Journal of Cellular Physiology

Thermal[Q1] Killing of Human Colon Cancer Cells Is Associated With the Loss of Eukaryotic Initiation Factor 5a

ALEXANDER GOSSLAU,[1] DAVID LI-EN JAO,[1] RENEE BUTLER,[1] ALICE Y.C. LIU,[2] AND KUANG YU CHEN[1]*

[1] *Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, New Jersey*

[2] *Department of Cell Development and Neuroscience, Rutgers University, Piscataway, New Jersey*

Heat-induced cell death appears to be a cell-specific event. Chronic heat stress was lethal to human colon cancer cells (Caco-2, HT29, and HCT116), but not to normal diploid fibroblasts and other cancer cells (BJ-T, WI38, HeLa, ovarian 2008, WI38VA). Acute heat stress (45–51°C, 30 min) caused cell death of colon cancer cells during recovery at physiological temperature. Thermal killing of Caco-2 cells was not mediated via oxidative stress since Caco-2 cells were much more resistant than HeLa and other cancer cells to H_2O_2-induced cell death. Acute heat stress caused a striking loss of eukaryotic initiation factor 5A (eIF5A) in colon cancer cells, but not in HeLa and other normal or transformed human fibroblasts. The heat-induced loss of eIF5A is likely to be due to changes in the protein stability. The half-life of eIF5A was changed from >20 h to less than 30 min during the acute heat stress. Sequence analysis of the eIF5A gene from Caco-2 and HeLa cells did not reveal any difference, suggesting that the change in stability in Caco-2 cells was not due to any eIF5A mutation. Pretreatment of cells with protease inhibitors such as phenylmethyl sulfonyl fluoride (PMSF) partially blocked the heat-induced loss of eIF5A and prevented heat-induced cell death. In light of the essential role of eIF5A in cell survival and proliferation, our results suggest that the stability of eIF5A may have an important role in determining the fate of the particular cell type after severe heat stress.
J. Cell. Physiol. 9999: 1–9, 2009. © 2008 Wiley-Liss, Inc.

Heat stress causes dramatic change in translation including transient halt of protein synthesis linking with a breakdown of polysomes, and the reformation of polysomes devoted to the synthesis of heat shock proteins (Li et al., 1995; Beere and Green, 2001). The repression of protein translation and the synthesis of heat shock proteins serve as a frontline protection of cells against the accumulation of unfolded or mis-folded protein molecules. Heat stress can also affect membrane, cytoskeleton, cell cycle and signal transduction pathways (Dikomey and Franzke, 1992; Roti et al., 1998; Sakaguchi et al., 1995; Stein et al., 1999). It has been shown that high temperatures can damage and kill cancer cells, usually with minimal injury to normal tissues (van der Zee, 2002). These specific temperature-dependent effects provide the rationale for the application of hyperthermia in combined therapeutic modalities. Several phase III trials have shown a beneficial effect of hyperthermia (Hildebrandt et al., 2002; Wust et al., 2002; Bergs et al., 2007).

Eukaryotic initiation factor 5A (eIF5A) contains hypusine, an unusual amino acid derived from the modification of a specific lysine residue by two enzymes, deoxyhypusine synthase and deoxyhypusine hydroxylase (Chen and Liu, 1997; Park et al., 1997; Park, 2006). Inhibition of deoxyhypusine synthase leads to growth arrest and cell death (Jakus et al., 1993; Park et al., 1994[Q2]; Chen et al., 1996). Disruption of the genes encoding either eIF5A or deoxyhypusine synthase leads to lethal phenotype in yeast *Saccharomyces cerevisiae* (Schnier et al., 1991; Sasaki et al., 1996). These studies indicate that eIF5A is essential for cell survival and proliferation. We have shown that eIF5A can serve as an RNA-binding protein (Xu and Chen, 2001; Xu et al., 2004). Moreover, we and others have recently shown that eIF5A interacts specifically with the translation active 80S ribosomes in vivo, suggesting that eIF5A may be involved in some aspects of translation (Jao and Chen, 2006; Zanelli et al., 2006). In light of the dramatic effect of heat stress on translation and the essential nature of eIF5A for cell survival and proliferation, it is tempting to speculate that eIF5A may have a role in the heat-induced cell death.

In the present study, we have investigated the effect of severe heat stress, on the eIF5A level and the survival of different cell lines. We found that heat-induced cell death correlated well with the loss of eIF5A protein during heat-stress. Thus, Caco-2 colon cancer cells exhibited almost complete loss of eIF5A after heat treatment and were most sensitive to thermal killing. In contrast, HeLa cells did not show any appreciable decrease in eIF5A after heat treatment and were resistant to thermal killing. The heat-induced loss of eIF5A in Caco-2 cells could be partially blocked by PMSF, an irreversible serine protease inhibitor. Importantly, PMSF and other protease inhibitors also partially protected Caco-2 cells from thermal killing. In summary, our study demonstrated that severe heat stress can lead to eIF5A loss in certain cancer cells and suggested that the fate of cancer cells in response to severe heat stress may be coupled to the level of eIF5A.

Contract grant sponsor: NCI, NIH;
Contract grant number: RO1 CA49695.

*Correspondence to: Kuang Yu Chen, Department of Chemistry and Chemical Biology, Rutgers University, 610 Taylor Road, Piscataway, NJ 08854. E-mail: kychen@rutgers.edu

Received 13 June 2008; Accepted 9 December 2008

Published online in Wiley InterScience (www.interscience.wiley.com.), 00 Month 2008.
DOI: 10.1002/jcp.21696

© 2008 WILEY-LISS, INC.

Materials and Methods

Materials and chemicals

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD). Oligo-dT, poly-dA, dNTPs, Superscript™ II reverse transcriptase were purchased from Invitrogen Life Technologies (Baltimore, MD). Chicken anti-human eIF5A antibody was generated as previously described (Jao and Chen, 2002[Q3]). Rabbit against chicken antibody conjugated to horseradish peroxidase (HRP-RAC) was from Amersham Pharmacia (Piscataway, NJ). Other biological chemicals were purchased from Sigma (St. Louis, MO). Complete™ Protease Inhibitor Cocktail Tablet was purchased from Roche Molecular Biochemicals (Indianapolis, IN).

Cell culture and stress treatment

Different normal, transformed, and cancer human cell lines such as WI38 (normal lung fibroblasts), BJ-T (normal foreskin fibroblasts transfected with telomerase), WI38VA (SV40 virally transformed lung fibroblasts), IMR-90SV (SV40 virally transformed fibroblast), Caco-2 (colon cancer), HT-29 (colon cancer), 2008 (ovarian cancer), HeLa (cervix cancer) were used for experiments. All cell lines were cultured at 37°C in a humidified, 10% CO_2 atmosphere in DMEM supplemented with 10% FBS. Cells were subcultured in culture flasks and passaged every 3 days. Viability of cells was routinely tested by the trypan blue exclusion assay. For heat treatment, cells were seeded in 60 and 35 mm culture dishes to achieve a confluent monolayer. Then, a heat shock at either 44 or 51°C was applied using a CO_2 incubator (for 44°C) or by transferring cell culture dishes into a water bath for 30 min (for 51°C) followed by different recovery in CO_2 incubator at 37°C. Oxidative stress was administered by incubating the cells with different concentrations of hydrogen peroxide.

Cellular proliferation assays and morphological analysis

For viability analysis, cells were treated in 35 mm cell culture dishes. Morphological analysis was performed by phase contrast light microscopy. For quantification, cell viability was measured by the MTT-method, which is based on the conversion of the tetrazolium salt (MTT, 3.(4.5-dimethylthiazol-2-yl)-2.5.-diphenyl-tetrazolium-bromide) to blue formazan by mitochondrial dehydrogenase activity (Berg et al., 1990). After treatment, 150 µl of MTT solution (5 mg/ml PBS) was added to the cells. The cultures were then incubated for 3 h at 37°C and the supernatant discarded. Solubilization of cells and formazan was achieved by adding 3 ml of lysis buffer (10% SDS in 50% dimethylformamide, pH 4.7). After shaking for 2 h, color development was documented by a scanner and quantified.

DNA fragmentation analysis

Confluent cultures were treated with either acute heat stress followed by recovery or oxidative stress. Cells were harvested and suspended in a lysis buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS and 100 µg/ml Proteinase K) for 20 h at 37°C. DNA was extracted with a phenol–chloroform mixture, precipitated by ethanol, dried, and dissolved in a TE buffer (10 mM Tris–HCl, pH 8.0, 25 mM EDTA). RNA was digested with 2 µg/ml of RNase Cocktail (Ambion[Q4]). The DNA samples were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide (0.5 µg/ml).

Western blot analysis

Cells were washed and scraped into 300 µl of lysis buffer (20 mM HEPES–KOH, pH 7.5, 1 mM EGTA, 5 mM $MgCl_2$, 10 mM KCl, 10% glycerol, 300 mM sorbitol, 2 mM DTT, and 1× Complete™ protease inhibitors (Roche). After a brief sonication, the homogenate was clarified by centrifugation at 14,000*g* for 5 min. The supernatant was recovered and protein concentration was determined by Bradford method (Bio-Rad[Q5]). Total proteins (5 µg) were subjected to 15% SDS–PAGE and Western blotting analysis using polyclonal antibodies against eIF5A (Jao et al., 2006[Q6]). The proteins were detected by enhanced chemical luminescence method (Amersham Pharmacia Biotech[Q7]).

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were harvested and the total RNA was prepared using RNeasy™ Total RNA Kit (Qiagen[Q8]). Total RNA (1 µg) was reverse transcribed into cDNA by incubating with SuperScriptTM RNase H reverse transcriptase (Invitrogen Life Technologies) using oligo(dT)$_{12\text{-}18}$ as primer. For PCR amplification, gene specific primers, both sense and antisense, were used. The sequences of these primers were:

(eIF5A-1 sense) = 5'-CTg ACA TgT TTT CTg ACg gC-3';
(eIF5A-1 antisense) = 5'-TCA gCC CAT CTT CTT CCA gA-3';
(eIF5A-2 sense) = 5'-ACT TgT ggC CCA gAT Agg CAC CCA g-3';
(eIF5A-2 antisense) = 5'-gCg ACT TCg CCg AgA TgT CCA gC-3';
(HSPA1A sense) = 5'-ACT TgT ggC CCA gAT Agg CAC CCA g-3';
(HSPA1A antisense) = 5'-gCg ACT TCg CCg AgA TgT CCA gC-3';
(GAPDH sense) = 5'-TgA AgC TCg gAg TCA Acg gAT TTg-3';
(GAPDH antisense) = 5'-CAT gTg ggC CAT gAg gTC CAC CAC-3'.

PCR conditions were chosen to ensure that the yield of the amplified product was linear with respect to the amount of input RNA. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an internal control. The PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

Half-life of eIF5A

Cultures of Caco-2 cells (80% confluent) were radiolabeled with [^{3}H]leucine at 5 µCi/ml for 48 h at 37°C. At that time the radioactive medium was removed and the monolayer cells were rinsed three times to remove free [^{3}H]leucine. The cultures were then replenished with fresh growth medium containing cycloheximide (50 µg/ml) and incubated at 37 or 51°C to initiate the chase with leucine at 4 mM for various time. At indicated time points following the leucine chase, cells were harvested and cell extracts prepared for acid-insoluble radioactive counting and for SDS–polyacrylamide gel. The radioactivity associated with the eIF5A was measured by scintillation counting of the band excised from the membrane. Half-life of eIF5A was estimated from the time course of the disappearance of radioactive eIF5A during leucine chase. The value of radioactive eIF5A was normalized against the total radioactivity associated with acid-insoluble protein in cell extracts used for gel electrophoresis with the value at time zero set as 1.0.

Cloning of eIF5A cDNA

Total RNA was isolated from HeLa and Caco-2 cells using RNeasy kit (Qiagen). Thereafter, cDNAs were synthesized from 1 µg of isolated total RNA by reverse transcriptase (RT) using oligo(dT) primers. To amplify eIF5A cDNA from each cell line, primers were designed to cover the entire open reading frame. The amplified eIF5A cDNA from each cell line was directly subjected to DNA sequencing using ABI PRISM® 3100 Genetic Analyzer (DNA Core Facility, UMDNJ).

Results

Chronic heat stress on cell survival

To determine whether there is cell-specific difference in response to chronic heat stress, we compared four different human cell lines, BJ-T, HeLa, WI38VA, and Caco-2 to direct thermal killing at 44°C over a time period up to 24 h. Figure 1 shows that among cells tested, Caco-2 was the most sensitive one. No viable attached cells were detected in the culture after 6 h under heat stress. In contrast, other cell types survived the heat treatment at 44°C and remained viable for over 24 h.

Acute heat shock and recovery

Since both HeLa and Caco-2 are of epithelial origin, we further compared their ability of recovering at physiological temperature (37°C) from acute heat stress. Both Caco-2 and HeLa cells were incubated at 51°C for 30 min. The treated cultures were then incubated at 37°C and the recovery was monitored by cell morphology and metabolic activity. As shown in Figure 2, HeLa cells remained attached and viable during the recovery period for up to 24 h. In contrast, Caco-2 cells failed to recover from the acute heat stress, with massive cell death ensued within 12 h. We also tested two other colon cancer cell lines HCT116 and HT29 in order to determine whether the sensitivity to thermal killing is a general feature for colon cancer cells. Figure 2 shows that all three colon cancer cell lines were sensitive to thermal killing, suggesting that human colon cancer cells are in general more vulnerable to heat-stress as compared to HeLa or other cell types.

The thermal killing effect is not due to oxidative stress

Although mild and transient heat stress can endow cells with thermotolerance due to the induction of heat shock proteins, acute heat-stress or chronic exposure of cells to a sub-lethal temperature can lead to cell death via apoptotic pathways or necrosis (Li and Werb, 1982; Parsell and Lindquist, 1993; Samali et al., 1999). Oxidative stress, resulting from an impairment of mitochondrial electron transfer, has been proposed to be involved in heat-induced cell death (Davidson and Schiestl, 2001; Larkindale and Knight, 2002; Vacca et al., 2004). To determine whether thermal killing of colon cancer cells is related to oxidative stress, we compared the effect of hydrogen peroxide on the growth of Caco-2 with HeLa and other transformed cell lines. Figure 3 shows that Caco-2 was quite resistant to the treatment of H_2O_2 as compared to HeLa and other transformed fibroblasts, suggesting that thermal killing of Caco-2 cells was not related to oxidative stress. To further determine whether apoptosis is involved in the heat-induced cell death, we compared the effects of acute heat stress and oxidative stress on chromosomal DNA integrity. Figure 4 shows that DNA fragmentation occurred during the recovery period after acute heat stress. In contrast, oxidative stress did not cause any appreciable DNA fragmentation up to 72 h, consistent with the finding that Caco-2 cells are sensitive to thermal killing but resistant to oxidative stress.

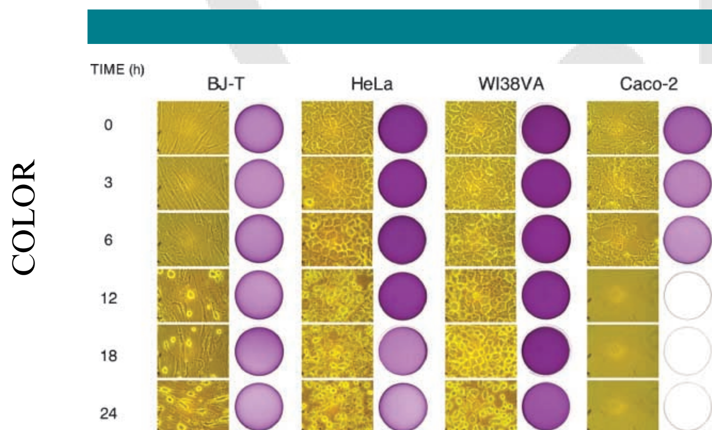

Fig. 1. Chronic heat stress and cell survival. Human BJ-T (normal foreskin fibroblasts transfected with telomerase), HeLa (cervix cancer), WI38VA (SV40 virally transformed lung fibroblasts), and Caco-2 (colon cancer) were exposed to heat stress at 44°C for various times as indicated. Phase contrast microscopic analysis and MTT assay were performed.

COLOR

Acute heat stress induced the loss of eIF5A

Since one of the earliest response of cells to heat stress is the translation repression and loss of active polysomal ribosomes (Panniers and Henshaw, 1984; Mariano and Siekierka, 1986; Duncan and Hershey, 1989), we wonder whether eIF5A, an essential protein that recently reported to bind translational active ribosomes (Jao and Chen, 2006), may be involved in heat induced cell death. In this regard, it can be noted that acute heat stress of pancreatic cancer cells at high temperature (51°C) has been reported to cause a decrease of eIF5A (Takeuchi et al., 2002). We therefore examined the effect of severe heat stress on the level of eIF5A in HeLa and Caco-2 cells. Figure 5A shows that although both Caco-2 and HeLa cells remained viable, there was a striking difference in eIF5A protein level in these two cell lines at the end of 30 min of heat stress. Thus, the eIF5A level was reduced by more than 90% in Caco-2 cells, but remained unchanged in HeLa cells. Since nearly all eIF5A molecules in mammalian cells exist in hypusine modified form that is functional (Duncan and Hershey, 1986; Taylor et al., 2007), we concluded that acute heat stress caused the loss of functional eIF5A in Caco-2 cells. In light of the essential nature of eIF5A for cell survival (Chen and Liu, 1997; Park et al., 1997), the loss of eIF5A may contribute to the heat-induced cell death and thus may explain why Caco-2 cells failed to recover from acute heat stress. We then determine whether the loss of eIF5A in Caco-2 cells is due to a decrease in gene expression at mRNA level. There are two genes encoding eIF5A in mammalian cells and other eukaryotes (Jenkins et al., 2001). Both eIF5A1 and eIF5A2 genes were expressed in Caco-2 cells, but only eIF5A1 was expressed in HeLa cells (Fig. 5B). The eIF5A1 transcript level was not significantly affected by heat treatment in HeLa cells, but was attenuated by about 20% in Caco-2 cells. This decrease, however, was not sufficient to account for the loss of more than 90% of eIF5A protein in Caco-2 cells after heat stress. With the consideration of the abundant literature evidence on the inhibitory effect of heat stress on general protein synthesis (Panniers and Henshaw, 1984; Mariano and Siekierka, 1986; Duncan and Hershey, 1989), our data suggested that the cause of heat-induced loss of eIF5A was most likely due to changes that occur at the posttranslational stage.

We also included a comparison of the expression of HSPA1A, the gene that encodes for heat shock 70 kDa protein 1A, to determine whether acute heat stress produces differential effect of on heat shock gene expression between Caco-2 and HeLa cells. Figure 5B shows that HSPA1A in both HeLa and Caco-2 cells was barely detectable at normal temperature and was not induced during the short duration of acute heat stress (Fig. 5B, lanes 7 and 8).

Dose–response and time course of the effect of heat stress

We further examined the dose-response and time course of the effect of heat stress on the level of eIF5A protein. Figure 6A shows that the eIF5A protein level in HeLa, WI38VA, WI38, and the 2008 ovarian cancer cells was about the same

COLOR

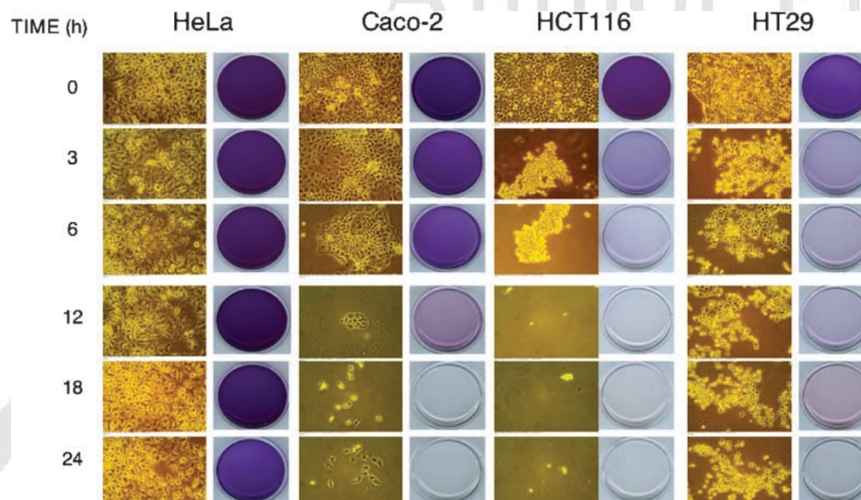

Fig. 2. Recovery from acute heat stress. HeLa, HCT116, HT29, and Caco-2 cells at confluence were exposed to heat stress at 51°C for 30 min and then transferred back to 37°C in the CO2 incubator for recovery. MTT assay and morphological analysis of cell cultures was performed at various times during the recovery period.

over the temperature range from 37 to 51°C. However, the level of eIF5A in Caco-2 showed progressive decrease as the incubation temperature increased. The heat-induced loss of eIF5A occurred rapidly; more than 80% of eIF5A disappeared within 30 min of acute heat stress (Fig. 6B). To further determine whether there was significant global degradation of cellular proteins during acute heat stress, we compared the SDS–polyacrylamide gel pattern of equal volumes of cell extracts derived from cultures before and during heat stress (51°C). As shown in Figure 6C, the protein pattern and staining intensity at the end of heat stress were nearly identical to that before heat treatment (Fig. 6C, lane 4 vs. lane 1), suggesting that there was no global loss of cellular proteins. We then estimated the effect of heat stress on the half-life of eIF5A. As shown in Figure 7, the half-life of eIF5A in Caco-2 at 37°C was estimated to be longer than 20 h, similar to that reported in the literature for other mammalian cells (Torrelio et al., 1987; Bergeron et al., 1998; Nishimura et al., 2005)[Q10]. Acute heat stress (51°C) shortened the half-life of eIF5A to less than 30 min (Fig. 6C), suggesting that the heat-induced loss of eIF5A was most likely

COLOR

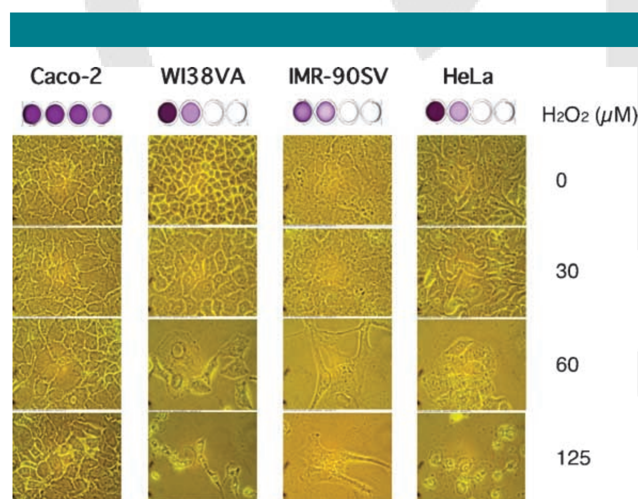

Fig. 3. Effect of oxidative stress on different human cell lines. Confluent[Q9] cultures of human Caco-2, WI38VA, IMR-90SV (SV40 virally transformed fibroblast), and HeLa cells were exposed to different concentrations of hydrogen peroxide as indicated. After 24 h of incubation, cell viability was determined by the MTT assay (upper row) and by morphological analysis with phase contrast light microscopy.

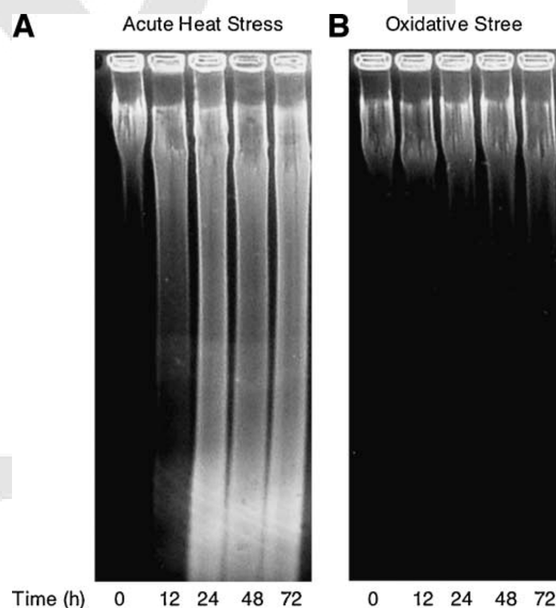

Fig. 4. DNA fragmentation analysis of the effects of acute heat stress and oxidative stress on Caco-2 cells. A: Caco-2 cells at confluence were subjected to acute heat stress for 30 min and then incubated at 37°C for various times as indicated. B: Caco-2 cells at confluence were treated with H_2O_2 (250 μM) for various time. Cells were harvested at the indicated times for DNA fragmentation analysis.

COLOR

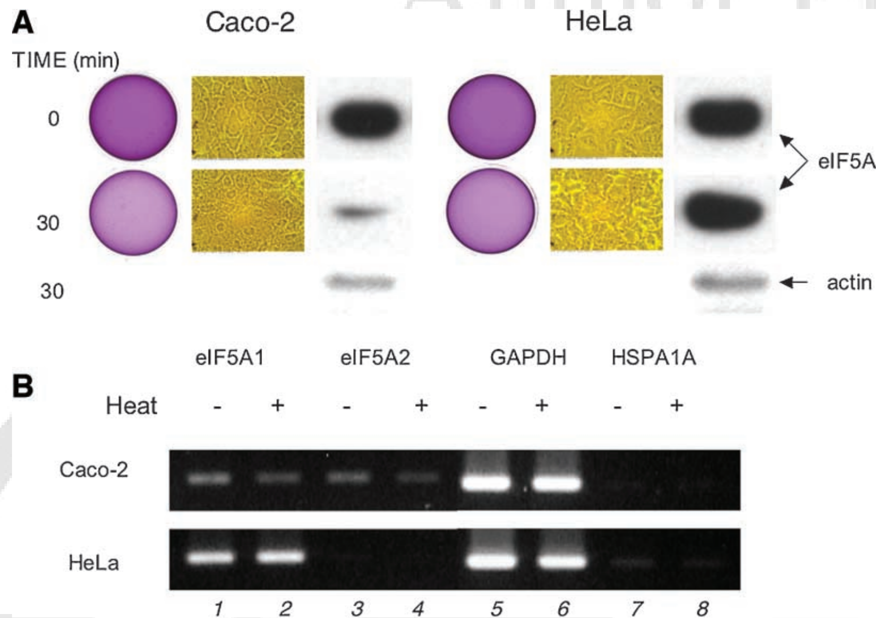

Fig. 5. Effect of acute heat stress on the level of eIF5A protein (A) and mRNA (B) in Caco-2 and HeLa. A: Cells were exposed to acute heat stress at 51°C for 30 min or left untreated as controls (Time 0). Cell viability was monitored by MTT assay (left column) and morphological analysis (middle column). Cells were then harvested and equal amount of cellular protein (5 μg) was analyzed by 15% SDS–page, followed by Western blot analysis (right column) using a specific chicken antibody against human eIF5A as described in Materials and Methods Section. B: Cells were exposed to acute heat stress at 51°C for 30 min or left untreated as controls as indicated. After RNA isolation and reverse transcription, gene expression of eIF5A1, eIF5A2, and HSPA1A was analyzed by RT-PCR using specific primers and conditions, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal standard.

due to either changes in eIF5A stability or an enhanced protein degradation. These results also indicate that in colon cancer cells, the heat-induced loss of eIF5A appeared to be one of the very earlier biological events in response to acute heat stress. It is certainly of interest to further investigate why heat-induced loss of eIF5A occurs in certain cell types such as colon cancer cells but not in other cell types.

Is the eIF5A in Caco-2 mutated?

We have previously shown that a single amino acid mutation in yeast eIF5A resulted in a temperature-sensitive phenotype, due to a significant change of eIF5A stability (Chatterjee et al., 2006). Thus, one possible cause for the heat-induced change in eIF5A stability may be due to a mutation of eIF5A gene in human colon cancer cells. To test this possibility, we have cloned and compared the sequences the eIF5A cDNA from Caco-2 and HeLa cells (Fig. 8). Both sequences were found to be identical to the wild type sequence (BC001832) in GenBank, indicating that gene mutation was not the cause for the heat-induced loss of eIF5A in Caco-2 cells. We concluded that the heat-induced loss of eIF5A could not be attributed to any conformational change that is unique to colon cancer cells.

Protease inhibitors block heat-induced loss of eIF5A and cell death

We reasoned that if enhanced protein degradation due to certain proteolytic system may account for the heat-induced loss of eIF5A, protease inhibitor may block such a degradation. We therefore examined the effect of PMSF, a serine protease inhibitor, on the heat-induced loss of eIF5A in Caco-2 cells. Figure 9A shows that PMSF could partially block the heat-induced loss of eIF5A, suggesting that protein degradation was the cause for the heat-induced loss of eIF5A in Caco-2 cells. This finding allows us to further test whether the heat-induced loss of eIF5A could account for the heat-induced cell death of Caco-2 cells. Figure 9B shows that indeed the heat-induced cell death of Caco-2 or HCT116 colon cancer cells could be blocked not only partially by PMSF but also more prominently by pan protease inhibitor cocktail (CPIP, Complete Protease Inhibitor Cocktail) that inhibits broad spectrum of serine, cysteine and metalloproteases as well as calpains (Fig. 9B). Together, these results support the contention that heat stress induced a rapid degradation of eIF5A and that this loss of eIF5A then contributed to the thermal killing of colon cancer cells.

Discussion

Environmental stress elicits diverse biological responses in living cells, including heat shock response and mitogen-activated protein kinase cascade. The ubiquitous and highly conserved heat shock proteins play a key role in protecting cells against the stress insult. On the other hand, MAPK pathways, particularly JNK and p38, are related to the apoptotic signaling (Beere and Green, 2001; Nollen and Morimoto, 2002). In addition to these general stress responses, different stress may modulate different genes within a given cell type (Kaltschmidt et al., 2002) and, for different cell types, different genes may be specifically regulated by a particular stress depending on the composition of the intracellular milieu (Woessmann et al., 1999; DeMeester et al., 2001). Thus, the ultimate fate of cells in response to a stress can be cell-specific.

In this study we have compared the effect of temperature on thermal killing of human cells under two conditions: (i) chronic heat stress at 44°C, and (ii) acute heat stress at the temperature range of 45–51°C. We found that Caco-2, but not HeLa, is highly susceptible to thermal killing under both conditions (Figs. 1 and 2). These findings indicate that the two cell types, Caco-2 and HeLa, respond differently to heat stress. Mitochondrial injury due to heat stress can lead to an

COLOR

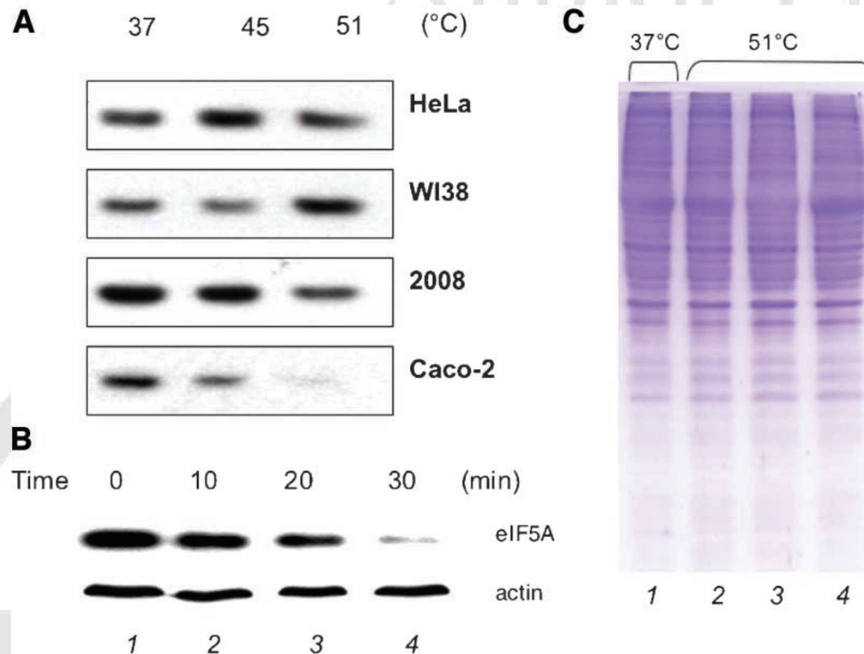

Fig. 6. Western blot analysis of eIF5A in four human cell lines at different temperatures. A: Human HeLa, WI38 (normal lung fibroblasts), 2008 (ovarian cancer), and Caco-2 cells were incubated at 37, 45, or 51°C for 30 min. Cells were then harvested and equal amount of cellular protein (5 μg) was analyzed by 15% SDS–page, followed Western blot analysis using anti-eIF5A antibody. B: Time course of the loss of eIF5A in Caco-2 cells at 51°C. Caco-2 cells were incubated at 51°C for 10, 20, and 30 min, or left untreated at 37°C as control (Time 0). Thereafter, protein levels of eIF5A were determined by Western blot analysis. Actin was used as a loading control. C: Heat stress on global protein profile. Caco-2 cells were either maintained at 37°C (lane 1) or stressed at 51°C for 10, 20, and 30 min (lanes 2, 3, and 4, respectively). Cells were rinsed and harvested in an equal volume of extraction buffer and processed for SDS–polyacrylamide gel electrophoresis. Equal volume of extract samples (5 μl) was loaded onto each lane.

accumulation of reactive oxygen species that generate oxidative stress (Davidson and Schiestl, 2001). It has also been reported that oxidative stress and antioxidant enzymes play a major role in heat-induced cell death in yeast (Davidson et al., 1996). However, oxidative stress does not appear to be the cause for the thermal killing of Caco-2 cells, as they are quite resistant to the cytotoxic effect of hydrogen peroxide up to 125 μM (Fig. 3). Together, our results demonstrate that for a given cell type (Caco-2 or HeLa), different stress may produce different physiological effects, and that for a given stress (heat or oxidative), different cell type may respond differently.

The thermal killing of colon cancer cells appears to be the result of apoptosis as suggested by DNA fragmentation analysis (Fig. 4). However, Takeuchi et al. (2002) reported that they did

COLOR

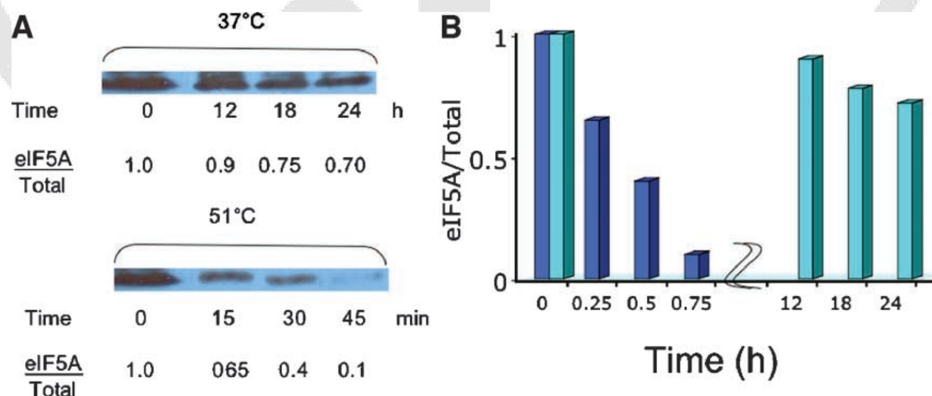

Fig. 7. Half-life of eIF5A. A: Western blot analysis of eIF5A protein. Caco-2 cells were radiolabeled for 48 h with [^{3}H]leucine (5 μCi/ml) and then chased with cold leucine (4 mM) in the presence of cycloheximide (50 μg/ml) at 37 or 51°C for various time as indicated. Cells were then analyzed by SDS–PAGE, the eIF5A band position was located by Western blotting. The ratio of radioactivity associated with eIF5A relative to the total radioactivity was determined at each time point with the value at time zero set at 1.0 as described in Materials and Methods Section. B: Loss of radiolabeled eIF5A as a function of time of leucine chase. The plot of the ratio eIF5A/Total versus the time of chase was used to estimate of the half-life of eIF5A in Caco-2 cells at 37°C (dark blue) and at 51°C (light blue).

HeLa	1	ATGGCAGATG	ACTTGGACTT	CGAGACAGGA	GATGCAGGGG	CCTCAGCCAC
Caco-2	1	ATGGCAGATG	ACTTGGACTT	CGAGACAGGA	GATGCAGGGG	CCTCAGCCAC
BC001832		ATGGCAGATG	ACTTGGACTT	CGAGACAGGA	GATGCAGGGG	CCTCAGCCAC
HeLa	51	CTTCCCAATG	CAGTGCTCAG	CATTACGTAA	GAATGGCTTT	GTGGTGCTCA
Caco-2	51	CTTCCCAATG	CAGTGCTCAG	CATTACGTAA	GAATGGCTTT	GTGGTGCTCA
BC001832		CTTCCCAATG	CAGTGCTCAG	CATTACGTAA	GAATGGCTTT	GTGGTGCTCA
HeLa	101	AAGGCCGGCC	ATGTAAGATC	GTCGAGATGT	CTACTTCGAA	GACTGGCAAG
Caco-2	101	AAGGCCGGCC	ATGTAAGATC	GTCGAGATGT	CTACTTCGAA	GACTGGCAAG
BC001832		AAGGCCGGCC	ATGTAAGATC	GTCGAGATGT	CTACTTCGAA	GACTGGCAAG
HeLa	151	CACGGCCACG	CCAAGGTCCA	TCTGGTTGGT	ATTGACATCT	TTACTGGGAA
Caco-2	151	CACGGCCACG	CCAAGGTCCA	TCTGGTTGGT	ATTGACATCT	TTACTGGGAA
BC001832		CACGGCCACG	CCAAGGTCCA	TCTGGTTGGT	ATTGACATCT	TTACTGGGAA
HeLa	201	GAAATATGAA	GATATCTGCC	CGTCAACTCA	TAATATGGAT	GTCCCCAACA
Caco-2	201	GAAATATGAA	GATATCTGCC	CGTCAACTCA	TAATATGGAT	GTCCCCAACA
BC001832		GAAATATGAA	GATATCTGCC	CGTCAACTCA	TAATATGGAT	GTCCCCAACA
HeLa	251	TCAAAAGGAA	TGACTTCCAG	CTGATTGGCA	TCCAGGATGG	GTACCTATCA
Caco-2	251	TCAAAAGGAA	TGACTTCCAG	CTGATTGGCA	TCCAGGATGG	GTACCTATCA
BC001832		TCAAAAGGAA	TGACTTCCAG	CTGATTGGCA	TCCAGGATGG	GTACCTATCA
HeLa	301	CTGCTCCAGG	ACAGCGGGGA	GGTACGAGAG	GACCTTCGTC	TCCCTGAGGG
Caco-2	301	CTGCTCCAGG	ACAGCGGGGA	GGTACGAGAG	GACCTTCGTC	TCCCTGAGGG
BC001832		CTGCTCCAGG	ACAGCGGGGA	GGTACGAGAG	GACCTTCGTC	TCCCTGAGGG
HeLa	351	AGACCTTGGC	AAGGAGATTG	AGCAGAAGTA	CGACTGTGGA	GAAGAGATCC
Caco-2	351	AGACCTTGGC	AAGGAGATTG	AGCAGAAGTA	CGACTGTGGA	GAAGAGATCC
BC001832		AGACCTTGGC	AAGGAGATTG	AGCAGAAGTA	CGACTGTGGA	GAAGAGATCC
HeLa	401	TGATCACGGT	GCTGTCTGCC	ATGACAGAGG	AGGCAGCTGT	TGCAATCAAG
Caco-2	401	TGATCACGGT	GCTGTCTGCC	ATGACAGAGG	AGGCAGCTGT	TGCAATCAAG
BC001832		TGATCACGGT	GCTGTCTGCC	ATGACAGAGG	AGGCAGCTGT	TGCAATCAAG
HeLa	451	GCCATGGCAA	AATAA			
Caco-2	451	GCCATGGCAA	AATAA			
BC001832		GCCATGGCAA	AATAA			

Fig. 8. Comparison of the eIF5A cDNA sequences of HeLa and Caco-2 cells. Specific primers designed to amplify the eIF5A gene were used in PCR using cDNAs reverse-transcribed from HeLa and Caco-2 cells as the template. The cloned eIF5A1 PCR products from each cell line were directly subjected to DNA sequencing. The sequences were then aligned, along with the wild-type human sequence (accession number BC001832).

COLOR

not detect any DNA fragmentation after heat stress at 45 or 51°C. This apparent discrepancy is likely due to the difference in the time course chosen for chromosome integrity analysis. We note that they have limited their detection window to less than 12 h following the stress, which appears to be too short for any significant DNA fragmentation to occur.

Since acute heat stress (51°C) has been reported to cause eIF5A loss (Takeuchi et al., 2002), it is certainly plausible to speculate that the differential response of HeLa and Caco-2 cells to thermal killing (Fig. 2) may be related to differences in eIF5A content after heat stress. Indeed, the correlation of heat-induced loss of eIF5A and thermal killing is striking (Fig. 5A). It is surprising that, even though both cell types are of epithelial origin, heat stress induced a rapid loss of eIF5A only in Caco-2, not in HeLa cells (Fig. 5A). The fact that such a loss does not occur in tumor cells such as HeLa and ovarian 2008 or in normal cells suggests that this phenomenon is unique to some but not all transformed cells (Fig. 6A). The heat-induced loss of eIF5A represents an early response of colon cancer cells to heat stress (Fig. 6B), suggesting that it may impact some downstream events that are related to heat stress response. Although the precise function of eIF5A has not been clearly defined, abundant literature evidence indicates that eIF5A correlates with proliferation and is essential for cell survival (Chen and Liu, 1997; Park, 2006). Thus, the loss of eIF5A could be a major contributing factor for the inability of Caco-2 cells to recover from the acute heat stress at lethal temperature. Similarly, the reason why HeLa and other cells are resistant to thermal killing can be explained, at least in part, by the fact that eIF5A level remains unchanged during heat stress (Figs. 5A and 6A).

There are three possible explanations for the selective loss of eIF5A in colon cancer cells in response to acute heat stress: (i) eIF5A gene in Caco-2 is mutated and thus the gene product becomes susceptible to protease digestion at higher

COLOR

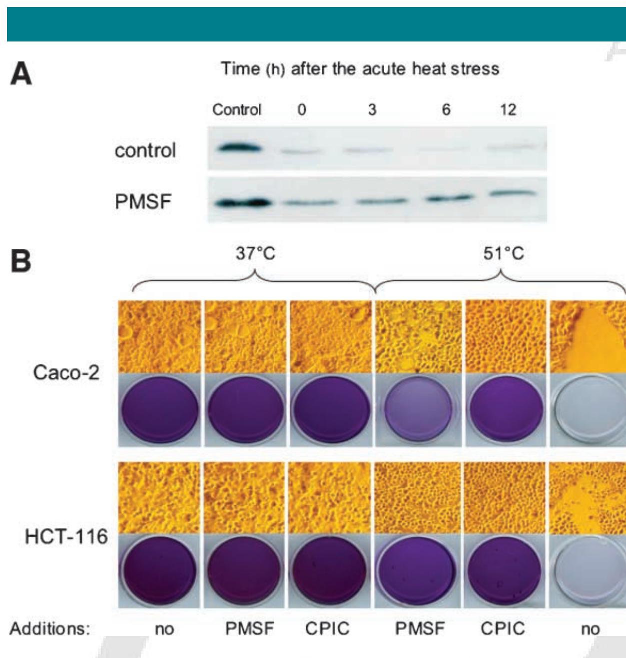

Fig. 9. Effect of protease inhibitors on eIF5A level and cell survival. A: Caco-2 cells were treated with PMSF (250 μM) prior to acute heat stress (51°C, 30 min). Cell cultures were then shifted back to 37°C for various time as indicated. The protein levels of eIF5A were determined by Western blot analysis using chick anti-heIF5A antibody at 1:1,000 and HRP-RAC antibody (1:1,000). B: Caco-2 and HCT116 colon cancer cells were maintained at 37°C or treated by acute heat stress (51°C, 30 min) either without or with pretreatment of PMSF (250 μM) or Complete Protease Inhibitor Cocktail (CPIC, final concentrations: pronase 2 μg/ml, thermolysin 0.2 μg/ml, chymotrypsin 1.2 μg/ml, papain 132 μg/ml, pancreas extract 8 μg/ml, EDTA 0.4 mM). Cell cultures were then incubated at 37°C for 12 h and the cell viability was monitored by morphological analysis using phase contrast light microscopy and by metabolic MTT assay. The study has been repeated three times and representative data were presented.

temperatures; (ii) heat induces a selective secretion of eIF5A to the medium; and (iii) heat stress activates a particular proteolytic system which selectively targets eIF5A. Since the eIF5A sequence of Caco-2 cells is identical to that of HeLa cells and other normal human cells (Fig. 8), protein mutation could not be the cause for heat-induced loss of eIF5A in Caco-2. Selective secretion of eIF5A cannot be the cause either, because we did not observe any secretion of eIF5A before or after heat treatment of Caco-2 cells (data not shown). Our finding that PMSF partially blocks the heat-induced loss of eIF5A Caco-2 cells suggests that a certain heat-activated protease may be involved (Fig. 9A). The finding that PMSF and other protease inhibitors also block the thermal killing of colon cancer cells Caco-2 and HCT116 (Fig. 9B) is consistent with the notion that eIF5A is essential for cell survival and proliferation. The nature of this heat-sensitive proteolytic system remains to be investigated. Nonetheless, it is of interest to note a parallel observation that we have made with a temperature sensitive yeast mutant, whose eIF5A, with a single amino acid substitution, is stable at permissive temperature, but rapidly decays at restrictive temperature. Here, we also found that PMSF inhibits the degradation of eIF5A and partially suppresses the temperature-sensitive growth arrest phenotype in this yeast strain (Chatterjee et al., 2006).

Hyperthermia is a type of cancer treatment in which body tissue is exposed to high temperatures. Research has shown that high temperatures can damage and kill cancer cells, usually with minimal injury to normal tissues (Hildebrandt et al., 2002; van der Zee, 2002). The finding that heat induces a rapid loss eIF5A in colon cancer cells may provide a mechanistic basis for the effectiveness of using hyperthermic regimen. In this context, it is noteworthy that DFMO has been reported to increase the sensitivity of CHO cells to the cytotoxic effect of elevated temperature (Gerner et al., 1980; Fuller and Gerner, 1982). DFMO inhibits ornithine decarboxylase, the key enzyme for the biosynthesis of putrescine, the precursor of spermidine which is required for hypusine formation on eIF5A (Chen and Liu, 1997; Park et al., 1997). Thus, the effect of DFMO in enhancing the cytotoxic effect of hyperthermia could be mediated via the inhibition of hypusine formation and hence inactivation of eIF5A. It will be of interest to determine whether the synergistic interaction of hyperthermia in bimodality or trimodality treatment may also involve eIF5A. In view of our study here, we propose that manipulation of eIF5A stability or hypusine formation can be used to enhance the efficacy of hyperthermia in treating cancer.

Acknowledgments

The work was supported in part by United States Public Health Services Grant RO1 CA49695 awarded by the National Cancer Institute, NIH.

Literature Cited

Beere HM, Green DR. 2001. Stress management—Heat shock protein-70 and the regulation of apoptosis. Trends Cell Biol 11:6–10.
Berg K, Hansen MB, Nielsen SE. 1990. A new sensitive bioassay for precise quantification of interferon activity as measured via the mitochondrial dehydrogenase function in cells (MTT-method). Apmis 98:156–162.
Bergs JW, Franken NA, Haveman J, Geijsen ED, Crezee J, van Bree C. 2007. Hyperthermia, cisplatin and radiation trimodality treatment: A promising cancer treatment? A review from preclinical studies to clinical application. Int J Hyperthermia 23:329–341.
Chatterjee I, Gross SR, Kinzy TG, Chen KY. 2006. Rapid depletion of mutant eukaryotic initiation factor 5A at restrictive temperature reveals connections to actin cytoskeleton and cell cycle progression. Mol Genet Genomics 275:264–276.
Chen KY, Liu AY. 1997. Biochemistry and function of hypusine formation on eukaryotic initiation factor 5A. Biol Signals 6:105–109.
Chen ZP, Yan YP, Ding QJ, Knapp S, Potenza JA, Schugar HJ, Chen KY. 1996. Effects of inhibitors of deoxyhypusine synthase on the differentiation of mouse neuroblastoma and erythroleukemia cells. Cancer Lett 105:233–239.
Davidson JF, Schiestl RH. 2001. Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in Saccharomyces cerevisiae. Mol Cell Biol 21:8483–8489.
Davidson JF, Whyte B, Bissinger PH, Schiestl RH. 1996. Oxidative stress is involved in heat-induced cell death in Saccharomyces cerevisiae. Proc Natl Acad Sci USA 93:5116–5121.
DeMeester SL, Buchman TG, Cobb JP. 2001. The heat shock paradox: Does NF-kappaB determine cell fate? FASEB J 15:270–274.
Dikomey E, Franzke J. 1992. Effect of heat on induction and repair of DNA strand breaks in X-irradiated CHO cells. Int J Radiat Biol 61:221–233.
Duncan RF, Hershey JW. 1986. Changes in eIF-4D hypusine modification or abundance are not correlated with translational repression in HeLa cells. J Biol Chem 261:12903–12906.
Duncan RF, Hershey JW. 1989. Protein synthesis and protein phosphorylation during heat stress, recovery, and adaptation. J Cell Biol 109:1467–1481.
Fuller DJ, Gerner EW. 1982. Polyamines: A dual role in the modulation of cellular sensitivity to heat. Radiat Res 92:439–444.
Gerner EW, Holmes DK, Stickney DG, Noterman JA, Fuller DJ. 1980. Enhancement of hyperthermia-induced cytotoxicity by polyamines. Cancer Res 40:432–438.
Hildebrandt B, Wust P, Ahlers O, Dieing A, Sreenivasa G, Kerner T, Felix R, Riess H. 2002. The cellular and molecular basis of hyperthermia. Crit Rev Oncol Hematol 43:33–56.
Jakus J, Wolff EC, Park MH, Folk JE. 1993. Features of the spermidine-binding site of deoxyhypusine synthase as derived from inhibition studies. Effective inhibition by bis- and mono-guanylated diamines and polyamines. J Biol Chem 268:13151–13159.
Jao DL, Chen KY. 2006. Tandem affinity purification revealed the hypusine-dependent binding of eukaryotic initiation factor 5A to the translating 80S ribosomal complex. J Cell Biochem 97:583–598.
Jenkins ZA, Haag PG, Johansson HE. 2001. Human eIF5A2 on chromosome 3q25-q27 is a phylogenetically conserved vertebrate variant of eukaryotic translation initiation factor 5A with tissue-specific expression. Genomics 71:101–109.
Kaltschmidt B, Heinrich M, Kaltschmidt C. 2002. Stimulus-dependent activation of NF-kappaB specifies apoptosis or neuroprotection in cerebellar granule cells. Neuromol Med 2:299–309.
Larkindale J, Knight MR. 2002. Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. Plant Physiol 128:682–695.
Li GC, Werb Z. 1982. Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. Proc Natl Acad Sci USA 79:3218–3222.
Li GC, Mivechi NF, Weitzel G. 1995. Heat shock proteins, thermotolerance, and their relevance to clinical hyperthermia. Int J Hyperthermia 11:459–488.
Mariano TM, Siekierka J. 1986. Inhibition of HeLa cell protein synthesis under heat shock conditions in the absence of initiation factor eIF-2 alpha phosphorylation. Biochem Biophys Res Commun 138:519–525.
Nollen EA, Morimoto RI. 2002. Chaperoning signaling pathways: Molecular chaperones as stress-sensing 'heat shock' proteins. J Cell Sci 115:2809–2816.

Panniers R, Henshaw EC. 1984. Mechanism of inhibition of polypeptide chain initiation in heat-shocked Ehrlich ascites tumour cells. Eur J Biochem 140:209–214.
Park MH. 2006. The post-translational synthesis of a polyamine-derived amino acid, hypusine, in the eukaryotic translation initiation factor 5A (eIF5A). J Biochem 139:161–169.
Park MH, Lee YB, Joe YA. 1997. Hypusine is essential for eukaryotic cell proliferation. Biol Signals 6:115–123.
Parsell DA, Lindquist S. 1993. The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. Annu Rev Genet 27:437–496.
Roti Roti JL, Kampinga HH, Malyapa RS, Wright WD, vanderWaal RP, Xu M, 1998. Nuclear matrix as a target for hyperthermic killing of cancer cells. Cell Stress Chaperones 3:245–255.
Sakaguchi Y, Stephens LC, Makino M, Kaneko T, Strebel FR, Danhauser LL, Jenkins GN, Bull JM. 1995. Apoptosis in tumors and normal tissues induced by whole body hyperthermia in rats. Cancer Res 55:5459–5464.
Samali A, Holmberg CI, Sistonen L, Orrenius S. 1999. Thermotolerance and cell death are distinct cellular responses to stress: Dependence on heat shock proteins. FEBS Lett 461:306–310.
Sasaki K, Abid MR, Miyazaki M. 1996. Deoxyhypusine synthase gene is essential for cell viability in the yeast Saccharomyces cerevisiae. FEBS Lett 384:151–154.
Schnier J, Schwelberger HG, Smit-McBride Z, Kang HA, Hershey JW. 1991. Translation initiation factor 5A and its hypusine modification are essential for cell viability in the yeast Saccharomyces cerevisiae. Mol Cell Biol 11:3105–3114.
Stein U, Rau B, Wust P, Walther W, Schlag PM. 1999. Hyperthermia for treatment of rectal cancer: Evaluation for induction of multidrug resistance gene (mdr1) expression. Int J Cancer 80:5–12.
Takeuchi K, Nakamura K, Fujimoto M, Kaino S, Kondoh S, Okita K. 2002. Heat stress-induced loss of eukaryotic initiation factor 5A (eIF-5A) in a human pancreatic cancer cell line, MIA PaCa-2, analyzed by two-dimensional gel electrophoresis. Electrophoresis 23:662–669.
Taylor CA, Sun Z, Cliché DO, Ming H, Eshaque B, Jin S, Hopkins MT, Thai B, Thompson JE. 2007. Eukaryotic translation initiation factor 5A induces apoptosis in colon cancer cells and associates with the nucleus in response to tumour necrosis factor α signaling. Exp Cell Res 313:437–449.
Vacca RA, de Pinto MC, Valenti D, Passarella S, Marra E, De Gara L. 2004. Production of reactive oxygen species, alteration of cytosolic ascorbate peroxidase, and impairment of mitochondrial metabolism are early events in heat shock-induced programmed cell death in tobacco Bright-Yellow 2 cells. Plant Physiol 134:1100–1112.
van der Zee J. 2002. Heating the patient: A promising approach? Ann Oncol 13:1173–1184.
Woessmann W, Meng YH, Mivechi NF. 1999. An essential role for mitogen-activated protein kinases, ERKs, in preventing heat-induced cell death. J Cell Biochem 74:648–662.
Wust P, Hildebrandt B, Sreenivasa G, Rau B, Gellermann J, Riess H, Felix R, Schlag PM. 2002. Hyperthermia in combined treatment of cancer. Lancet Oncol 3:487–497.
Xu A, Chen KY. 2001. Hypusine is required for a sequence-specific interaction of eukaryotic initiation factor 5A with postsystematic evolution of ligands by exponential enrichment RNA. J Biol Chem 276:2555–2561.
Xu A, Jao DL, Chen KY. 2004. Identification of mRNA that binds to eukaryotic initiation factor 5A by affinity co-purification and differential display. Biochem J 384:585–590.
Zanelli CF, Maragno AL, Gregio AP, Komili S, Pandolfi JR, Mestriner CA, Lustri WR, Valentini SR. 2006. eIF5A binds to translational machinery components and affects translation in yeast. Biochem Biophys Res Commun 348:1358–1366.

Q1: Author: The Journal's copyeditors have taken care to format your authorship according to journal style (First name, Middle Initial, Surname). In the event a formatting error escaped their inspection, or there was insufficient information to apply journal style, please take a moment to review all author names and sequences to ensure the accuracy of the authorship in the published article. Please note that this information will also affect external indexes referencing this paper (e.g., PubMed).

Q2: Author: Please add in the reference list.

Q3: Author: Please add in the reference list.

Q4: Author: Please provide complete location.

Q5: Author: Please provide complete location.

Q6: Author: Please add in the reference list.

Q7: Author: Please provide complete location.

Q8: Author: Please provide complete location.

Q9: Author: Subpart A is not provided in the artwork of Figure 3. Hence we have deleted subpart A from the caption. Please check.

Q10: Author: Please add in the reference list.

WILEY
Publishers Since 1807

COLOR REPRODUCTION IN YOUR ARTICLE

These proofs have been typeset using figure files transmitted to production when this article was accepted for publication. Please review all figures and note your approval with your submitted proof corrections. You may contact the journal production team at **JCPprod@wiley.com** if you wish to discuss specific concerns.

Because of the high cost of color printing, we can only print figures in color if authors cover the expense. If you have submitted color figures, please indicate your consent to cover the cost on the table listed below by marking the box corresponding to the approved cost on the table. The rate for this journal is $500 USD per printed page of color, regardless on the number of figures appearing on that page.

Please note, all color images will be reproduced online in Wiley *InterScience* at no charge, whether or not you opt for color printing.

You will be invoiced for color charges once the article has been published in print.

Failure to return this form with your article proofs may delay the publication of your article.

JOURNAL **JOURNAL OF CELLULAR PHYSIOLOGY** MS. NO. ______ NO. COLOR PAGES ______

MANUSCRIPT TITLE ______

AUTHOR(S) ______

No. Color Pages	Color Charge	No. Color Pages	Color Charge	No. Color Pages	Color Charge
☐ **1**	**$500**	☐ **5**	**$2500**	☐ **9**	**$4500**
☐ **2**	**$1000**	☐ **6**	**$3000**	☐ **10**	**$5000**
☐ **3**	**$1500**	☐ **7**	**$3500**	☐ **11**	**$5500**
☐ **4**	**$2000**	☐ **8**	**$4000**	☐ **12**	**$6000**
*****Contact JCPprod@wiley.com for a quote if you have more than 12 pages of color*****					

☐ Please print my figures color ☐ Please print my figures in black and white

☐ Please print the following figures in color ______

and convert these figures to black and white ______

Approved by ______

Billing Address ______ E-mail ______

______ Telephone ______

______ Fax ______

REPRINT BILLING DEPARTMENT • 111 RIVER STREET • HOBOKEN, NJ 07030
FAX: (201) 748-7670
E-MAIL: reprints@wiley.com

PREPUBLICATION REPRINT ORDER FORM

Please complete this form even if you are not ordering reprints. This form **MUST** be returned with your corrected proofs and original manuscript. Your reprints will be shipped approximately 4 weeks after publication. Reprints ordered after printing will be substantially more expensive.

JOURNAL *JOURNAL OF CELLULAR PHYSIOLOGY* VOLUME ______ ISSUE ______
TITLE OF MANUSCRIPT ______________________
MS. NO. ______ NO. OF PAGES ______ AUTHOR(S) ______________________

No. of Pages	100 Reprints	200 Reprints	300 Reprints	400 Reprints	500 Reprints
	$	$	$	$	$
1-4	**336**	**501**	**694**	**890**	**1052**
5-8	**469**	**703**	**987**	**1251**	**1477**
9-12	**594**	**923**	**1234**	**1565**	**1850**
13-16	**714**	**1156**	**1527**	**1901**	**2273**
17-20	**794**	**1340**	**1775**	**2212**	**2648**
21-24	**911**	**1529**	**2031**	**2536**	**3037**
25-28	**1004**	**1707**	**2267**	**2828**	**3388**
29-32	**1108**	**1894**	**2515**	**3135**	**3755**
33-36	**1219**	**2092**	**2773**	**3456**	**4143**
37-40	**1329**	**2290**	**3033**	**3776**	**4528**

**REPRINTS ARE ONLY AVAILABLE IN LOTS OF 100. IF YOU WISH TO ORDER MORE THAN 500 REPRINTS, PLEASE CONTACT OUR REPRINTS DEPARTMENT AT (201) 748-6353 FOR A PRICE QUOTE.

☐ Please send me ____________________ reprints of the above article at $ ______

Please add appropriate State and Local Tax for United States orders only. (Tax Exempt No.____________________) $ ______

Please add 5% Postage and Handling $ ______

TOTAL AMOUNT OF ORDER** $ ______

***International orders must be paid in currency and drawn on a U.S. bank*

Please check one: ☐ Check enclosed ☐ Bill me ☐ Credit Card
If credit card order, charge to: ☐ American Express ☐ Visa ☐ MasterCard

Credit Card No ______ Signature ______ Exp. Date ______

BILL TO:
Name ______
Institution ______
Address ______

Purchase Order No. ______

SHIP TO: (Please, no P.O. Box numbers)
Name ______
Institution ______
Address ______

Phone ______ Fax ______
E-mail ______

COPYRIGHT TRANSFER AGREEMENT

Date: ____________________ Contributor name: ____

Contributor address: ____

Manuscript number (Editorial office only): ____

Re: Manuscript entitled ____

____ (the "Contribution")

for publication in ____ (the "Journal")

published by ____ ("Wiley-Blackwell").

Dear Contributor(s):
Thank you for submitting your Contribution for publication. In order to expedite the editing and publishing process and enable Wiley-Blackwell to disseminate your Contribution to the fullest extent, we need to have this Copyright Transfer Agreement signed and returned as directed in the Journal's instructions for authors as soon as possible. If the Contribution is not accepted for publication, or if the Contribution is subsequently rejected, this Agreement shall be null and void. **Publication cannot proceed without a signed copy of this Agreement.**

A. COPYRIGHT

1. The Contributor assigns to Wiley-Blackwell, during the full term of copyright and any extensions or renewals, all copyright in and to the Contribution, and all rights therein, including but not limited to the right to publish, republish, transmit, sell, distribute and otherwise use the Contribution in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so.

2. Reproduction, posting, transmission or other distribution or use of the final Contribution in whole or in part in any medium by the Contributor as permitted by this Agreement requires a citation to the Journal and an appropriate credit to Wiley-Blackwell as Publisher, and/or the Society if applicable, suitable in form and content as follows: (Title of Article, Author, Journal Title and Volume/Issue, Copyright © [year], copyright owner as specified in the Journal). Links to the final article on Wiley-Blackwell's website are encouraged where appropriate.

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor's Employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution.

C. PERMITTED USES BY CONTRIBUTOR

1. Submitted Version. Wiley-Blackwell licenses back the following rights to the Contributor in the version of the Contribution as originally submitted for publication:

a. After publication of the final article, the right to self-archive on the Contributor's personal website or in the Contributor's institution's/employer's institutional repository or archive. This right extends to both intranets and the Internet. The Contributor may not update the submission version or replace it with the published Contribution. The version posted must contain a legend as follows: This is the pre-peer reviewed version of the following article: FULL CITE, which has been published in final form at [Link to final article].

b. The right to transmit, print and share copies with colleagues.

2. Accepted Version. Re-use of the accepted and peer-reviewed (but not final) version of the Contribution shall be by separate agreement with Wiley-Blackwell. Wiley-Blackwell has agreements with certain funding agencies governing reuse of this version. The details of those relationships, and other offerings allowing open web use, are set forth at the following website: http://www.wiley.com/go/funderstatement. NIH grantees should check the box at the bottom of this document.

3. Final Published Version. Wiley-Blackwell hereby licenses back to the Contributor the following rights with respect to the final published version of the Contribution:

a. Copies for colleagues. The personal right of the Contributor only to send or transmit individual copies of the final published version in any format to colleagues upon their specific request provided no fee is charged, and further-provided that there is no systematic distribution of the Contribution, e.g. posting on a listserve, website or automated delivery.

b. Re-use in other publications. The right to re-use the final Contribution or parts thereof for any publication authored or edited by the Contributor (excluding journal articles) where such re-used material constitutes less than half of the total material in such publication. In such case, any modifications should be accurately noted.

c. Teaching duties. The right to include the Contribution in teaching or training duties at the Contributor's institution/place of employment including in course packs, e-reserves, presentation at professional conferences, in-house training, or distance learning. The Contribution may not be used in seminars outside of normal teaching obligations (e.g. commercial seminars). Electronic posting of the final published version in connection with teaching/training at the Contributor's institution/place of employment is permitted subject to the implementation of reasonable access control mechanisms, such as user name and password. Posting the final published version on the open Internet is not permitted.

d. Oral presentations. The right to make oral presentations based on the Contribution.

4. Article Abstracts, Figures, Tables, Data Sets, Artwork and Selected Text (up to 250 words).

a. Contributors may re-use unmodified abstracts for any non-commercial purpose. For on-line uses of the abstracts, Wiley-Blackwell encourages but does not require linking back to the final published versions.

b. Contributors may re-use figures, tables, data sets, artwork, and selected text up to 250 words from their Contributions, provided the following conditions are met:

(i) Full and accurate credit must be given to the Contribution.
(ii) Modifications to the figures, tables and data must be noted. Otherwise, no changes may be made.
(iii) The reuse may not be made for direct commercial purposes, or for financial consideration to the Contributor.
(iv) Nothing herein shall permit dual publication in violation of journal ethical practices.

D. CONTRIBUTIONS OWNED BY EMPLOYER

1. If the Contribution was written by the Contributor in the course of the Contributor's employment (as a "work-made-for-hire" in the course of employment), the Contribution is owned by the company/employer which must sign this Agreement (in addition to the Contributor's signature) in the space provided below. In such case, the company/employer hereby assigns to Wiley-Blackwell, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in paragraph A above.

2. In addition to the rights specified as retained in paragraph B above and the rights granted back to the Contributor pursuant to paragraph C above, Wiley-Blackwell hereby grants back, without charge, to such company/employer, its subsidiaries and divisions, the right to make copies of and distribute the final published Contribution internally in print format or electronically on the Company's internal network. Copies so used may not be resold or distributed externally. However the company/employer may include information and text from the Contribution as part of an information package included with software or other products offered for sale or license or included in patent applications. Posting of the final published Contribution by the institution on a public access website may only be done with Wiley-Blackwell's written permission, and payment of any applicable fee(s). Also, upon payment of Wiley-Blackwell's reprint fee, the institution may distribute print copies of the published Contribution externally.

E. GOVERNMENT CONTRACTS

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government, U.K. Government, and other government employees: see notes at end)

F. COPYRIGHT NOTICE

The Contributor and the company/employer agree that any and all copies of the final published version of the Contribution or any part thereof distributed or posted by them in print or electronic format as permitted herein will include the notice of copyright as stipulated in the Journal and a full citation to the Journal as published by Wiley-Blackwell.

G. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that the Contribution is the Contributor's original work, all individuals identified as Contributors actually contributed to the Contribution, and all individuals who contributed are included. If the Contribution was prepared jointly, the Contributor agrees to inform the co-Contributors of the terms of this Agreement and to obtain their signature to this Agreement or their written permission to sign on their behalf. The Contribution is submitted only to this Journal and has not been published before. (If excerpts from copyrighted works owned by third parties are included, the Contributor will obtain written permission from the copyright owners for all uses as set forth in Wiley-Blackwell's permissions form or in the Journal's Instructions for Contributors, and show credit to the sources in the Contribution.) The Contributor also warrants that the Contribution contains no libelous or unlawful statements, does not infringe upon the rights (including without limitation the copyright, patent or trademark rights) or the privacy of others, or contain material or instructions that might cause harm or injury.

CHECK ONE BOX:

☐ Contributor-owned work

ATTACH ADDITIONAL SIGNATURE PAGES AS NECESSARY

Contributor's signature ______________________ Date ________

Type or print name and title ______________________

Co-contributor's signature ______________________ Date ________

Type or print name and title ______________________

☐ Company/Institution-owned work (made-for-hire in the course of employment)

Company or Institution (Employer-for-Hire) ______________________ Date ________

Authorized signature of Employer ______________________ Date ________

☐ U.S. Government work

Note to U.S. Government Employees
A contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. Government publication, is called a "U.S. Government work," and is in the public domain in the United States. In such case, the employee may cross out Paragraph A.1 but must sign (in the Contributor's signature line) and return this Agreement. If the Contribution was not prepared as part of the employee's duties or is not an official U.S. Government publication, it is not a U.S. Government work.

☐ U.K. Government work (Crown Copyright)

Note to U.K. Government Employees
The rights in a Contribution prepared by an employee of a U.K. government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown. U.K. government authors should submit a signed declaration form together with this Agreement. The form can be obtained via http://www.opsi.gov.uk/advice/crown-copyright/copyright-guidance/publication-of-articles-written-by-ministers-and-civil-servants.htm

☐ Other Government work

Note to Non-U.S., Non-U.K. Government Employees
If your status as a government employee legally prevents you from signing this Agreement, please contact the editorial office.

☐ NIH Grantees

Note to NIH Grantees
Pursuant to NIH mandate, Wiley-Blackwell will post the accepted version of Contributions authored by NIH grant-holders to PubMed Central upon acceptance. This accepted version will be made publicly available 12 months after publication. For further information, see www.wiley.com/go/nihmandate.