Cirp protects against tumor necrosis factor-α-induced apoptosis via activation of extracellular signal-regulated kinase

Toshiharu Sakurai a,b, Katsuhiko Ito b, Hiroaki Higashitsuji a, Kohsuke Nonoguchi a, Yu Liu a, Hirohiko Watanabe a, Tadasu Nakano a, Manabu Fukumoto c, Tsutomu Chiba b, Jun Fujita a,⁎

a Department of Clinical Molecular Biology, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawaharacho, Sakyo-ku, Kyoto 605-8507, Japan
b Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawaharacho, Sakyo-ku, Kyoto 605-8507, Japan
c Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, Japan

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Abstract

Mild hypothermia shows protective effects on patients with brain damage and cardiac arrest. To elucidate the molecular mechanisms underlying these effects, we analyzed the effects of low culture temperature (32 °C) and cold-inducible RNA-binding protein (Cirp) expression on apoptosis in vitro. In BALB/3T3 cells treated with tumor necrosis factor (TNF)-α and cycloheximide, the down-shift in temperature from 37 °C to 32 °C increased the expression of Cirp and suppressed the apoptosis. Activation of caspase-8 was suppressed, and the level of phosphorylated extracellular signal-regulated kinase (ERK) was increased. Transduction of Cirp into the Cirp-deficient mouse fibroblasts increased the level of phosphorylated ERK and suppressed the TNF-α-induced apoptosis both at 37 °C and 32 °C. The ERK-specific inhibitor PD98059 decreased the cytoprotective effect of Cirp as well as that of low culture temperature. These data suggest that mild hypothermia protects cells from TNF-α-induced apoptosis, at least partly, via induction of Cirp, and that Cirp protects cells by activating the ERK pathway.

Abbreviations: Cirp, cold-inducible RNA-binding protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; Rbm3, RNA binding motif protein3; TNF, tumor necrosis factor

⁎ Corresponding author. Tel.: +81 75 751 3751; fax: +81 75 751 3750.
E-mail address: jfujita@virus.kyoto-u.ac.jp (J. Fujita).

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1. Introduction

Cellular stress causes an imbalance of the cellular homeostasis, which in turn initiates a complex cascade of stress responses in an attempt to return the cell to its previous equilibrium. Apoptosis is induced if damage exceeds the capacity of repair mechanisms. Apoptosis typically proceeds through one of two general signaling pathways [1–3]. In the intrinsic apoptotic pathway, when the mitochondria receive appropriate apoptotic signals, cytochrome c is released into the cytosol, resulting in activation of caspase-9. Caspase-9 in turn processes and activates other caspases to orchestrate the biochemical execution of apoptosis [1–3]. In the extrinsic apoptotic pathway, binding of specific death ligands such as Fas ligand and tumor necrosis factor (TNF)-α to their receptors causes oligomerization of death receptors, and the recruitment of adaptor molecules involved in the activation of caspase-8. In type I cells, active caspase-8 alone is sufficient to robustly induce caspase-3 activity and the execution of apoptosis [4]. In type II cells, caspase-8-mediated activation of caspase-3 is inefficient and the apoptotic process therefore depends on a mitochondria-dependent amplification loop.

In bacteria and plants, cold stress induces the synthesis of several cold-shock proteins, which are involved in various cellular processes including transcription and translation [5]. Although clinical application of mild hypothermia (32–35 °C) for patients with brain injury and cardiac arrest has been carried out with promising results [6,7], no extensive studies have been performed on the effects of cold temperatures in mammalian cells in vitro. Most mammalian studies have used whole animals or isolated organs and temperatures below 15 °C with or without rewarming [8,9]. Previously, we reported the
identification of the first mammalian cold shock protein Cirp (cold-inducible RNA-binding protein) in the mouse testis [10]. Cirp is an RNA-binding protein belonging to a glycine-rich RNA-binding protein family, and possibly binds to DNA as well [11]. Cirp is induced by mild cold stress (32 °C) in most cell lines, but not by temperatures below 20 °C, and mediates the cold-induced suppression of cell growth [10]. Recently, we have found that culture of mouse BALB/3T3 cells at 32 °C protects them from a variety of stress conditions including DNA damage (adriamycin), endoplasmic reticulum stress (thapsigargin), high osmolarity, and H₂O₂ by p53-dependent and p53-independent mechanisms [12]. In the present study, we analyzed the effects of low temperature (32 °C) on TNF-α-induced apoptosis in mouse BALB/3T3 cells. By utilizing Cirp-null mouse embryonic fibroblasts (Cirp⁻/⁻ MEFs), we further assessed the involvement of Cirp in the cytoprotective effect of hypothermia.

2. Materials and methods

2.1. Cell culture

Mouse BALB/3T3 fibroblasts, MEFS established from 17-day-old embryo of Cirp⁻/⁻ genotype, and human hepatoma Huh-7 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum at 32 °C or 37 °C in a humidified atmosphere of 5% CO₂ in air. The Cirp⁻/⁻ mice were viable and fertile and displayed no obvious phenotype under normal laboratory conditions (Nonoguchi, K. and Fujita, J., unpublished). For the induction of apoptosis, confluent cultures of cells were incubated with TNF-α (50 ng/ml) in the presence of cycloheximide (10 μg/ml), and the cultures were continued at 37 °C or transferred to 32 °C. In some experiments, apoptosis was induced by TNF-α and cycloheximide in the presence of SB203580 (20 μM, Calbiochem, San Diego, CA), SP600125 (30 μM, Calbiochem) or PD98059 (30 μM, Calbiochem), the specific inhibitor of p38, c-Jun N-terminal kinase (JNK) or extracellular signal-regulated kinase (ERK), respectively, or vehicle alone. Cirp⁻/⁻ MEF clones stably expressing Cirp or RNA binding motif protein (Rbm3) were obtained by transfecting the cells with Cirp or Rbm3 cDNA in the expression vector pcDNA3.1. As a control, the vector alone was transfected. Three clones each were used in the present experiments. Numbers of viable cells were estimated by the MTT assay.

2.2. Western blot analysis

Western blot analysis was performed as described [12]. Briefly, 20 μg of proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto polyvinyl difluoride membranes (Millipore, Tokyo, Japan), and treated with the antibody. The mouse monoclonal antibodies used were anti-phospho p44/42 mitogen-activated protein kinase (MAPK)/ERK antibody (Cell Signaling Technology), anti-phospho SAPK/JNK antibody (Cell Signaling Technology), and anti-β-actin antibody (Chemicon International, Temecula, CA). The polyclonal antibodies used were rabbit anti-phospho p38 antibody (Cell Signaling Technology), rabbit anti-p44/42 MAPK/ERK antibody (Cell Signaling Technology), and anti-Cirp antibody [10]. Protein concentrations were determined using the Bradford reagent (BioRad Labs, Hercules, CA). Bound antibody was detected using horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies (DAKO, Kyoto, Japan) and the enhanced chemiluminescence system (ECL, Amersham Life Science).

2.3. Measurement of caspase activities

Approximately 4 × 10⁵ cells were used for measurement of caspase activities. Caspase-8- and caspase-9-like activities were measured using IETD-p-nitroanilide (MBL, Nagoya, Japan) and LEHD-p-nitroanilide (MBL) as substrates, respectively [12]. The fluorescence of the released p-nitroanilide was measured with an excitation wavelength of 360 nm and an emission wavelength of 530 nm.

2.4. Reporter gene assay

The effect of Cirp on the NF-κB signal transduction pathway was analyzed by using PathDetect In Vivo Signal Transduction Pathway Reporting Systems (Stratagene, La Jolla, CA) as described [13]. cis-reporter vector containing NF-κB binding sites in the promoter (pNF-κB-Luc) was co-transfected with pRL-TK that directs expression of Renilla luciferase onto Cirp-expressing Cirp⁻/⁻ MEF clones and Cirp-deficient Cirp⁻/⁻ MEF clones. Cultures were continued at 37 °C for 24 h, and then cells were treated with TNF-α (50 ng/ml) and cycloheximide (10 μg/ml). After 20 min of further incubation at 37 °C, luciferase activities were determined using a Dual-Luciferase Reporter Assay System (Promega, Tokyo, Japan) following the manufacturer’s protocol. The reporter activity was normalized to that of Renilla luciferase.

2.5. Statistical analysis

All statistical analyses were performed using the JMP (SAS Institute, Cary, NC) on a microcomputer. One-way analysis of variance (ANOVA) was used to compare the values between groups at points apparently different between them. Differences with a P value of less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of low temperature (32 °C) on apoptosis

TNF-α in combination with cycloheximide induced apoptosis within 6 h in mouse BALB/3T3 fibroblasts at 37 °C (Fig. 1A). The number of surviving cells at 12 h after TNF-α-treatment was significantly higher when cells were cultured at 32 °C compared with 37 °C. Similar cytoprotective effect of low culture temperature was observed with the human hepatoma Huh-7 cells as well (Fig. 1B). As reported previously [10], the level of Cirp protein was increased in BALB/3T3 cells cultured for 6 h, but not 3 h, at 32 °C (data not shown). TNF-α is known to activate caspase-8 and caspase-9 [4,14]. As shown in Fig. 1C, the activation of caspase-8 was significantly suppressed at 8 h after TNF-α-treatment and temperature shift to 32 °C compared with that at 37 °C. The activation of caspase-9 was not affected by incubation at 32 °C (data not shown). These results indicate that the death receptor-mediated apoptosis is inhibited by the low culture temperature.

The MAPK cascades play critical roles in cellular proliferation, differentiation, and apoptosis [15]. We examined the activation states of MAPKs, p38, JNK and ERK in BALB/3T3 cells treated with TNF-α and cycloheximide. When cells were cultured at 37 °C, the level of phosphorylated ERK was increased at 20 min after TNF-α treatment (Fig. 2A). A higher level of phosphorylated ERK was observed when cells were cultured at 32 °C after TNF-α treatment. The level of phosphorylated JNK was also increased by TNF-α treatment at 37 °C, but the level was not different from those treated with TNF-α and cultured at 32 °C (data not shown). Phosphorylated p38 was not detected under the present conditions. Without TNF-α treatment, culturing cells at 32 °C for 20 min did not increase the level of phosphorylated ERK, but after 3 h of incubation at
32 °C a slight increase compared with those at 37 °C was observed (Fig. 2B and data not shown).

To determine whether the activation of ERK contributes to the anti-apoptotic effect of lower culture temperature, we utilized the specific ERK inhibitor PD98059. As shown in Fig. 2C, PD98059 significantly suppressed the anti-apoptotic effect. In contrast, the specific inhibitor of p38 or JNK, SB203580 or SP600125, respectively, did not suppress the effect of low temperature. These data suggest that low culture temperature shows anti-apoptotic effects, at least partly, via activation of ERK.

3.2. Anti-apoptotic effect of Cirp at 32 °C

Culturing cells at 32 °C increases expression of Cirp [8,10]. To address whether Cirp contributes to the hypothermia-induced activation of ERK and cytoprotection, we utilized the Cirp−/− MEFs. In contrast to BALB/3T3 cells, the shift of culture temperature from 37 °C to 32 °C did not further increase the level of phosphorylated ERK in Cirp−/− MEFs treated with TNF-α and cycloheximide (Fig. 3A), which suggest that Cirp is necessary for the hypothermia-induced increase in the level of phosphorylated ERK. The level of phosphorylated ERK was higher in Cirp-expressing MEFs cultured at 32 °C compared with those in Cirp-deficient MEFs. Exogenous expression of Cirp increased the survival of TNF-α-treated Cirp−/− MEFs cultured at 32 °C (Fig. 3B), and the caspase-8 activity was decreased in them. Notably, culturing the Cirp−/− MEFs at 32 °C increased the survival compared with that at 37 °C even in the absence of Cirp expression (Fig. 3B), indicating the presence of a Cirp-independent protective mechanism(s) of hypothermia. The exogenous Cirp protein levels expressed in the Cirp−/− MEFs were almost the same as that physiologically induced in BALB/3T3 fibroblasts cultured at 32 °C (data not shown). These results suggest that Cirp plays a major role in the protective effects of low culture temperature against TNF-α-induced apoptosis.

3.3. Anti-apoptotic effect of Cirp at 37 °C

In order to examine whether induction of other cold shock protein(s) is necessary for Cirp to show the anti-apoptotic effect in cells at 32 °C, we assessed the effect of Cirp at 37 °C. As shown in Fig. 3B and C, expression of Cirp increased the survival of and decreased the caspase-8 activity in the TNF-α-treated Cirp−/− MEFs at 37 °C. Although Rbm3 is structurally similar to Cirp and inducible at 32 °C in BALB/3T3 cells [16], overexpression of Rbm3 did not increase the survival of Cirp−/− MEFs.
MEFs treated with TNF-α and cycloheximide (data not shown). At 37 °C, the level of phosphorylated ERK was higher in Cirp-expressing MEFs than Cirp-deficient MEFs after TNF-α treatment (Fig. 4A). The ERK-specific inhibitor PD98059 decreased the survival of the TNF-α-treated MEFs expressing Cirp, whereas SP600125 did not, indicating that the cytoprotective effect of Cirp is mediated by the ERK activation (Fig. 4B). NF-κB is a transcription factor that usually shows anti-apoptotic activity [17]. In Cirp−/− MEFs, the activity of NF-κB was increased by expression of Cirp (Fig. 4C). These results suggest that Cirp shows protective effect against TNF-α-induced apoptosis, at least partly, via activation of ERK and NF-κB in the absence of other cold shock proteins.

4. Discussion

TNF-α acts by binding to its receptors, TNF-R1 (p55) and TNF-R2 (p75), on the cell surface [4]. Most cells express TNF-R1, which is believed to be the major mediator of the cytotoxicity of TNF-α. Engagement of the death receptors, such as TNF-R1, Fas, TNF-related apoptosis-inducing ligand (TRAIL)-R1 and TRAIL-R2 delivers a powerful and rapid pro-apoptotic signal through a death domain-mediated recruitment of the adaptor proteins such as FADD and TRADD, and the formation of the so-called death-inducing signaling complex (DISC) [1,18]. FADD in turn mediates the recruitment and the activation of procaspase-8. Culturing HeLa cells at 32 °C suppresses activation of caspase-8 and apoptosis induced by anti-Fas antibody [12]. In mouse primary hepatocytes treated with soluble Fas ligand, however, culturing at 32 °C suppresses activation of caspase-9 and cytochrome c release, but not activation of caspase-8 [19], indicating cell type-specific effects of hypothermia. In the present study, culturing mouse BALB/3T3 fibroblasts at 32 °C suppressed activation of caspase-8 as well as apoptosis induced by TNF-α and cycloheximide. The suppression of caspase-8 activation was not apparent for the initial 4 h after TNF-α-treatment and temperature down-shift (Fig. 1C). The reason for this time-lag is unclear, but probably

Fig. 3. Effects of Cirp at 32 °C. Cirp−/− MEFs expressing (+, black bars) or not expressing (−, white bars) exogenous Cirp were cultured at 37 °C or 32 °C with media containing TNF-α and cycloheximide, or vehicle alone. (A) Decreased level of phosphorylated ERK in Cirp-deficient cells. Cell lysates were prepared at the indicated times after initiation of the TNF-α-treatment and temperature shift, and analyzed by western blotting using the indicated antibodies. Representative results are shown. (B) Decreased survival of Cirp-deficient cells. Numbers of viable cells were estimated by the MTT assay at 12 h after initiation of the TNF-α-treatment. The values are shown relative to those not treated with TNF-α. Results are mean±S.E.M. of triplicates. *P<0.05 vs. Cirp-deficient MEFs treated with TNF-α and cultured at the same temperature. (C) Increased caspase-8 induction in Cirp-deficient cells. Cytosolic extracts were analyzed for caspase-8 activities at 12 h after initiation of the TNF-α-treatment. The activity is shown relative to that in Cirp-deficient MEFs treated with vehicle alone and cultured at 37 °C. Results are mean±S.E.M. of 3 clones. *P<0.05 vs. Cirp-deficient MEFs treated with TNF-α and cultured at the same temperature.

Fig. 4. Effects of Cirp at 37 °C. (A) Increased level of phosphorylated ERK in Cirp-expressing cells. Cirp−/− MEFs expressing (+) or not expressing (−) exogenous Cirp were cultured at 37 °C in the presence (+) or absence (−) of TNF-α and cycloheximide as indicated. Cell lysates were prepared at 20 min of incubation and analyzed by western blotting using the indicated antibodies. (B) Suppression of cytoprotective effect of Cirp by ERK inhibitor. Cirp−/− MEFs expressing (black bars) or not expressing (white bars) exogenous Cirp were cultured at 37 °C with media containing the specific inhibitor of JNK (SP600125, 30 μM) or ERK (PD98059, 30 μM), or vehicle alone (control) in addition to TNF-α and cycloheximide. After 12 h of incubation, viable cell numbers were determined by the MTT assay, and expressed as relative to those at the start of incubation with TNF-α. Results are mean±S.E.M. of 3 clones. *P<0.05 vs. control Cirp-expressing MEFs. (C) Increased NF-κB induction in Cirp-expressing cells. Cirp−/− MEFs expressing (+, black bar) or not expressing (−, white bar) exogenous Cirp were cotransfected with NF-κB-responsive luciferase reporter and pRL-Tk. Cultures were continued at 37 °C for 24 h, and then cells were treated with TNF-α and cycloheximide. 20 min later, the reporter activity was determined and normalized to that of Renilla luciferase. The values are expressed relative to those in Cirp-deficient cells. Results are mean±S.E. M. of 3 clones. *P<0.05.
related to the fact that between 3 to 6 h is necessary for induction of Cirp and possibly other cold-inducible proteins by low culture temperature [10,20]. One of other possibilities is that Cirp affects the inhibitor(s) of caspase-8 which must first accumulate before taking effect. Since activation of caspase-8 is not suppressed in BALB/3T3 cells treated with thapsigargin or adriamycin at 32 °C [12], the observed effect on caspase-8 does not seem to be due to a general suppression of enzyme activity at lower temperature. One possible mechanism is interference with the interaction between procaspase-8 and FADD, but the exact mechanism remains to be clarified.

Death receptors are in principle able to activate both the apoptotic pathway and the anti-apoptotic NF-κB pathway [4]. Activation of NF-κB could increase the expression of a number of survival gene products and inhibit the TNF-α-induced apoptosis [17]. TNF-R1 also activates JNK in almost every cell line treated with TNF-α, but the role of JNK for TNF-induced apoptosis is poorly understood [4]. MEFs deficient in JNK1 or JNK2 show increased sensitivity to TNF-α-induced apoptosis [21]. In other cell types, however, inhibitors of the JNK pathway increases survival after TNF-α treatment [22]. In the present study, hypothermia suppressed TNF-α-induced apoptosis, but did not affect the JNK activation in BALB/3T3 cells. Furthermore, the JNK specific inhibitor did not affect the anti-apoptotic effect of hypothermia, suggesting no significant involvement of the JNK pathway in the protection. Compared to the TNF-α-induced activation of JNK, TNF activation of ERKs is, in most cells, absent or only moderate [4]. However, we found that culturing BALB/3T3 cells at 32 °C increases basal as well as TNF-α-induced ERK activation, and that the inhibitor of ERK suppresses the protective effect of low culture temperature against TNF-α-induced apoptosis. These data suggest that the increased ERK signaling is involved in the cytoprotective effect of hypothermia under the present conditions. Depletion of intracellular polyamines increases basal as well as TNF-α-induced ERK, and decreases apoptosis induced by TNF-α in IEC-6 intestinal epithelial cells [23]. Recently, these effects have been attributed to the decreased protein phosphatase 2A activity induced by the polyamine depletion [24]. Whether hypothermia affects the protein phosphatase 2A activity remains to be determined.

Beneficial effects of mild hypothermia on brain and liver damages have been noted in patients and experimental animals. In a rat cardiac arrest-resuscitation model, mild hypothermia reduces neuronal loss and increases ERK activity, which is due to increased level of brain-derived neurotrophic factor [25]. Although a down-shift of culture temperature from 37 °C to 32 °C suppresses the protein synthesis in general [8], some proteins such as Cirp and Rbm3 are induced at the low temperature [10,20]. Recently, cytoprotective effects of Cirp and Rbm3 against UV- and polyglutamine-induced cell death, respectively, have been demonstrated in vitro [26,27]. In the present study, we demonstrated that expression of Cirp activates the ERK pathway and blocks the TNF-α-induced apoptosis in Cirp−/− MEFs both at 32 °C and 37 °C. Expression of Cirp was necessary for hypothermia-induced activation of ERK. The inhibitor of ERK partially suppressed the anti-apoptotic effects of Cirp and hypothermia. Furthermore, we found that Cirp increases the NF-κB activity in TNF-α-treated cells. Taken together, these results suggest that Cirp contributes to the anti-apoptotic effects of hypothermia through ERK signaling and NF-κB upregulation independently of other cold shock proteins. Further elucidation of the mechanisms underlying the cytoprotective effects of Cirp and mild hypothermia will help lead to the future development of novel therapeutic modalities against diseases such as brain damages.

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References


