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The Cytoprotection of Nicotinamide Adenine Dinucleotide (NADH) in the Mitochondria Regulation Mechanism*

Xu Meng, Zhang Jiren[1], Sarah SC Hui[2]

Department of Oncology, Nan Fang Hospital, The First Military Medical University, Guangzhou 510515, [1]Department of Oncology, Zhu Jiang hospital, [2]School of Traditional Chinese Medicine, The University of Hong Kong, Hong Kong SAR, P.R. China

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NADH is an essential component of enzymes for many metabolic reactions and energy production in cell. Mitochondria are well known to have a critical function in energy metabolism and damage to mitochondria has been related to apoptosis. Cytoprotection of NADH in apoptotic damage induced by Cisplatin (DDP) was explored to clarify the mechanism of mitochondria regulation pathway. Laser scanning confocal microscope was employed to detect mitochondria membrane potential $\Delta\psi m$ with fluorescent probe R123, intracellular free Ca^{2+} value with probe Flu-3-AM, pH value with probe SNARF-1-AM and reduced oxygen species (ROS) value with probe H_2DCF in hepatocytes. The expression of cytochrom e c and poly (ADP-ribose) polymerase (PARP) protein was detected by Western blot. Mitochondrial oxidative phosphorylation was measured polarographically by determining oxygen consumption rate state 3 and state 4, respiratory control rate (RCR) and ADP/O ratio. Compared with the group of control, in the group of DDP the fluorescence intensity of R123, Fluo-3/AM, SNARF-1-AM and H_2DCF was raised obviously, which indicated that the reduction of mitochondria membrane potential, the improvement of intracellular Ca^{2+} and ROS value was kept. In the group of control, the expression of cytochrome c was released from mitochondria matrix to cytoplasm in the group of DDP and cytochrome c was not released in the group of NADH/DDP. 113 kDa PARP was detected in the group of control, but in the group of DDP PARP was broken into 89 kDa fragment. PARP kept integrated in the group of NADH/DDP. Compared with the group of control, the value of S_3, RCR and ADP/O reduced more than 38%, 35% and 40% in the group of DDP. There was a significant difference between the group of NADH/DDP and DDP. The change of S_3 and mitochondria RCR reduction resulted in hepatocyte injury induced by DDP. NADH could prevent DDP-induced mitochondria impairment. Improving mitochondrial function represents a novel therapeutic strategy in cytoprotection of chemotherapy.

Introduction

Chemotherapy has provided better clinical remission and cure for patients with some malignant tumors, such as skin cancer, chorioepithelioma and retinoblastoma. However, chemotherapy for cancer often has severe side effects that limit its efficacy, such as myelosuppression, mucositis, nephrotoxicity, neurotoxicity and cardiotoxicity[1]. As any cytoprotector in chemotherapy, it should be shown to reduce normal tissue damage from these cytotoxic therapies. The coenzmye NADH helps many enzymes involved in energy production within mitochondria and metabolic reaction in cells. NADH plays a vital role in the generation of adenosine triphosphate. NADH is the most biologically potent antioxidant in the nature and has the strongest positive effect in fighting the damage of free radicals. NADH increases the body¥s creation of depleted brain chemicals called neurotransmitters. NADH not only alleviated the impairment on motor skills caused by Parkinson¥s[2], but also effectively treated the corresponding cognitive dysfunction. Apoptosis could be triggered by a variety of antitumor drugs. In many types of antineoplastic-induced damages, toxic effects are thought to happen by effects on the mitochondria[3]. Mitochondria are presented as the important executioner of apoptosis. Anti-apoptotic members probably function as mitochondria membrane stabilizing molecular. New chemotherapeutic strategy was provided to develop the latest cytoprotector in chemotherapy, establish new usage of coenzyme NADH and avoid the chemotherapy-induced toxicity of normal tissues as far as possible. The role of NADH in cytoprotection by mitochondria regulation mechanism was elucidated.

Materials and Methods

Hepatocyte treated with NADH and DDP

Human hepatic cell line L02 was provided by Institute of Cell Biology, Chinese Academy of Medical Science. Cell was cultured in RPMI-1640 supplemented with 10% heated-inactivated fetal calf serum, 4mM glutamine, 100 U/ml penicillin, 1 μg/ml glucose, 0.25 U/ml insulin under 37 C, 5% CO_2, 95% air and 95% humidity. Exponentially growing hepatocytes on log phase were treated as follows: (1) in the group of DDP, cells were incubated in medium containing 1 μM DDP for 12h to 48h; (2) in the group treated with NADH and DDP, cells were incubated in medium containing 0.8 mM NADH for 6h and then 1 μM DDP; (3) in the group of control, cells were incubated in RPMI-1640 medium for 48h.

Determination of mitochondrial membrane potential ($\Delta\psi\eta$), reduced oxygen species (ROS) production, intracellular free Ca2+ and pH value by confocal microscope

Cells were fixed with 4% paraformaldehyde for 5 min and then washed in PBS buffer. Treated or untreated cells were incubated with 5 μg/ml Rhodamine 123 for $\Delta\psi\eta$, 5 μg/ml H_2DCF for ROS generation, 20 μM Flu-3-AM for intracellular free Ca^{2+} value and 10 μM SNARF-1-AM for pH value separately for 15 min at 37 C in a 5% CO_2 humidified incubator. In all cases, overslips were mounted on glass slides and followed by analysis on a confocal microscope (ACASS570, Meridian).

Western blot analysis of PARP cleavage and cytochrome c

Crude extracts were obtained by suspending about 10^6 cells in 150 μl extraction buffer. Samples were centrifuged at 14000 rpm for 20 min at 4 C.

Cytosolic extracts were removed and added to sample buffer. The lysates were separated through 15% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Cleavage of PARP was determined using the PARP polyclonal antibody. Cytochrome c was detected by H-104 polyclonal antibody. Following extensive washing with TBS containing 0.05% Tween-20, blots were stained with the chromogenic detection of alkaline phosphatase-labeled antibodies on western blots.

Measurement of oxidative phosphorylation

The hepatocytes were removed into a breaker containing ice-cold 0.25 M sucrose buffer. Three passes of the glass-teflon homogeniser were used. The homogenise was centrifuged at 12000 rpm for 10 min in refrigerated centrifuge. The supernatant was poured off and the mitochondrial pellet was resuspended. Biuret method was used to determine protein concentration of mitochondrial suspension. The oxygen electrode of biological oxygen monitor was set up. 0.25 M sucrose buffer, 0.1 M potassium dihydrogen phosphate, 150 mM sodium succinate and hepatocyte mitochondria were placed into the reaction chamber. Oxygen consumption was measured.

Results

The alteration of mitochondrial membrane potential $\Delta\psi\eta$, ROS, intracellular free Ca2+ and pH

Compared with the group of control, in the group of DDP the fluorescence intensity of $\Delta\psi\eta$, Ca^{2+}, pH and ROS increased obviously. In the group of DDP, the fluorescence intensity of Rhodamine 123 was 1827.27 ± 197.75, but in the group treated with NADH and DDP it was 900.33 ± 9.50 ($P < 0.05$). An increase of the mitochondrial fluorescence of Rhodamine 123 represents an increase of mitochondria depolarization, meaning more negative membrane potential and a reduction of mitochondrial membrane potential. It indicated a fall of $\Delta\psi\eta$ occurred during cell damage induced by DDP. In the group of DDP, ROS was 2704.27 ± 155.95 vs 1014.88 ± 85.72 compared with the group treated with NADH and DDP ($P < 0.05$), meaning the involvement of mitochondrial ROS signaling in cell apoptosis pathways.

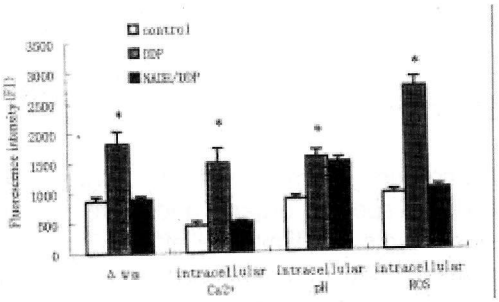

Fig. 1. Levels of hepatocytes $\Delta\psi\eta$, ROS, intracellular free Ca2+ and pH value
* Compared with the group of control, $P < 0.05$

The expression of PARP and cytochrome c

We found that the 113kD PARP was cleaved during apoptosis into 89kD fragments, which served as an early specific marker of apoptosis. In the group treated with NADH and DDP, those phenomenons were not observed. The cytosolic fractions of 15 kD cytochrome c were detected in the group of DDP, which was responsible for the release of cytochrome c from mitochondria during apoptosis.

The effect of NADH and DDP on function of mitochondria oxidative phosphorylation

Mitochondrial respiratory function (S_3: oxygen consumption stimulated by ADP; S_4: oxygen consumption in the absence of ADP, RCR: ratio of S_3 and S_4; ADP/O ratio

between the nanomoles of ADP phosphorylated to ATP and nanoatoms of oxygen consumed) was measured by polarographic methods. Compared with the group of control, the value of S_3, RCR and ADP/O reduced more than 38%, 35% and 40% in the group of DDP. There was a significant difference between the group of NADH/DDP and DDP. The change of S_3 and mitochondria RCR reduction resulted in hepatocyte injury induced by DDP. NADH could prevent DDP-induced mitochondria impairment.

Table 1: Oxidative phosphorylation in the mitochondria of hepatocyte

	S3	S4	RCR	ADP/O
Control	4.50 ± 0.50	1.61 ± 0.02	2.82 ± 0.25	3.48 ± 0.30
DDP	2.78 ± 0.23*	1.65 ± 0.34	1.75 ± 0.44*	2.08 ± 0.29*
NADH/DDP	3.85 ± 0.35	1.97 ± 0.13	1.96 ± 0.15	2.94 ± 0.09

Compared with the group of control, *$p < 0.05$

Discussion

Numerous chemotherapeutic drugs are toxic to specific organs and have severe side effects that limit their efficacy, such as myelotoxicity and gastrointestinal toxicity. Evidence is increasing that some chemotherapeutic agents kill both healthy and cancer cells by causing injury to specific cellular targets[4]. Our previous reports showed that NADH could inhibit apoptosis of human normal cells induced by chemotherapy drugs, such as Cisplatin and Doxorubicin[5-7]. Apoptosis is essential in many physiological processes. In oncology, extensive interest in apoptosis comes from the mode of cell death that is triggered by a variety of antitumor drugs. Cell death may result from mitochondrial respiratory dysfunction. Mitochondrial membrane potential was monitored with the fluorescence probe Rhodamine 123. In the group of DDP an increase of the mitochondrial fluorescence of Rhodamine 123 represents an increase of mitochondria depolarization, meaning more negative membrane potential and a reduction of mitochondrial membrane potential. Mitochondria are presented as the important executioner of apoptosis. This crucial position of mitochondria in apoptosis control is proven by the results obtained from the changes of mitochondrial membrane potential and ROS production[8]. The level of intracellular ROS is activated to commit cells to apoptosis. However, NADH could inhibit this kind of ROS signal. NADH can inhibit the production of ROS, indicating that it can scavenge the superoxide free radical. PARP has all the hallmarks of a DNA damage signal protein. Like p53, it recognizes DNA strand breaks and interconnects with other components of signal pathways. PARP generates an input signal into the p53 pathway, and is an obligatory signal for some apoptotic responses. The most critical and extensively studied role of PARP is its participation in DNA base excision repair. Following binding to damaged DNA, PARP uses NADH to synthesize branched polymers of poly(ADP-ribose) on nuclear target proteins. PARP contains an N-terminal DNA-binding domain (DBD), a central automodification domain that accepts poly(ADP-ribose) and a C-terminal catalytic domain. PARP is one of the substrate proteins targeted by caspase-3 during apoptosis. Normally, cytochrome c is localized exclusively in the space between the outer and inner membranes of mitochondria. It is released into the cytoplasm during apoptosis because mitochondrial permeability[9, 10]. NADH can directly reduce cytochrome c, showing that it is an antioxidant. We found that the 113 kD PARP was cleaved during apoptosis

into 89 kD fragments and the cytosolic fractions of 15 kD cytochrome c were detected into 89 kD fragments and the cytosolic fractions of 15 kD cytochrome c were detected in the group of DDP, in the group treated with NADH and DDP, those phenomena were not observed. NADH can prevent DDP-induced mitochondria impairment.

Mitochondria are well known to have a critical function in energy metabolism. Mitochondria are responsible for ATP provision by oxidative phosphorylation. Our results demonstrate disruption of mitochondrial function induced by DDP: an initial phase of uncoupling of oxidative phosphorylation and a late phase with damage to mitochondrial function. To study ATP synthesis coupled to respiratory electron flow it is necessary to use intact mitochondria. The mitochondria are separated from the homogenate by sequential centrifugation. Isolated mitochondria are suspended in a buffer containing ADP, Pi and oxidizable substrate such as succinate. Three easily measured processes occur: 1) the substrate is oxidized, 2) O_2 is consumed and 3) ATP is synthesized. O_2 consumption is measured using a oxygen electrode. Oxygen consumption and ATP synthesis are dependent upon substrate oxidation and the ADP/O quotient is determined for three different substrates. Because the energy of substrate oxidation drives ATP synthesis in mitochondria, it is not surprising that inhibitors of the passage of electrons to O_2 block ATP synthesis. The change of S_3 and mitochondria RCR reduction resulted in hepatocyte injury induced by DDP. NADH could prevent DDP-induced mitochondria impairment. The alteration in mitochondrial electron transport and oxidant production associated with loss of cytochrome c could activate the occurrence of apoptosis[11, 12].

Improving mitochondrial function represents a novel therapeutic strategy in cytoprotection of chemotherapy. These data suggest the existence of mitochondria signaling pathway in which NADH acts its anti-apoptosis function. Currently available cytoprotective agents applicable to cancer chemotherapy include Amifostine[13], indicated for regimens containing Cisplatin, and Dexrazoxane[14], a cardioprotor indicated for regimens containing Doxorubicin. The agents have the potential to lessen the toxic effects of chemotherapeutic drugs on normal tissues and preserve the function of normal tissues. Moreover, elucidating real mechanims contribute to mitochondrial dysfunction induced by chemotherapy drugs will provide better treatment and diagnosis tools[15].

References

1. Powis G, Hacker MP. The toxicity of anticancer drugs. New York: Pergamon Press, 1990, 10-62.
2. Birkmayer JGD, Vrecko C, Volc D, et al. Nicotinamide adenine dinucleotide (NADH) ñ a new therapeutic approach to Parkinson¥s disease, Comparison of oral and parenteral application. Acta Neurol Scand, 1993, 87:32-35.
3. Hickman JA, Caroline D. Apoptosis and cancer chemotherapy. New Jersey: Humana Press, 1999, 21-280.
4. Schiller JH. Lung Cancer: therapeutic modalities and cytoprotection. Lung 1998, 176:145-164.
5. Zhang JR, Vrecko K, Nadlinger K, et al. Coenzyme Nicotinamide Adenine Dinucleotide (NADH) repair DNA damage of PC12 cells induced by Doxorubicin. J Tumor Marker Oncology, 1998, 13(4):5-17.
6. Zhang JR, Vrecko K, Nadlinger K, et al. Coenzyme Nicotinamide Adenine Dinucleotide (NADH) repair DNA damage of PC12 cells induced by Doxorubicin. J Tumor Marker Oncology, 1998, 13(4):5-17.
7. Xu Meng, Zhang Jiren, Li Peng. The molecular mechanisms of NADH in inhibiting human liver cells from apoptosis induced by Cisplatin.

Journal of Tumor Marker Oncology, 2000, 15(2):139-145.
8. Mignotte B, Vayssiere JI. Mitochondria and apoptosis. Eur J Biochem 1998; 252:1-15. Cai Jiyang, Jie Yang, Jones DP. Mitochondrial control of apoptosis: the role of cytochrome c. Biochimica Acta, 1998, 1366:139-149.
9. Cai J, Yang J, Jones DP. Mitochondrial control of apoptosis; the role of cytochrome c. Biochim Biophys Acta, 1998, 1366:139-49.
10. Zhivotovsky B, Orrenius S, Brustugun OT, et al. Injected cytochrome c induces apoptosis. Nature, 1998, 391:449-50.
11. Susin SA, Zamzami N, Kroemer G. Mitochondria as regulators of apoptosis: doubt no more. Biochim Biophys Acta, 1998, 1366:151-65.
12. Dykens JA, Davis RE, Moos WH. Introduction to mitochondrial function and genomics. Drug Development Res, 1999, 46:2-13.
13. Spencer CM, Goa KL. Amifostine: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential as a radioprotector and cytotoxic chemoprotector. Drug, 1995, 50(6): 1001-1031.
14. Giantris A, Abdurrahman L, Hinkle A, et al. Anthracyline-induced cardiotoxicity in children and young adults. Critical Rev in Oncology Hematology, 1998, 27:53-68.
15. Lenaz G. Role of mitochondria in oxidative stress and ageing. Biochim Biophys Acta, 1998, 1366:53-67.

Correspondence to:
Dr. Xu Meng
Department of Oncology
Nan Fang Hospital
The First Military Medical University
Guangzhou 510515
P.R. China
Tel. 8620-85141654
Fax: 8620-87726110
e-mail: xug@fimmu.edu.cn