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Inhibition of glycerophosphate-dependent H_2O_2 generation in brown fat mitochondria by idebenone

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Abstract

The established protective effect of coenzyme Q (CoQ) analogs is dependent on the location of reactive oxygen species (ROS) generation. One of these analogs—idebenone (hydroxydecyl-ubiquinone) is used as an antioxidative therapeutic drug. We tested its scavenging effect on the glycerophosphate (GP)-dependent ROS production as this enzyme was shown as a new site in the mitochondrial respiratory chain where ROS can be generated. We observed that idebenone inhibits both GP- and succinate-dependent ROS production. Idebenone and CoQ_1 were found to be more efficient in the scavenging activity (IC_{50}: 0.052 and 0.075 μM, respectively) than CoQ_3 (IC_{50}: 45.8 μM). Idebenone also inhibited ferricyanide (FeCN)-activated, GP-dependent ROS production. Our data thus extend previous findings on the scavenging effect of idebenone and show that it can also eliminate GP-dependent ROS generation.
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Coenzyme Q (CoQ) as an obligatory component of the mitochondrial respiratory chain plays an important role in the oxidative phosphorylation process. It is the only non-protein component of the mitochondrial respiratory chain. In comparison with other respiratory-chain complexes, its molar ratio is in high excess [1]. As a mobile electron carrier, the oxidized CoQ (ubiquinone) is reduced to ubiquinol by various dehydrogenases. Reduced ubiquinol is reoxidized to ubiquinone by Complex III [2,3].

Besides this well-known function of CoQ in the respiratory chain, there is growing evidence for its new roles in cellular metabolism [4,5]. CoQ forms a component of extra-mitochondrial redox chains [6], it is an obligatory factor for uncoupling protein activation [7,8], and it regulates mitochondrial permeability transition pore [9–11]. Moreover, CoQ participates in protection against reactive oxygen species (ROS) generation and serves as an important antioxidant in both mitochondria and different membranes in the cell [12]. The latter function is also exerted indirectly by recycling tocopherol [13].

The actions outlined for CoQ can explain its broad range of beneficial effects in animal experiments or human therapy when the synthesis of CoQ is disturbed [14]. Because CoQ is highly hydrophobic, synthetic analogs with lower hydrophobicity were prepared and idebenone (hydroxydecyl-ubiquinone), was found to be very effective in the therapy of respiratory chain diseases [14–16]. However, it was found that various CoQ analogs behave differently when reacting with various dehydrogenases [17,18]. Their antioxidative capacity was highly dependent on the region of ROS generation in the respiratory chain [19,20].

Recently, it was found that mitochondrial glycerophosphate dehydrogenase (mGPDH) localized on the outer

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surface of the inner membrane represents a new location in the mammalian respiratory chain where ROS are generated. This glycerophosphate (GP)-dependent ROS production by brown fat mitochondria was strongly inhibited by CoQ_3 [21]. GP-dependent ROS production in mammals was detected not only in brown fat mitochondria [21–23], but also in rat liver mitochondria [24], in mitochondria from human placenta [25], and in prostate cancer cell lines [26]. Because there is no information on the interaction of idebenone with mGPDH which belongs to mitochondrial CoQ reacting complexes, in this study we tested to what extent idebenone can act as a scavenger of GP-dependent hydrogen peroxide generation.

Materials and methods

Isolation of brown adipose tissue mitochondria. The experiments were performed on mitochondria isolated from adult male Syrian hamsters (*Mesocricetus auratus*) adapted to 4 °C for 3 weeks. Brown adipose tissue mitochondria were prepared by differential centrifugation in 250 mM sucrose, 10 mM Tris–HCl, and 1 mM EDTA (pH 7.4) as described by Hittelman et al. [27].

Measurement of oxygen consumption. Oxygen consumption by mitochondria was measured with a High Resolution Oxygraph (Oroboros, Austria) in a KCl medium containing 80 mM KCl, 10 mM Tris–HCl, 5 mM K-phosphate, 3 mM $MgCl_2$, and 1 mM EDTA (pH 7.4) using 0.15–0.3 mg of mitochondrial protein in the individual experiments. The oxygraphic curves presented are the first derivative of oxygen tension changes. For calculation of oxygen uptake and for presentation of oxygraphic curves OROBOROS software Datlab2 was used [28]. Oxygen consumption is expressed as pmole oxygen/s/mg protein.

Determination of enzyme activities. Enzyme activities were determined spectrophotometrically at room temperature as cytochrome *c* oxidoreductases following cytochrome *c* reduction at 550 nm or as 2,6-dichlorophenol-indophenol (DCIP) oxidoreductases, following the DCIP reduction at 600 nm. In 1 ml of medium containing 50 mM KCl, 20 mM Tris–HCl, 1 mM EDTA, and 2 mM KCN (pH 7.4) mitochondrial protein concentration was 0.055–0.075 mg. The reaction was started by addition of 25 mM succinate or 25 mM glycerophosphate. An extinction coefficient of 19 and 21 $mmol^{-1}\,cm^{-1}$ was used for cytochrome *c* reductase and DCIP reductase, respectively.

Fluorometric detection of reactive oxygen species production. ROS production was measured at 5-min intervals at room temperature by Victor^2 1420 (Wallac) multiwell fluorometer using a fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Molecular Probes) at the final concentration of 1 μM. The excitation/emission wavelengths were 485 nm (bandwidth 15 nm)/535 nm (bandwidth 30 nm). The assay was performed with 0.1 mg of frozen-thawed mitochondria in KCl medium (80 mM KCl, 10 mM Tris–HCl, 5 mM K-phosphate, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.4) supplemented with 10 mM GP, 1 μg/ml antimycin A, and appropriate CoQ analog at the specified concentration.

Protein determination. Proteins were determined according to Lowry et al. [29] using bovine serum albumin as standard.

Results

Inhibition of GP-dependent reactive oxygen species production by idebenone

As demonstrated in Fig. 1, we tested the scavenging effect of idebenone on GP- and succinate-dependent hydrogen peroxide generation by frozen-thawed brown fat mitochondria using the fluorescent probe CM-H_2DCFDA. The probe is routinely used in intact cells, being taken up and deacetylated by endogenous hydrolases to a form that is then oxidized by peroxides (including H_2O_2) to fluorescent dichlorofluorescein. It has been shown [30] that mitochondria and submitochondrial particles (SMP) can deacetylate the probe and oxidize it in the presence of ROS production. Since the quantitative detection of superoxide is critical in the planned experiments, it is required that deacetylation of the probe by SMP and conversion of superoxide, produced by the reaction of the respiratory chain with molecular oxygen, to hydrogen peroxide, proceed at a rate that is not rate-limiting with respect to superoxide production. Indeed, addition of hydrogen peroxide enhances the probe fluorescence at a rate largely exceeding the rate obtained with respiratory substrates, showing that the non-reactive acetyl ester is cleaved at a rate higher than that of natural H_2O_2 production. On the other hand, even if the first product of reaction of respiratory chain with oxygen is superoxide, it is readily converted to hydrogen peroxide by spontaneous or SMP-catalyzed dismutation. The addition of superoxide dismutase only slightly increases the conversion of superoxide anion to hydrogen peroxide (data not shown).

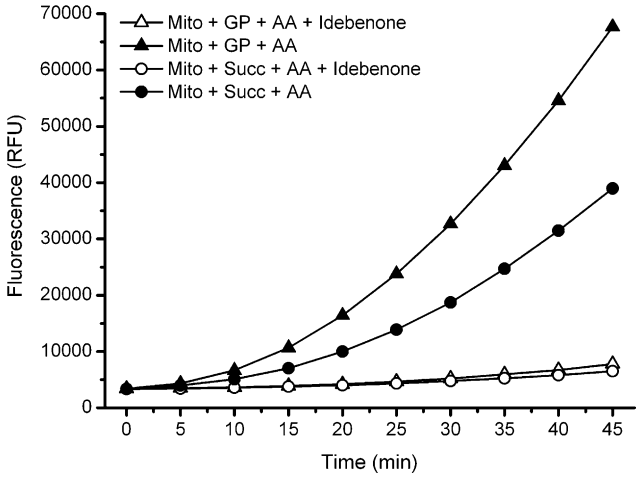

Fig. 1. The effect of idebenone on glycerophosphate- and succinate-dependent hydrogen peroxide generation. Frozen-thawed brown fat mitochondria (Mito) were incubated at room temperature in a KCl medium containing 10 mM glycerophosphate (GP) or succinate (Succ), 1 μg/ml antimycin A (AA), 12.5 μM idebenone, and 1 μM CM-H_2DCFDA. Changes of fluorescence (RFU) at 485/535 nm (excitation/emission) were detected at 5-min intervals by Victor^2 multiwell fluorometer.

Addition of 12.5 μM idebenone to the incubation medium strongly inhibited GP- and succinate-dependent hydrogen peroxide production. The rate of GP-dependent hydrogen peroxide production was approximately twice as high as that dependent on succinate. Similar were the enzyme activities of glycerophosphate and succinate oxidoreductases when measured under the same experimental conditions in the absence of BSA as cytochrome *c* oxidoreductase (279 ± 7 and 188 ± 8 nmol/min/

mg protein, respectively) and DCIP oxidoreductase (109 ± 7 and 86 ± 8 nmol/min/mg protein, respectively).

In addition, we tested the concentration dependence of idebenone on the inhibition of GP-induced peroxide production. We compared the inhibitory effect (IC_{50}) of idebenone with the IC_{50} of CoQ_1 and CoQ_3. As shown in Fig. 2A a high inhibitory effect can be obtained already at 100 nM idebenone or CoQ_1. CoQ_3 was found to be less efficient. In Fig. 2B the IC_{50} values for idebenone, CoQ_1, and CoQ_3 are compared. The IC_{50} of idebenone and CoQ_1 were quite similar, 0.052 and 0.075 μM, respectively. However, CoQ_3 as an antioxidant was less efficient having an IC_{50} thousand-fold higher (45.80 μM).

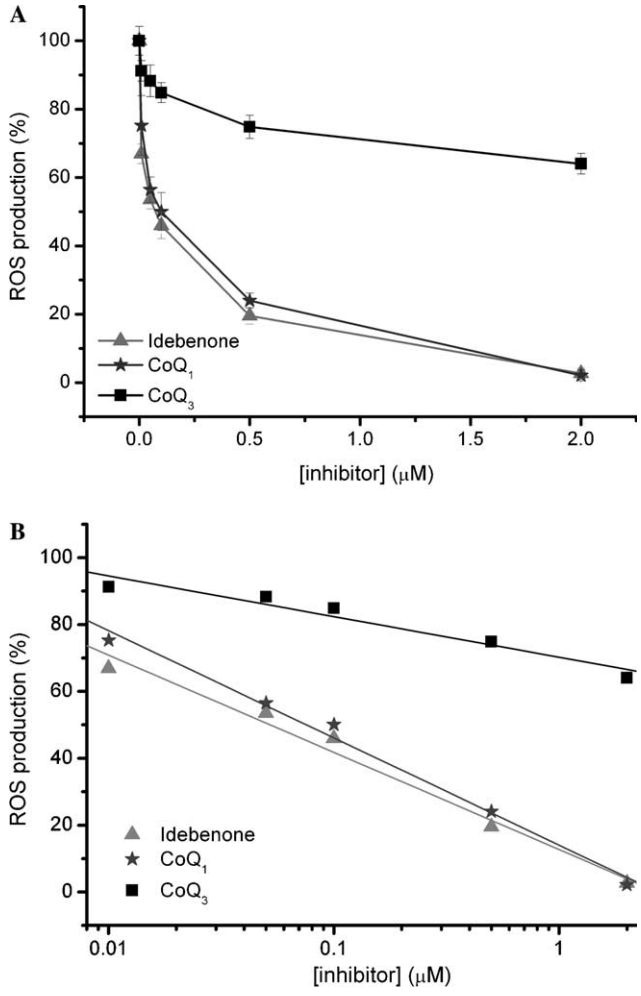

Fig. 2. Comparison of the inhibitory effect of idebenone, CoQ_1, and CoQ_3 on hydrogen peroxide generation by brown fat mitochondria. Brown fat mitochondria (frozen-thawed) were incubated in a KCl medium containing 10 mM glycerophosphate, 1 μg/ml antimycin A, 1 μM CM-H_2DCFDA, and the appropriate CoQ analog at the final concentration specified in the figure. Changes of fluorescence at 485/535 nm (excitation/emission) were detected after 60 min of incubation at room temperature by Victor^2 multiwell fluorometer. (A) Inhibition of ROS production by idebenone, CoQ_1, and CoQ_3. (B) Linear regression of data from A with inhibitor concentration displayed on logarithmic scale. Calculated inhibitory constants (IC_{50}) for idebenone, CoQ_1, and CoQ_3 are shown in the text.

Inhibition of ferricyanide (FeCN)-activated, GP-dependent hydrogen peroxide production by idebenone

In our previous reports, we found that GP-dependent hydrogen peroxide generation in brown fat mitochondria detected as KCN-insensitive oxygen uptake was five- to seven-fold stimulated by the one-electron acceptor FeCN and this peroxide generation was inhibited by CoQ_3 [21,22]. Fig. 3A shows the conditions under which KCN-insensitive oxygen uptake was measured. Oxidation of GP was inhibited by KCN, addition of FeCN induced a high increase of oxygen uptake, after its complete reduction, the rate of oxygen uptake returned to original

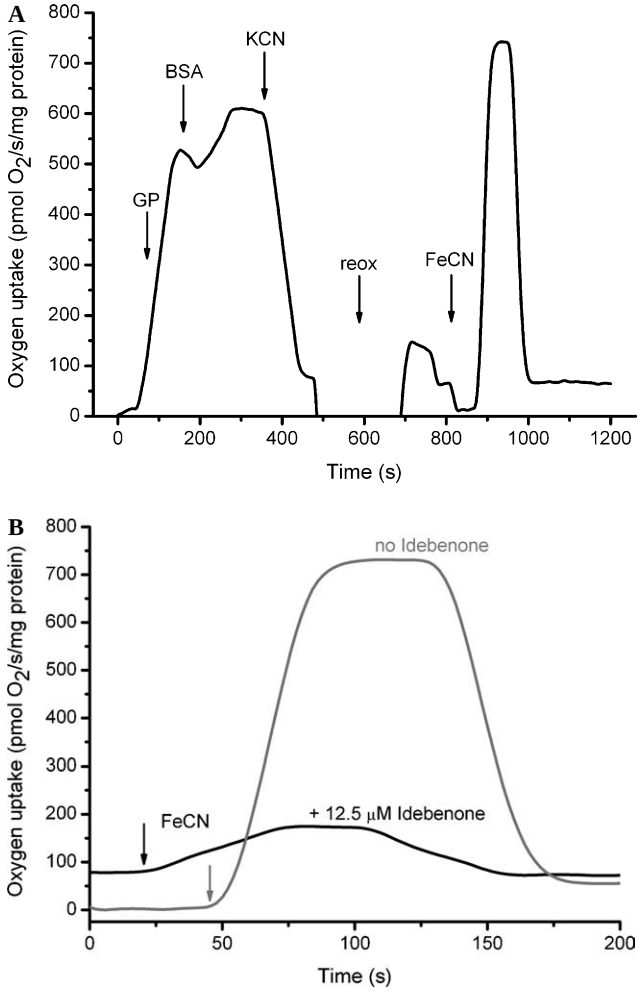

Fig. 3. Inhibition of ferricyanide-activated, GP-dependent hydrogen peroxide generation by idebenone. Hydrogen peroxide generation was measured as KCN-insensitive oxygen uptake at 30 °C in a KCl medium. Mitochondrial protein was 0.23 mg/ml. (A) Inhibition of GP oxidation by KCN and induction of oxygen uptake by ferricyanide. Where indicated, 10 mM glycerophosphate (GP), 1 mg bovine serum albumin/ml (BSA), 0.5 mM KCN, and 0.0625 mM potassium ferricyanide (FeCN) were added, reox indicates reoxidation of the medium to original oxygen tension. (B) The inhibitory effect of idebenone on the ferricyanide-induced oxygen uptake. To the mitochondrial suspension KCN (0.5 mM), BSA (1 mg/ml) and 10 mM glycerophosphate (GP) were added and the reaction was started by addition of 0.0625 mM potassium ferricyanide (FeCN). Where indicated, 12.5 μM idebenone was added.

Table 1
The inhibition of the ferricyanide (FeCN)-induced glycerophosphate-dependent oxygen uptake, total oxygen uptake after addition of FeCN and O/FeCN ratio by idebenone

Idebenone (μM)	Rate (pmol/s/mg protein)	Total oxygen uptake (ngAtO/mg protein)	O/FeCN (ngAtO/nmolFeCN)
0	724.0 (100%)	123.0 (100%)	0.450 (100%)
3.12	425.7 (59%)	58.9 (49%)	0.215 (48%)
6.25	301.9 (42%)	49.0 (40%)	0.180 (40%)
12.50	175.3 (24%)	17.8 (14%)	0.060 (13%)

FeCN-induced oxygen uptake was measured as described in Fig. 3.

values before FeCN addition. Other additions of the same amount of FeCN induced the same response as we demonstrated in previous papers [21,25]. Fig. 3B shows the strong inhibition of GP-dependent hydrogen peroxide generation activated in the presence of 12.5 μM idebenone.

From the curve obtained after FeCN addition the maximum rate of oxygen uptake, the total amount of oxygen consumed during FeCN reduction as well as the ratio between added FeCN and oxygen consumed could be calculated. In Table 1, the maximum rate of oxygen uptake, the total oxygen uptake, and the O/FeCN ratio at various concentrations of idebenone are presented. It is evident that idebenone has a strong inhibitory effect on all the above mentioned parameters (Table 1). However, under these experimental conditions the concentration of idebenone required for 50% inhibition of the rate of FeCN stimulated oxygen uptake was higher (4.4 μM) than that required for the non-stimulated peroxide generation (0.05 μM).

Discussion

Idebenone and CoQ_{10} serve as pharmacological antioxidative compounds to treat diseases where there is an oxidative stress component [14]. However, it has been found that various ubiquinones and their analogs differ in their ability to protect against oxidative damage caused by mitochondrial ROS generation and also react differently with various mitochondrial Q reacting systems [17–19]. These different pharmacokinetic properties result from different hydrophobicity, redox properties of the particular ubiquinones, and from the different localization of various dehydrogenases generating reducing equivalents in the lipoprotein structure of the inner mitochondrial membrane. Therefore, when mGPDH was characterized as a new location in the respiratory chain where ROS can be generated [21] we tested the protection effect of idebenone on GP-dependent ROS generation because mGPDH, in contrast to succinate- and NADH-dehydrogenase, is located on the outer surface of the inner membrane. There are also indications that the transfer of electrons from mGPDH to CoQ pool is less protected to electron leak than that from succinate- and NADH-dehydrogenases [21,31,32]. In our previous report, we found that CoQ_3 strongly inhibits FeCN-stimulated, GP-dependent ROS generation [21]. In the present communication, we measured the IC_{50} for CoQ_1, CoQ_3, and idebenone for both non-stimulated and FeCN-stimulated ROS generation. For non-stimulated ROS generation we observed that idebenone and CoQ_1 are more potent as scavenging agents than CoQ_3. For FeCN-stimulated ROS generation we found a similar scavenging effect for idebenone, CoQ_1, and CoQ_3 with the IC_{50} about 5 μM which is much higher than that obtained in the case of non-stimulated ROS generation (Fig. 2). This lower efficiency can be explained by several factors. By the 5–6-fold higher rate of hydrogen peroxide generation in the presence of FeCN, and also by oxido-reduction interactions of FeCN and idebenone that could be involved and decrease its scavenging efficiency.

In summary, we have shown that idebenone potently inhibits the GP-dependent ROS generation thus extending the previous findings indicating that idebenone is a very effective electron acceptor for GPDH [19]. This phenomenon could constitute part of idebenone beneficial therapeutic effects, particularly in situations where high GPDH activity can be involved in tissue oxidative damage.

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