under vacuum by evaporating off acetone up to the point when no trace of the organic solvent could be detected.

Drug-loaded PECA nanocapsules were centrifuged at 20,000 × *g* for 1 h at 4°C to remove the untrapped idebenone. The floating nanocapsules were collected and suspended in double-distilled water. This washing procedure was carried out four times. After purification, no presence of both the Tween 80 (20) and idebenone was observed. Final drug-loaded PECA nanocapsule colloidal systems, suspended in sterile double-distilled water, were made isotonic with NaCl.

Drug Loading Determination

After purification, nanocapsule suspensions were lyophilized overnight. Freeze-dried nanocapsules were solubilized in an acetone-methanol (9:1 v/v) organic mixture and made up to 25 ml. This solution was filtered through 0.45-μm polytetrafluorine-ethylene membranes (Sartorius, Göttingen, Germany) and were submitted to HPLC analysis to determine the amount of entrapped idebenone. A Hewlett-Packard (Milan, Italy) model 1050 liquid chromatographic system equipped with a 20-μl injection valve (model 7125, Rheodyne, Cotati, CA) was used. Substance detection was carried out at 282 nm. Chromatogaphic analyses were performed at room temperature with a reverse-phase ODS Hypersil C_{18} column (5 μm, 125 mm × 4 mm i.d.; Merck, Darmstadt, Germany). The mobile phase was an acetonitrile/water mixture (70:30 v/v) with a flow rate of 1 ml/min. The eluent mixture was filtered through 0.22-μm poly-tetrafluorine-ethylene membranes (Sartorius). The amount of idebenone was calculated using a calibration curve, reporting concentration and peak area. No interference of the other nanocapsule components was observed. Results are expressed as encapsulation yield (percentage of the starting drug that became encapsulated). The drug release from PECA nanocapsules was determined by incubating the colloidal suspension in the cell culture medium. At time intervals, the suspension was centrifuged and the supernatant was assayed by HPLC.

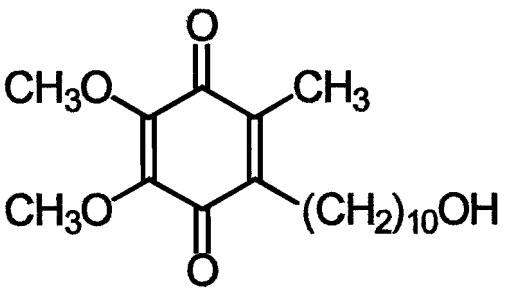

Fig. 1. Chemical structure of idebenone, 6-(10-hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzo-quinone.

PECA Nanocapsule Colloidal Characterization

Dimensional analysis of PECA nanocapsule suspensions was carried out by photon correlation spectroscopy with a Zetamaster (Malvern Instruments Ltd, Sparing Lane South, Worcs, England). A solid-state laser was used as the light source. This laser diode had a nominal power of 4.5 mW with a maximum output of 5 mW at 670 nm. The photon correlation spectroscopy measurements were carried out at a scattering angle of 90°. As a correlation function, a third-order cumulant fitting was applied to obtain mean particle diameter and polydispersity. Samples were suitably diluted with filtered water (0.22-μm membrane filters, Sartorius) and were placed in quartz cuvettes. Thirty measurements per sample were carried out.

Electrophoretic mobility and zeta potential distribution were measured with the Zetamaster particle electrophoresis analyzer set-up (Malvern) equipped with a 5-mW HeNe laser (633 nm). Zeta limits ranged from –120 to 120 V. A Smoluchowsky constant F(Ka) of 1.5 was used to achieve zeta potential values from electrophoretic mobility.

Morphologic characterization was carried out by freeze-fracture electron microscopy, as reported elsewhere (16).

Cell Cultures

Our study was carried out *in vitro* on human nonimmortalized fibroblast cell lines that were kindly donated by Professor A. Simeone (Istituto Internazionale di Genetica Molecolare, CNR Naples, Italy). The cells were cultured in humidified atmosphere (5% CO_2 at 37°C) in 35-mm dishes containing a final volume of 2 ml made up of Dulbecco's modified eagle's medium plus 10% (v/v) fetal calf serum, 1 mM glutamine, and 100 μl penicillin-streptomycin. When confluence was reached, the cells were challenged by exposure to 0.5 mM DEM for 60 min or 0.1 mM H_2O_2 for 30 min. DEM was dissolved in dimethyl sulfoxide, which was assayed alone in the cultures. The employed concentrations of the two oxidants and the times of the treatments were selected on the basis of our previous study (unpublished data). Free or encapsulated idebenone was added to the cultures at 0.5 μM

concentration at the same time as DEM or H_2O_2 and was maintained in the culture for 60 min. The total amount of PECA added to the culture medium was 1 μg.

ROS Determination

Intracellular ROS was determined both directly in the dishes and in scraped and resuspended cells using 5,6-carboxy-2^I,7^I-dichlorodihydrofluorescine diacetate bis acetomethyl ester (Molecular Probes) as a fluorescent probe. This probe, diffusing through cell membranes, reaches the cytosol. At this level, the probe is hydrolyzed by esterases to 5,6-carboxy-2^I,7^I-dichlorodihydrofluorescine, which is oxidized to fluorescent 5,6-carboxy-2^I,7^I-dichlorofluorescine by ROS. The fluorescence intensity is proportional to the amount of intracellular ROS (21). The 5,6-carboxy-2^I,7^I-dichlorodihydrofluorescine diacetate bis acetomethyl ester was added to the cultures after DEM or H_2O_2 treatment and was incubated for 30 min. Thereafter, the medium was discharged, the dishes were washed with phosphate-buffered saline solution (PBS), and the fluorescence intensity was both qualitatively and quantitatively evaluated by fluorescence microscopy and by spectrofluorimetry at λ_{ex} 475 nm and λ_{em} 525 nm (F-2000, Hitachi, Tokyo, Japan), respectively. 5,6-carboxy-2^I,7^I-dichlorofluorescine-stained cells were not exposed to light before measurement.

Lactate Dehydrogenase Release

Lactate dehydrogenase (LDH) activity was determined as a kinetic reduction of $NADH(H^+)$ to the oxidized compound NAD^+. The reduction of the amount of $NADH(H^+)$ is followed by ultraviolet adsorption at λ_{max} 340 nm (22). The oxidation of $NADH(H^+)$ to NAD^+ is due to the presence of LDH, which catalyzes the reduction from piruvate to lactate using $NADH(H^+)$ as a cofactor. The release of LDH was tested for each sample, both in the dish medium and in the corresponding cells, following cell disruption by sonication. The percentage of LDH released in the medium (reflecting cytotoxicity) was calculated considering the total LDH activity, namely, that measured in the medium plus that measured in the sonicated cells.

Tetrazolium Salt Test

The tetrazolium salt test (MTT), a calorimetric assay, was used to indirectly monitor cell growth. The cells were cultured in 200-μl microplates for 12 h. Cells were then washed with PBS and incubated either with DEM or with H_2O_2. At the end of incubation, MTT (5 mg/ml) was added to each well and incubation was continued for a further 24 h. The concentration of tetrazolium salts, following conversion to the colored product formazan, was determined at 570 nm with a Titertek Multiskan microplate spectrophotometer (Flow Laboratories, Milan, Italy) (23). The amount of formazan is directly proportional to the number of living cells present in the culture.

Caspase-3 Activity

The caspase-3 (CPP) activity was quantitatively measured by the Casp-ACE assay system according to Promega's protocol. This kit provides fluorometric reagents to quickly measure the activity of the CPP-32 enzyme. Cell extract enzymes were assayed. We used a tetrapeptide substrate labelled with a fluorochrome, which is released upon cleavage by CPP-32-like enzymes. The yellow-green fluorescence was proportional to the amount of CPP-32 activity present in the sample. Fluorescence intensity was determined at λ_{em} 460 nm by using a Hitachi F-2000 spectrofluorometer.

COMET Assay

DNA damage was evaluated using single-cell gel electrophoresis (24). Briefly, the cells were scraped, washed, and resuspended in a low volume of PBS. 10 μl (about $10–20 \times 10^4$ cells) were embedded in 75 μl 0.5% (w/v) low-melting-point agarose and spotted as a second layer on a first layer of normal melting point agarose 1% (w/v) covering a microscope slide. Finally a third layer was applied with 75 μl of low-melting-point agarose without cells. The slides were immersed first in pH 10 lysing buffer at 4°C for 1 h, and then they were placed in a horizontal electrophoresis tank (Bio-Rad, Burlington, Massachusetts). The tank was filled with alkaline buffer (300 mM NaOH and 1mM Na_2EDTA at pH 13), and an electrophoretic run was performed at 4°C for 20 min applying a 300-mA current with a potential difference of 25 V. The slides then were neutralized in 0.4 M Tris HCl pH 7.5, stained with ethidium bromide, and examined under a fluorescence microscope. During these experiments, yellow light was used to prevent additional DNA damage. DNA damage was measured on 100 counted DNA molecules (representative of 100 cells) with regard to tail length as follows: 0, no tail; 1, short tails; 2, medium tails; 3, long tails; and 4, very long tails. The percentage of tailed DNA molecules (cells) was also determined.

Apoptosis Detection

The presence of apoptotic cell death was investigated using the TdT-mediated dUTP nick-end labeling assay (Promega) in which terminal deoxynucleotidal transferase was used to add fluorescein-12-dUTP to the 3' OH ends of fragmented DNA present in the cells. Before analysis, cells were scraped, fixed on slides, and labelled both with propidium iodide and fluoresceine, according to Promega's protocol. The apoptotic cells were visualized within a cell population by fluorescence microscopy. In particular, green nuclei contained fragmented apoptotic DNA, whereas red nuclei contained intact propidium iodide-stained DNA.

Statistical Analysis

Data are presented as means ± SD and were analyzed by Student's *t* test and one-way analysis of variance (ANOVA) with *a posteriori* Bonferroni test. Data with $P < 0.05$ are considered to be significant.

RESULTS

PECA Nanocapsule Preparation and Characterization

In this investigation, nanocapsules (Fig. 2) were prepared by using acetone as the organic phase, as reported in a previous article (17). The influence of the Tween 80 concentration in the polymerization medium on the formulation parameters of the colloidal carrier was evaluated. As reported in Table 1, the Tween 80 concentration noticeably influenced

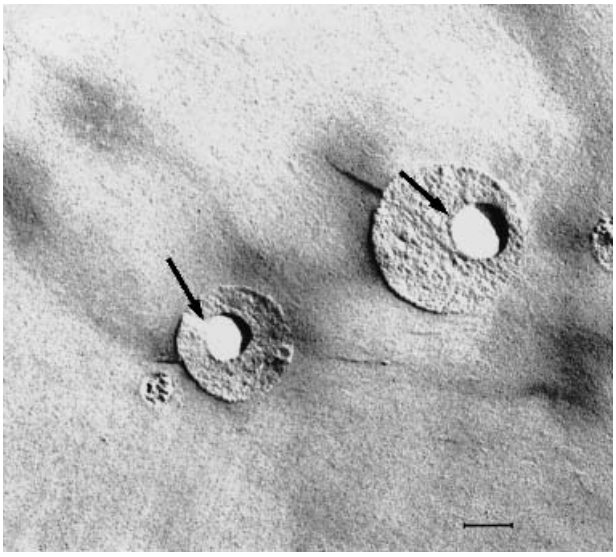

Fig. 2. Freeze-fracture electron micrograph of idebenone-loaded Tween 80-coated PECA nanocapsules. The black arrow shows the internal oil (Mygliol 812) droplet surrounded by the polymeric shell. The bar is 100 nm.

the mean size of PECA nanocapsules. The higher the Tween 80 concentration, the smaller the colloidal suspension mean size up to a surfactant concentration of 3% (w/v). At Tween 80 concentrations higher than 3% (w/v), no significant size variation of the nanocapsule colloidal suspension was observed. The effect of Tween 80 on the emulsion formation was evaluated by determining the size distribution of the emulsion droplets without monomer and with increasing concentrations of the surfactant. Tween 80 was able to reduce the hydrodynamic size of the emulsion droplets as a function of its concentration (Table 1). At concentrations of Tween 80 higher than 3% (w/v), the mean size of the emulsion droplets was not influenced. Beyond a concentration of 3% (w/v), a second colloidal population is clearly observed by photon correlation spectroscopy and is characterized by a very small size, showing the presence of micelles. Increasing Tween 80 concentrations, a reduction of the polymeric wall thickness of PECA nanocapsules was observed (data not reported), according to our previously reported observations (17). The

Table I. Light Scattering Analysis of Colloidal Dispersions of PECA Nanocapsules and Emulsion Droplets Prepared in the Presence of Different Concentrations of Tween 80 in a Polymerization Medium at pH 7[a]

Tween 80 (% w/v)	PECA nanocapsules		Emulsion droplets[b]	
	Size (nm)	PI[c]	Size (nm)	PI[c]
0.5	312.3 ± 21.1	0.21 ± 0.04	289.8 ± 14.5	0.16 ± 0.05
1.0	247.8 ± 13.4	0.15 ± 0.02	217.2 ± 12.1	0.14 ± 0.03
3.0	185.1 ± 18.6	0.11 ± 0.02	179.2 ± 13.8	0.06 ± 0.01
6.0	183.5 ± 15.7	0.23 ± 0.03	175.4 ± 11.6	0.09 ± 0.02
9.0	176.9 ± 20.9	0.17 ± 0.04	177.3 ± 12.9	0.11 ± 0.03

[a] Each value is the average of four different experiments ± SD.
[b] Emulsion droplets obtained without the monomer.
[c] Polydispersity index. This value represents the dimensional homogeneity of the Tween 80-coated PECA nanocapsule colloidal suspensions.

presence of different amounts (from 20 mg up to 200 mg) of idebenone had no influence on the physicochemical characteristics of the colloidal suspensions (data not reported).

Surface properties of Tween 80-coated PECA nanocapsules are characterized by a zeta potential of –13.1 ± 3.4 mV. A lower zeta potential value should be observed, but the presence of the nonionic surfactant Tween 80 on the PECA nanocapsule surface behaved like a polyoxyethylene shield around the particles, which elicited a reduction of the zeta potential. This parameter was poorly influenced by the various preparation conditions.

Idebenone presented an encapsulation yield within nanocapsules of 97% with respect to the amount added during preparation. This drug, due to its highly lipophilic character, is solubilized within the oil core of PECA nanocapsules. The poor water solubility and the high affinity for the oil core of PECA nanocapsules of idebenone also justify the poor release from PECA nanocapsules in an aqueous environment. In fact, after 12 h only 2.7% of the entrapped amount of idebenone is released into the cell culture medium. For *in vitro* assays, idebenone-loaded PECA nanocapsules were prepared in the presence of 3% (w/v) Tween 80.

Experiments on Human Fibroblasts

For all the *in vitro* experiments, a control assay was carried out to measure the effects of unloaded PECA nanocapsules. The results showed that, according to the literature (25–27), the addition of nanocapsules to the fibroblasts elicited no modification in the cell cultures during the different assays (data not shown). The experiments were carried out by administering *in vitro* free or encapsulated idebenone at a concentration of 0.5 μM. To evaluate whether the presence of the carrier can modulate the action of the drug, a physical mixture of the free drug and an unloaded nanocapsule were also assayed. In this case, the effects on the various parameters were similar to those obtained for the free drug (data not reported).

ROS Determination

Our data (Fig. 3a) showed, with respect to the control (untreated cells), a more severe oxidative damage for H_2O_2-treated cells than for DEM-treated cells. Dimethyl sulfoxide, the solvent used for DEM, elicited no significant modification under our experimental conditions.

At the drug concentration investigated, no free idebenone antioxidant effect was observed in the case of cells treated with stress-inducing substances (DEM or H_2O_2). Conversely, idebenone-loaded PECA nanocapsules were able to exert an effective antioxidant activity (Fig. 3a).

COMET Assay

The stress conditions are associated with nuclear DNA damage as shown by the COMET assay (Fig. 3b). Similarly to the ROS experiment, H_2O_2 treatment triggered a more severe damage of genomic DNA than that observed after DEM treatment. DEM caused DNA damage ranging from 1 to 2 as regards tail length, whereas H_2O_2 damage was from 3 to 4.

Also in this case, the idebenone encapsulated in PECA nanocapsules was much more effective than the free drug regarding the reduction of DNA fragmentation (Fig. 3b), par-

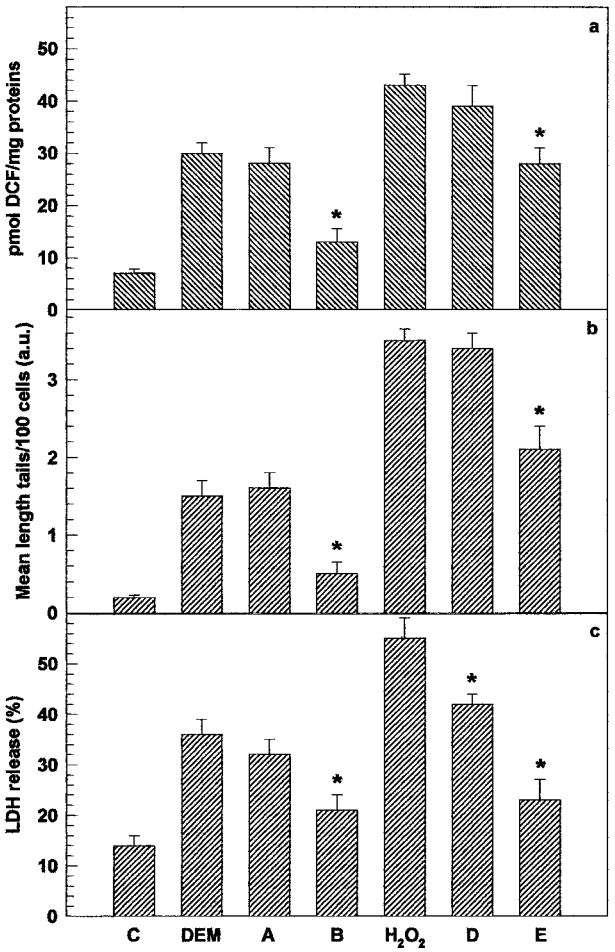

Fig. 3. Evaluation of ROS production (a), DNA damage (COMET assay) (b), and LDH release (c) of stress-induced, human, nonimmortalized fibroblasts. C, untreated control cells; DEM, cells treated with DEM (0.5 mM) for 60 min; A, DEM-stressed cells treated with free idebenone; B, DEM-stressed cells treated with idebenone-loaded PECA nanocapsules; H_2O_2, cells treated with H_2O_2 (0.1 mM) for 30 min; D, H_2O_2-stressed cells treated with free idebenone; E, H_2O_2-stressed cells treated with idebenone-loaded PECA nanocapsules. Histograms show the mean value of five different experiments ± SD. *Student's *t*-test $P < 0.001$ compared with the respective stressing treatment (DEM or H_2O_2). All sample values were significant ($P < 0.001$) compared to the control. ANOVA is $P < 0.001$.

ticularly in the case of DEM-treated cells. Further evidence of this came from LDH release experiments.

LDH release

This cytotoxicity assay measures the activity of LDH as a stable cytosolic enzyme that is released into the culture medium following the disruption of the cellular cytoplasmatic membrane. An increase of LDH leakage was observed in the case of stress-inducing molecule treatment (Fig. 3c). Also in this experiment, the damage was more severe after H_2O_2 treatment.

Free idebenone was not able to significantly reduce the LDH release. Conversely, idebenone-loaded nanocapsules reduced the LDH release by ~50% for both DEM-stressed and H_2O_2-stressed cells.

MTT Assay

The cell viability test (MTT) was also carried out to evaluate the effects of the stress-inducing substances and of the antioxidant agent on cell growth. In fact, this test determined the number of viable cells. As shown in Fig. 4, a moderate decrease in cellular viability (–10%) was observed after DEM treatment. The damage was clearly evident after H_2O_2 treatment (–60%). The antioxidant idebenone was able to repair the damage induced by DEM but only partially repaired that induced by H_2O_2, when administered encapsulated in PECA nanocapsules.

Apoptosis Detection

Because oxidative damage causing single-strand or double-strand DNA breakage may be responsible for cell death, apoptosis or necrosis, depending on the severity of the induced stress, was evaluated by Promega's apoptosis detection system. This assay is designed for the specific detection of apoptotic cells within a cell population by revealing DNA fragmentation at the level of the single cell.

The results concerning apoptotic experiments obtained in the presence of free and encapsulated idebenone are presented in Fig. 5. After DEM treatment, a diffused presence of green apoptotic nuclei within cells, which seemed to have intact plasmatic membranes, was observed. After H_2O_2 treatment, more severe DNA damage with different diffused green domains and an alteration of cellular structure was observed, which is in agreement with the results obtained by the COMET assay and LDH release. Idebenone showed a more effective antioxidant activity toward DEM-treated fibroblasts, particularly when administered as idebenone-loaded PECA nanocapsules.

CPP Activity

Usually, two or more independent methods should be used to confirm apoptotic cell death. For this reason, CPP-

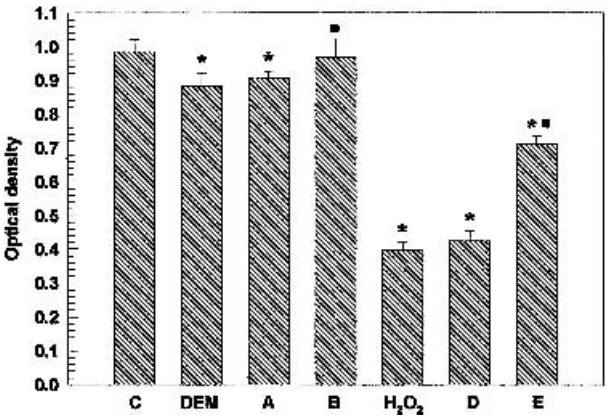

Fig. 4. Cell viability evaluation by MTT assay in human nonimmortalized fibroblasts stressed with DEM (0.5 mM) for 60 min or H_2O_2 (0.1 mM) for 30 min. C, untreated control cells; A, DEM-stressed cells treated with free idebenone; B, DEM-stressed cells treated with idebenone-loaded PECA nanocapsules; D, H_2O_2-stressed cells treated with free idebenone; E, H_2O_2-stressed cells treated with idebenone-loaded PECA nanocapsules. Histograms show the mean value of five different experiments ± SD. *Student's *t* test $P < 0.001$ compared with the control. ■ Student's *t* test $P < 0.001$ compared with the respective stressing treatment (DEM or H_2O_2). ANOVA is $P < 0.001$.

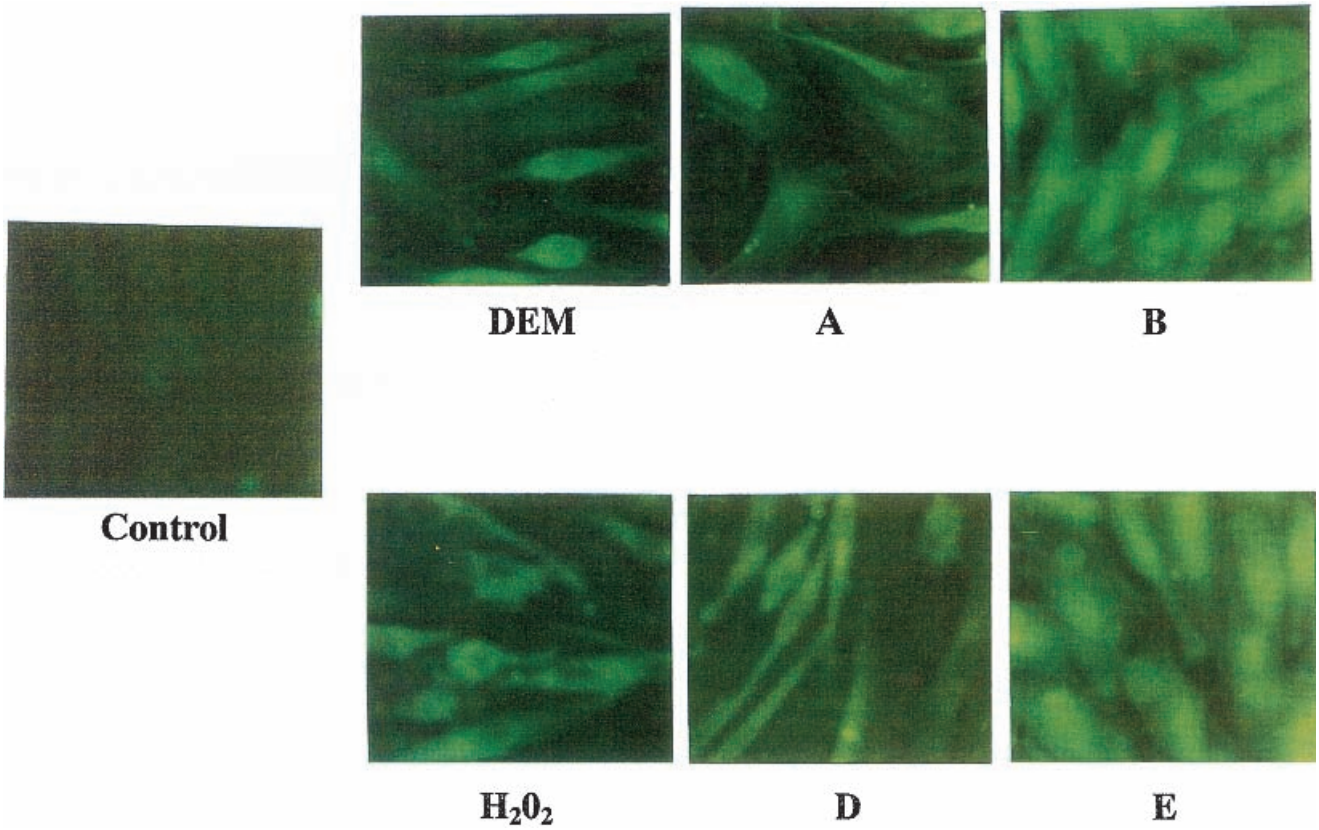

Fig. 5. Fluorescence microscopy detection of double-stained (propidium iodide and fluorescein), human, nonimmortalized fibroblasts. Apoptotic nuclei (green) were detected by TdT-mediated dUTP nick-end labeling-like analysis carried out with Promega's apoptosis detection kit. Fibroblasts were stressed with DEM (0.5 mM) for 60 min or H_2O_2 (0.1 mM) for 30 min. Control, untreated cells; A, DEM-stressed cells treated with free idebenone; B, DEM-stressed cells treated with idebenone-loaded PECA nanocapsules; D, H_2O_2-stressed cells treated with free idebenone; E, H_2O_2-stressed cells treated with idebenone-loaded PECA nanocapsules.

like activity was measured. Caspase (interleukin-1β-converting enzyme ICE/CED-3) is a family of cysteine aspartic acid-specific proteases. These types of enzymes have been shown to play fundamental roles in the inflammation and apoptosis processes of mammalian cells (28). The assay system used a specific tetrapeptide substrate that is labelled with 7-amino-4-methyl-coumarin (AMC). AMC is released from the substrate upon cleavage of a CPP-like enzyme, thus producing fluorescence. The amount of fluorescence is proportional to the amount of CPP-like activity present in the sample. The results (Fig. 6), contrary to those obtained for the LDH release, showed a noticeable increase of CPP activity after DEM treatment, whereas a moderate increase was observed after H_2O_2 treatment. The increase of caspase activity was reduced by both the free drug (0.5 μM) and idebenone-loaded PECA nanocapsules (0.5 μM) in the case of both DEM-treated and H_2O_2-treated cells. Idebenone antioxidant action was more efficacious for DEM-stressed cells than for those stressed with H_2O_2. In particular, idebenone-loaded PECA nanocapsules were more effective than the free drug.

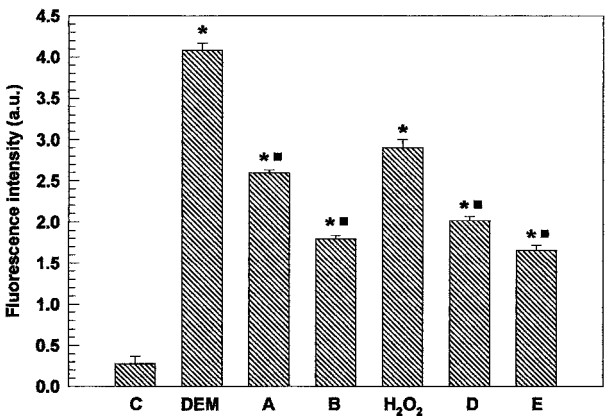

Fig. 6. Fluorimetric evaluation of CPP-like protein activity of human non immortalized fibroblasts stressed with DEM (0.5 mM) for 60 min or with H_2O_2 (0.1 mM) for 30 min. C, untreated control cells; A, DEM-stressed cells treated with free idebenone; B, DEM-stressed cells treated with idebenone-loaded PECA nanocapsules; D, H_2O_2-stressed cells treated with free idebenone; E, H_2O_2-stressed cells treated with idebenone-loaded PECA nanocapsules. AMC fluorescence intensity was determined at λ_{em} 460 nm. Histograms show the mean value of five different experiments ± SD. *Student's *t* test $P < 0.001$ compared with the control. Student's *t* test $P < 0.001$ compared with the respective stressing treatment (DEM or H_2O_2). ANOVA is $P < 0.001$.

DISCUSSION

The preparation procedure herein reported allowed the formation of nanocapsules in which Tween 80 is adsorbed into the PECA polymeric shell, drastically reducing the pres-

ence of free surfactant in the aqueous suspension. Nanocapsule colloidal systems, that is, an oil core surrounded by a polymeric shell, are more suitable colloidal carriers than nanoparticles for the delivery of lipophilic substances such as idebenone, which can be solubilized in the oil compartment. The size of this colloidal carrier can be controlled within a certain interval by regulating the Tween 80 concentration. In fact, a nanocapsule size reduction was achieved by increasing the surfactant concentration. This finding is mainly due to two factors: (1) the size reduction of the emulsion droplets where polymerization takes place; and (2) the increase of the number of the droplets. This situation happens up to a 3% (w/v) concentration of Tween 80, because beyond this value Tween 80 forms micelles and is not able to further reduce the droplet size and increase the number of the Miglyol 812 emulsion droplets.

We demonstrated the greater effectiveness of idebenone-loaded PECA nanoparticles toward human fibroblasts than the free drug by investigating the *in vitro* antioxidant effect under different stress conditions (DEM or H_2O_2).

DEM is a depleting agent of the cellular GSH/GSSG ratio, thus eliciting a moderate increase of ROS and some alterations in the electron transport chain (29). These alterations seem to be responsible for DNA damage and can be partially antagonized by some free radical scavenging species, such as idebenone.

H_2O_2, by increasing the number of radical species in cells, reduces the antioxidant cellular defences and triggers a depletion of ATP concentration (30), thus causing a severe cellular insult in both the cytosol and the mitochondria (31). Under these conditions, cellular macromolecules and genomic DNA can be altered in a lethal way.

Our results show that idebenone-loaded nanocapsules exert a greater protective antioxidant effect after DEM-induced stress than that after H_2O_2 treatment. This action is probably due to two different contrasting factors: (1) a massive involvement of mitochondria in DEM-induced damage; and (2) the electron carrier activity of idebenone. As regards the molecular mechanism of action of idebenone, its protective effect, under our experimental conditions, seems to be exerted more on DNA damage and membrane breakdown than on ROS production. This activity could be due to an idebenone-dependent activation of protective pathways (32) that is exerted more at the level of mitochondria than of cytosolic ROS. Electron carrier and antioxidant activities of idebenone maintain mitochondria membrane integrity, so contrasting the release from the organelles of dangerous pro-apoptotic factors (i.e., cit C, APO 1, and AIF), which, in turn, activate caspases and poly-A-ribosyl-polymerase, inducing apoptotic DNA fragmentation.

The difference in the biological action between the free drug and idebenone-loaded PECA nanocapsules is probably due to a mechanism based on the adherence of nanospheres to the cells, thus allowing the lipophilic drug, i.e., idebenone, to diffuse into cellular and subcellular structures. Our findings show that idebenone encapsulation within Tween 80-coated PECA nanocapsules may be useful both to ensure a greater drug effectiveness by reducing the concentration to be used, by allowing an easier passage through biological barriers, and by drastically reducing the restrictive binding of the drug with serum proteins, and to avoid the useless accumulation of the drug. In our experiments the dosage of idebenone-loaded PECA nanocapsule was 0.5 μM, which is lower than plasmatic levels of patients submitted to oral drug treatments (33).

The increased CPP activity, which was particularly high after DEM-induced stress, shows that (at the dosage used in our experiments) oxidative damage is prevalently of the apoptotic type, because caspase activation is required for development of the complete apoptotic phenotype in different stress conditions. In the case of H_2O_2-induced stress, we hypothesize that, in an initial phase, an apoptotic cellular death occurred, followed by tardy necrotic damage (see LDH release). This situation can explain the minor extent of CPP activity achieved for H_2O_2-treated fibroblasts and the highest percentage of LDH release in the medium. Because the oxidative damage is partially recovered by the idebenone-loaded PECA nanocapsules in the case of DEM-stressed fibroblasts and a good percentage of recovery is obtained after H_2O_2 treatment, idebenone can also be considered both as an antiapoptotic and an antinecrotic agent in some physiopathologic conditions.

In conclusion, our study provides further important evidence about the antioxidant effect of idebenone, which is exerted particularly at the mitochondrial level, and shows the improved effect of this drug when encapsulated in Tween 80-coated PECA nanocapsules. This colloidal drug delivery system can be efficaciously used in many conditions in which oxidative damage occurs. In particular, these colloidal systems may allow not only the oral administration of idebenone, thus resulting in a greater drug bioavailability, but also intravenous administration (a useful therapeutic approach in acute treatment). Colloidal nanocapsule suspensions may provide an easy passage of the drug through the blood-brain barrier (34) and can be formulated in a such a way as to have active targeting properties. Furthermore, PECA nanocapsules could also increase the life span of idebenone in the circulation (35). To our knowledge, this is the first report in which idebenone is considered as a possible antiapoptotic agent. Our results open different and interesting ways both for the investigation of the antiapoptotic activity of idebenone and for the possibility of developing new pharmacologic approaches.

ACKNOWLEDGMENTS

This work was financially supported by Italian MURST LOFIM 2001. The authors are very grateful to Dr. Antony Bridgewood for his revision of the language of this article.

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