Archives of Biochemistry and Biophysics 478 (2008) 154–160

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Acetyl-L-carnitine suppresses apoptosis of thioredoxin 2-deficient DT40 cells

Xiaoping Zhu [a], Eisuke F. Sato [a,*], Yi Wang [a], Hajime Nakamura [b], Junji Yodoi [b], Masayasu Inoue [a]

[a] Department of Biochemistry & Molecular Pathology, Osaka City University Medical School, 1-4-3 Asahimachi, Abeno, Osaka 545-8585, Japan
[b] Department of Biological Responses, Institute for Virus Research, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

ARTICLE INFO

Article history:
Received 4 June 2008
and in revised form 30 July 2008
Available online 7 August 2008

Keywords:
Thioredoxin 2
Mitochondria
Reactive oxygen species
Acetyl-L-carnitine
Adenine nucleotide translocator
DT40 cells

ABSTRACT

To elucidate the mechanism by which L-carnitine and related metabolites inhibited mitochondria-dependent apoptosis, we used conditional TRX2-knockout DT40 cells ($TRX2^{-/-}$) and compared the properties of signaling pathways leading to apoptosis in the wild and $TRX2^{-/-}$ cells. Caspase-3 and 9, but not caspase-8, were strongly activated in $TRX2^{-/-}$ cells but not in wild cells. $TRX2^{-/-}$ cells generated large amounts of reactive oxygen species that markedly decreased cellular glutathione levels both in cytosol and mitochondria. We found that the critical thiol groups of adenine nucleotide translocator (ANT) were oxidized more easily in $TRX2^{-/-}$ cells than in wild cells and that the reduced form, but not oxidized form, of ANT selectively bound to TRX2. Cytochrome *c* and SOD1 were released from mitochondria more easily in $TRX2^{-/-}$ cells than in wild cells. All these phenomena observed with $TRX2^{-/-}$ cells were effectively inhibited by acetyl-L-carnitine but not L-carnitine. Thus, acetyl-L-carnitine effectively suppressed the oxidative stress in and around mitochondria thereby preventing mitochondrial signaling pathway leading to apoptosis.

Thioredoxin (TRX) is a multi-functional protein (12-kD) that has a redox-active disulfide/dithiol group within the conserved active site sequence Cys–Gly–Pro–Cys. TRX2 cooperates with nicotinamide adenosine dinucleotide phosphate (NADPH) and thioredoxin reductases as an efficient reducing system for exposed protein disulfides to maintain their reduced form required for the protection of cells from oxidative stress [1]. Tanaka et al. [2] reported that conditional-TRX2[1]-deficient chicken DT40 cells ($TRX2^{-/-}$ cells) spontaneously generated ROS, activated caspase-9 and 3, and released cytochrome *c* from mitochondria to induce apoptosis. Lack of TRX2 caused severe apoptosis of a wide variety of cells and led to early embryonic death of the $TRX2^{-/-}$ mice, suggesting a crucial role of TRX2 in the survival of animals [3]. Cre-mediated inactivation of TRX-2 reductase also causes embryonic death. The body weight of TRX2 reductase-deficient embryos is small as compared with wild animals [4]. These animals also showed severe anemia and increased apoptosis of cells in the liver. These observations indicate the critical role of mitochondrial TRX2 system in the regulation of cell apoptosis during embryogenesis and maturation.

L-Carnitine is a naturally occurring nutrient that plays essential role in the beta-oxidation of fatty acids to generate ATP. We previously reported that L-carnitine effectively prevented mitochondrial injury caused by oxidative damage *in vivo* [5]. L-Carnitine also showed strong inhibition of mitochondria-dependent apoptosis both *in vivo* and *in vitro* [6,7]. The protective effects of carnitine and related metabolites have been postulated to be due to improved energy metabolism, inhibition of electron leakage from mitochondrial electron transport systems [8], and enhanced repair of oxidized membrane/lipid bilayers [9,10]. However, critical roles of carnitine and acetyl-carnitine in mitochondria-dependent apoptosis remain obscure.

To elucidate the mechanism by which L-carnitine and its acetyl metabolite inhibited mitochondria-dependent apoptosis of cells, we used conditional TRX2-knockout DT40 cells and compared the properties of signaling pathways leading to apoptosis in the wild and TRX2-deficient cells.

Materials and methods

Chemicals

RPMI-1640 was purchased from Wako Co. (Osaka, Japan). Fetal calf serum (FCS) and chicken serum were from Equitech-Bio (Kerrville, TX) and Bioscience (Colorado), respectively. Doxycycline was obtained from Sigma (St. Louis, MO). Hyperfilm ECL was purchased from GE Healthcare (Buckinghamshire, UK). Mouse anti-cytochrome *c* (clone 7H8.2C12) and sheep anti-SOD1 antibodies (JM-3458-3) were purchased from PharMingen (San Diego, CA) and MBL (Nagoya, Japan), respectively. Mouse anti-β-actine (clone AC-15) and rabbit anti-TRX2 antibody was purchased from Sigma Co. (St. Louis, MO) and Redox Bioscience Co. (Kyoto, Japan), respec-

* Corresponding author. Fax: +81 6 6645 3721.
E-mail address: sato@med.osaka-cu.ac.jp (E.F. Sato).

[1] *Abbreviations used:* TRX2, thioredoxin-2; NADPH, nicotinamide adenosine dinucleotide phosphate; SOD, superoxide dismutase; ROS, reactive oxygen species; ANT, adenine nucleotide translocator; OCTN, organic cation transporter; NEM, *N*-ethylmaleimide, DCFH, 2′,7′-dichlorofluorescein diacetate; MPT, mitochondrial permeability transition.

0003-9861/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2008.07.024

tively. HRP-labeled anti-mouse (P0399) and rabbit IgG antibodies (A0424) were purchased from DAKO Glostrup (Denmark) and ECL kit was from GE Healthcare (Buckinghamshire, UK). L-Carnitine and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR) and Wako Co. (Osaka, Japan), respectively. Acetyl-L-carnitine, *N*-ethylmaleimide (NEM), and ceramide (C2 ceramide, A7191) were purchased from Sigma Co. (St. Louis, MO). Protease inhibitor cocktail was purchased from Nacalai Tesque. (Kyoto, Japan).

Cell culture

TRX2-deficient chicken DT40 cells were cultured at 39 °C under 5% CO_2 in RPMI 1640 medium supplemented with 1% penicillin and streptomycin, 10% FCS and 1% chicken serum (Biosciences). Doxycycline (Sigma) was used to suppress cellular expression of TRX2. In the presence or absence of 1 μg/ml doxycycline, cell viability was evaluated by Trypan Blue exclusion test. Carnitine or acetyl-L-carnitine was added 30 min before doxycycline treatment.

Caspase activity

Activities of caspase-3, 8, and 9 were measured by caspase kits. Briefly, cells were collected and washed twice with ice-cold PBS (−), re-suspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 10 mM EGTA and 50 μM digitonin) and then incubated at 39 °C for 10 min. Cell lysates were centrifuged at 15,000 rpm for 3 min, and the supernatants were collected. A total of 10 μg protein was incubated with 50 μM Ac-DEVD-afc (caspase-3 substrate), Ac-IETD-afc (caspase-8 substrate), and Ac-LEHD-afc (caspase-9 substrate) at 39 °C and the released 7-amino-4-methyl-coumarin (AMC) was monitored by a spectrofluorometer (Gemini, Molecular Devise) using an excitation and emission wavelength of 400 and 505 nm, respectively. As a negative control, 10 μl of ICE reaction buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 10 mM EGTA) containing 50 μM Ac-DEVD-AMC was used.

Assay for ROS generation using DCFH-DA

Intracellular generation of ROS was measured by using oxidation-sensitive fluorescent dye DCFH-DA [11]. DT40 cells (1×10^6) were washed with ice-cold PBS and centrifuged at 1000 rpm for 5 min at 4 °C. The cells were re-suspended in 1 ml PBS, and then 1 μl of DCFH-DA (5 mM in DMSO) was added to the cell suspension and the mixture was incubated at 39 °C for 15 min. Subsequently, the cells were kept on ice and immediately analyzed by flow cytometry using FACS Calibur (Becton Dickenson Laboratory). Data was analyzed by using BD CELL Quest software.

Fractionation of cytosolic and mitochondria

After washing DT40 cells (1×10^6) in ice-cold PBS by centrifugation at 1700 rpm for 5 min, cells were lysed at 4 °C for 5 min in 100 μl of cell lysis and mitochondria intact buffer (250 mM Sucrose, 70 mM KCl, 200 μg/ml digitonin in PBS) to isolate cytosolic and mitochondrial fractions as described previously [12]. The cell lysate was centrifuged at 1100 rpm for 5 min at 4 °C. The pellet was re-suspended in 100 μl of universal immunoprecipitation buffer (50 mM Tris–HCl, pH7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, 0.3% NP-40 and protease inhibitors) and placed on ice for 10 min. The supernatant fraction was stored at −80 °C and used as the cytosolic fraction. The suspension was centrifuged at 10,000 rpm at 4 °C for 10 min. The precipitate was re-suspended in 100 μl of the same buffer, stored at −80 °C and used as a mitochondrial fraction.

Determination of mitochondrial and cellular glutathione

Each fraction was assayed for total glutathione (GSH + 2 GSSH) to quantitate its amount in mitochondria and cytosol. Total amount of glutathione was determined by the enzymatic method as described previously [13]. Protein concentration was measured by the Lowry's method (Bio-Rad protein assay) using BSA as the standard.

Binding of ANT to a phenylarsine oxide-conjugated column

The binding of ANT to a phenylarsine oxide-conjugated column was analyzed as described previously [14]. DT40 cells (0.5 mg protein/ml) were incubated in the standard medium containing 5 mM of either succinate or glutamate at 39 °C for 10 min. After centrifugation at 8000*g* for 10 min, the pellets were suspended in a 50 mM Hepes buffer (pH 7.2) containing 150 mM Na_2SO_4, 1 mM EDTA, 3% Triton X-100 (w/v), and 1% protease inhibitor cocktail. The solubilized cell samples were passed through a 0.5 ml phenylarsine oxide column. The column was washed with 10 ml of a solubilization buffer containing 0.25% Triton X-100 (w/v) and 10 mM dithiothreitol. The eluted fractions were analyzed by SDS–PAGE and Western blotting using rabbit anti-ANT antibody (1:1000).

Preparation of recombinant proteins

Recombinant His 6-tagged TRX2 protein was prepared under denaturing conditions according to the manufacture's instructions (Promega, Madison, MI). *Escherichia coli* strain XL1 Blue MRF, transformed with a mature form of mouse TRX2 (amino acids 60–166) expression vector derived from pQE-31, was treated with 1 mM isopropyl-D-thiogalactoside for 4 h. The His 6-tagged TRX2 protein was purified using a Ni^{2+}-nitrilotriacetic acid-agarose column. The size and purity of the eluted protein was analyzed by SDS polyacrylamide gel electrophoresis. The anti-TRX2 antibody was prepared by immunization of His 6-tagged TRX2 protein as described previously [15]. The immune serum was purified further by an affinity column coupled to His 6-tagged TRX2 protein with CNBr-activated Sepharose 4B (GE Healthcare UK Ltd.).

Protein binding assay

DT40 cells grown in RPMI1640 medium were centrifugated at 1000*g* for 5 min and the supernatant was discarded. After isolation of mitochondrial fractions, they were lysed with 0.1% digitonin buffer (0.1% digitonin, 10 mM triethanolamine, 150 mM NaCl, 10 mM iodoacetamide, 1 mM EDTA, 10 mg/ml aprotinine, pH 7.5) at 4 °C for 30 min. The lysates were centrifuged at 10,000*g* for 15 min. The supernatant fractions were incubated overnight at 4 °C with 20 ml protein A-Sepharose (GE Healthcare UK Ltd.) and 5 ml normal rabbit serum to block non-specific binding proteins. After pre-clearing with protein A-Sepharose, samples were incubated with recombinant His 6-TRX2 with or without 1 mM NEM at 4 °C for 2 h, and then with protein A-Sepharose for additional 1 h. Samples were then centrifuged and washed five times with 0.1% digitonin buffer. The precipitated proteins were subjected to Western blot analysis using anti-ANT antibody.

Analysis of cytochrome c and SOD1

The releases of cytochrome *c* and SOD1 from mitochondria were analyzed using anti-cytochrome *c* and anti-SOD1 antibodies, respectively. Both mitochondrial and cytosolic fractions were added with 0.2 volume of 125 mM Tris–HCl buffer (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol, 25% glycerol and 0.002% bromophenol blue. After incubation at 95 °C for 5 min, the samples