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Mild heat shock switches glucose depletion-induced necrosis to apoptosis in A549 lung cancer cells

Graduate School of Chosun University

Department of Bio New Drug Development

Duong Hong Quan

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A549 폐암세포에서 포도당 결핍에 의해 유도되는 괴사의 저온열충격에 의한 자멸사로의 전환

Advisor: Prof. Sung-Chul Lim

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Department of Bio New Drug Development

Duong Hong Quan

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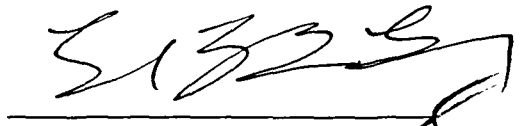
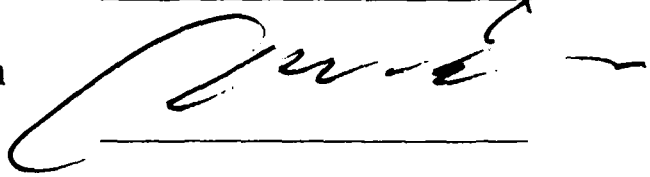
Approved by the Guidance Committee:

Prof. Cheol-Hee Choi
Chosun University



Prof. Sung-Chul Lim
Chosun University



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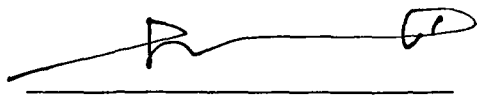

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ABBREVIATIONS

GD	Glucose depletion
OGD	Oxygen and glucose depletion
DHE	Dihydroethidium
DCFH-DA	2',7'-dichlorofluorescein diacetate
DSF	Disulfiram
HO/PI	Hoechst 33342/Propidium Iodide
ERK1/2	Extracellular signal-regulated kinase
Akt/PKB	Protein kinase B
MEK1/2	Mitogen-activated and extracellular regulated kinase kinase
PI-3K	Phosphatidylinositol-3 kinase
MnSOD	Manganese superoxide dismutase
CuZnSOD	Copper/zinc superoxide dismutase
ROS	Reactive oxygen species
PARP	Poly (ADP ribose) polymerase
HMGB1	High mobility group B 1
RT-PCR	Reverse transcription-polymerase chain reaction
HS	Heat shock

Con	Control
CAT	Catalase

국문초록

A549 폐암세포에서 포도당 결핍에 의해 유도되는 괴사의 저온열충격에 의한 자멸사로의 전환

Duong Hong Quan
지도교수: 임 성 철
바이오신약개발학과
조선대학교 대학원

세포사멸의 주요한 유형 중 자멸사(apoptosis)는 세포막의 파괴 없이 세포가 응축되어 염증반응을 수반하지 않고 주위세포와 조직에 미치는 영향을 최소화하는 세포사멸의 형태이다. 이와는 달리 괴사(necrosis)는 세포막이 파괴되어 세포내부의 구성 물질들을 세포 외 공간으로 방출함으로써 주변 조직에 염증반응을 수반하는 다른 유형의 세포사멸인데, 이러한 염증 반응은 결국 암의 성장을 촉진하고 성상을 더욱 악성으로 심화시키는 것으로 보고되고 있다. 따라서, 각종 항암치료의 결과 초래되는 세포사멸의 유형이 치료효과에 큰 영향을 미칠 것으로 판단된다.

암을 치료하기 위한 여러 가지 시도 중에 온열 요법이 방사선치료나 항암제치료와 병행될 때 긍정적인 효과를 나타낸다는 보고들이 있으나, 온열 효과의 메커니즘은 잘 알려져 있지 않다. 본 연구자는 암세포가 암 조직 내에서 직면하기 쉬운 미세 환경인 포도당(glucose) 결핍상태에서 폐암세포주인 A549 가 괴사로 사멸할 때 고온 환경이 유발하는 효과를 조사하였다.

저온열충격(mild heat shock)은 caspase-9, -3 와 PARP 분열을 유도하였고, HMGB1 의 방출을 억제하였으나, 고온열충격(harsh heat shock)은 이런 현상이 나타나지 않았다. 고온열충격은 포도당결핍에 의한 암세포의 괴사를 촉진하는 반면, 저온열충격은 CuZnSOD 의 저하를 억제하고, 활성산소 발생을 억제함으로써 괴사를 자멸사로 전환시켰다.

따라서, heat stress는 그 크기에 따라 포도당 결핍 시 나타나는 암세포의 괴사를 촉진하거나 억제하는 상반된 효과를 나타낼 수 있으므로 이를 이용한 암치료 시도시 저온열충격이 효과적일 것으로 사료된다.

INTRODUCTION

Cells typically die by either apoptosis or necrosis [1-4]. Apoptotic cell death is characterized by nuclear condensation and fragmentation, cleavage of chromosomal DNA into inter-nucleosomal fragments and package of the deceased cells into apoptotic bodies without plasma membrane breakdown [5]. Unlike apoptosis that is a genetically controlled programmed cell death that involves a proteolytic system consisting of caspases [1, 6, 7], necrosis is regarded as an accidental cell death and thereby is not programmed and uncontrollable [1-4]. Necrotic cell death is characterized by rapid swelling, a loss of plasma membrane integrity and frequently in connection with dramatic irreversible drops in ATP levels [8, 9]. Necrotic cells trigger an increase in the secretion of proinflammatory cytokines from independently activated macrophages. It is also conceived that the release of cytokines or other factors from the necrotic cells themselves may be crucial for an inflammatory response.

Recent data indicate that apoptosis and necrosis are not totally distinct and independent entities that share a common pathway referred to as necrapoptosis [2]. In necrapoptosis, determination of cell death mode into either apoptosis or necrosis most notably depends on the levels of cellular ATP, Ca^{2+} and reactive oxygen species (ROS) [2]. Cellular damage by ROS is determined by the levels of defense antioxidant enzymes such as cytosolic copper/zinc superoxide dismutase (CuZnSOD) and mitochondrial manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPx), and catalase [10, 11]. However the consequences of apoptosis and necrosis are quite different for a whole organism. In the case of apoptosis, the cell content remains packed in the apoptotic bodies that are removed by marcrophages, and thereby inflammation does not occur; during

necrosis, the cell membrane is ruptured, and the cytosolic constituents e.g. A nuclear protein high mobility group box 1 (HMGB1) are released into the extracellular space causing a massive inflammatory response [1, 12].

Recently, necrosis and inflammation has been suggested to promote tumor growth and angiogenesis through the tumor promoting activity of HMGB1 and by increasing probability of proto-oncogenic mutation [12-15]. In solid tumors, necrosis is commonly found in the core region in response to oxygen and glucose depletion (OGD) due to insufficient vascularization [16-18]. OGD and microenvironmental acidosis have been suggested to combine to enhance both the survival and aggressiveness of cancer cells, and they can adversely impact on some forms of treatment [12, 16, 18]. We suspect that necrosis may contribute to OGD-mediated tumor growth and aggressiveness through a tumor-promoting cytokine HMGB1. Thus, it is crucial to determine whether cells die by necrosis or apoptosis particularly with respect to the development of tumor in an organism.

Hyperthermia has been used for the treatment of many kinds of resistant tumors and efforts have concentrated on combining heat with other anti-tumor modalities, principally ionizing radiation and some chemotherapeutic drugs [19-21]. Although significant improvement in clinical outcome by hyperthermia may be linked to its ability to induce cell cycle arrest and apoptosis, to activate the immune system and to cause increases in blood flow and tumor oxygenation [19-21], its mechanism is still unclear.

In this study we examined the effects of mild heat shock on glucose depletion (GD)-induced cell death mode. We have previously shown that in A549 lung carcinoma cells, GD induced necrosis and ROS plays a critical role(s) in determination of GD-induced cell death mode. Here we show that mild heat shock switched GD-induced necrosis to apoptosis through ERK1/2-dependent prevention of GD-induced CuZnSOD

degradation and ROS production, whereas harsh heat shock accelerated GD-induced necrosis. Based on these results, we suggest that necrosis-to-apoptosis switch may be one of the mechanisms underlying tumor-suppressive activities of hyperthermia.

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Cell culture

A549 lung adenocarcinoma cells (KCLB 10185) were grown in RPMI 1640 with 10% FBS and antibiotic-antimyotic solution (10,000 unit/ml penicillin, 10 mg/ml streptomycin and 25 μg amphotericin B) (100X) in 5% CO_2 at 37°C.

2.1.2. Reagents

LY294002	A.G. Scientific
UO126 (U-1026)	A.G. Scientific
PD98059 (P-1019)	A.G. Scientific
Catalase (C-1345)	Sigma

2.1.3. Antibodies

Cleaved caspase-3 (Asp175) antibody: 9661	Cell signaling
Cleaved caspase-9 (Asp315) antibody: 9505	Cell signaling
Cleaved caspase-8 (Asp384) antibody: 9748	Cell signaling
CuZnSOD antibody (FL-154): SC-11407	Santa Cruz Biotechnology
MnSOD antibody (FL-222) : SC-30080	Santa Cruz Biotechnology
Anti-PARP antibody: 556494	BD pharmingen
Total Akt1/2 antibody (H-136):SC-8312	Santa Cruz Biotechnology

p-AKT (Ser473) antibody:9271	Cell signaling
Total ERK1/2 antibody	Santa Cruz Biotechnology
p-ERK1/2 antibody (E-4): SC-7383	Santa Cruz Biotechnology
Goat anti-rabbit IgG-HRP antibody	Santa Cruz Biotechnology

2.1.4. Materials for cell culture

Glucose-free RPMI 1640 medium	Gibco, Invitrogen
RPMI 1640 medium	Gibco, Invitrogen
DPBS (Dulbecco's Phosphate Buffered Saline) (10X)	Welgene
Trypsin-EDTA (10X)	Welgene
Antibiotic-antimycotic solution (10,000 unit/ml penicillin, 10 mg/ml streptomycin and 25 μg amphotericin B) (100X)	Welgene
Fetal Bovine Serum (FBS)	Welgene

2.1.5. Materials for detection of Apoptosis and Necrosis

Hoechst 33342	Calbiochem
Propidium Iodide (PI)	Calbiochem
Paraformaldehyde	Sigma
Gel/Mount	Biomeda
Laboratory Glassware	Marienfeld

2.1.6. Materials for SDS-PAGE and Western Blot analysis

Enhanced Chemilumenescent (ECL) detection kit	Pierce, Rockford, USA
Developer	Vivid
Rapid fixer	Vivid
Films	Kodak
Glycine	Sigma
99.8% Methanol	Burdick & Jackson
Milk powder	Invitrogen
Nitrocellulose transfer membrane, Protran	Whatman
Ponceau S	Amresco
TEMED	Amresco
Tris-HCl	Sigma
Tween -20	Sigma
Whatman paper	Whatman
NaCl	Amresco
Triton X-100	Sigma
PMSF	Sigma
β- Mercaptoethanol	Sigma
30% Acrylamide/Bis solution (29:1)	Bio-Rad

2.1.7. Materials for SOD activity assay

Na_2HPO_4	Sigma
KH_2PO_4	Sigma
Sucrose	Sigma
EDTA	Sigma
Triton X-100	Sigma
NBT (Nitroblue tetrazolium)	Sigma
Riboflavin	Sigma
TEMED	Amresco
99% Glycerol	Sigma
30% Acrylamide/Bis solution (29:1)	Bio-Rad
Tris-HCl	Sigma
Glycine	Sigma

2.2. METHODS

2.2.1. Morphological detection of Apoptosis and Necrosis by Hoechst 33342 and PI assay

At various time points after indicated treatment, Hoechst 33342 (5 μg/mL) and PI (10 μg/mL) were added to the culture medium and incubated for 15 minutes at 37°C with 5% CO_2. After that, A549 cells were harvested by trypsinization with 1X trypsin for 10 min at 37°C. As some apoptotic and necrotic cells detached from the culture substratum into the medium, these cells were collected by centrifugation at 1,500 rpm for 5 min. The

cell pellet was also washed with cool 1X PBS and centrifuged again as described above. The cell pellet was solved in 500 μl of 3.7% paraformaldehyde and incubated for 5 min. The fraction of suspension was centrifuged in cytospinner (Cellspin, Hanil, Korea). Slides were immediately fixed. The slides were then washed and dried, covered surely with a glass coverslip, and viewed under fluorescence microscopy (Leica DFC480, Germany). A total of 500 cells or 800 cells from several randomly chosen fields were counted, and the number of apoptotic cells or necrotic cells was counted and presented as a percentage of the total number of counted cells.

2.2.2. SDS-PAGE and Western blot analysis

At various time points after indicated treatment, A549 cells were collected and washed with 1X cold PBS. And then, A549 cell pellets were lysed in lysis buffer (50 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 20 mM NaF, 50 mM PMSF, 1 mM Na_3VO_4, 100 μg/ml leupeptin, and 10 μg /ml aprotinin). Cell debris was removed by centrifugation (13,000 rpm, 15 min, 4°C). Protein concentration was determined by using the Bio-Rad protein assay, and the samples were boiled for 10 min. The boiled samples were loaded onto a 10%, 12% or 15% SDS-PAGE gel in accordance with molecular weight of proteins, and electrophoresis was fixed at 100 V and run for two and a half hours. Protein was electrophoretically transferred onto 0.22 μM nitrocellulose transfer membrane and immunoblotted with various primary antibodies against different proteins. All immunoblots were stained with horseradish peroxidase-linked secondary antibodies and visualized by enhanced chemiluminescence reagent. For presentation, immunoblots were digitally scanned at 600 dpi by using Photoshop 6.0 and their color was removed and figures were generated with Microsoft Power-Point.

2.2.3. SOD activity assay

For the preparation of samples for the SOD activity assay, A549 cells were collected and washed with cold 1X PBS at various time points after indicated treatment. After that, A549 cell pellets were lysed in extraction buffer (0.05 M KH_2PO_4, 0.25 M Sucrose, 1 mM EDTA and 0.2% Triton X-100). The samples were centrifuged at 13,000 rpm for 15 min at 4°C. Protein concentration was determined by using the Bio-Rad protein assay. Electrophoresis was carried out at 4°C with 1.5 mm of 10% polyacrylamide mini-slap gel without 10% SDS in standard tris-glycine buffer (pH 8.3). Samples were loaded with 40 μg protein into each well and then electrophoresed at 100 V for 2 hours. After eletrophoresis, the gel was firstly soaked with 20 ml of solution A (0.3 mM NBT, 260 μM riboflavin and 0.1 mM EDTA in phosphate buffer pH 7.2) in the dark for 20 min, briefly washed 2 times with water and 1 time with phosphate buffer, then soaked with 20 ml of solution B (86 mM TEMED and 0.1 mM EDTA in phosphate buffer pH 7.2) for 10 min. The gel was briefly washed 2 or 3 times again with water [22]. For presentation, gel was digitally scanned at 600 dpi by using Photoshop 6.0 and their color was removed and figures were generated with Microsoft Power-Point.

2.2.4. RT-PCR

Total cellular RNA was isolated from A549 cells at various time points of treatment with agents using Trizol following manufacture's protocol. The complimentary DNA strand (cDNA) was synthesized using Superscript II reverse transcriptase enzyme (Invitrogen). Reverse transcription polymerase chain reaction (RT-PCR) was used to analyse the expression of mRNA for MnSOD and β-actin. MnSOD expression was detected with sense and antisense primers [23] corresponding to nucleotides 65-84 (5'-

GGCATCAGCGGTAGCACCAG-3') and 324-305 (5'-TCTCCCTTGGCCAACGCCTC-3'), respectively, yielding a 260 bp PCR product. β-actin expression as a quantitative control was detected with sense and antisense primers [24] corresponding to nucleotides 1912-1932 (5'-GACTATGACTTAAGTTGCGTTA-3') and 2412-2392 (5'-GCCTTCATACATCTCA-AGTTG), respectively yielding a 501 bp PCR product. 23 cycles of PCR reaction of MnSOD were carried out as follows: denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 1 min. 18 cycles of PCR reaction of β-actin were carried out as follows: denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s. After last cycle, all PCR products were subjected to final extension at 72°C for 5 min. The amplified products were mixed with 10X DNA dye (5 mg/ml bromophenol blue, 50% glycerol, 100 mM Tris, 20 mM NaCl and 1 mM EDTA) and separated by DNA electrophoresis on 2% agarose gel containing 0.2% ethidium bromide. For presentation, gel was digitally scanned at 600 dpi by using Photoshop 6.0 and their color was removed and figures were generated with Microsoft Power-Point.

2.2.5. Measurement of intracellular ROS

To determine production of intracellular H_2O_2 and O_2^-, cells were plated in 48-well plates with 2 x 10^4 cells/well. Cells were incubated at 37°C, 41°C or 43°C with 5% CO_2 incubator for 3h and then medium was replaced by GD medium or normal medium for control. Cells were further incubated in 37°C with 5% CO_2 incubator and loaded with 2'-7'-dichlorofluorescein diacetate (DCFH-DA, Molecular probes, 50 μM), or dihydroethidium (DHE, Molecular probes, 10 μM) for 30 min after the indicated times. Fluorescence measurements were obtained by Fluorocount plate reader (MQX200) with excitation at 490 nm and emission at 530 nm for DCFH-DA or excitation at 540 nm and emission at 610 nm.

2.2.6. Treatment of A549 cells

2.2.6.1. Treatment of A549 cells with mild heat shock

A549 cells were treated with mild heat shock at 41°C and several heat shocks at 43°C with 5% CO_2 for 3 hours. After that, heat shock cells were gently rinsed two times with 1X PBS solution and treated with glucose-free RPMI 1640 medium with 5% dialyzed FBS and antibiotic-antimycotic solution or glucose-complete RPMI 1640 medium with 5% dialyzed FBS and antibiotic-antimycotic solution for several time points in accordance with the design of each experiment at 37°C with 5% CO_2.

2.2.6.2. Treatment of A549 cells with glucose depletion

A549 cells were gently rinsed two times with PBS solution and treated with glucose-free RPMI 1640 medium with 5% dialyzed FBS and antibiotic-antimycotic solution for several time points in accordance with the design of each experiment at 37°C with 5% CO_2.

2.2.7. Protein qualification

As following the Bio-Rad protein assay, the standard curve was set up with BSA dilution in water as final concentration of 0, 1.25, 2.5, 5, 7.5, and 10 µg/µl. 2 µl of each sample and 798 µl sterilized water were added with 200 µl of Bio-Rad protein assay reagent, vortexed and mixed well. And then, take 100 µl of each sample mixture and move into 96 well plate. Light absorbance was read at 595 nm, compared to the standard curve to determine the protein concentration of the samples [25].

2.2.8. Data analysis

Comparison of the effects of various treatments was performed by using one way analysis of variance and a two-tailed t test. Differences with a P value <0.05 were considered statistically significant. Experiment results shown are the means of multiple individual points (±standard errors of the means [S.E.M.]).

RESULTS

3.1. Mild heat shock switches GD-induced necrosis to apoptosis, whereas harsh heat shock enhances necrosis

In this study, the cell death mode in response to GD is investigated. GD induced necrosis in A549 cells as determined by HO/PI staining method. This double staining method involves the use of DNA-binding dyes HO33342 which is known to cross the plasma membrane of all cells, whether they are damaged or not, causing a blue fluorescence of their nuclei and PI that only penetrates cells with damaged membranes and leads to nuclear fluorescence. Thus, intact blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei, and intact pink nuclei were considered viable, early apoptotic, late apoptotic (secondary necrotic), and necrotic cells, respectively. GD increased the population of the cells that had intact pink nuclei (Fig. 1A). Necrosis was further confirmed by release of HMGB1 into the extracellular space in response to GD (Fig. 2A) and by AV/PI double staining methods (data not shown). As demonstrated previously [26, 27], it was also observed that GD significantly enhanced production of intracellular H_2O_2 and O_2^- (Fig. 3). As shown in Fig. 1A, catalase, a specific antioxidant for H_2O_2 significantly decreased the population of PI-positive cells and increased cells having condensed/fragmented blue nuclei. In addition, GD-induced HMGB1 release was suppressed by antioxidant treatments (Fig. 2A). Thus, ROS appeared to play a critical role(s) in GD-induced cell death mode determination.

Previously, our group has shown that protein kinase C-ERK1/2 signal pathway switches GD-induced necrosis to apoptosis by regulating SOD and suppressing ROS production in A549 cells. Here, the effects of heat shock on GD-induced necrosis were

examined. As shown in Fig. 1A, mild heat shock markedly prevented GD-induced necrosis and switched the cell death mode to apoptosis. As observed in cells treated with catalase, mild heat-potentiated GD-induced apoptosis was mediated by caspase 9 and caspase 3, but not by caspase 8 and p85 fragment of PARP, a well-known caspase-3 substrate, was detected when mild heat shock-treated cells were exposed to GD (Fig. 4). In contrast, harsh heat shock accelerated GD-induced necrosis. GD-induced HMGB1 release was also suppressed by treatment of mild heat shock, but not harsh heat shock (Fig. 2B).

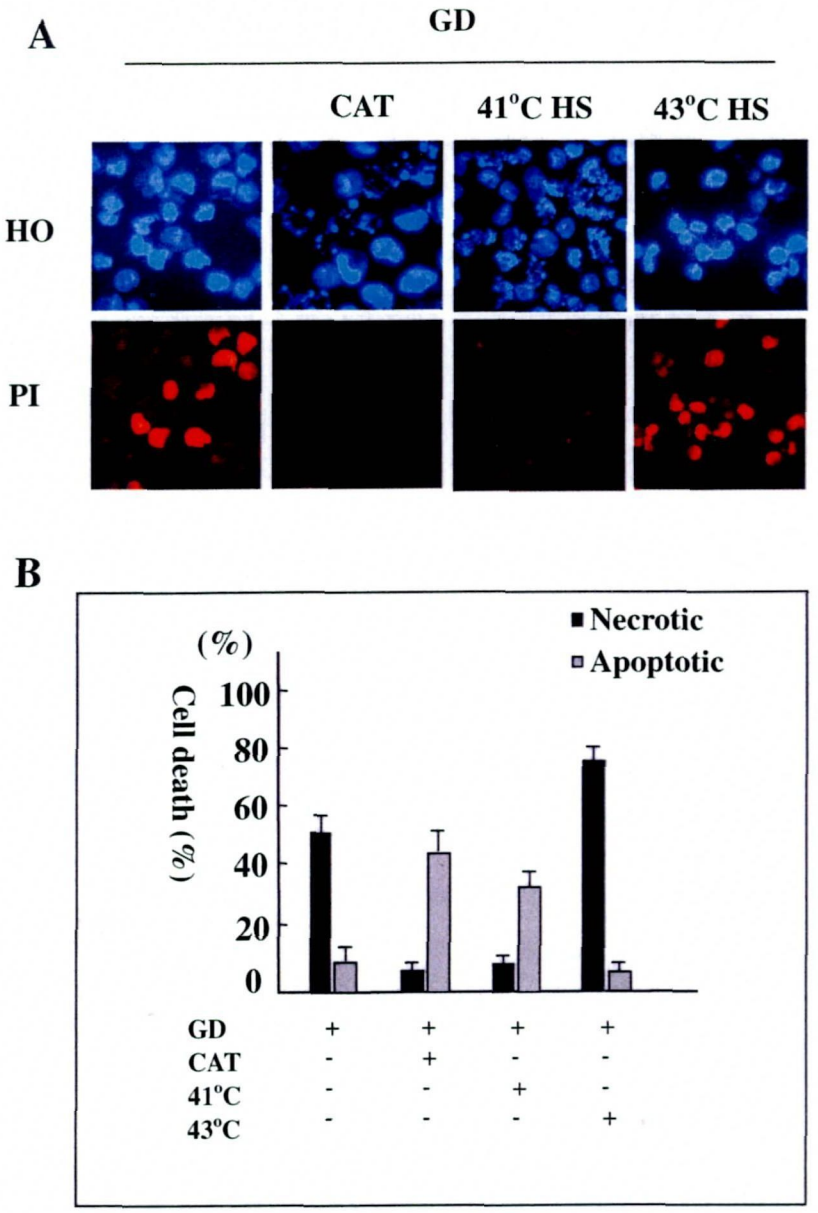

A
GD
CAT
41°C HS
43°C HS
HO
PI
B
Necrotic
Apoptotic
(%)
Cell death (%)
100
80
60
40
20
0
GD + + + +
CAT - + - -
41°C - - + -
43°C - - - +

Fig. 1: Mild heat shock or catalase switches GD-induced necrosis to apoptosis. A549 cells were pretreated with catalase (CAT, 1000 unit/ml) for 1 h, mild heat shock or harsh heat shock for 3 h and then exposed to GD medium for the indicated times. **A**: The cells were stained with Hoechst 33342/PI and observed under a fluorescence microscope, the apoptotic cells were observed as intense signal after staining with Hoechst 33342 (GD-induced catalase or GD-induced mild heat shock). **B**: Apoptotic and necrotic cells were scored. In these analyses, 500 to 800 cells in each group were counted. Data are the means ± S.E.M from three independent experiments.

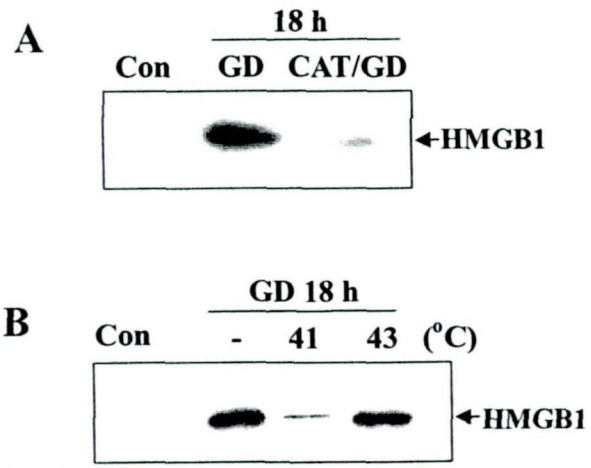


Fig. 2: Mild heat shock or catalase switches GD-induced necrosis to apoptosis. A549 cells were pretreated by catalase (CAT, 1000 unit/ml) for 1 h, mild heat shock or harsh heat shock for 3 h and then exposed to GD medium for 18 h. **A&B**: The culture medium was filtered through Centricon YM-100 (Millipore) to clear sample from cell debris and macromolecular complexes formed during clotting. Samples then were concentrated and analyzed by Western blotting using antibody to HMGB1.

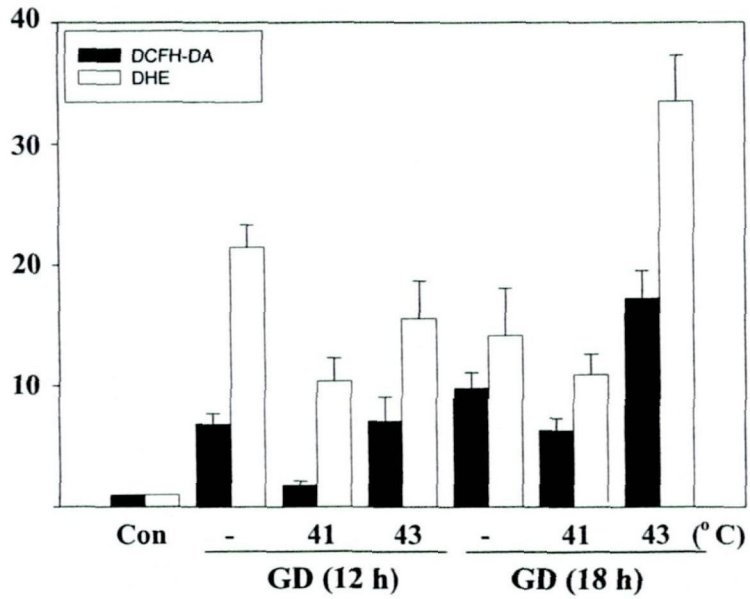


Fig. 3: Mild heat shock suppresses GD-induced ROS production. A549 cells were exposed to mild heat shock or harsh heat shock for 3 h and then exposed to GD medium for the indicated times such as 12 and 18 h, respectively. The A549 cells were treated with DCFH-DA and DHE (fluorescent probes) and determined by fluorocount plate reader.

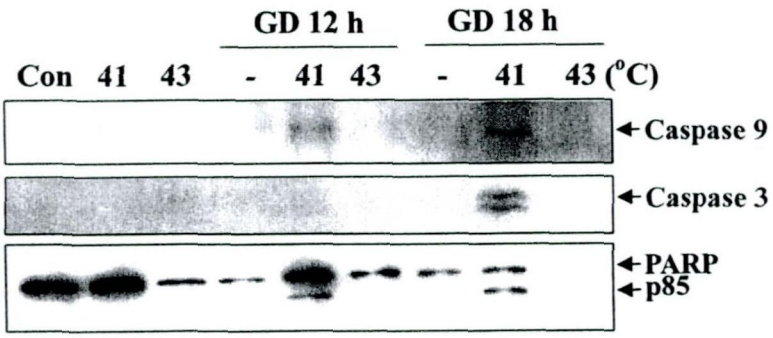
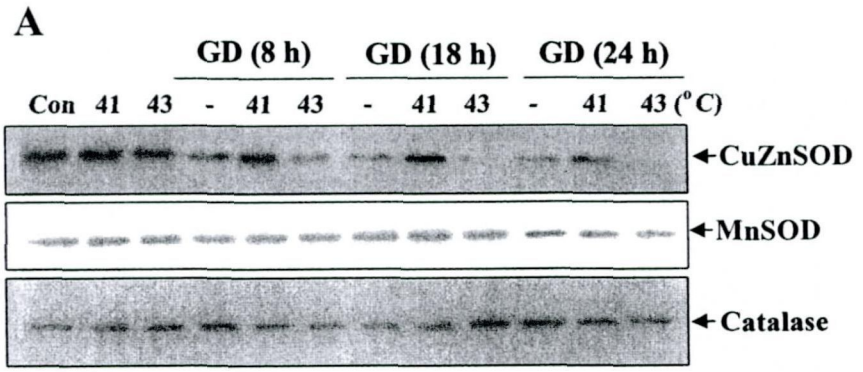


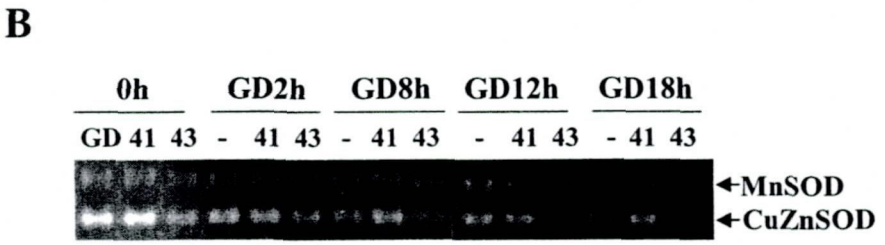
Fig. 4: Mild heat shock switches GD-induced necrosis to apoptosis. A549 cells were pretreated by mild heat shock or harsh heat shock for 3 h and then exposed to GD medium for the indicated times. The A549 cells were collected at the time points indicated and lysed. The cellular proteins were analysed by Western blotting with antibodies to active caspase-9, active caspase-3 and PARP.

3.2. Mild, but not harsh, heat shock suppresses GD-induced ROS production through inhibition of GD-induced CuZnSOD degradation

It was further investigated whether the effects of heat shock are linked to regulation of GD-induced ROS production. As shown in Fig. 4, mild, but not harsh, heat shock prevented production of intracellular H_2O_2 and O_2^- in response to GD. We hypothesized that mild heat shock regulates levels of antioxidant enzymes thereby influencing cellular damage by ROS. There are 3 major antioxidants including SODs, GPx, and catalase in cells. The levels of CuZnSOD, but not MnSOD and catalase, were decreased upon GD (Fig. 5A). Pretreatment of mild, but not harsh, heat shock significantly suppressed GD-mediated CuZnSOD decrease, whereas it caused no significant alterations in CuZnSOD expression as determined by RT-PCR (Fig. 5C).

Disulfiram (Sigma, 1 μM), a CuZnSOD inhibitor [28], prevented the cell death mode switch effects of mild heat shock, indicating critical roles of CuZnSOD in mild heat shock-mediated cell death mode switch (Fig. 6).


A
GD (8 h)
GD (18 h)
GD (24 h)
Con 41 43 - 41 43 - 41 43 - 41 43 (°C)
CuZnSOD
MnSOD
Catalase


B
0h
GD2h
GD8h
GD12h
GD18h
GD 41 43 - 41 43 - 41 43 - 41 43 - 41 43
MnSOD
CuZnSOD

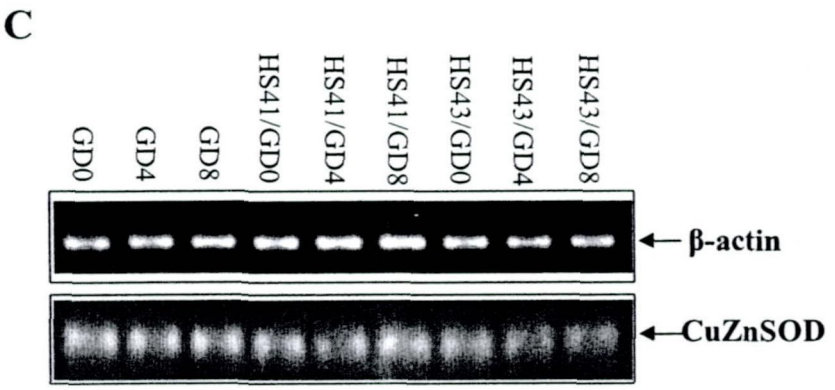
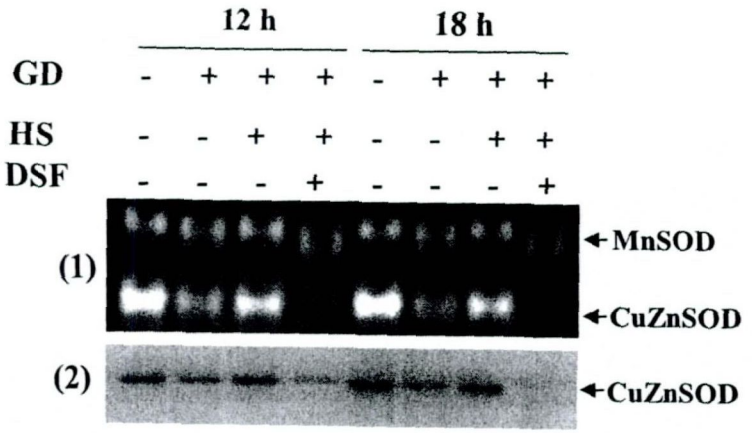

C
GD0
GD4
GD8
HS41/GD0
HS41/GD4
HS41/GD8
HS43/GD0
HS43/GD4
HS43/GD8
β-actin
CuZnSOD

Fig. 5: Mild heat shock suppresses GD-induced CuZnSOD degradation. A549 cells were exposed to mild heat shock or harsh heat shock for 3 h and then exposed to GD medium for the indicated times such as 0, 2, 4, 8, 12, 18, and 24 h. **A**: For Western Blotting. The A549 cells were collected at the indicated times such as 0, 8, 18, and 24 h and lysed by lysis buffer. The cellular proteins were analysed by Western blotting with antibodies to CuZnSOD, MnSOD and catalase. **B**: For SOD activity assay. The A549 cells were collected at the indicated times such as 0, 2, 8, 12, and 18 h. The enzyme activity assay to MnSOD and CuZnSOD was carried out as the protocol described in Materials and Methods, respectively. **C**: For RT-PCR: The cells were collected at the indicated time points and extracted mRNA by Trizol method. cDNA reverse transcribed from mRNA was amplified with primer pair for β-actin and CuZnSOD.

A


12 h
18 h
GD - + + + - + + +
HS - - + + - - + +
DSF - - - + - - - +
(1)
MnSOD
CuZnSOD
(2)
CuZnSOD

B

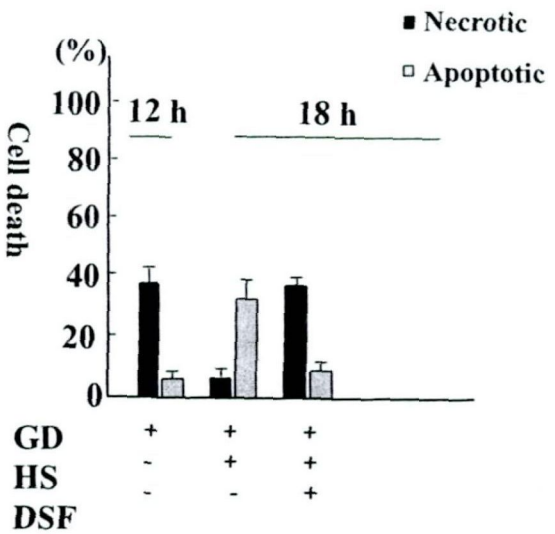

Necrotic
Apoptotic
(%)
100
80
60
40
20
0
Cell death
12 h
18 h
GD + + +
HS - + +
DSF - - +

Fig. 6: The effect of disulfiram (DSF), a CuZnSOD inhibitor enhances GD-induced CuZnSOD degradation under mild heat shock condition. A549 cells pretreated with DSF (1 μM) and exposed to mild heat shock for 3 h and then exposed to GD medium for the indicated times such as 12 and 18 h. **A**: The enzyme activity assay for MnSOD and CuZnSOD was carried out as the protocol described in Materials and Methods, respectively (panel 1). The cellular proteins were analyzed by Western blotting with antibodies to CuZnSOD (panel 2). **B**: The cells were stained with HO/PI and observed under a fluorescence microscope. Apoptotic and necrotic cells were scored. In these analyses, 500 to 800 cells in each group were counted. Data are the means ± S.E.M. from three independent experiments.

3.3. ERK1/2 is involved in mild heat shock-induced necrosis-to-apoptosis switch and prevention of GD-induced CuZnSOD degradation

We investigated the signal pathways by which mild heat shock induces switch of necrosis to apoptosis and prevents GD-induced CuZnSOD degradation. As demonstrated previously [29], pretreatment of mild, but not harsh, heat shock activated ERK1/2 and Akt/PKB (Fig. 7A). Pretreatment of U0126 suppressed inhibition of CuZnSOD decrease as revealed by Western blotting and enzyme activity assay (Fig. 7B, C). Inhibition of MEK1/2 by U0126, an MEK1/2 inhibitor, and in a lesser extent, PD98059, an MEK1 inhibitor, but not LY294002, a PI3K inhibitor, suppressed the cell death mode switch effects of mild heat shock and reversed the cell death mode to necrosis (Fig. 7D). Thus, mild heat shock is likely to exert cell death mode switch activities through ERK1/2-dependent CuZnSOD regulation. In this study it was shown that although harsh heat shock accelerated necrosis by GD in A549 cells, mild heat shock switched the cell death mode to apoptosis. The effects of mild heat shock appeared to be linked to ERK1/2-depedent inhibition of GD-induced CuZnSOD level decrease and suppressing ROS production.

Our results suggest that heat shock may accelerate or suppress GD-induced necrosis depending on the strength and the duration of applied heat shock.

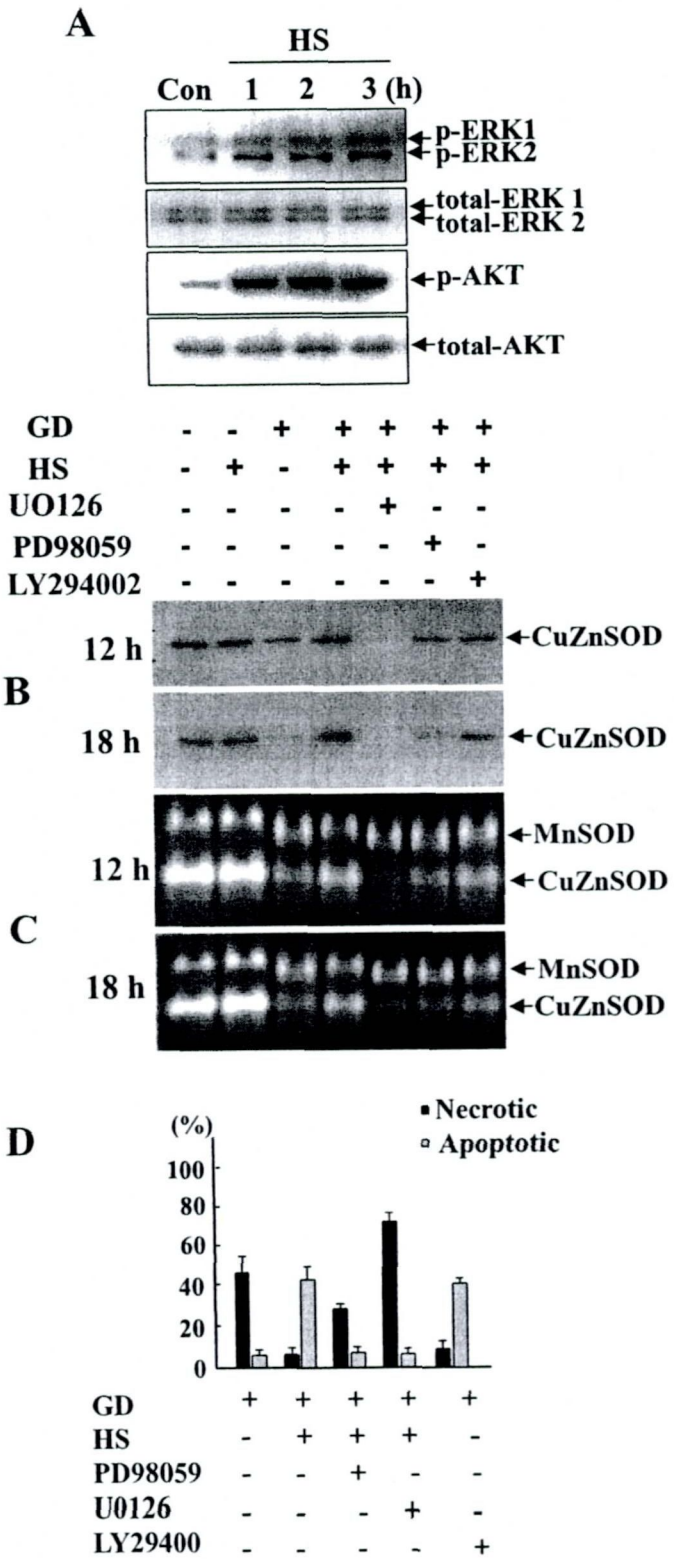

A
HS
Con 1 2 3 (h)
p-ERK1
p-ERK2
total-ERK 1
total-ERK 2
p-AKT
total-AKT
GD - - + + + + +
HS - + - + + + +
UO126 - - - - + - -
PD98059 - - - - - + -
LY294002 - - - - - - +
B
12 h
CuZnSOD
18 h
CuZnSOD
C
12 h
MnSOD
CuZnSOD
18 h
MnSOD
CuZnSOD
D
(%)
100
80
60
40
20
0
Necrotic
Apoptotic
GD + + + + +
HS - + + + -
PD98059 - - + - -
U0126 - - - + -
LY29400 - - - - +

Fig. 7: The effect of various PKC inhibitors enhances GD-induced CuZnSOD degradation under mild heat shock condition. A549 cells pretreated with UO126, a MEK1/2 inhibitor (10 μM) or PD98059, a MEK1 inhibitor (50 μM) or LY294002, a PI-3K inhibitor (50 μM) and exposed to mild heat shock for 3h and then exposed to GD medium for the indicated times such as 12 h or 18 h. **A**: The cellular proteins were analyzed by SDS-PAGE and Western blotting with antibodies to p-ERK1/2, total-ERK1/2, p-AKT and total-AKT. **B**: The cellular proteins were analyzed by SDS-PAGE and Western blotting with antibodies to CuZnSOD, 12 h or 18 h, respectively. **C**: The enzyme activity assay for MnSOD and CuZnSOD was carried out as the protocol described in Materials and Methods, 12 h or 18 h, respectively. **D**: The cells were stained with HO/PI and observed under a fluorescence microscope. Apoptotic and necrotic cells were scored. In these analyses, 500 to 800 cells in each group were counted. Data are the means ± S.E.M. from three independent experiments.

DISCUSSIONS

Necrosis is found in core region of solid tumors and induced by combined effects of glucose depletion, hypoxia, and acidic pH that are commonly found characteristics of tumor microenvironment. Since necrosis has been suggested to promote tumor growth through the tumor promoting activity of HMGB1, it is important to understand the mechanism for necrotic cell death in solid tumors for their treatment and prevention. In the previous study, our group suggested that GD-induced necrosis was switched to apoptosis through PKC-ERK signal pathway by regulating SOD and suppressing ROS production in A549 cells. In the present study, the effects of heat shock on GD-induced necrosis were examined. The effect of mild heat shock or harsh heat shock on GD-induced cell death has shown extremely different result. Mild heat shock markedly prevented GD-induced necrosis and switched the cell death mode to apoptosis and suppressed GD-induced HMGB1 release. In contrast, harsh heat shock accelerated GD-induced necrosis and HMGB1 release. Similar differential response to mild or harsh heat shock has been demonstrated in aspect of cell cycle. In most cases, heat shock has been thought to act as a proteotoxic stress that causes protein denaturation in cells and exerts a variety of antiproliferative effects in mammalian cells. It has been demonstrated that whereas severe heat shock has been shown to lead to cell cycle arrest and apoptosis [30-32], mild heat shock is presumed to positively regulate cell cycle progression and differentiation [29, 33]. Thus, the effects of heat shock on the cell death appeared to depend on the strength and the duration of applied heat shock [34, 35]. It is very difficult to define the terms 'mild' and 'harsh', since the effects of heat shock are determined by both heat temperature and exposure time: as temperature increases by 1°C, the time required for the same extent of

the heat shock response is reduced by 2-fold. Furthermore, heat shock sensitivity varies depending on biological factors including cell types, tissue origin, developmental stage, and cell cycle phase of the cell line analyzed and the cellular events measured. Thus, the criteria for grading heat shock should be considered in both arithmetic and biological aspects [34, 35].

The effects of heat shock appeared to be closely linked to the regulation of GD-induced ROS production. Mild heat shock prevented production of intracellular H_2O_2 and O_2^- in response to GD and pretreatment of mild heat shock significantly recorverd CuZnSOD level in response to GD, whereas it caused no significant alterations in CuZnSOD expression level. Treatment of disulfiram (Sigma, 1 μm), a CuZnSOD inhibitor [29], reversed the cell death mode switch effects of mild heat shock, indicating a critical roles of CuZnSOD in mild heat shock-mediated cell death mode switch. CuZnSOD may detoxify O_2^- that is produced from the electron transport chain in mitochondria and can be released to cytosol possibly through Complex III [36]. Collectively, mild heat shock seems to contribute to maintain CuZnSOD levels.

The signal pathway by which mild heat shock induces necrosis to apoptosis switch seems to be related with ERK1/2 activation. As demonstrated previously [29], pretreatment of mild heat shock, but not harsh heat shock, activated ERK1/2 and Akt/PKB and pretreatment of U0126 suppressed inhibition of CuZnSOD decrease. Inhibition of MEK1/2 by U0126, an MEK1/2 inhibitor, and in a lesser extent, PD98059, an MEK1 inhibitor, but not LY294002, a PI3K inhibitor, suppressed the cell death mode switch effects of mild heat shock and reversed the cell death mode to necrosis. Thus, mild heat shock is likely to exert cell death mode switch activities through ERK1/2-dependent CuZnSOD regulation. Recently, the MEK1 inhibitors U0126 and PD184161 have been shown to aggravate

necrotic death of glucose-deprived cells by inhibiting ATP synthase function [37]. In this study it was shown that although harsh heat shock accelerates necrosis by GD in A549 cells, mild heat shock switched the cell death mode to apoptosis. The effects of mild heat shock appeared to be linked to ERK1/2-depedent inhibition of GD-induced CuZnSOD level decrease and suppressing ROS production.

Hyperthermia has been used for the treatment of many kinds of resistant tumors with good results and efforts have concentrated on combining heat with other anti-tumor modalities, principally ionizing radiation and some chemotherapeutic drugs [19-21]. The synergistic effects of hyperthermia combined with radiotherapy or chemotherapy have been demonstrated for tumors of the head and neck, breast, and lung [19-21]. Although hyperthermic improvement in clinical outcome is suggested to be linked to its ability to induce cell cycle arrest and apoptosis, and to activate the immune system and to cause increases in blood flow and tumor oxygenation, its mechanism is still unclear. Our results suggest that heat shock may accelerate or suppress GD-induced necrosis depending on the strength and the duration of applied heat shock.

CONCLUSION

In conclusion, mild heat shock prevents GD-induced necrosis and switches GD-induced necrosis to apoptosis through ERK1/2-dependent prevention of GD-induced CuZnSOD degradation and ROS production in A549 cells. However, harsh heat shock accelerates GD-induced necrosis.

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