Genome-wide Identification and Quantitative Analysis of Cleaved tRNA Fragments Induced by Cellular Stress

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Background: Regulation of stress-induced tRNA cleavage by angiogenin is not well studied.

Results: tRNA fragment accumulation was higher during oxidative than hypertonic stress.

Conclusion: tRNA cleavage is regulated by the availability of angiogenin and tRNA substrate, levels of RNH1, and the rates of protein synthesis.

Significance: Stress-specific tRNA cleavage mechanisms and patterns will provide insights into novel stress signaling pathways.

Certain stress conditions can induce cleavage of tRNAs around the anticodon loop via the use of the ribonuclease angiogenin. The cellular factors that regulate tRNA cleavage are not well known. In this study we used normal and eIF2α phosphorylation-deficient mouse embryonic fibroblasts and applied a microarray-based methodology to identify and compare tRNA cleavage patterns in response to hypoxic stress, oxidative stress (arsenite), and treatment with recombinant angiogenin.

Most stress conditions decrease translation by inhibiting initiation (8–10) via phosphorylation of the initiation factor eIF2, which delivers the initiator tRNA (initiator-tRNA Met) to the translation initiation complex (8). Phosphorylation of the α subunit (eIF2α) on Ser-51 sequesters eIF2 in an inactive complex, thus impeding translation initiation (11). A family of protein kinases is known to phosphorylate eIF2α in response to various stresses in mammalian cells (12–18). Although most studies show that the global inhibition of protein synthesis during cellular stress is mediated through the phosphorylation of eIF2α, some stress conditions involve eIF2α phosphorylation-independent mechanisms that can also shut down global protein synthesis (17, 19).

Regulation of cleavage of transfer RNAs (tRNAs) has been suggested as a mechanism for decreasing protein synthesis during specific stress conditions (20).

tRNAs play an important role during the cellular adaptation to stress (21, 22). During amino acid starvation uncharged tRNAs act as signaling molecules to activate the Gcn2 kinase which phosphorylates eIF2α (23, 24). Recent reports have indicated that during oxidative stress, tRNAs are endonucleolytically cleaved around the anticodon, giving rise to small RNA species (tRNAs, 30–40 nt in length) which may enter various stress response pathways (25–27). tRNA fragments are present in most organisms even in unstressed cells, but they increase during stress (28–33). Stress-induced tRNA cleavage occurs via specific ribonucleases that are secreted from the stressed cells. These are RNY1 in yeast (34) and angiogenin (ANG) in mammalian cells (35, 36). In resting cells ANG is sequestered in vacuoles, in the nucleus or bound to its inhibitor RNH1 (37, 38). The percentage of tRNA cleavage during stress is very small defects have been implicated in various pathological states including cancer, diabetes, and inflammatory diseases (6, 7).

The cellular response to diverse stresses often includes inhibition of global protein synthesis, as a measure to conserve energy for repair and recovery. This response involves transcriptional and translational reprogramming (1–5). Translational reprogramming during stress is crucial for cell survival;
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(1–2%); hence, tRNA cleavage will not shrink the cellular tRNA pool significantly and decrease translation rates directly. However, it has been shown that when a pool of endogenous tRNAs is transfected into U2OS cells, protein synthesis rates decreased by ~20% (36). Recent reports show that transfection of cells with natural or selected synthetic 5’ tRNAs (5’ tRNAAla and 5’ tRNA<sup>Cys</sup>) can inhibit protein synthesis by displacing eIF4G/elf4A from both uncapped and capped RNAs (20) and also trigger assembly of stress granules (39). These exciting findings indicate that tRNAs can play an important role in fine tuning mRNA translation during stress.

Stress-induced tRNA cleavage is not a general stress response. For example, tRNA cleavage does not occur in mammalian cells subjected to caffeine or etoposide, which is known to induce apoptosis (36). In this study we have shown that tRNA cleavage occurs when mammalian cells are subjected to hypertonic stress, which causes cell shrinkage and macromolecular crowding. Increased osmolarity is often associated with inflammation and can have pathological consequences (40–46). Earlier work from this laboratory showed that during severe hypertonic stress there is a global shutdown of protein synthesis, and this translational arrest does not require phosphorylation of eIF2α (19). This important observation led us to investigate the mechanism and patterns of tRNA cleavage during cellular stress.

Using mouse embryonic fibroblasts (MEFs) from C57BL/6 mice (S/S) and mice with a homozygous mutation in eIF2α (S51A) that abolishes the phosphorylation site (A/A) we have established the patterns of tRNA cleavage during oxidative stress, hypertonic stress, and treatment with recombinant ANG. We have also determined the effects of eIF2α phosphorylation on the extent and specificity of cleavage. In all three scenarios MEFs deficient in eIF2α phosphorylation showed a higher accumulation of tRNAs including those derived from initiator-tRNAMet. The extent of tRNA cleavage was also dependent on the type and intensity of stress. We found that tRNA accumulation was higher during oxidative stress compared with hypertonic stress in both S/S and A/A cells. A trigger for tRNA cleavage during oxidative stress was the increased availability of ANG, due to the decreased levels of its inhibitor RNH1. Higher tRNA cleavage also correlated with higher rates of protein synthesis. This was further supported by a decrease in tRNA accumulation in ANG treated cells in the presence of protein synthesis inhibitors.

We propose that the accessibility of tRNAs to ANG-mediated cleavage during stress is higher when protein synthesis is active and tRNAs transit more frequently between the ribosome-bound (EF1A-bound) and the aminoacyl-tRNA synthetase-bound states (47–49). In support of this hypothesis, we did not observe tRNA binding to translation factors eIF2α and EF1α in ANG-treated cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—S/S and A/A MEFs were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Media were supplemented with sucrose to give the desired osmolality, assuming that the standard medium is 300 mosmol/liter. Unless otherwise specified, hypertonic medium was 600 mosmol/liter, obtained by addition of 300 mM sucrose to standard medium. S/S and A/A cells were treated with 500 µM sodium arsenite for the times specified. S/S and A/A cells were treated with 0.5 µg/ml recombinant ANG for 90 min unless otherwise specified. Hippuristanol (Hipp) (gift from J. Tanaka) treatments were done in combination with ANG for 90 min with concentrations as specified.

**siRNA Transfection**—Transfection of control siRNA and siRNH1 (Dharmacon, Thermo Scientific) by Lipofectamine 2000 (Invitrogen) was performed according to the manufacturer’s protocol. The cells were plated in DMEM with 10% FBS for 24 h and used at 70–80% confluence. Plates were washed twice and changed to Opti-MEM (GIBCO) without serum before transfection. After transfection cells were allowed to grow in normal media before the experiments.

**Isolation and Labeling of tRNA Fragments**—Total RNA enriched in small RNAs was obtained using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s manuals. Total RNA (5 µg) was labeled with radioactive [γ-<sup>32</sup>P]ATP and separated on 10% Tris-Borate-EDTA-urea acrylamide gels. Gel fractions containing cleaved tRNA fragments were visualized and isolated using intensities evaluated with a PhosphorImager and were eluted in 450 µl of crush and soak buffer (50 mM KOAc, 200 mM KCl, pH 7.5) with rocking at 4 °C overnight. After centrifugation, ethanol precipitation was performed to obtain the cleaved tRNA fractions. These were then hybridized onto the microarrays.

**Hybridization and Microarray Analysis**—The custom tRNA microarray contained a total of 95 oligonucleotide probes and one empty slot (for buffer only), each repeated eight times. There were 45 probes for mouse nuclear-encoded tRNAs, and other yeast and *Escherichia coli* tRNA probes for hybridization and normalization controls. The oligonucleotide probes were sequences complementary to the full-length tRNA sequences as reported previously (50, 51). The oligonucleotides were printed manually to generate large array spots necessary for radioactive detection. The array hybridization protocol has been described previously for the analysis of tRNA abundance and charging levels (52, 53).

Immediately prior to hybridization, microarray slides were immersed in boiling water for 2 min to remove undesired particles and uncross-linked oligonucleotides. Total RNA from cells was isolated using the mirVana miRNA isolation kit that allows an enrichment of small RNAs. Four bacterial tRNA transcripts (5’ and 3’ halves of *E. coli* tRNA<sup>Asp</sup>, 5’ and 3’ halves of *E. coli* tRNA<sup>Met</sup>) of sizes 35–43 nt were then added to the total RNA. The sequence of these transcripts were: *E. coli* tRNA Lys 5’; GGGUCGUGUAGCCUCAGUU; *E. coli* tRNA Tyr 3’; AAAUCUCCGUUGCAGGUCAGAGAAU; *E. coli* tRNA Tyr 3’, AAAUCUGCCGUUGCAGGUCAGAGAAU; *E. coli* tRNA Tyr 3’. The tRNA fragments were separated from full-length tRNA by running the samples in denaturing polyacrylamide-urea gels. The bands corre-
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Corresponding to the size of 35–40 nt were cut out of the gel and purified. For a size comparison $^{32}$P-labeled bacterial tRNA halves were run on the same gel. The RNA was extracted from the gel pieces by overnight elution with crush and soak buffer at 4 °C followed by ethanol precipitation. The labeled tRNA sample was then dissolved in microarray hybridization buffer (Sigma-Aldrich) containing 20 μg of salmon sperm DNA and 10 μg of poly(A). This was then applied to the hybridization chamber of the GeneTAC Hyb4 station (Genomic Solutions, Ann Arbor, MI). The following program was used for hybridization of tRNA fragments: 75 °C (2 min), 60 °C (probe introduction), 90 °C (5 min), 60 °C (16 h hybridization). Following hybridization, the slides were washed on the Hyb4 station twice with 2×SSC, 0.1% (w/v) SDS at 50 °C, twice with 0.1×SSC, 0.1% (w/v) SDS at 42 °C, and twice with 0.1×SSC at 42 °C. Slides were then removed from the station, rinsed with 0.1×SSC, and dried by centrifugation. They were then exposed to the PhosphorImager to visualize the signals deriving from the hybridized tiRNAs. Microarray slides were imaged using a Fuji BAS scanner. $^{32}$P intensities were quantified and corrected for background using Fuji BAS software. The median values for eight replicate spots were obtained for each tRNA and divided by the median value of the two tRNA standards added (E. coli tRNA$^{Lys}$ and E. coli tRNA$^{Tyr}$).

Immunoprecipitation—S/S cells were treated with recombinant ANG as specified. Total cell extracts were immunoprecipitated with 5 μg of EF1A (Millipore; catalog number 05-235) or eIF2α (Santa Cruz Biotechnology; catalog number sc133227), or Ago2 (Millipore; catalog number MABE56), or mouse control antibody (Santa Cruz Biotechnology) as described previously (19).

Bioinformatics Analysis—A human RNA transcripts database was downloaded from GenBank. Each tRNA probe sequence was divided into 30-nucleotide windows at each sequence position. This created a database of small tRNA fragments that were matched against all human RNA transcripts. The goal was to find all genomic loci that are similar to tRNA sequences (and complementary to the probes in the tRNA array). The matching of the tRNA fragments to the human RNA transcripts database allowed at most one mismatch in matches of at least 28 nt.

Other Methods—Western blot analysis was performed using standard procedures. Polyclonal rabbit anti-RNH1 was obtained from Proteintech Group, Inc. The anti-ANG antibody was bought from Santa Cruz Biotechnology (catalog number sc1409). Polyrribosome analysis by sucrose gradient sedimentation was carried out as described previously (19). Total RNAs from fractions of polyribosome analysis were prepared using TRIzol (Invitrogen). For quality control, ribosomal RNAs were visualized by agarose gel electrophoresis. mRNAs were monitored by RT-qPCR using Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sets used were: ANG_forward, GGCAACAAAGGC-AACATCAAGG, and ANG_reverse, AGGCCAACATTCT-TCACAGGC; RNH1_forward, GCTGGAGTCACGTGG-GATAAAGACC and RNH1_reverse, GTGGGTGTGCTGCT-ATTTGC; McI1_forward, TCAAGATGCCGTAACAAA-CCTGG and McI1_reverse, CCCGTTCGTCTTACAAGAAC.

RESULTS

Hypertonic Stress Induces tRNA Cleavage Independently of eIF2α Phosphorylation—It was previously shown that severe hypertonic stress (600 mosmol/liter) caused a global arrest of protein synthesis in S/S and A/A MEFs that is independent of eIF2α phosphorylation (19). We used these MEFs to determine the rates of protein synthesis under increasing intensity of hypertonic stress. Mild hypertonic stress (400 mosmol/liter) caused only ~10% decrease in protein synthesis rates in both S/S and A/A cells. Both cell types showed dramatic decreases in protein synthesis rates with increased stress intensity; ~50% decrease when subjected to 500 mosmol/liter and >80% when subjected to 600 mosmol/liter (Fig. 1A). We also determined the kinetics of protein synthesis inhibition during severe hypertonic stress. Protein synthesis dropped to ~30% after 5 min and decreased by >80% after 15 min (Fig. 1B). These data indicate that a signaling pathway is rapidly activated by severe hypertonic stress. This signaling is independent of eIF2α phosphorylation, given the similar responses for S/S and A/A cells.

It has been suggested that stress-induced tRNA cleavage plays a role in signaling protein synthesis inhibition during oxidative stress (20). To examine whether tRNA cleavage occurs during hypertonic stress and its dependence on eIF2α phosphorylation, we examined the induction of cleavage in stressed S/S and A/A cells (600 mosmol/liter). Because stress causes cleavage of ~1% of the total tRNA pool (36), we used radioactive labeling to increase the sensitivity of detection of the cleaved tiRNAs. The RNAs were end-labeled with radioactive [32P]ATP, and the stress-induced tiRNAs were separated from full-length tRNA on denaturing polyacrylamide-urea gels. In both S/S and A/A cells, two discrete RNA bands corresponding to ~30 and 40 nt were observed after 3 h of hypertonic stress (Fig. 1C). Although we observed bands of similar size in untreated S/S cells, the intensity of the bands was much higher in stressed cells.

It has been shown previously that oxidative stress in mammalian cells caused by arsenite treatment induced tRNA cleavage via the ribonuclease enzyme ANG (36). Consistent with previous results, treatment of S/S and A/A cells with arsenite or recombinant ANG resulted in accumulation of RNA bands of 30–40 nt (Fig. 1D), similar to the ones observed during hypertonic stress (Fig. 1, C and D). We concluded that hypertonic stress caused an increase in the accumulation of small RNAs which include tiRNAs. To compare the level of fragment accumulation during the three treatments (hypertonic media, arsenite, and recombinant ANG), each fragment band in control and treated cells was normalized to the sum of fragment and full-length tRNA bands, and the resulting value for each treatment was normalized to control. As shown in Fig. 1E, the highest cleavage was observed in cells treated with recombinant ANG (~1.6-fold in S/S and ~1.7-fold in A/A cells), followed by arsenite (~1.3-fold in both S/S and A/A) and hypertonic treatment (~1.1-fold in A/A cells). Although these data suggest that the increase in tiRNAs is not significant during hypertonic stress, they also show that the degree of tRNA cleavage is dependent on the kind of stress applied. These data also suggest that tRNA cleavage during hypertonic stress does not require
In fact, eIF2α phosphorylation might inhibit this stress-induced cleavage.

**Detection and Quantification of All Nuclear Encoded tRNA Fragments in Mouse Cells during Stress**—To determine whether there is differential accumulation of tRNAs during stress, we performed a microarray analysis on S/S and A/A cells subjected to three treatments (hypertonic media, arsenite, and recombinant ANG). We applied a multistep strategy to identify and quantitatively analyze all nuclear encoded tRNAs that undergo cleavage during cellular stress (Fig. 2, A and B, and “Experimental Procedures”). Gel elution of RNA corresponding to size ~30–45 nt and stringent hybridization conditions ensured that only RNA fragments corresponding to tRNA halves were detected in the array. In addition, we performed two control experiments to ensure that the majority of the signals on the arrays were derived from cleaved mammalian tRNAs. First, we hybridized only the four *E. coli* tRNA standards to the microarray (Fig. 2 C). In this case, only 36 spots exhibited signals above background. As expected, the most intense signals (16 spots corresponding to two tRNAs) were derived from the two *E. coli* tRNAs. Of the remaining 20 spots, 16 spots corresponded to mouse tRNAs Lys-TTT and Tyr-
TGA. Sequence analysis showed that the 5’ half of the *E. coli* tRNA^{Lys} has 60% sequence identity in a 30-nt overlap with the mouse tRNA^{Lys} and 60% sequence identity in a 32-nt overlap with the mouse tRNA^{Tyr} (Lalign server). The signal from the remaining 4 nonspecific spots was very weak, so these spots were not considered in our analysis.

We also tested the specificity of the array hybridization. We hybridized the tRNA fragments generated during arsenite treatment in the presence of a molar excess of the tRNA^{Gly} probe. This RNA should compete only with the tRNAs from Gly-sCC tRNAs. Indeed we saw that the signal from Gly-sCC tRNA was selectively diminished by the competitor RNA, but signals obtained from treated samples were again normalized to control samples. TreeView images were generated from these values where green indicated a lower and red a higher accumulation of fragments (Fig. 3A). Stress-induced tRNAs were found to be more abundant in A/A cells compared with S/S cells (Fig. 3A), again indicating that eIF2α phosphorylation is not essential for tRNA cleavage and may have a protective function.

Microarray signals were grouped according to the type of amino acid carried by the tRNAs. In S/S cells, most tRNAs carrying polar and charged amino acids gave rise to tiRNAs at 15 min and 1 h. In contrast, tRNAs carrying hydrophobic and small amino acids did not give rise to tiRNAs at the same time points. At the 3 h time point we observed accumulation of fragments derived from hydrophobic and small amino acid carrying tRNAs. In A/A cells, signals for tiRNAs originating from all four groups (hydrophobic, small, charged, and polar) were higher at 15 min and 1 h of treatment compared with control. At 3 h of treatment, tiRNAs derived from hydrophobic and small amino acid carrying tRNAs were still higher than control; however, tiRNAs derived from charged and polar amino acid carrying
tRNAs were lower than control. The initiator-tRNA$^{\text{Met}}$ fragment was observed at all three time points in A/A cells but only at the 3 h time point in S/S cells (blue arrow, Fig. 3A). These data suggest that the nature and availability of tRNA substrates may be among the limiting factors in the generation of tiRNAs during stress.

Within the limitations posed by the tRNA microarrays (inability to deduce absolute values), we have made quantitative comparisons to generate patterns of cleavage for each stress condition in both S/S and A/A cells. This was done to allow comparisons of cleaved tRNAs between different stresses. For each set of microarray data we normalized each tRNA fragment signal to the median value for that set. Signals indicating propensity toward cleavage were designated as High (red), tRNAs with fragment signal closer to median value were designated Medium (black), and tRNAs with fragment signals lower than median value were designated Low (blue).

Data from the Median analysis of S/S and A/A cells subjected to hypertonic stress is shown in Fig. 3B. As expected, the median value for the cleavage signal was higher in A/A than S/S cells. Interestingly at 15 min, initiator tRNA$^{\text{Met}}$ showed a High signal. The following conclusions can be drawn from our data: (i) the highest total signal for tRNA cleavage was observed at the
15 min time point of treatment for both cell types. (ii) In S/S cells, a higher accumulation with increased time of treatment was observed for tiRNAs derived from hydrophobic amino acid carrying tRNAs. In contrast, tiRNAs derived from polar and charged amino acid-carrying tRNAs showed lower accumulation. (iii) The total microarray signal for tRNA cleavage was higher in A/A cells, compared with S/S cells, independent of the time of treatment.

**Stress-induced tRNA Cleavage Is More Pronounced during Arsenite-induced Oxidative Stress**—tRNA cleavage during oxidative stress has been previously reported (35, 36). To determine whether there are stress-specific patterns of tRNA cleavage we subjected S/S and A/A cells to arsenite treatment (500 μM sodium arsenite for 2 h) and analyzed the resulting tiRNAs on the microarrays. The signals were normalized in the manner described above, and TreeView images were generated for S/S and A/A cells (Fig. 4A). tiRNA fragments were much more abundant in A/A than in S/S cells (Fig. 4A). In both cell lines we saw that tRNA^Gly^ (sCC/TCC) and tRNA^Pro^ (hGG) were extensively cleaved. However, the cleavage of initiator-tRNA^Met^ was only observed in A/A cells (Fig. 4A, blue and purple arrows). This observation was consistent with tRNA cleavage data reported earlier for mammalian cells subjected to arsenite-induced oxidative stress (36). Interestingly, the initiator-tRNA^Met^ cleavage was observed in A/A cells in both hypertonic and oxidative stress.

**FIGURE 4.** Stress-induced tRNA cleavage is more pronounced when S/S and A/A cells are subjected to arsenite-induced oxidative stress. A, accumulation of tRNA fragments by oxidative stress (500 μM sodium arsenite, 2 h) in S/S and A/A cells shown as TreeView image. All values are relative to untreated control. TreeView image colors and their relevance to tRNA cleavage were described in Fig. 3. Initiator-tRNA^Met^ fragment is only observed in A/A cells. Purple arrows indicate tRNA fragments which were shown to undergo cleavage in U2OS cells when treated with sodium arsenite (36). B, comparative cleavage profile of S/S and A/A cells during hypertonic stress. The designation of High (red), Medium (black), and Low (blue) signals is as described in Fig. 3.
Median analysis showed different cleavage profiles between S/S and A/A cells (Fig. 4B). We conclude the following: (i) tRNA cleavage induced by arsenite treatment was more pronounced compared with hypertonic treatment. (ii) Similar to hypertonic stress, the total microarray signal for tRNA cleavage and the median values were higher in A/A than S/S cells. (iii) We did not find a clear trend for cleavage of specific groups of tRNAs, as observed with hypertonic stress treatment. Our data suggest that the type of stress, the availability of substrate tRNAs, and the availability/activity of the nuclease ANG are among the factors that regulate tRNA cleavage in response to stress.

**A/A Cells Show Higher tRNA Cleavage Than S/S Cells during Recombinant ANG Treatment** —We have shown above that hypertonic and arsenite-induced stress caused higher tRNA cleavage in A/A than S/S cells. It is well established that tRNA cleavage occurs via the nuclease ANG, which can be secreted from cells and be taken up by adjacent cells (37). In mammalian cells, ANG is also present in the cytoplasm but is rendered inactive by the inhibitor RNH1 (38). We therefore determined whether the differential response of S/S and A/A cells to stress was also observed in ANG-treated cells. Recombinant ANG induced tRNA cleavage in both cell types. tRNAs corresponding to initiator-tRNAMet were observed in both S/S and A/A cells (Fig. 5A, blue arrow). Consistent with oxidative and hypertonic stress treatments, tRNA cleavage was higher in A/A cells (Fig. 5A). Fragments corresponding to all tRNAs except tRNA1ω(TAT) were observed (Fig. 5A, green arrow). The median value for A/A cells was 9.0-fold higher than S/S cells (Fig. 5B). Our data indicate that S/S cells were more resistant to ANG-mediated tRNA cleavage than A/A cells.

**In vitro studies in cell free lysates and Xenopus oocytes** have shown that recombinant ANG cleaves cellular tRNAs nonspecifically (54). However, our data point toward a more selective mechanism in vivo. Not all tRNAs in S/S cells showed accumulation of fragments, and loss of eIF2α phosphorylation (A/A cells) rendered cells more sensitive to cleavage. Interestingly, the degree of cleavage during arsenite treatment was similar to the ANG treatment in S/S cells. In A/A cells, the median value for cleavage for ANG treatment was 5-fold higher than hypertonic treatment and 3.5-fold higher than arsenite treatment. These data suggest that the differences in tRNA cleavage patterns in S/S and A/A cells during hypertonic and arsenite stress may be due to the regulation of ANG levels and activity during these two stress conditions.

**Knockdown of the ANG Inhibitor RNH1 Enhanced Production of tRNA Fragments in Both S/S and A/A Cells during Oxidative Stress but Only in A/A Cells during Hypertonic Stress** —It has been reported that RNH1 can bind to and inhibit ANG in the cytoplasm (55). In U2OS cells siRNA-mediated knockdown of RNH1 induced greater tRNA cleavage during oxidative stress (36). To determine whether ANG availability is an important factor in the generation of tRNAs during hypertonic and oxidative stress we used our microarrays to investigate the effect of RNH1 knockdown on tRNA generation in S/S and A/A cells during hypertonic and oxidative stress. Knockdown of RNH1 was anticipated to increase the level of active ANG in the cytoplasm and enhance stress-induced tRNA cleavage. S/S and A/A cells were transiently transfected with siRNAs, the cells were subjected to stress, and tRNAs were analyzed on microarrays (Fig. 6). In S/S cells transfected with si-RNH1 and treated with arsenite, there was a marked increase in tRNAs compared with si-CON-transfected cells. During hypertonic stress we did not observe any difference between si-CON- versus si-RNH1-transfected S/S cells (Fig. 6B, left). In A/A cells during hypertonic stress, accumulation of tRNAs was higher in cells transfected with si-RNH1 than si-CON. Because arsenite-induced cleavage was already very high in A/A cells the difference between si-RNH1 and si-CON cells was not significant (Fig. 6B, right). A/A cells transfected with si-RNH1 had ~1.3-fold higher accumulation of tRNAs compared with A/A cells transfected with si-CON even at the resting state (data not shown). We did not observe an increase of cleavage in S/S cells. This observation is similar to the finding with exogenous ANG, where we observed higher accumulation of cleaved tRNAs in A/A than in S/S cells. These data might be a reflection of lower siRNA transfection efficiency in S/S cells. However, two alternative hypotheses may also explain our findings: (i) eIF2α phosphorylation may prevent tRNA cleavage during hypertonic stress, under conditions where there is increased ANG availability due to lower levels of the inhibitor; and (ii) availability of the substrate tRNAs may be lower in S/S cells during hypertonic stress. The latter was supported by the observation that S/S cells treated with increasing amounts of ANG did not show increased tRNA cleavage (data not shown). Therefore, availability of tRNA substrates for cleavage may be a limiting factor for tRNA accumulation during stress.

In agreement with prior reports for mammalian cells our data showed that arsenite-induced tRNA cleavage increased upon knockdown of the ANG inhibitor (35, 36). Knockdown of RNH1 also increased tRNAs during hypertonic stress in A/A cells but not in S/S cells. These data further strengthen our hypothesis that stress-induced tRNA cleavage is regulated differently during different stress responses. To understand the mechanisms that regulate tRNA cleavage during different stress conditions, we studied the regulation of ANG and its inhibitor RNH1.

**Stress-induced tRNA Cleavage Is Regulated by the ANG Inhibitor RNH1** —Higher levels of tRNAs during oxidative stress than in hypertonic stress indicated that ANG and its inhibitor RNH1 are regulated differently during these two stress conditions. We first determined the effect of oxidative stress and hypertonic stress on the levels of ANG and RNH1 proteins in S/S and A/A cells. ANG levels showed a small decrease during oxidative stress in both cell types. In contrast, during hypertonic stress we did not observe any change in ANG levels (Fig. 7A). The levels of RNH1 decreased considerably during oxidative stress in both S/S and A/A cells but remained the same during hypertonic stress (Fig. 7A).

We also examined the levels of ANG and RNH1 mRNAs during oxidative and hypertonic stress (Fig. 7B). A significant increase was observed for ANG mRNA levels in A/A cells during arsenite treatment and in both S/S and A/A cells during hypertonic stress. Arsenite treatment caused a slight decrease in the levels of the RNH1 mRNA in A/A cells, but no change was observed in S/S cells. The levels of RNH1 mRNA increased in both S/S and A/A during hypertonic stress (~1.2-fold).
These data suggested that sustained mRNA levels for ANG and RNH1 may contribute to the observed tRNA cleavage during hypertonic stress. Also, the decreased levels of RNH1 protein in S/S and A/A cells during arsenite treatment are not the result of decreased mRNA levels.

To determine whether differences in the translation efficiency of the RNH1 mRNA can account for the different levels of the RNH1 protein in the two stress conditions, we evaluated the distribution of the RNH1 mRNA in polyribosome fractions during oxidative and hypertonic stress. Arsenite treatment caused a more dramatic decrease of global protein synthesis in S/S cells compared with A/A cells (Fig. 7C). This is consistent with a decrease in abundance of polyribosomes in S/S cells and a less pronounced decrease in A/A cells (Fig. 8A), suggesting that eIF2α phosphorylation during arsenite treatment contributes to the decreased rates of protein synthesis. It has been shown earlier that hypertonic stress decreases the abundance of polyribosomes in both S/S and A/A cells, and our data are in full
agreement (19). Hypertonic stress caused attenuation of the translation of the RNH1 mRNA in both S/S and A/A cells. Polyribosome-associated RNH1 mRNA decreased by 43 and 38% in S/S and A/A cells, respectively (Fig. 8B). Arsenite treatment of cells also caused translational attenuation of RNH1 mRNA, although not to the same degree as hypertonic stress (Fig. 8B). Polyribosome-associated RNH1 mRNA decreased by 20 and 18% in S/S and A/A cells, respectively. These data suggest that the dramatic reduction in RNH1 protein in S/S and A/A cells treated with arsenite could not be correlated with less efficient translation of the RNH1 mRNA. In contrast, the RNH1 mRNA was more efficiently translated in cells treated with arsenite as compared with hypertonic stress treatments (Fig. 8B). As a control we also examined the distribution of the cap-dependent Mcl-1 mRNA during hypertonic and oxidative stress. During hypertonic stress, polyosome-associated Mcl-1 mRNA decreased by 23 and 27% in S/S and A/A cells respectively, similar with previous reports (19). As expected, during arsenite treatment polysome-associated Mcl-1 mRNA decreased by 16 and 9% in S/S and A/A cells, respectively (Fig. 8B), in agreement with the kinetics of inhibition of global protein synthesis rates during oxidative stress (Fig. 7C).

In summary, our data indicated that ANG mRNA and protein levels in S/S and A/A MEFs showed minimal changes during both hypertonic and oxidative stress. However, the translation of the RNH1 mRNA was attenuated in both cell types during both stresses. The translational attenuation was higher during hypertonic stress than in oxidative stress, which is in agreement with the inhibition of global protein synthesis by these stresses. However, during oxidative stress there was a marked decrease in RNH1 protein levels in both S/S and A/A cells whereas the protein levels did not change during hyper-

![Graphical representation of RNH1 and Mcl-1 mRNA translation during stress conditions.](image)
totic stress. This decrease in RNH1 protein levels can contribute to the higher tRNA cleavage during oxidative stress.

**Global Protein Synthesis Rates Modulate ANG-mediated tRNA Cleavage by Regulating Substrate Availability**—The differences in tRNA cleavage during oxidative stress and hypertonic stress can be attributed to the differential regulation of RNH1 by the two stresses. To explain the differences in cleavage between the two cell types (S/S and A/A) and at different times of treatment (with hypertonic stress) we hypothesized that the rates of protein synthesis in the cells play a regulatory role. ANG-mediated cleavage of tRNAs should depend on accessibility of tRNA substrates. We thought that during active protein synthesis there is increased shuttling of tRNA between the aminoacylated and nonaminoacylated states, and this shuttling increases susceptibility to cleavage (47–49). Based on this hypothesis we expected that active protein synthesis and increased ANG levels would result in higher tRNA cleavage. In contrast, dramatic inhibition of protein synthesis as observed during hypertonic stress treatment should result in lower cleavage due to slower recycling of tRNAs. We also hypothesized that translation factors such as EF1A and elf2α should bind only full-length tRNAs and not tiRNAs. This hypothesis was tested next.

We first tested the effect of Hipp, an inhibitor of translation initiation (56–58), on global protein synthesis rates in S/S cells in the presence and absence of ANG. In both cases we saw an 80% decrease of protein synthesis (Fig. 9A). We next subjected S/S cells to increasing concentrations of ANG to enhance tRNA cleavage. We made the important observation that tiRNA accumulation did not increase beyond a certain level even in the presence of increasing ANG (data not shown). This further validates our hypothesis that ANG-mediated tRNA cleavage is limited by the amount of available tRNA substrates in the cells.

To test the hypothesis that ANG-mediated tRNA cleavage should decrease with inhibition of protein synthesis, we studied the effect of Hipp on ANG-treated cells. Our data showed that increasing concentrations of Hipp decreased protein synthesis and ANG-mediated tRNA cleavage in S/S cells (Fig. 9, B and C). To further validate our hypothesis we performed microarray analysis of tiRNAs from S/S cells treated with ANG alone or ANG in the presence of Hipp. In agreement with our hypothesis, we saw lower levels of tiRNAs in cells treated with ANG in the presence of Hipp. The total microarray signal data showed a difference of 1.4-fold (Fig. 9D).

Finally, to test our hypothesis that tiRNAs do not associate with the tRNA recycling proteins EF1A and EF1α, we immunoprecipitated EF1A and elf2α from ANG-treated S/S cells. We labeled either RNA isolated from the total cell extracts (input) or the immunoprecipitations (Fig. 9, E and F). The results show that only full-length tRNAs were bound to EF1A and elf2α in ANG-treated cells (Fig. 9F). These data support the conclusion that regulation of ANG and tRNA-substrate availability are critical factors in the generation of tiRNAs during stress.
DISCUSSION

It is well established that the function of tRNAs in both prokaryotic and eukaryotic cells is not limited to delivering amino acids to the translation machinery. In the emerging field of tRNA biology, one of the most recent discoveries is the existence of tRNA cleavage pathways that are activated during various cellular stresses (20, 26, 35, 36). Stress-induced tRNA fragments are not random degradation intermediates, but instead these constitute a novel class of small RNAs that participate in several cellular signaling pathways including microRNA-mediated signaling (59, 60).

During severe hypertonic stress, shutdown of global protein synthesis is very rapid (Fig. 1B); there is a >80% decrease in protein synthesis by 15 min. This translational arrest is independent of eIF2α phosphorylation. In our search for novel mechanisms involved in cellular adaptation to hypertonic stress, we discovered the hitherto unreported response of tRNA cleavage. Using quantitative high throughput tRNA microarrays we identified and quantified all tRNA fragments that were generated during hyperosmotic stress in wild type S/S and eIF2α phosphorylation mutant A/A MEFs. We applied the same analysis to S/S and A/A cells during arsenite-induced oxidative stress and treatment with...

FIGURE 8. Translation of RNH1 mRNA is attenuated during oxidative stress and hypertonic stress. A, S/S and A/A cells were treated for 3 h with hypertonic medium and for 2 h with 500 μM sodium arsenite, and polyribosome profiles were analyzed by sucrose gradient centrifugation (69, 70). Gradients were fractionated, and absorbance at 254 nm was recorded (upper panels). The positions of 80S ribosomes and polyribosomes are indicated. Equal volumes of RNA isolated from the gradient fractions were run on agarose gels to analyze ribosomal RNAs (lower panels). B, equal volumes of fractions from A were analyzed for the distribution of mRNAs by RT-qPCR. The percentage of RNH1 and Mcl-1 mRNAs on polyribosomes (pooled fractions 7–12) is shown. The significance of differences among means of stress treatments versus control was evaluated using Student’s t test; error bars, S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
recombinant ANG. Comparison of the cleavage profiles of all microarray data suggested that the extent of tRNA cleavage is governed by the nature of stress. The tRNA cleavage profiles during oxidative and hypertonic stress and recombinant ANG treatment were all unique with certain convergent patterns. Accumulation of stress-induced tiRNAs was higher during oxidative stress than hypertonic stress. The same stress (hypertonic) when applied for different times created different patterns (Fig. 3A).

The eIF2α/H9251 phosphorylation status of the cells was an important factor for tRNA cleavage. In all three stresses, the mutant
A/A cells showed higher cleavage than wild type S/S cells. For example, fragments derived from initiator-tRNA\textsubscript{Ala} accumulated in A/A cells during hypertonic (at all time points) and oxidative stress; however, the same was not true for S/S cells (Figs. 3 and 4). In a recent study, 5′ tiRNAs halves derived from tRNA\textsubscript{Ala} and tRNA\textsubscript{Cys} were shown to inhibit protein synthesis via the use of 5′-terminal oligoquanine (TOG) motifs (20). We observed that tiRNAs from tRNA\textsubscript{Ala} accumulated during all stress conditions in both S/S and A/A cells. However, fragments from tRNA\textsubscript{Cys} were only observed in A/A cells. Using sequences from the tRNA database, we found two more human tRNA species that contain the 5′-TOG motif: tRNA\textsubscript{Tyr}(GTA) (database id: tdbD00011647) and tRNA\textsubscript{Val}(AAC) (database id: tdbD00010986). Our microarray data show that similar to tRNA\textsubscript{Ala}, tiRNAs corresponding to tyrosine (GTA) and valine (mAC) are not observed in S/S cells during any stress treatments (Figs. 3 and 4). They are also protected from recombina nt ANG treatment (Fig. 5). Further studies are required to determine the stress-specific functions of tRNAs that contain the TOG motif.

Stress-mediated tRNA cleavage has been suggested to occur near the anticodon leading to fragments of 30–40 nt. tRNAs are extensively modified around the anticodon (61, 62). We speculate that the tRNA modifications around the anticodon loop can play an important role in determining the propensity toward cleavage. To our knowledge, only one study reported on the role of tRNA modifications in stress-induced cleavage. In Drosophila, methylation by Dnmt2 can protect certain tRNAs from stress-induced cleavage (63). Modification of tRNAs is a dynamic process, and not all tRNAs are modified to the same degree. In a resting cell a fraction of tRNAs is always hypomodified (51). Regulation of tRNA modifications during the stress response has not been studied. These facts make it difficult to correlate modification patterns of tRNAs with stress-induced cleavage. Due to our great interest in this subject we have made an attempt to compare all of the known tRNA anticodon loop sequences for human and mouse with the tRNA data obtained in this study (64). The majority of known mouse and human tRNAs are modified at the anticodon loop (Table 1). At this time we do not have enough literature reports or experimental data to conclude as to the significance of tRNA modifications on stress-induced cleavage.

In this report we also studied the underlying mechanisms of stress-induced tRNA cleavage. Our data showed that during both hypertonic and oxidative stress responses, translation of RNH1 mRNA was attenuated. However, the level of RNH1 protein only decreased during oxidative stress (Fig. 7A). Decrease of RNH1 during oxidative stress will lead to an increase in available free ANG which in turn will cause higher cleavage. This can explain the observed difference in tRNA cleavage between oxidative and hypertonic stress. The decrease in RNH1 protein during oxidative stress could be due either to the degradation of the protein during arsenite-treatment or to the inhibition of protein synthesis. If the latter was true we would expect to see a similar decrease of RNH1 during hypertonic stress, but our data say otherwise. It has been reported that the proteasome action is inhibited during hypertonic stress; if RNH1 undergoes proteasome-mediated decay this would explain why the decrease in protein levels occurs only during oxidative stress (65). However, we did not observe a stabilization of RNH1 by treatment with a proteasome inhibitor (data not shown), which indicates absence of proteasome-mediated decay for RNH1. Alternatively, this stabilization may involve interaction with another protein during osmotic stress. Our study is the first to report this differential regulation of RNH1 during cellular stress and its effect on tRNA cleavage. Recently, several reports implicated ANG in pathogenesis of neurodegenerative diseases such as Parkinson disease and amyotrophic lateral sclerosis (66, 67) as well as stress responses (68). Our finding may promote increased understanding of ANG regulation in disease states.

Another novel finding of this study is the cross-talk between global protein synthesis rates and stress-induced tRNA cleavage. In a resting cell, the majority of the endogenous tRNAs are involved in mRNA translation (47–49). During the stress response tRNA cleavage is triggered by the activation of ANG, either via release from the nucleus or by the decay of its inhibitor RNH1 (Fig. 7A). Once the enzyme has been activated, tRNA substrate availability may be the limiting factor for the cleavage. We propose that this availability is controlled by the rate of protein synthesis. Modest decreases in protein synthesis rates during stress will allow more available tRNAs that are accessible to active ANG. However, when protein synthesis is sharply inhibited, most tRNAs will be bound in complexes, thus reducing cleavage. We have shown that the rate of tRNA cleavage decreases when protein synthesis is inhibited by Hipp (Fig. 9, C and D). Further support on the limited availability of tRNA substrate for cleavage during stress came from the finding that there was no parallel increase in tRNA cleavage with increasing concentrations of ANG in unstressed cells (data not shown).

The extent of tRNA cleavage during hypertonic and oxidative stress showed a positive correlation with the rate of protein synthesis. We have observed that during oxidative stress, tRNA cleavage and protein synthesis rates are higher in A/A than S/S cells (Figs. 4A and 7C). Both S/S and A/A cells undergo rapid decrease in global protein synthesis during hypertonic stress. It is therefore expected that the lower substrate availability coupled with the lower concentration of active ANG will result in lower tRNA cleavage during hypertonic stress. Dependence of tRNA cleavage on protein synthesis rates can also explain the higher cleavage in both S/S and A/A cells during early hypertonic stress when the protein synthesis rate is higher than late times of treatment (Fig. 3A). Our overall data suggest that factors that influence the intensity and pattern of tRNA cleavage during stress include (i) type of stress, (ii) ANG availability and activity, (iii) tRNA substrate availability, and (iv) global protein synthesis rates.

The three different stress conditions of this study generated different cleavage profiles. Significant differences were also observed among isoacceptors of the same tRNA throughout this study. For example, although most tRNA fragments accumulated in A/A cells during oxidative, hypertonic, and/or ANG treatment, fragments corresponding to tRNA\textsubscript{Ile}(TAT), which codes for the rare isoleucine codon AUA, was conspicuously absent in all three conditions. This finding indicates that either tRNA\textsubscript{Ile}(TAT) is present at very low abundance in A/A cells or this tRNA is protected from stress-mediated cleavage. Another
### TABLE 1

**tiRNA data and corresponding anticodon loop sequences**

TiRNA data were obtained from the microarrays of S/S and A/A cells treated with hypertonic stress (3 h), oxidative stress, or recombinant ANG. Signals higher than control are denoted as +, and signals lower than control are denoted as −. In cases where there are multiple isoacceptors for the same tRNA, specific isoacceptors are indicated within parentheses. The anticodon loop sequences, including the modification codes for human and mouse tRNAs, were obtained from the tRNA database (64). The tRNAs whose anticodon loop sequences are unknown are denoted as u/k.

| tRNA | Anticodon loop sequence | Hyperosmotic stress | Oxidative stress | Angiogenin |
|------|-------------------------|---------------------|------------------|------------|
| Ala  | JUGOCP u/k              | + (IGC) + (hGC)    | + (IGC) + (hGC) | + (IGC)   |
| Arg  | UICGKA u/k              | - (IGC) - (hGC)    | + (IGC) + (hGC) | - (IGC)   |
| Asn  | UGAA u/k                | - (GTG) - (GGG)    | + (GTG) + (GGG) | - (GTG)   |
| Asp  | U/k u/k                 | + (GCT) + (GGG)    | + (GCT) + (GGG) | + (GCT)   |
| Cys  | U/k u/k                 | - (GCC) - (GGG)    | + (GCC) + (GGG) | - (GCC)   |
| Gin  | UGA/GAA u/k             | + (TGG) + (GGG)    | + (TGG) + (GGG) | + (TGG)   |
| Glu  | CUCACU CUCACUC       | - (ATC) - (ATG)    | + (ATC) + (ATG) | + (ATC)   |
| Gly  | UCCCAUC UGCAUC       | + (GCT) + (GGG)    | + (GCT) + (GGG) | + (GCT)   |
| His  | PUGUGC u/k              | + (GTG) + (GGG)    | + (GTG) + (GGG) | + (GTG)   |
| Ile  | U/k UAU6A               | + (ATG) + (GGG)    | + (ATG) + (GGG) | + (ATG)   |
| Ini-Met | CCA6A CCA6A     | + (ATG) + (GGG)    | + (ATG) + (GGG) | + (ATG)   |
| Leu  | CAKPK u/k               | - (AGG) - (AGG)    | - (AGG) - (AGG) | - (AGG)   |
| Lys  | UC6U6A CUC6A           | + (GGG) + (GGG)    | + (GGG) + (GGG) | + (GGG)   |
| e-Met | CUBAU6A CUBAU6A   | + (GGG) + (GGG)    | + (GGG) + (GGG) | + (GGG)   |
| Phe  | BUA6A/BUA6A            | - (GAA) - (GAA)    | - (GAA) - (GAA) | - (GAA)   |
| Pro  | U/k U/A/K               | - (AGG) - (AGG)    | - (AGG) - (AGG) | - (AGG)   |
| Ser  | UUGAAU/GKA             | - (GAG) + (GGG)    | + (GAG) + (GGG) | + (GAG)   |
| Thr  | U/K U/K                | - (GGG) - (GGG)    | - (GGG) - (GGG) | - (GGG)   |
| Trp  | U/K U/K                | - (CCG) - (CCG)    | - (CCG) - (CCG) | - (CCG)   |
| Tyr  | U/GPAKA u/k             | - (GTA) + (GTA)    | + (GTA) + (GTA) | + (GTA)   |
| Val  | UCACAC BUCA6A          | - (MAC) + (MAC)    | + (MAC) + (MAC) | + (MAC)   |
| Sec  | CUNCA+A CUNCA+A        | - (TCA2) - (TCA2)  | - (TCA2) - (TCA2)| - (TCA2) |
striking observation in S/S cells was that tRNA corresponding to only one Ala isoacceptor (Ala-hGC) exhibited high cleavage during both hypertonic and oxidative stress. Ala tRNA has been previously reported to inhibit protein synthesis in vitro, but the specific isoacceptors were not tested (20). Interestingly, ANG treatment caused higher cleavage of both isoacceptors in S/S cells. However, the increased cleavage of the two Ala isoacceptors in ANG treatments did not correlate with a higher inhibition of protein synthesis when compared with the two stress treatments (ANG treatment in S/S cells caused ~20%, inhibition of protein synthesis whereas hypertonic and oxidative stress caused >70% inhibition). These findings suggest that cleaved tRNAs in vivo may not affect protein synthesis as described by in vitro studies. It is more likely that tRNAs participate in specific signaling pathways during the cellular stress response, which are still to be discovered.

Previous reports have speculated that tRNAs may participate in the microRNA signaling pathway (59, 60). Although we did not extensively study the intersection of stress-induced tRNA cleavage and the microRNA signaling pathway, our preliminary studies showed that a key member of the RNA-induced silencing complex (RISC), Ago2, can form complexes during stress which contain certain tRNAs. Using an Ago2 antibody we carried out immunoprecipitation experiments in control and ANG-treated S/S cells. We performed microarray analyses of the immunoprecipitated tRNAs and performed bioinformatics analysis on 10 tRNA candidates, which showed increased association with Ago2 upon ANG treatment (Leu-wAG, Leu-CAA, Val-TAC, Ala-IGC, Arg-ICG, Arg-yCG, Arg-ICG, Val-CA, Ala-CA, and Arg-CA). The 3′ end of this tRNA matched almost perfectly to the 5′ end of the human microRNA miR-3676. These data suggest that tRNA fragments could be potential cellular signaling molecules. Interesting questions can be derived from these findings such as: how do these fragments get loaded in the RISC complex and are they competent for gene silencing? Because tRNAs account for as much as 15% of total RNA, tRNA fragments loaded in RISC could be a fast way to monopolize and shut down the miRNA silencing machinery. Further studies beyond the scope of the current work are needed and are being currently pursued to answer some of these very important questions. We propose that tRNA cleavage is a tightly regulated stress response, which requires simultaneous activation of ANG and availability of tRNAs. The tRNAs may play a role in downstream signaling pathways.

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