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Archives of Biochemistry and Biophysics 454 (2006) 114–122
ABB
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Avicins, natural anticancer saponins, permeabilize mitochondrial membranes ☆

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Received 8 July 2006, and in revised form 5 August 2006
Available online 23 August 2006

Abstract

Avicins are a class of natural saponins with selective pro-apoptotic activity in cancer cells. In this work, we studied the influence of avicins on metabolic state of rat liver mitochondria. Avicin-treated mitochondria underwent a significant decrease in oxygen consumption rate that was completely restored by addition of exogenous cytochrome *c*. On the other hand, avicins increased the rotenone-insensitive oxidation of external NADH in the presence of exogenous cytochrome *c*, long before high amplitude swelling of mitochondria was observed. The increase in external NADH oxidation was cyclosporin A-insensitive. Avicin G significantly accelerated hydroperoxide-induced oxidation of mitochondrial endogenous NAD(P)H, the drop of the inner membrane potential and the high amplitude swelling of mitochondria. The obtained data might explain selective induction of apoptosis in tumor cells by avicins. Based on other studies showing that tumor cells generate hydroperoxides with a very high rate, avicins could provide a new strategy of anticancer therapy by sensitizing cells with high levels of reactive oxygen species to apoptosis.

Keywords: Mitochondria; Outer mitochondrial membrane; NADPH; Cytochrome *c*; Avicin; Hydroperoxides; Cancer; Apoptosis

Avicins represent a new class of plant stress metabolites that exhibit selective pro-apoptotic [1–4] and cytotoxic activity [5] in tumor cells, as well as anti-inflammatory [6,7] and antioxidant properties [8–10]. Our previous finding that avicins induce apoptosis in Jurkat cells by a direct perturbation of mitochondria [1], prompted us to study their effects on the oxidative phosphorylation system of rat liver mitochondria.

Most common effects of anti-tumor drugs, by which they target mitochondrial structure and functions, are revealed in direct permeabilization of the outer mitochondrial membrane (OMM[1]) to cytochrome *c*, or in oxidative stress and mitochondrial swelling. The rupture or direct permeabilization of the OMM results in release of cytochrome *c* and other pro-apoptotic factors from mitochondria, as well as in disturbance of oxidative phosphorylation system [11,12]. Allosteric interactions of various factors with the voltage-dependent anion channel that increase the probability of its closure under some physiological and pathological conditions, have been also considered as a possible way to induce tumor cells death [13,14]. Thus, mitochondria play a central role in cancer survival and are one of the main targets for developing anticancer drugs [11–15].

☆ Financial support for this work was provided by the: Clayton Foundation for Research; Biomedical Research Foundation; Abraham J. and Phyllis Katz Foundation; Colciencias (Colombia) Grant #2213-05-16851.
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[1] *Abbreviations used:* OMM, outer membrane of mitochondria; IMM, inner membrane of mitochondria; ROS, reactive oxygen species; TMPD,*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl-cyanide-*p*-trifluoromethoxy phenylhydrazone; DNP, 2,4-dinitrophenol; tBH, *tert*-butylhydroperoxide.

0003-9861/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2006.08.008

In this work, we demonstrate that anticancer drugs avicins induce the OMM permeabilization, which leads to release of cytochrome *c* and inhibition of respiratory chain in rat liver mitochondria. Another effect of avicins is the creation of hypersensitivity of mitochondria to hydroperoxides. It was revealed in a faster exhaustion of endogenous NAD(P)H, accelerated swelling of mitochondria, and in a faster drop of the inner membrane potential induced by *tert*-butylhydroperoxide (tBH). Mitochondrial NADPH-dependent metabolism of hydroperoxides [16,17] is coupled to the inner membrane potential through the energy-dependent trans-hydrogenase. Thus, the decreased generation of the inner membrane proton motive force, as result of cytochrome *c* release, should result in accumulation of hydroperoxides in the cells up to cytotoxic levels. The presence of high levels of reactive oxygen species (ROS) in most cancer cells [18–22] might sensitize tumor cells to the cytotoxic effects of avicins. Avicins therefore, could have great potential as an anticancer drug, either by itself or in combination with other drugs, based on the synergistic effect of avicins and hydroperoxides.

Materials and methods

Materials

Avicin D and G were obtained from ground seedpods of *Acacia victoriae* as described earlier [23]. The solutions of 1 mg/ml in 10% DMSO were stored at −20 °C before use in experiments. Sucrose, D-mannitol, digitonin, Hepes, Trizma base, EDTA, EGTA, bovine heart cytochrome *c*, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), rotenone, antimycin A, myxothiazol, carbonyl-cyanide-*p*-trifluoromethoxy phenylhydrazone (FCCP), 2,4-dinitrophenol (DNP), β-NADH, *tert*-butylhydroperoxide, succinic acid, glutamate, malate, and other salts were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of analytical grade.

Isolation of mitochondria

Liver mitochondria from male Sprague–Dawley rats (4–6 month, starved overnight) were isolated according to the method of Schneider and Hogeboom [24] with modifications. Cooled liver was homogenized in medium containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA–Tris, 2.5 mM $MgCl_2$, 10 mM Hepes–Tris, pH 7.2, at 2–4 °C. The homogenization medium contained $MgCl_2$ to prevent cytochrome *c* adsorption on the mitochondria [25]. After washing mitochondria twice, they were finally suspended in medium containing 210 mM mannitol, 70 mM sucrose, 0.02 mM EGTA, and 0.3 mg/ml bovine serum albumin (fraction V, free fatty acids), 10 mM Hepes–Tris, pH 7.2, at 2–4 °C. Protein content was evaluated by the biuret method as described earlier [26] using bovine serum albumin as the standard and Na^+-cholate.

Measurement of respiration

The rate of oxygen consumption by rat liver mitochondria was measured using Clark-type oxygen electrode as described earlier [25]. Incubation medium was composed of 100 mM sucrose, 75 mM KCl, 10 mM Hepes–Tris, pH 7.2 (SKH medium). Succinate–Tris, pH 7.2 (6.5 mM), glutamate–Tris, pH 7.2 (4 mM) plus malate–Tris, pH 7.2 (1 mM), or NADH (0.6 mM) were added to the medium as substrates of oxidation, wherever indicated. After inhibition of glutamate–malate-dependent respiration, 10 mM ascorbate–Tris, pH 7.2, was added to initiate the cytochrome *c*- or TMPD-dependent respiration. Avicin D or avicin G (5 μg/ml), 5 mM phosphate–Tris, pH 7.2, 400 μM ADP, 2.5 μM rotenone, 0.5 μM antimycin A, 0.5 μM myxothiazol, 0.5 μM FCCP, 100 μM DNP, 1 μg/ml oligomycin, 20 μM cytochrome *c*, and 0.5 mM TMPD were added to the medium wherever indicated.

Monitoring of the redox state of mitochondrial pyridine nucleotides

The level of endogenous NAD(P)H of mitochondria was monitored fluorimetrically (340 nm excitation, 450 nm fluorescence). To minimize the influence of light dispersion on fluorescence measurements, the exciting and emitting light beams were focused only on the 1.5 × 1.5 mm corner of the cuvette [27] as shown in Fig. 1. Mitochondria were added at a concentration of 0.7 mg protein/ml to the incubation medium (100 mM sucrose, 75 mM KCl, 10 mM Hepes–Tris, and 6.5 mM succinate–Tris, pH 7.2). Avicins at final concentration of 5 μg/ml, 5 mM phosphate–Tris and 120 μM tBH were added wherever indicated.

Measurement of the inner membrane potential

The inner membrane potential of mitochondria was monitored using the potential-sensitive fluorescent probe safranin O (520 nm excitation, 580 nm fluorescence) [28]. Mitochondria were added at a concentration of 0.7 mg protein/ml to the incubation medium (100 mM sucrose, 75 mM KCl, 10 mM Hepes–Tris, pH 7.2, 6.5 mM succinate–Tris, 5 mM phosphate–Tris and 10 μM safranin O) without or with avicins (5 μg/ml).

Monitoring of mitochondrial swelling

The swelling of rat liver mitochondria was determined by monitoring the apparent light absorbance at 640 nm using the SP-850 spectrophotometer (USA), additionally equipped with the magnetic stirrer and thermostatic chamber. The absorbance curve was recorded with the Linseis recorder (USA), connected to the spectrophotometer. Avicins (5 μg/ml) were added to the SKH medium, supplemented with 6.5 mM succinate–Tris and

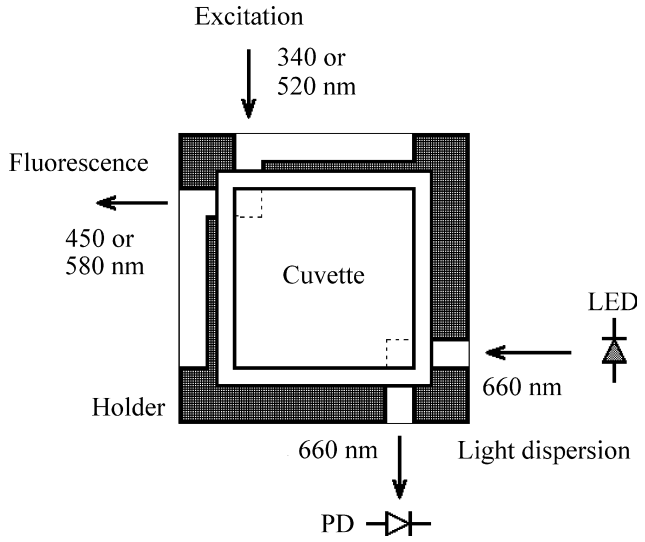

Fig. 1. Two channel simultaneous monitoring of the fluorescence and light dispersion using Aminoc-Bowman-2 luminescence spectrometer. To minimize the influence of light dispersion on fluorescence measurements, the exciting and emitting light beams were focused only on the 1.5 × 1.5 mm corner of the cuvette [27]. To follow mitochondrial swelling during fluorimetric monitoring of mitochondrial NAD(P)H or of the inner membrane potential, the cuvette holder was equipped with a red light emitted diode (LED) and a photodiode (PD) allowing 90°-dispersion of light using the 2 × 2 mm cuvette corner, which is diagonally opposite to the fluorescence corner: the dispersed light, received by PD, is monitored through the Aux-2 additional channel of the spectrometer.