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Expression and distribution of HuR during ATP depletion and recovery in proximal tubule cells

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Abstract

Human antigen R (HuR) is a nucleocytoplasmic shuttling protein that binds to and stabilizes mRNAs containing adenine- and uridine-rich elements. Under normal growth conditions, the bulk of HuR is maintained in the nucleus, but under conditions of cell stress, HuR may become more prevalent in the cytosol, where it can stabilize mRNA and regulate gene expression. We have studied the behavior of HuR in LLC-PK_1 proximal tubule cells subjected to ATP depletion and recovery. ATP depletion resulted in detectable net movement of HuR out of the nucleus, followed by net movement of HuR back into the nucleus on reversion to normal growth medium. In addition, HuR protein levels increased during energy depletion. This increase was inhibited by cycloheximide and was independent of HuR mRNA levels, since no change was noted in the quantity of HuR transcript. In contrast, recovery in normal growth medium resulted in increased HuR mRNA, while protein levels decreased to baseline. This suggested a mechanism by which previously injured cells maintained normal levels of HuR but were primed to rapidly translate increased amounts of protein on subsequent insults. Indeed, a second round of ATP depletion resulted in heightened HuR protein translation at a rate more rapid than during the first insult. Additionally, the second insult produced increased HuR levels in the cytoplasm while still maintaining high amounts in the nucleus, indicating that nuclear export may not be required on subsequent insults. These results suggest a role for HuR in protecting kidney epithelia from injury during ischemic stress.

Keywords

mRNA; gene expression; renal ischemia; kidney tubule; human antigen R protein

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The cellular response to stress triggers broad modulations of gene expression. Transcription levels are altered (50), and global translation is suppressed, usually via a highly conserved mechanism by which the translation initiation factor eIF2α is phosphorylated, leading to reduced levels of an $eIF2{-}GTP{-}tRNA_i^{met}$ complex required for initiation (20,23). Paradoxically, selective translation of specific stress-induced products can proceed and subsequently upregulate downstream pathways that provide protection to the cell (21). Thus multiple mechanisms are involved in protecting a cell from stress events. It follows that, since regulated turnover of certain mRNAs is an important form of gene expression in mammalian cells, then stress-induced alterations in mRNA stability are likely to play a role in promoting cell survival.

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A number of RNA-binding proteins have been identified that are important in regulating mRNA stability under normal growth conditions. The most widely studied of these bind to adenine- and uridine-rich elements (AU-rich elements; AREs) in the 3′-untranslated regions of select mRNAs. Some of these, such as the ubiquitously expressed AUF-1 (hnRNP D), bind to ARE-containing mRNAs and recruit proteins that hasten degradation of these transcripts. Other ARE-binding proteins include tristetraprolin, TIA-1, TIAR, and the Hu [embryonic lethal/abnormal vision (ELAV)] proteins, which can modulate mRNA half-life and/or translatability. AREs initially were described in short-lived mRNAs such as those encoding protooncogenes and cytokines and were thought to function strictly as destabilizing elements (46,47) because of the activity of AUF-1 and proteins of similar activity. More recent data show that the total number of mammalian transcripts containing these sequences may number in the thousands and include both short- and long-lived mRNAs (3,17). It has become apparent that the ARE-mediated half-life of a transcript is a function of both destabilizing proteins such as AUF-1 and mRNA-stabilizing proteins, most notably human antigen R (HuR) protein. The ubiquitously expressed HuR (also referred to as HuA or ELAV-1) also binds to ARE-containing transcripts as well as more degenerate uridine-rich sequences (34) but, unlike AUF-1, enhances the stability of its targets. Thus HuR and AUF-1, which demonstrate similar tissue distributions and bind overlapping pools of target mR-NAs, both can contribute to the ultimate half-life of a transcript, depending on their abundance and compartmentalization in the cell (30).

Through identification of its target transcripts, it has become clear that HuR plays a major function in cell division, carcinogenesis, immune responsiveness, and the response to cellular stress (18). HuR has been shown to stabilize ARE-containing mRNAs during hypoxia (32), UV irradiation (52), heat shock (19), treatment with actinomycin D (2), nutritional starvation (53), and, in our recent study (24), energy depletion. While HuR is localized predominantly to the nucleus under normal conditions, stabilization of ARE-containing mRNAs is associated with a net redistribution of HuR from the nucleus to the cytoplasm, allowing this protein to protect cytoplasmic mRNAs from degradation and perhaps alter their translatability. In addition, HuR itself may facilitate nucleocytoplasmic trafficking of these mRNAs (15,45), although this has not been demonstrated directly.

Although the activity of HuR has been studied considerably under several forms of cell stress, its potential role in recovery from ischemic injury/energy depletion has not been well explored. We previously showed that HuR is required for stable expression of V-ATPase mRNAs in the porcine proximal tubule cell line LLC-PK_1 during ATP depletion (24). This well-established model of energy depletion results in changes that mimic many of the effects of ischemic injury on proximal tubule epithelia in whole animal models, including disruption of the actin cytoskeleton (8,9,16,49) and activation of heat shock proteins (HSPs) (35,42,49) and protein kinases (36,42,43). LLC-PK_1 cells also can undergo preconditioning events that protect from simulated ischemia (36). In this study, we investigated the behavior of HuR during ATP depletion and recovery in these cells. We observed that HuR not only undergoes the characteristic nucleocytoplasmic redistribution that is seen during other cellular stresses, but that expression of this RNA-binding protein and its associated mRNA transcript is tightly regulated during ATP depletion and recovery. During energy depletion, HuR levels rise even in the face of severe ATP loss. Furthermore, recovery from ATP depletion results in increased expression of HuR mRNA but not protein. On a second insult, HuR protein levels are very rapidly upregulated, suggesting that the increases in HuR mRNA levels during recovery act as a preconditioning event to prevent loss of critical mRNA species. Because thousands of mRNAs may be stabilized by HuR, including stress-induced proteins such as HSP70 (34), this RNA-binding protein is likely to play a major role in protection against ischemic injury.

MATERIALS AND METHODS

Cell culture, ATP depletion, and media additives

LLC-PK_1 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing penicillin-streptomycin, supplemented with fetal bovine serum (10%), at 37°C in 5% CO_2. For ATP depletion, the culture medium was replaced with DMEM base supplemented with L-glucose, sodium bicarbonate, and 0.1 μM antimycin A. For recovery, depletion medium was replaced with normal growth medium. Cellular ATP levels were assayed using the Sigma Bioluminescent somatic cell assay kit protocol (Sigma, St. Louis, MO).

Cycloheximide was dissolved in DMSO and used at a final concentration of 10 μg/ml; actinomycin D was dissolved in ethanol and used at a final concentration of 5 μg/ml (both from Sigma).

Immunocytochemistry

LLC-PK_1 cells, cultured as above, were fixed and permeabilized in 2% formaldehyde, 0.2% Triton X-100, and 0.5% deoxycholate in a physiological salt solution. Cells underwent treatment with 10 mM sodium borohydride to reduce free aldehydes and were preblocked in a solution of 0.8% bovine serum albumin, 0.1% gelatin, and 10 μg/ml goat IgG in phosphate-buffered saline. Monoclonal anti-HuR antibody 3A2 (Santa Cruz Biotechnology, Santa Cruz, CA) was added to a solution of 0.8% bovine serum albumin, 0.01% gelatin, and 10 μg/ml goat IgG in phosphate-buffered saline. Cells were washed and then incubated with Alexa488-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR). After further washing and mounting, cells were visualized with a Zeiss 510 META laser-scanning microscope at the Campus Microscopy and Imaging Facility of The Ohio State University.

Western blot analysis

Whole cell lysates of LLC-PK_1 cells were obtained by incubating cells with M-PER buffer containing Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL) and centrifuging to remove cell debris. Lysates in sample buffer were loaded onto precast polyacrylamide gels (Bio-Rad, Hercules, CA) and run under standard conditions. Proteins were transferred to Hybond-P membranes (Amersham Biosciences, Piscataway, NJ). The monoclonal primary antibody against HuR (Santa Cruz Biotechnology; 3A2) was used at a 1:1,000 dilution, and a mouse monoclonal antibody reactive to β-actin (Sigma) was used at 1:1,000. Both were detected using anti-mouse horseradish peroxidase-conjugated antibody and enhanced chemiluminescence reagent (Amersham Biosciences).

Cytoplasmic and nuclear extracts from LLC-PK_1 cells were obtained using the Paris kit (Ambion, Austin, TX). Equivalent samples from each fraction were loaded in precast polyacrylamide gels (Bio-Rad) and visualized by Western blotting analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected using a monoclonal antibody from Ambion, while histone H3 was detected with a polyclonal antibody from Cell Signaling Technology (Danvers, MA). Quantitation of Western blots using whole cell or fractionated extracts was performed using a Chemi-Doc image analysis system with Quantity One software. Statistical analysis was performed using Student's *t*-test.

Competitive RT-PCR

An internal standard for competitive RT-PCR of porcine HuR was synthesized using a previously published method (31). Total RNA from LLC-PK_1 cells was isolated using RNA Bee (Tel-Test, Friendswood, TX), following the manufacturer's instructions. A mixture of

internal standard RNA (1 pg) and LLC-PK_1 RNA (5 μg) was reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA). The resulting cDNA was subjected to PCR using Platinum Taq High Fidelity (Invitrogen) with the following primers under the following conditions: 5′-GGTTAT-GAAGACCACATGGCCG-3′ (sense) and 5′-AAGCCATAGC-CCAAGCTGT-3′ (antisense); 2 min at 94°C; then 30 cycles of 94°C for 30 s, 54°C for 30 s, 68°C for 30 s; followed by a final extension at 68°C for 10 min. The PCR reaction was electrophoresed on a 2% agarose gel and visualized and quantified using a Chemi-Doc image analysis system with Quantity One software (Bio-Rad).

RESULTS

ATP depletion in LLC-PK_1 cells

Treatment of LLC-PK_1 cells with antimycin A in DMEM base provides an effective cellular model of ATP depletion (8–10,16,35,36,42,43,49). To characterize the efficacy of our system, ATP levels were measured in cells subjected to depletion for 0–4 h and in cells recovered in normal media for 0–4 h. As expected, cellular ATP levels decreased drastically within 1 h of depletion (Fig. 1, *top*) and remained at ~1% of normal levels throughout the treatment. Cells that were ATP depleted for 1 h were able to recover 55% of normal ATP levels following 1 h in normal medium; within 4 h, this rose to 75% (Fig. 1, *bottom*). Cells that were ATP depleted for 2 h before recovery also rebounded to similar levels but at a slower initial rate. These results are consistent with other studies in proximal tubule epithelia in which the rebound of ATP levels was shown to be incomplete after only a few hours of recovery (8,10,48).

Relocalization of HuR following ATP depletion

To determine whether energy depletion induces redistribution of HuR, immunocytochemistry was performed on LLC-PK_1 cells undergoing 1–4 h of ATP depletion. Under normal conditions, HuR gave a characteristic nuclear staining that was retained during 4 h of mock treatment (Fig. 2*A*, *top*). After 1 h in depletion medium, HuR was less abundant in the nucleus and showed some redistribution into the cytoplasm. During the course of ATP depletion, redistribution of HuR to the cytoplasm appeared to increase; by 4 h of depletion, many nuclei appeared empty of HuR, and the staining was almost entirely cytoplasmic (Fig. 2*A*, *bottom*). Subcellular fractionation and Western analysis of ATP-depleted cells over the 4-h time course confirmed the immunofluorescence findings (Fig. 2*B*). In contrast, mock-treated cells demonstrated no change in nuclear/cytoplasmic distribution of HuR over time (not shown).

To establish whether the nucleocytoplasmic redistribution of HuR was reversible, depleted cells were allowed to recover in normal growth medium, and immunolocalization of HuR was performed (Fig. 2*C*). As described above, cells depleted of ATP for 1 or 2 h displayed an increased cytoplasmic staining that was accompanied by decreased nuclear staining. When cells were recovered for 2 h in normal growth media, HuR staining decreased in the cytoplasm and increased in the nucleus. After 4 h of recovery, HuR staining returned to a nearly exclusive nuclear localization, demonstrating the reversible nature of HuR redistribution during energy depletion. To ensure that this redistribution was not a result of alterations in nuclear membrane integrity during ATP depletion and recovery, LLC-PK_1 cells were separated into nuclear and cytosolic fractions. Proteins from these fractions were subjected to Western analysis and probed for the presence of nuclear and cytoplasmic proteins (histone H3 and GAPDH, respectively). As shown in Fig. 2*D*, histone H3 was present primarily in the nuclear fraction throughout ATP depletion and recovery, whereas GAPDH was maintained in the cytosolic fraction. These results indicate that nuclei

remained intact during treatment and that changes in HuR distribution were the result of specific transport events.

HuR expression during ATP depletion and recovery

We next analyzed HuR mRNA and protein levels to determine whether ATP depletion caused alterations in HuR gene expression. Figure 3*A* demonstrates that ATP depletion induced a steady increase of HuR protein compared with untreated and mock-treated cells. This treatment resulted in an ~2.3-fold elevation in expression over the 4-h time course, as graphed in Fig. 3*B*. During the same time course, β-actin showed no change in expression (Fig. 3*A*). In contrast, when total cellular RNA was analyzed for HuR message using a competitive RT-PCR method, neither mock-treated nor ATP-depleted cells demonstrated increased mRNA levels, indicating that heightened HuR protein was not mediated by an elevation in mRNA content (Fig. 3*C*). To determine whether the augmented HuR protein levels were a result of new translation rather than decreased protein degradation, HuR expression during ATP depletion was assessed in the presence or absence of cycloheximide (Fig. 3*D*). Vehicle (DMSO)-treated cells produced the gradual increase in HuR first observed in Fig. 3*A*, but cycloheximide-treated cells showed no increase in protein over the 4-h ATP depletion period. These experiments are both illustrated in representative Western blots (*top*) and quantified as an average of five separate trials (*bottom*). These findings suggest that, even though the LLC-PK_1 cultures were severely depleted of energy stores, translation of HuR was increased. Upregulation of HuR during ATP depletion is potentially indicative of a mechanism by which limited cellular resources were utilized specifically for production of this gene product.

To establish how recovery in normal growth medium might affect HuR expression, cells were ATP depleted and then assayed for HuR protein and mRNA during a 4-h recovery period. Figure 4*A* demonstrates that the slightly amplified levels of HuR protein during 1 h of ATP depletion returned to normal levels during recovery. Similar results were found when cells were depleted for 2 h before recovery (data not shown). Unexpectedly, HuR mRNA levels, which were unchanged during ATP depletion, actually increased over the 4 h of recovery (Fig. 4*B*) to levels approximately fourfold that of the ATP-depleted cells (Fig. 4*C*). This change occurred whether the cells were initially ATP-depleted for 1 h, as in Fig. 4, or for 2 h (not shown). These results demonstrate a decoupling of HuR mRNA and protein levels; that is, HuR protein was maintained at baseline levels in the face of increasing mRNA levels. To determine whether increased mRNA expression was the result of new transcription or heightened stability, cells were allowed to recover in the absence or presence of actinomycin D, an inhibitor of new transcription. Figure 4*D* shows that this treatment resulted in a suppression of the recovery-induced increase in HuR mRNA, indicating that new transcription, rather than enhanced mRNA stability, mediated this change.

HuR during a second ATP depletion event

Increased transcription of HuR mRNA during the recovery phase suggested a mechanism by which cells may be primed for enhanced HuR production on a second cellular stress event. To establish whether this increase in HuR mRNA prepared the cell for a subsequent insult, cells that underwent 2 h of ATP depletion and 4 h of recovery were exposed to a second ATP depletion (Fig. 5*A*). As previously demonstrated, little change in HuR protein was noted within the first hour of an initial insult. However, within 15 min of the second insult, HuR protein levels rapidly began to increase and peaked at 30–45 min, with a 2.3-fold induction, as graphed in Fig. 5*A*. The increase in expression was followed by a reproducible decrease in protein by 1 h of ATP depletion. This rapid augmentation was in contrast to the

first injury, in which HuR protein increased to the same extent (2.3-fold) gradually over 4 h (Fig. 3, *A* and *B*).

We next analyzed HuR mRNA levels to discern whether they changed during a second insult. Figure 5*B* demonstrates that the increased quantity of transcript brought on by recovery remains at a constant, elevated level during a second ATP depletion, indicating that new transcription does not play a role in response to additional insults. Finally, to determine whether the rapidly increased HuR levels demonstrated in Fig. 5*A* were products of new translation rather than suppressed degradation, Western analysis was performed on LLC-PK_1 cells that had suffered a second insult in the presence of cycloheximide or vehicle (DMSO). Figure 5*C* illustrates that cycloheximide inhibited the rapid elevation in HuR protein normally demonstrated during this event. Both representative Western blots and graphical representation of five separate experiments are shown. These results demonstrate that translation of newly increased HuR mRNA is likely to account for the rapid amplification of protein during the second insult. Furthermore, this finding suggests that cells were preconditioned to mount a rapid response such that HuR levels were immediately heightened on induction of a subsequent ATP depletion and that the mRNA newly transcribed during the recovery period may facilitate this response. Indeed, addition of actinomycin D to ATP-depleted cells during the recovery phase abolished the rapid increase in HuR protein triggered by a second insult (Fig. 5*D*).

Immunocytochemistry demonstrates a dramatic difference in HuR distribution between the first and second insults (Fig. 6). Figure 6*A* shows cells treated with a single ATP depletion event for 1 h. As previously shown in Fig. 2*A*, HuR began to redistribute from nuclei to the cytoplasm. In contrast, cells subjected to a second ATP depletion for 1 h (Fig. 6*F*) still showed intense staining of the nuclei at levels similar to those for mock-treated or recovered (Fig. 6*B*) cells. However, there was a notable increase in cytoplasmic HuR levels for cells undergoing a second insult compared with baseline staining. This cytoplasmic staining became evident at ~30 min into the second ATP depletion (Fig. 6*D*) and was maintained through the 45-min (Fig. 6*E*) and 1-h (Fig. 6*F*) time points. Notably, this increased cytoplasmic staining correlated with increased HuR protein levels as demonstrated in Fig. 5*A*. It seems likely that the elevated levels of HuR allowed maintenance of abundant nuclear protein while also maintaining high levels in the cytoplasm. Indeed, it is possible that the cytoplasmic staining demonstrated in multiply stressed cells may be accounted for by new translation without the need for nuclear export.

These findings suggest that regulation of HuR expression and activity is mediated by multiple means during energy depletion and recovery, including transcriptional and translation mechanisms, as well as nucleocytoplasmic shuttling. A summary of our model of HuR regulation is presented in Table 1.

DISCUSSION

Following a stress event, cells must express a variety of gene products to aid in the repair and restoration of the cellular status quo. In addition, gene products required for cell survival must be maintained in the absence of normal transcription and translation. These stress-induced functions can be achieved in part by stabilization of mRNA transcripts. Several RNA-binding proteins exist that are involved in either the stabilization or destabilization of AU-rich mRNAs. HuR, a ubiquitously expressed protein, relocalizes from the nucleus to the cytoplasm where it binds ARE-containing mRNAs and stabilizes them. Here, we showed that HuR reversibly shuttles from a primarily nuclear to a primarily cytoplasmic distribution during energy depletion of renal epithelia. This result, coupled with our previous studies showing that HuR does indeed stabilize ARE-containing V-ATPase mRNAs during ATP

depletion in LLC-PK_1 cells (24), indicates a role for HuR in protecting renal cells from the stress of ischemic injury. More significantly, this study is, to our knowledge, the first detailed analysis of the mechanisms behind HuR gene regulation during cell stress and recovery. We found that HuR protein and mRNA levels are not coupled during these processes and that each phase of the cellular stress (first insult, recovery, and second insult) results in a stage-specific regulation of the mRNA and protein. Although the data from this study were obtained using an in vitro model of renal injury, our preliminary data from rat kidneys subjected to ischemia-reperfusion indicates that similar alterations in HuR distribution and expression may occur in vivo (not shown).

Rather remarkably, HuR protein increases via new translation during a 4-h period in which cellular ATP levels are reduced to <1% of normal. The mechanism by which this might occur during energy depletion is unknown. In other models of cell stress, phosphorylation of the α-subunit of translation initiation factor eIF2 serves as a convergent checkpoint to suppress overall translation levels. Paradoxically, translation of some proteins, such as the yeast transcription factor Gcn4p or the mammalian transcription factor ATF4, is promoted by this event (20,22). It is not clear whether eIF2α phosphorylation occurs in our system, although our preliminary data (not shown) are suggestive of this process. In other cell culture systems, it has been reported that ATP depletion does not lead to eIF2α phosphorylation but may simply prohibit formation of $eIF2{-}GTP{-}tRNA_i^{met}$ complexes required for translation initiation (27). However, endoplasmic reticulum (ER) stress, which can lead to activation of the eIF2 kinase PERK and eIF2α phosphorylation, has been shown to accompany ischemic injury in several tissues, including kidney epithelia (4,7,29,44). More recently, renal ischemia-reperfusion was shown directly to induce phosphorylation of eIF2α via PERK (37). Translational repression from ER stress has long been known to be a transient phenomenon (6,25), and recent data have shown that dephosphorylation of eIF2α by a phosphatase complex containing the stress-inducible protein GADD34 correlates with translation of stress-induced genes (40). Thus it possible that the slow increase in HuR protein over an initial 4-h ATP depletion period might result from this delayed phosphatase activity. More experimentation will be required to determine the role of eIF2 kinases and phosphatases in this model system.

We were further intrigued by regulation of HuR during the phase in which cells recovered from energy depletion. Although HuR protein levels returned to normal from their stress-augmented levels, HuR mRNA levels rose steadily during the recovery phase. This decoupling of protein and mRNA expression suggested that elevated mRNA levels might provide a pool of template for rapid translation on the onset of future insults. Indeed, the rapid upregulation of HuR protein produced by a second ATP depletion appears consistent with this hypothesis. This notion is further supported by our results showing that abolition of recovery-induced HuR transcription suppresses subsequent upregulation of HuR protein levels. Interestingly, decoupled mRNA and protein levels had been noted in previous surveys of HuR expression. Both normal and malignant tissues demonstrate a lack of correlation between HuR mRNA and protein expression (38,41), and it had been suggested that translational control of HuR plays a large role in regulating overall levels. However, as we have shown here, transcriptional control, especially during recovery from cell stress, may also be a key player.

One mechanism by which HuR mRNA levels may rise in the absence of their translation is sequestration of these transcripts in stress granules, which are complexes of stalled translational components that can form during some types of cell stress and recovery (1,28). It is believed that most mRNAs are contained in these complexes during stress events, but that critical stress-response transcripts, such as those encoding heat shock proteins, are excluded (39). Energy depletion previously was shown to induce formation of these

complexes in DU145 cells (27), and our preliminary data suggest that LLC-PK_1 cells transiently form stress granule-like complexes early in recovery from ATP depletion but that these dissipate over the recovery period (not shown). The timing of putative stress granule formation in LLC-PK_1 cells is consistent with recent studies in hippocampal neurons subjected to ischemia-reperfusion injury, in which it was shown that these complexes formed within the first 10 min of reperfusion and slowly decreased in number for up to 4 h (26). Thus it is possible that HuR mRNA transcripts temporarily may be unavailable for translation by localization to these compartments early in recovery, and may be freed within a matter of hours for translation on a second ATP depletion event. Nonetheless, more studies will be needed to understand the fates of HuR mRNAs in this model system.

A few other studies have examined changes in HuR protein expression following various forms of cell stimuli, including growth factor addition and activation through cell surface ligands. Changes in HuR expression and distribution were shown to occur in both serum-stimulated fibroblasts and anti-CD3-activated T cells (2). In both cases, HuR protein expression increased, albeit over the course of 18–24 h, in contrast to the rapid increases we detected in our energy depletion model. In a stress model of amino acid starvation, HuR redistributed from the nucleus to the cytoplasm over the course of at least 9 h; however, overall protein levels were diminished by 60% over a 12-h period (53). Thus the regulation of HuR gene expression varies widely with specific cellular stimuli and, likely, with specific cell types. Our model of ATP depletion is a very severe form of cell stress; with only minimal energy stores available for physiological processes, cells must respond quickly to ensure survival. It would be of interest to determine the threshold values of cellular ATP required to produce the expression and distribution changes in HuR we observed.

The mechanisms by which HuR levels are regulated are of interest not only for understanding the ways in which cells manage stress but also for their potential roles in cell cycle control and proliferation. It has been well documented that increased cytoplasmic abundance of HuR is associated with oncogenicity (5,11,13,14,33,38) because of its stabilization of growth- and differentiation-associated mRNAs, including those encoding c-*fos* (45), c-*myc*, tumor necrosis factor-α (12), and cyclin A and B1 (51). Because of the decoupling of mRNA and protein levels, HuR translational control mechanisms may play a large role in mediating carcinogenesis. Although many of the studies of HuR and malignancy were based on immunohistochemical analysis and were not strictly quantitative, a few studies showed that only a fewfold difference in cytoplasmic HuR can affect cell cycle control. In lung tissue, benign and malignant tumors showed cytoplasmic HuR levels of 4.3 and 6.7 times that of wild-type tissue, respectively (5). Furthermore, chronic overexpression of HuR by only three- to fivefold in colon cancer cell lines resulted in significantly increased tumor size when these cells were injected into nude mice (33). Therefore, because of the capacity of HuR for affecting the stability of thousands of mRNAs, including those involved in cell cycle control, defining the mechanisms that affect its expression will be of great utility in understanding cellular responses to external stimuli.

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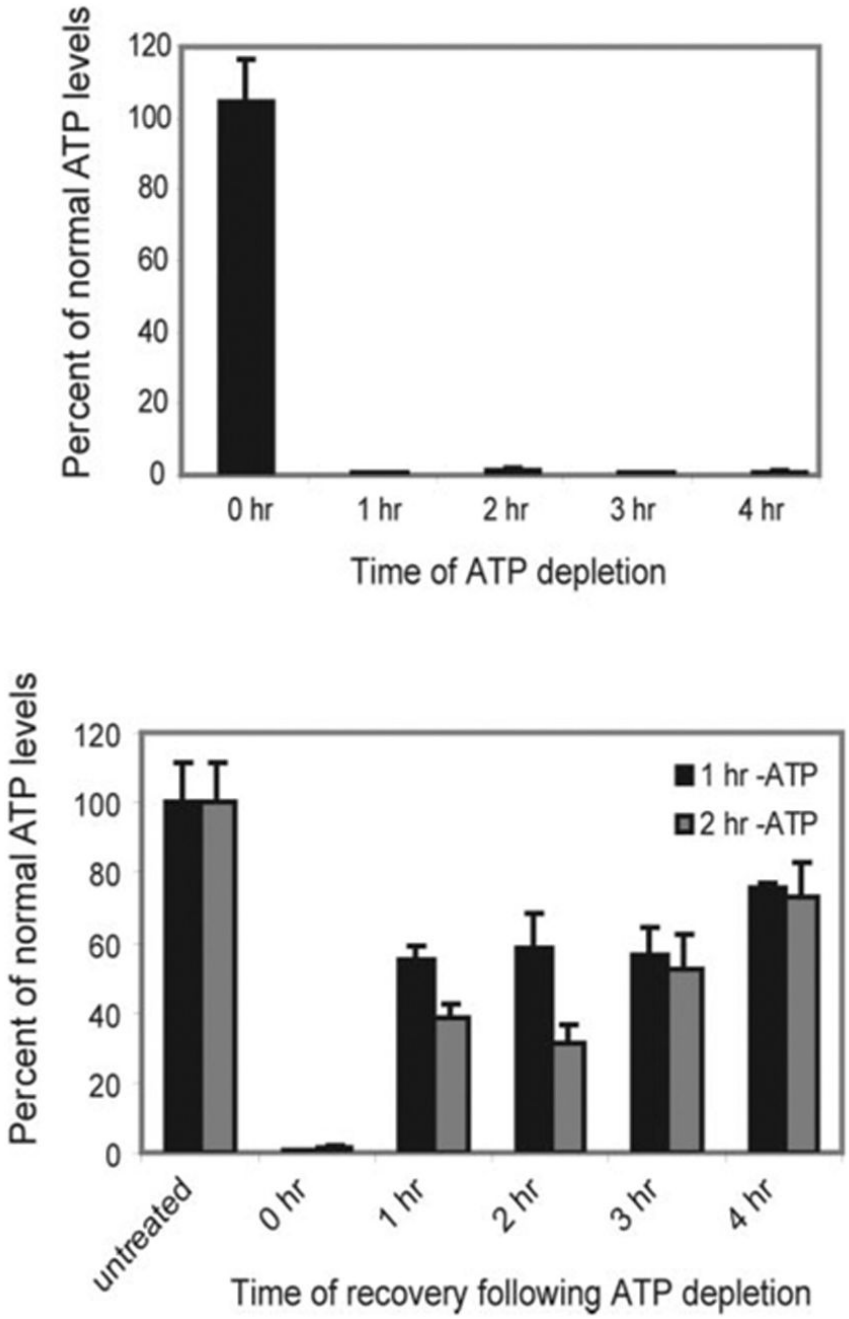

Fig. 1.

Cellular ATP levels in the model depletion system. *Top*: LLC-PK_1 cells were cultured in depletion medium for 0–4 h and assayed as described in Materials and methods. *Bottom*: LLC-PK_1 cells were ATP depleted for 1 or 2 h, allowed to recover for 0–4 h in normal culture medium, and assayed for ATP levels. In the experiments shown, normal ATP levels were measured at 79.2 pmol/μg protein, in line with previously published results (10). Bars represent means of triplicate samples ± SD.

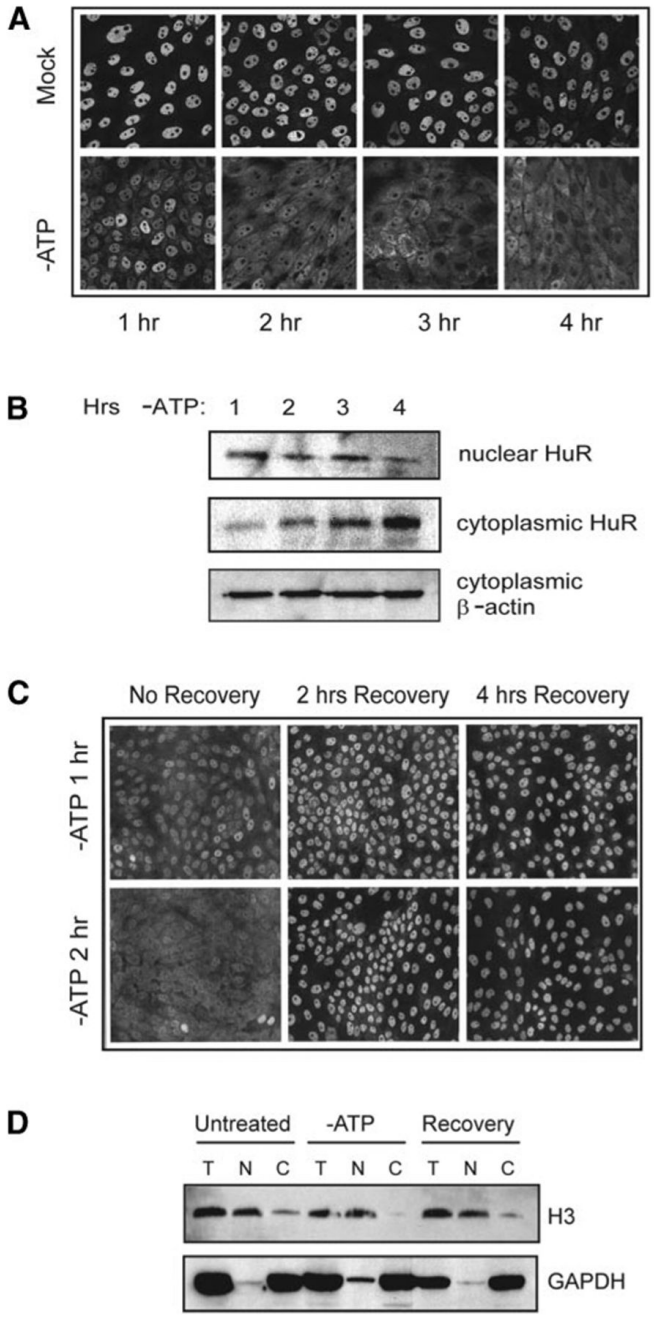

Fig. 2.

Nucleocytoplasmic shuttling during energy depletion and recovery. *A*: LLC-PK$_1$ cells were ATP depleted or mock treated for 1–4 h and labeled for HuR. *B*: ATP-depleted cells were fractionated into nuclear and cytoplasmic portions and assayed for HuR distribution by Western analysis. β-Actin was included as a loading control for the cytoplasmic fraction. *C*: LLC-PK$_1$ cells were ATP depleted for 1 or 2 h, allowed to recover for 2–4 h in normal culture medium, and labeled for HuR. *D*: LLC-PK$_1$ cells were left untreated, ATP depleted for 2 h, or ATP depleted for 2 h and then allowed to recover for 2 h more in normal growth medium. Total cellular protein (T) and nuclear (N) and cytoplasmic (C) fractions were

obtained and Western blotted for the nuclear protein histone H3 or the cytoplasmic glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

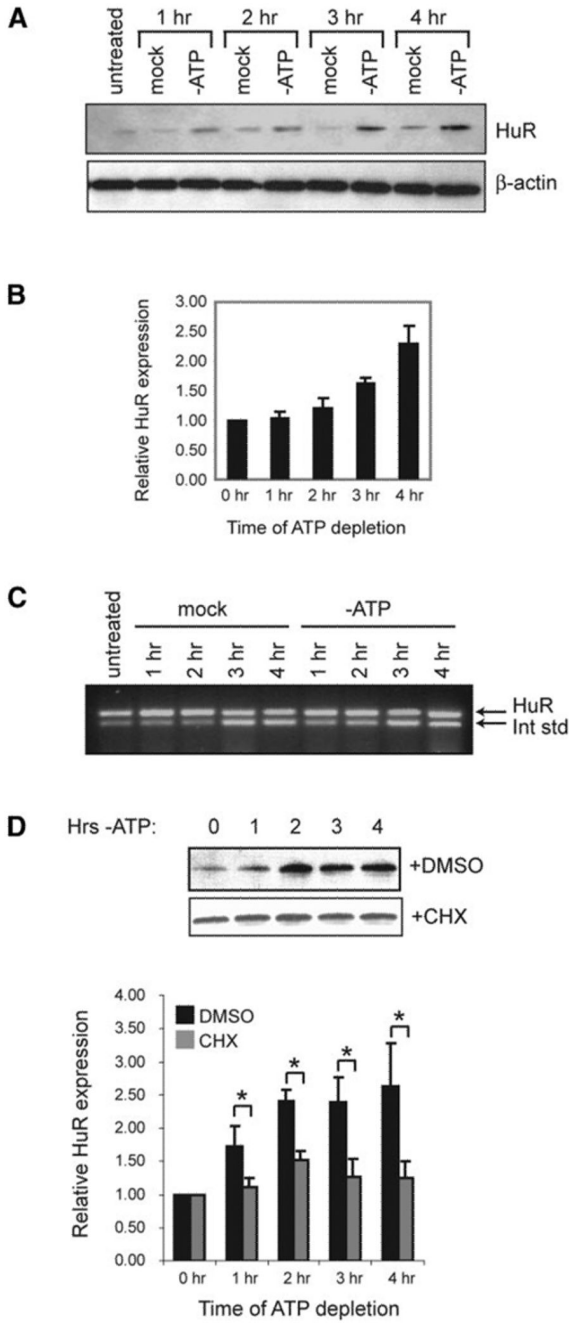

Fig. 3.

Expression of HuR protein and mRNA levels during ATP depletion. *A*: cells were ATP depleted or mock treated for 1–4 h, and HuR and β-actin were assayed by Western analysis. *B*: changes in HuR levels induced by ATP depletion in 3 separate experiments were calculated. Bars represent means ± SE. *C*: cells were treated as in *A*, and HuR mRNA levels were determined by competitive RT-PCR as described in Materials and methods. Int std, internal standard. *D*: LLC-PK$_1$ cells were ATP depleted for 0–4 h in the presence of 10 μg/ml cycloheximide (CHX) or vehicle (DMSO), and relative HuR expression was quantified by Western analysis. Representative blots are shown at *top*. The graphical analysis indicates

relative HuR levels from 7 separate experiments; bars represent means ± SE. *Statistically significant differences between CHX treatment and vehicle controls ($P < 0.05$).

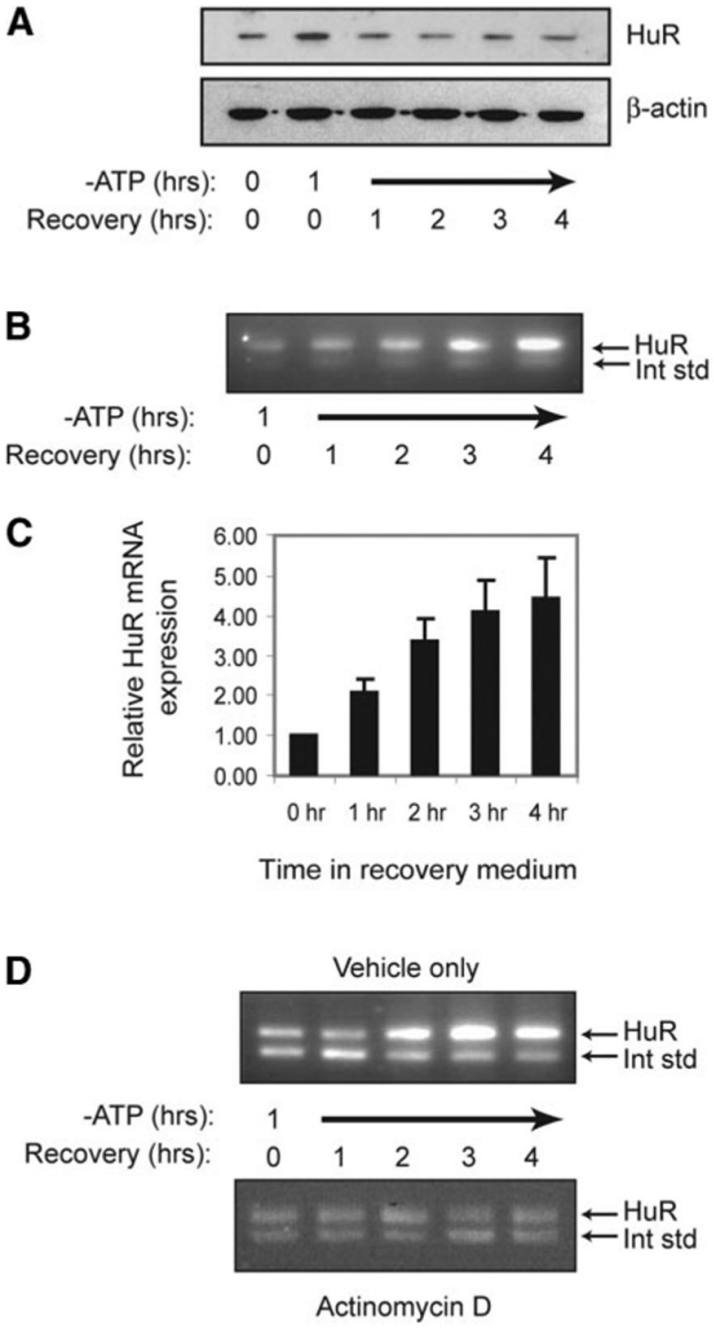

Fig. 4.

Expression of HuR protein and mRNA levels during recovery from ATP depletion. *A*: cells were ATP depleted for 1 h and allowed to recover in normal culture medium for 1–4 h, and HuR and β-actin were assayed by Western analysis. *B*: cells were ATP depleted and allowed to recover as in *A*, and HuR mRNA levels were determined by competitive RT-PCR. *C*: results of 5 experiments as in *B* were quantified and charted. Bars represent mean HuR expression levels ± SE. *D*: LLC-PK_1 cells were ATP depleted for 1 h and allowed to recover for 1–4 h in the presence of 5 μg/ml actinomycin D or vehicle (ethanol). HuR mRNA levels were assayed by competitive RT-PCR.

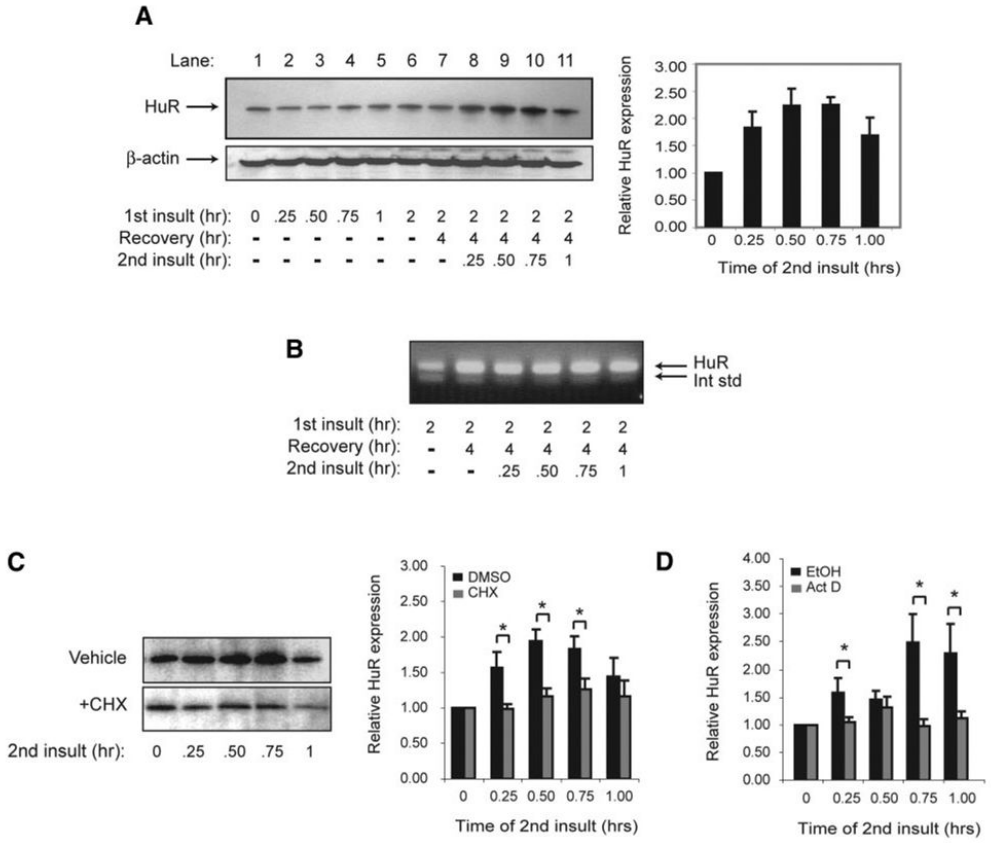

Fig. 5.

Expression of HuR protein and mRNA levels during a second ATP depletion event. *A*: cells were ATP depleted for up to 2 h, allowed to recover for 4 h, and replaced in depletion medium for a second insult. HuR and β-actin levels were assayed by Western analysis, as shown at *left*. At *right*, relative HuR levels during a second ATP depletion were quantified and charted. Bars indicate mean changes ± SE ($n = 4$). *B*: cells were ATP depleted for 2 h, allowed to recover for 4 h, and replaced in depletion medium for a second insult. HuR mRNA levels were assayed by competitive RT-PCR. *C*: cells were subjected to an initial ATP depletion of 2 h, allowed to recover for 4 h, and then depleted of ATP for an additional 1-h period. Either 10 μg/ml CHX or vehicle (DMSO) was added during the second insult. At *left*, representative Western blots of HuR levels from the second ATP depletion are shown. At *right*, relative HuR levels from 5 separate experiments were quantified and are shown in graphical format. Bars represent means ± SE. *Statistically significant differences between CHX treatment and vehicle controls ($P < 0.05$). *D*: LLC-PK$_1$ cells were subjected to multiple insults as in *A–C*, but actinomycin D (Act D) or vehicle (ethanol; EtOH) was added to cells during the 4-h recovery phase. Protein samples were obtained during the second insult and assayed by Western blot for relative HuR expression. Bars indicate means ± SE ($n = 7$). *Statistically significant differences between Act D and vehicle controls ($P < 0.05$).

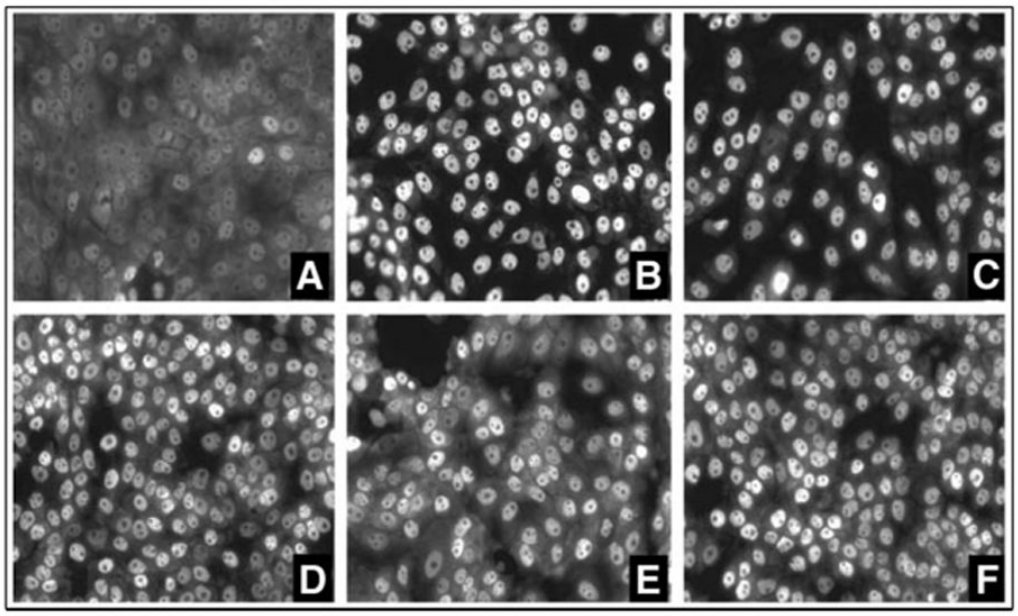

Fig. 6.

Redistribution of HuR during a second ATP depletion event. LLC-PK_1 cells were subjected to an initial ATP depletion of 1 h (*A*) and allowed to recover for 4 h (*B*). Cells were then subjected to a second ATP depletion for 15 min (*C*), 30 min (*D*), 45 min (*E*), or 1 h (*F*). HuR was detected by standard immunocytochemistry.

Table 1

Summary of HuR expression and distribution in ATP-depleted LLC-PK_1 cells

	1st ATP Depletion	Recovery	2nd ATP Depletion
mRNA levels	No change	Increase (transcriptional)	Maintenance at elevated levels
Protein levels	Slow increase (translational)	Return to baseline	Rapid increase (translational)
Distribution	Nucleus→cytoplasm	Cytoplasm→nucleus	Nuclear and cytoplasmic

HuR, human antigen R protein.