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Characterization of RNA damage under oxidative stress in *Escherichia coli*

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Abstract

We have examined the level of 8-hydroxyguanosine (8-oxo-G), an oxidized form of guanosine, in RNA in *Escherichia coli* under normal and oxidative stress conditions. The level of 8-oxo-G in RNA rises rapidly and remains high for hours in response to hydrogen peroxide (H₂O₂) challenge in a dose-dependent manner. H₂O₂ induced elevation of 8-oxo-G content is much higher in RNA than that of 8-hydroxydeoxyguanosine (8-oxo-dG) in DNA. Under normal conditions, the 8-oxo-G level is low in RNA isolated from the ribosome and it is nearly three times higher in non-ribosomal RNAs. In contrast, 8-oxo-G generated by a short exposure to H₂O₂ is almost equally distributed in various RNA species, suggesting that although ribosomal RNAs are normally less oxidized, they are not protected against exogenous H₂O₂. Interestingly, highly folded RNA is not protected from oxidation because 8-oxo-G generated by H₂O₂ treatment *in vitro* increases to approximately the same levels in tRNA and rRNA in both native and denatured forms. Lastly, increased RNA oxidation is closely associated with cell death by oxidative stress. Our data suggests that RNA is a primary target for reactive oxygen species and RNA oxidation is part of the paradox that cells have to deal with under oxidative stress.

Keywords

cell viability; 8-hydroxyguanosine (8-oxo-G); oxidative stress; RNA damage; RNA oxidation

Introduction

Many studies have revealed that DNA damaging agents also cause RNA damage (Li et al., 2006; Wurtmann and Wolin, 2009). Reactive oxygen species (ROS), which become more abundant under oxidative stress (OS), are common nucleic acid damaging agents. In the few cases when oxidative damages to DNA and RNA were compared by measuring the content of 8-hydroxyguanosine (8-oxo-G), oxidation was much greater in RNA than in DNA in mammalian cells and tissues, especially when exogenous oxidants such as hydrogen peroxide (H₂O₂), were introduced to cause oxidative stress (Fiala et al., 1989; Shen et al., 2000; Hofer et al., 2005, 2006). In a study using reverse-transcriptase PCR to measure the damage of *Escherichia coli* 16S rRNA, H₂O₂ treatment caused RNA damage that stopped reverse transcription up to 50% in 1 kilobase distance (Gong et al., 2006). These findings suggest that RNA oxidation is a pre-dominant feature under conditions of ROS attack.

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There is increasing evidence regarding RNA dysfunction caused by oxidation. mRNA oxidized *in vitro* causes a reduction in protein synthesis and the formation of aggregated protein products (Shan et al., 2003, 2007). Oxidative damage of mRNA could cause ribosome stalling during translation elongation (Shan et al., 2007). Consistent with these observations, truncated proteins were made from oxidized mRNA (Tanaka et al., 2007). Oxidation also causes ribosome dysfunction (Ding et al., 2005; Honda et al., 2005). Cells must have developed various mechanisms that control the quality of RNA and minimize the deleterious effect of RNA oxidation. RNA surveillance activities could reduce the incorporation of damaged nucleotides into RNA (Taddei et al., 1997; Hayakawa et al., 1999; Ishibashi et al., 2005) and degrade or repair damaged RNA (Li et al., 2006; Wurtmann and Wolin, 2009; Kong and Lin, 2010). Consistent with this notion, an important RNA degrading exoribonuclease, polynucleotide phosphorylase, was shown to reduce RNA oxidation and protect *E. coli* and HeLa cells under oxidative stress (Wu and Li, 2008; Wu et al., 2009).

Despite the increasing interest in studying RNA oxidative damage and quality control, little is known about the level of RNA damage under oxidative stress and whether different RNA species and structures are differentially oxidized by ROS. In a growing *E. coli* cell, highly structured rRNA and tRNA account for nearly 80% and 15% of the total RNA, respectively. In addition, most of the rRNA molecules are present in ribosomes where rRNAs are tightly bound with ribosomal proteins. One would expect that RNA would be protected from oxidation by the presence of highly folded structures or by association with proteins. In this work, we examined the level and distribution of oxidized RNA in *E. coli* under normal and oxidative stress conditions to answer these questions.

Results

H₂O₂ causes a quick and dosage-dependent increase of 8-oxo-G in cellular RNA

In order to determine the level of RNA oxidation in response to H₂O₂ treatment, we have measured 8-oxo-G content in cellular RNA in a time course after addition of H₂O₂ in *E. coli* cultures. Under conditions described in Materials and methods, the basal level of 8-oxo-G in *E. coli* RNA is slightly lower than one 8-oxo-G per 10⁵ G. After adding H₂O₂ to the cultures, the 8-oxo-G level increases in only minutes and remains high for hours (Figure 1A). It should be noted that the concentration of H₂O₂ reduces rapidly in the culture media, becoming close to the basal level 5 min after addition of the oxidant (Wu et al., 2009). Interestingly, after a pulse treatment with H₂O₂, the level of 8-oxo-G initially rises and then quickly drops to almost normal level when the cells were subsequently grown in fresh medium (Figure 1A). This observation suggests that the sustained high level of 8-oxo-G in cultures of continuous H₂O₂ treatment is likely caused by oxidized components of the medium.

The increase of 8-oxo-G in RNA depends on the dosage of H₂O₂, from three 8-oxo-G/10⁵ G when treated with 1 mM H₂O₂ to nearly ten 8-oxo-G/10⁵ G in the presence of 5 mM H₂O₂ (Figure 1B). Cells grown in rich and minimal media contain similar levels of basal and H₂O₂ induced 8-oxo-G in RNA (data not shown).

H₂O₂ induces higher levels of 8-hydroxyguanine in RNA than in DNA

To compare the level of oxidation in DNA and RNA, we measured 8-oxo-G content in RNA and 8-hydroxydeoxyguanosine (8-oxo-dG) in DNA simultaneously in the same culture treated with or without H₂O₂. This was done by a modified procedure that enabled us to extract the DNA and RNA together. The nucleic acids were digested and the resulting nucleosides were applied to HPLC analysis under conditions that guanosine (G),

deoxyguanosine (dG), 8-oxo-G and 8-oxo-dG were separately detected in a single sample. As shown (Figure 2), this modified procedure caused an elevation in the basal level of 8-oxo-G in RNA to above three 8-oxo-G/ 10^5 G, probably due to spurious oxidation during preparation of the nucleic acids (de Souza-Pinto and Bohr, 2002). Nonetheless, treatment with 5 mM H_2O_2 for 15 min increases 8-oxo-G to approximately ten per 10^5 G in RNA when both RNA and DNA were prepared simultaneously (Materials and methods). This result is consistent with the level of 8-oxo-G induction by the same H_2O_2 concentration following the procedure for RNA isolation only (Figure 1B).

The basal level of 8-oxo-dG in DNA is lower than 8-oxo-G in RNA (Figure 2). However, it must still be higher than the actual level in DNA due to the same spurious oxidation observed in RNA. Importantly, the level of 8-oxo-dG in DNA only increases slightly after treatment with 5 mM H_2O_2 , contrasting the large increase of 8-oxo-G in RNA. Similarly, treatment with 1 mM H_2O_2 causes moderate increase of 8-oxo-G in RNA but no change of 8-oxo-dG in DNA (data not shown). This data suggests that at a steady state, OS induced oxidative damage of RNA is higher than that of DNA, consistent with the results from similar studies using mammalian samples (Fiala et al., 1989; Shen et al., 2000; Hofer et al., 2005, 2006).

The distribution of 8-oxo-G in various RNA species

We first tried to understand the oxidation level of RNA of different sizes. Long and short RNAs were isolated from total RNA. The long RNA is predominantly composed of rRNA, and the short RNA is almost pure tRNA. Under normal conditions, 8-oxo-G level in the long RNA fraction is slightly lower than that in the short RNA. After cells were treated with 3 mM H_2O_2 , both RNA fractions contain elevated levels of 8-oxo-G (Figure 3A). These results suggest that RNA size does not significantly affect oxidation level and that structured RNA species can be oxidized efficiently. The latter point was examined further below.

The rRNA species present in ribosomes constitute the majority of cellular RNA. In order to understand if the rRNAs' highly folded structure and tight association with ribosomal proteins protect RNA from oxidation, we have determined 8-oxo-G levels in RNA from ribosome and nonribosome fractions. In order to isolate the RNAs, cells were opened by freeze and thaw, after which, the ribosome was pelleted as described in Materials and methods. RNA samples were prepared from both ribosomal and non-ribosomal (supernatant) fractions and were examined by gel electrophoresis. The RNA from ribosome fractions contains essentially pure rRNA. RNA from the non-ribosomal fraction contains all RNA species including rRNAs that are not incorporated into ribosomes in addition to RNA degradation intermediates. Non-ribosomal RNAs can also be further fractionated to long and short RNAs; the latter is almost pure tRNA (Deutscher and Hilderman, 1974; Li and Deutscher, 2009).

Under normal conditions, RNA isolated from ribosomes contains approximately 0.4 8-oxo-G per 10^5 G (Figure 3B). This is significantly lower than the level of 8-oxo-G in total RNA (Figure 1). In contrast, the level of 8-oxo-G in RNA from the non-ribosomal fraction is almost three times higher than the level in ribosomal RNA and is also higher than the level in total RNA (Figure 3B). There is no difference between the long and short RNAs in the non-ribosomal RNA fraction. In various experiments, RNA isolated from ribosomes is approximately 60% of total RNA and RNA from non-ribosomal fractions constitute the remaining 40%.

After cells are exposed to H_2O_2 for 15 min, 8-oxo-G levels increase in all RNA fractions. Interestingly, RNA from ribosomes contains 8-oxo-G at the same and in some cases higher levels as non-ribosomal RNA depending on the H_2O_2 dosage (Figure 3B). The results

suggest that the exceeding complex structures do not protect rRNA from oxidation, nor do the surrounding ribosomal proteins. 8-oxo-Gs were also generated in non-ribosomal RNAs, without showing differences in the long and short RNA fractions. Note that in the experiments shown in Figure 3B, the levels of 8-oxo-G are generally higher than those shown in Figure 1, presumably due to differences in cell growth and treatment conditions.

Highly folded structure does not protect RNA from being oxidized *in vitro*

In order to examine if RNA structure has any protective role against oxidation, we have determined the levels of 8-oxo-G generated by H₂O₂ treatment *in vitro* of tRNA and rRNA in either native or denatured forms. Such treatment introduces hundreds of 8-oxo-G per 10⁵ G using mM levels of H₂O₂ (Figure 4). Surprisingly, native tRNA contains slightly more 8-oxo-G than denatured tRNA at every concentration of H₂O₂ used in this experiment (Figure 4A). As for rRNA, 8-oxo-G level in the native form is not significantly different from that in the denatured form, although the latter appears slightly lower at the 1 mM and 10 mM H₂O₂ dosages (Figure 4B). The results are consistent with the observation of RNA oxidation in ribosomes *in vivo* (Figure 3) and are against the notion that RNA structure can protect RNA from oxidative damage.

RNA fragmentation upon H₂O₂ treatment

Oxidation is also able to cause RNA strand breaks (Li et al., 2006). Alternatively, oxidized RNA might undergo degradation. These processes could cause elevated levels of RNA fragments. Here, we have examined if such fragments are produced and accumulated upon H₂O₂ treatment. After cells were treated with H₂O₂, RNA products increased below the full length 16S rRNA band (Figure 5, left panel). These can be fragments of 23S or 16S rRNAs. We analyzed 23S rRNA, 16S rRNA and their fragments by Northern Blotting as described in Materials and methods. As shown (Figure 5), middle and right panels, short products of 23S and 16S rRNAs are increased by H₂O₂ treatment, especially in areas below the full-length 16S rRNA band. Note that the increase of fragmentation by H₂O₂ treatment is not dramatic. Considering that most cells are viable under this condition (see below) and that some fragments of the rRNAs might be degraded, the detectable increase in rRNA fragmentation does present another problem that cells have to handle under OS.

Cell death in response to H₂O₂ challenge

Escherichia coli cell viability under H₂O₂ treatment was studied by measuring optical density of cultures and by determining colony forming unit (CFU). H₂O₂ causes reductions of cell density at concentrations of 2 mM or higher (Figure 6A). The slight reduction of density in response to 2 and 4 mM H₂O₂ mainly occurs in the first 30 min. Very little change in OD₅₅₀ was observed when H₂O₂ was added to 7 or 10 mM, suggesting heavy cell death (Figure 6A).

Viable cell count confirms that the reduction of culture density is actually due to cell death, which happens mainly in the first 60 min after H₂O₂ addition (Figure 6B). Similar to 8-oxo-G levels in RNA, cell death depends on the dosage of H₂O₂ (Table 1). When H₂O₂ was added at 1 to 5 mM, cells die at rates that increase depending on H₂O₂ concentration. A higher rate of cell death was observed at 60 min than at 30 min after H₂O₂ addition at every concentration. H₂O₂ at 10 mM kills cells completely, consistent with the results of cell density reduction (Figure 6A). We noted that 1 mM H₂O₂ causes detectable cell death based on CFU but it does not affect culture density. The difference between CFU and culture density reduction responding to 1 mM H₂O₂ is pre-sumably due to the fact that dead cells contribute to density reading of the cultures. As expected, H₂O₂ at 2 mM or higher concentrations caused a reduction to both CFU and density of culture.

CFU analysis also demonstrated that the effect of H_2O_2 depends on the density of the culture. As shown (Figure 6C), where $\text{OD}_{550} = 0.01$ cultures were treated with $2 \text{ mM } \text{H}_2\text{O}_2$, only a small percentage of cells (4-5%) survived after 60 min. CFU was also reduced by 60% in the presence of only $0.5 \text{ mM } \text{H}_2\text{O}_2$. It is likely that low density cultures hydrolyze H_2O_2 much slower than high density cultures, resulting in sustained oxidation and more cell death in low density cultures.

Discussion

In this work we have demonstrated that cellular RNAs are quickly and highly oxidized under oxidative stress, as indicated by the rise of 8-oxo-G levels in RNA, which is dependent on H_2O_2 dosage. Oxidation occurs to all RNA species. RNA structures and association with proteins do not appear to be able to protect RNA from being oxidized by H_2O_2 . In addition, a small amount of ribosomal RNA fragments can be identified upon H_2O_2 treatment.

Fenton reaction during preparation, storage and processing causes spurious oxidation of nucleic acids, resulting in variations of reported basal levels of oxidized nucleobases in the order of magnitude (ESCODD et al., 2005). This variation could also greatly interfere with the results in response to oxidants (de Souza-Pinto and Bohr, 2002). In order to reduce spurious oxidation in our experiments, we have adopted procedures to minimize exposure to oxygen and to reduce the level of contaminating metal ions (Shen et al., 2000; ESCODD et al., 2005; Hofer et al., 2006; Wu et al., 2009). However, it is likely that the true basal level of 8-oxo-G is lower than reported in this work because spurious oxidation might not be completely avoided during HPLC analysis (ESCODD et al., 2005). In fact, elevated basal levels were occasionally observed when an alternative preparation method was used (Figure 2). Nonetheless, our results demonstrated 8-oxo-G levels that respond well to all dosages of H_2O_2 .

Oxidation of cellular RNA by exogenous H_2O_2 takes a very short time to happen, suggesting that RNA is a direct target of ROS (Figure 1). After H_2O_2 addition, 8-oxo-G levels remain increased throughout the entire time course examined (Figure 1A), although H_2O_2 levels in the culture quickly became undetectable in minutes (Wu et al., 2009; data not shown). A small decrease in the level of 8-oxo-G 60 min after the addition of H_2O_2 has been consistently observed. The H_2O_2 -induced 8-oxo-G level reduces rapidly after the cells were shifted to fresh H_2O_2 -free medium. It is likely that degradation or repair reduces the level of 8-oxo-G containing RNA, whereas residual ROS derived from H_2O_2 would cause an increase in the level of 8-oxo-G. The observed steady state levels of 8-oxo-G in RNA induced by continuous H_2O_2 treatment must reflect the equilibrium of the two processes.

An unexpected observation was that rRNA and tRNA are not protected by their structures or by proteins associated with rRNA (Figure 3). The inability of RNA structure to protect RNA from oxidation was further shown *in vitro* by exposing purified RNA to H_2O_2 (Figure 4). These findings are surprising because one would expect that RNA structure and protein binding would limit the accessibility of RNA to ROS. Apparently, ROS can reach the bases in these RNAs efficiently. One possible explanation for the lack of protection is the association of the highly structured RNAs with Fe^{2+} , the ion known to generate oxidative chemicals and free radicals from H_2O_2 by Fenton chemistry (Wardman and Candeias, 1996). Indeed, such association of rRNA with Fe^{2+} has been reported to play a role in promoting rRNA oxidation and inactivation in translation (Honda et al., 2005).

Importantly, under normal conditions rRNAs isolated from the ribosomes contain much lower levels of 8-oxo-G than the rest of the cellular RNA (Figure 3), suggesting that rRNA

in ribosomes are normally kept with low oxidative damage. This phenomenon could be important for optimal ribosome function to support cell growth because oxidative damage might hinder protein synthesis or generate errors in the protein products.

A number of the observations in this work also suggest the existence of RNA quality control activities that remove oxidized RNA and support cells surviving OS insults. First, the high levels of 8-oxo-G generated by H₂O₂ treatment must eventually be reduced to a normal level, at least in the viable cells. Second, given the fact that ribosomal RNA normally contains low 8-oxo-G but is efficiently oxidized by H₂O₂, oxidized rRNA species might be identified and removed from ribosomes. Such mechanism(s) might not only be responsible for the removal of 8-oxo-G in ribosomes (Min Liu and Zhongwei Li, unpublished observations) but could also be used for reducing 8-oxo-G in rRNA under normal conditions.

Degradation of oxidized RNA can be a major quality control pathway because until now a repairing mechanism is only found on the alkylated RNA. As reported previously, *E. coli* polynucleotide phosphorylase (PNPase) plays a pivotal role in controlling 8-oxo-G levels and supporting cell viability under OS (Wu et al., 2009). In addition, other RNA degradation activities also are important for reducing 8-oxo-G and protecting cells under OS (unpublished results from our laboratory). In addition to elevated levels of 8-oxo-G, we have also observed increased fragmentation of rRNA in response to H₂O₂ treatment (Figure 5). These rRNA fragments might be removed from ribosomes and eventually degraded. The detailed molecular mechanisms for the elimination of oxidized RNA, possibly involving specific recognition and targeted degradation of such RNA molecules, are yet to be elucidated in future work.

Under the same conditions that H₂O₂ cause ~0.7 8-oxo-dG/10⁵ G in DNA, 8-oxo-G rises by 6-7 per 10⁵ G in RNA at steady state (Figure 2). The relatively lower level of H₂O₂ induced 8-oxo-dG in DNA might be due to strong DNA repair activities that quickly remove 8-oxo-dG after its formation. Such repair activities have not yet been reported for RNA. In contrast, oxidized RNA might undergo rapid degradation resulting in the observed steady state level of 8-oxo-G in RNA. Considering that the amount of RNA is approximately four times that of DNA, the oxidized guanine in RNA can be much higher than that in DNA under OS conditions used in this work. The presence of such large amount of oxidized nucleotides in RNA and the possible turnover of oxidized RNA might be an important antioxidant mechanism which consumes the majority of nucleotide oxidizing agents and reduces DNA damage (Radak and Boldogh, 2010). These potential mechanisms should be defined in further studies.

Moreover, increased RNA damage might attenuate cell growth. A correlation between the levels of RNA oxidation and cell growth reduction is proposed (Table 2). Assuming that RNA is oxidized randomly and that total damage in RNA is approximately ten times higher than the levels of 8-oxo-G (Gajewski et al., 1990; Rhee et al., 1995; Cooke et al., 2003; Li et al., 2006), a significant fraction of RNA molecules might be damaged by H₂O₂ at mM concentrations at steady state. It is likely that RNA oxidative damage contributes, at least partly, to the observed cell death under the same OS conditions (Table 2). Currently, there is no clear explanation about how oxidized RNA would reduce cell viability under oxidative stress conditions. It was previously reported that oxidized rRNA and mRNA are defective in protein synthesis *in vitro* or in cells transfected by oxidized RNA (Ding et al., 2005; Honda et al., 2005; Tanaka et al., 2007; Shan et al., 2007). Lack of protein synthesis and production of aberrant proteins due to RNA oxidation are likely a main cause of cell death. We have reported that PNPase, an exoribonuclease that binds to 8-oxo-G RNA with specificity (Hayakawa et al., 2001) and degrades defective RNA (Li et al., 2002), is important for

controlling the level of 8-oxo-G in RNA and for protecting *E. coli* cells under OS (Wu et al., 2009). In the absence of this enzyme, *E. coli* cells become hypersensitive of H₂O₂ and other oxidants, and contain elevated levels of 8-oxo-G in RNA (Wu et al., 2009). These findings strongly support the notion that oxidized RNA is deleterious to cell.

Knowledge about the nature and effect of RNA oxidation would eventually enhance our understanding of related diseases. In the last decade, a number of reports have demonstrated that increased RNA oxidation is implicated in the development of numerous degenerative disorders (Nunomura et al., 2009; Wurtmann and Wolin, 2009). Other studies suggest that oxidation of RNA, but not of DNA, was increased in diseases such as Alzheimer's (Nunomura et al., 1999; Abe et al., 2002; Ding et al., 2006), Parkinson's (Zhang et al., 1999; Kikuchi et al., 2002), dementia with Lewy bodies (Nunomura et al., 2002), myopathies (Tateyama et al., 2003), atherosclerosis (Martinet et al., 2004), amyotrophic lateral sclerosis (ALS) (Chang et al., 2008), hemochromatosis (Broedbaek et al., 2009), hepatic encephalopathy (Görg et al., 2010) and schizophrenia (Che et al., 2010). More recently, RNA oxidation adducts were shown to be differentially correlated with insoluble amyloid- β_{42} , a causative factor of Alzheimer disease (Weidner et al., 2011), suggesting that different forms of oxidized RNA might be involved in different stages of this disease (Abe et al., 2002; Weidner et al., 2011). Furthermore, increased RNA oxidation was reported under physiological conditions associated with aging (Liu et al., 2002; Hofer et al., 2008a,b; Seo et al., 2008). These findings suggest that RNA oxidation is a predominant feature under conditions of ROS attack. RNA oxidative damage might contribute to the process of aging and disease development. Whether RNA oxidation damage directly causes diseases or aging and how much it contributes to these processes remains to be revealed.

Materials and methods

Bacterial strain and growth conditions

Escherichia coli K12 strain CA244 *rna* (*lacZ*, *trp*, *relA*, *spot*, *rna*, *Tn10::tet^R*) was used in this study. Cultures were grown in Yeast extract-Trypton (YT) medium (BD Diagnostic Systems, Sparks, Maryland, USA). Tetracycline was added to the medium at 10 μ g/ml when needed.

Materials

Guanosine was obtained from Sigma-Aldrich (St. Louis, MO, USA). 8-hydroguanosine (8-oxo-G) was from Calbiochem (La Jolla, CA, USA). Tri Reagent was from Molecular Research Center (Cincinnati, OH, USA). Lysozyme was from Sigma-Aldrich (St. Louis, MO, USA). α -³²P-dATP was purchased from GE Healthcare Inc (Piscataway, NJ, USA). dNTPs and Taq DNA polymerase used in PCR reactions were from Invitrogen (Carlsbad, CA, USA). The following DNA oligonucleotides were synthesized at Integrated DNA Technologies (Coralville, IA, USA) and were used as primers for rRNA specific PCR products: 23S-1, 5'-AGC GAC TAA GCG TAC ACG GT-3'; 23S-2, 5'-AAG ACC AAG GGT TCC TGT CC-3'; 23S-3, 5'-TTA GAG GCT TTT CCT GGA AGC-3'; 23S-4, 5'-AGC CTC ACG GTT CAT TAG TAC C-3'; 16S-R, 5'-TAT TCA CCG TCC CAT TCT GA-3'; 16S-F, 5'-TGC AAG TCG AAC GGT AAC AG-3'. All other chemicals are reagent grade. Double distilled water (ddH₂O) was treated with diethyl pyrocarbonate (DEPC) and Chelex 100, both from Sigma-Aldrich, Saint Louis, MO, USA. Buffers, phenol (EMD Chemicals, Inc. Gibbstown, NJ, USA), ethanol (Sigma-Aldrich, Saint Louis, MO, USA) and isopropanol (ACROS Organics, Fair Lawn, NJ, USA) were also treated with Chelex 100 as appropriate.

Treatment of *E. coli* cultures with H₂O₂

Overnight cultures grown in YT medium with shaking at 37°C were diluted with fresh, pre-warmed YT medium and incubated until OD₅₅₀ reached 0.5. H₂O₂ was added to the culture at the desired final concentration. Equal volume of H₂O was added in control cultures. The cultures were continually incubated with shaking and samples were collected at various time points.

Isolation of RNA and DNA

Cells were collected and resuspended in lysis buffer [10 mM Tris-Cl, 10 mM EDTA pH 8.0, 1% SDS, 10% glycerol and supplemented with freshly added deferoxamine mesylate (DFOM, Sigma-Aldrich, Saint Louis, MO, USA) to a final concentration of 10 mM]. Total RNA was routinely prepared for 8-oxo-G analysis. The lysates were diluted 10 times with ddH₂O and an equal volume of a mixture of liquefied phenol (pH ~4) and chloroform (9:1) was added. The mixture was applied to a vortex intermittently for 10 min at room temperature. After centrifugation, the upper layer of aqueous phase was transferred to a new tube and was extracted one more time with phenol-chloroform. To the recovered aqueous phase, 1/10 volume of 3 M KAc (pH 5.2) and an equal volume of isopropanol were added. The tubes were filled with nitrogen, mixed well and kept at -80°C for 1 h. RNA was precipitated by centrifuging at 20 000 *g* for 10 min at 4°C. The RNA pellets were washed twice with 75% cold ethanol and were vacuum-dried for 10 min. The dried RNA was directly dissolved in a digestion mixture in which the RNA was converted to nucleosides (Wu et al., 2009). When RNA and DNA were isolated simultaneously, cell lysates were extracted with phenol and chloroform and then the nucleic acids were precipitated by ethanol, as previously described (Wu et al., 2009).

Determination of 8-oxo-G level in RNA and 8-oxo-dG level in DNA

After digestion, the 8-oxo-G level in RNA was determined by HPLC as previously described (Gong et al., 2006 ; Wu et al., 2009). For simultaneous detection of 8-oxo-G in RNA and 8-oxo-dG in DNA, digestion was carried out under the same conditions for RNA. The resolving time of HPLC is 70 min to allow the separation of both DNA and RNA nucleosides. Chemical standards of G, dG, 8-oxo-G and 8-oxo-dG were used. The normalized level 8-oxo-G/10⁵ G in RNA or 8-oxo-dG/10⁵ dG in DNA was calculated.

Preparation of ribosomal and non-ribosomal RNA

Exponentially growing cells were harvested by centrifugation at 10 000 *g* for 3 min. The cell pellet was resuspended in 1 ml saline solution, transferred into an Eppendorf tube and was pelleted at 10 000 *g* for 1 min. The pellet was resuspended in 800 µl cell extract buffer (10 mM Tris-HCl, pH 7.75, 100 mM NH₄Cl and 1 mM DFOM) (Vaidyanathan et al., 2007). The cell suspension was supplemented with 12.5 µl lysozyme (40 mg/ml) and the mixture was incubated for 1 min at room temperature (Ron et al., 1966). The mixture was frozen in dry ice/ethanol bath and then thawed completely in a water bath at 37°C. The freeze-thaw process was repeated three times in order to completely lyse the cells (Ron et al., 1966). To the cell lysate, 30 µl of 10% sodium deoxycholate was added and the solution was incubated in an ice water bath for 3 min (Ron et al., 1966). The lysate was then centrifuged at 25 000 *g* for 40 min at 4°C. The supernatant was transferred to an ultracentrifuge tube (Microcentrifuge Polyallomer, Part No. 357448, Beckman Coulter, Indianapolis, IN, USA) and centrifuged at 200 000 *g* using the Beckman TLA-100.4 rotor and ultracentrifuge at 4°C for 2 h to pellet the ribosome (Vaidyanathan et al., 2007, Székely et al., 1973). The supernatant was transferred to an Eppendorf tube. Subsequently, the ribosome pellet was briefly rinsed with cell extract buffer, and re-suspended in the same buffer supplemented with 0.5% SDS (Li and Deutscher, 2009). RNA was isolated from the ribosome suspension

and the non-ribosomal supernatant fraction as previously described (Li and Deutscher, 2009).

Separation of long and short RNA species

Long and short RNAs were separated by differential precipitation using isopropanol (Deutscher and Hilderman, 1974; Li and Deutscher, 2009). RNA was added NaAc (pH 7.0) to 0.3 M and DFOM to 1 mM. To the RNA solution, 0.54 volume of isopropanol was added and mixed immediately. The mixture was centrifuged at 21 100 g for 15 min. The RNA pellet contained long RNA, mainly rRNA and mRNA species. The aqueous phase was transferred into a new Eppendorf tube, and additional isopropanol was added to a final 0.98 volume. The mixture was kept at -80°C for at least 1 h. RNA was pelleted by spinning at 21 100 g for 10 min. The resulting small RNA was almost pure tRNA (Deutscher and Hilderman, 1974). The pellets of long and short RNAs were washed twice with pre-cooled 75% ethanol at 21 100 g for 3 min before further analysis.

RNA denaturation and oxidation *in vitro*

The highly structured rRNA and tRNA were isolated by the method described above. RNA dissolved in ddH₂O was denatured by incubating at 95°C for 2 min, followed by chilling immediately in an ice water bath. *In vitro* oxidation was performed as described previously with modifications (Gong et al., 2006). Briefly, 40 µg of native or denatured RNA were incubated in a buffer containing 100 mM H₂PO₄/HPO₄, pH 7.4, 1 µM CuSO₄, 10 µM ascorbic acid and various concentrations of H₂O₂ at 37°C for 1 h. RNA was then precipitated and washed before analysis of 8-oxo-G level.

Determination of rRNA fragmentation

Total RNA was extracted by TRI Reagent and was dissolved in ddH₂O. The RNA solution was added to 1/5 volume of 6× RNA loading buffer containing 0.25 % Bromophenol blue, 0.25 % xylene cyanol, 30 % glycerol, 1.2 % SDS, 60 mM sodium phosphate (Kevil et al., 1997). RNA in the loading mixture was denatured by incubating at 75 °C for 5 min and immediately chilled in an ice water bath (Cheng and Deutscher, 2003). RNA was separated by electrophoresis on a 1.5 % agarose gel in 0.5×TBE buffer, first at 60 V for 10 min and then at 100 V for 1 h. The gel was stained with SYBR Gold and photographed under UV light. Northern analysis was carried out according to the procedure described previously (Cheng and Deutscher, 2003) with some modifications. RNA in the gel was transferred to GeneScreen Plus® membrane (Pall Life Science, Pensacola, FL, USA) by electroblotting at 15 V overnight in 1×TBE buffer. RNA was fixed to the membrane by UV irradiation for 2 min, prehybridized for 2.5 h at 68 °C in 1×Denhardt's solution and then hybridized overnight at 55 °C with probes specific for 23S or 16S rRNA. The 23S and 16S specific DNA products were generated by PCR using specific primers and *E. coli* genomic DNA. The PCR products cover the entire rRNA sequences. Because 23S rRNA is nearly 3 kilobases in length, we first made two halves using the two pairs of primers. 23S-1 and 23S-3 oligos were one pair of primers to synthesize the 5' end part products. 23S-2 and 23S-4 oligos were another pair for 3' end part. Then labeled [³²P]-labeled probes were generated by PCR reactions using the PCR product for specific rRNA as template, the primer complementary to the rRNA (23S-3, 23S-4, and 16S-R) and dNTPs plus α-[³²P]-dATP. RNAs in the membrane were hybridized with two 23S probes in one chamber to label the related rRNA products. A 16S rRNA probe was used to hybridize with another membrane and separately labeled the 16S rRNA products. After hybridization, the membrane was washed twice for 10 min at 55 °C, each with 1×SSC and 0.1 % SDS, prior to autoradiography.

Determination of cell viability

When the OD₅₅₀ of the culture reached 0.5, H₂O₂ or H₂O was added. 100 µl of the cultures were dispensed into each well of a 96-well plate. The plate was incubated at 37 °C by shaking at 150 rpm. The optical density value at 550 nm was detected by a plate reader in a time course.

CFU was determined in a time course after addition of H₂O₂ to exponentially grown cultures at different densities. 10 % (v/v) AlamarBlue (Life Technologies, Grand Island, NY, USA) stock solution (final concentration of resazurin: 17.5 mM) was made in YT medium. The serially 10-times diluted AlamarBlue was also prepared and kept on ice. 100 µl of each dilution was placed in all wells of a 96-well plate and one well contained 100 µl stock AlamarBlue as the blank. When a desired concentration of H₂O₂ was added to the cell in the exponential phase, at each time point, a 10 µl culture was transferred into one well containing the supplemented AlamarBlue dilutions. The plate was put in a plate reader (Molecular Devices SpectraMax M5e, Sunnyvale, CA, USA) and the fluorescence was detected (excitation, 544 nm; emission, 590 nm, automix, 5 s) in arbitrary units (FSU). Finally, the cell culture would reach the maximum fluorescence. FSU of each well was normalized by subtracting the fluorescence data of the blank. The linear curve of FSU and time was plotted. On the curve, the time to F₅₀ (half of maximal fluorescence), T₅₀, was pinpointed for each dilution. Then T₅₀ was plotted versus log CFU per ml.

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Figure 1.

H₂O₂ treatment causes quick, dosage-dependent increase of 8-oxo-G content in cellular RNA.

(A) 8-oxo-G levels in RNA in a time course after addition of H₂O₂ or H₂O. Exponentially grown cultures of *E. coli* CA244 *ma* strain (OD₅₅₀=0.5) were treated with 1 mM H₂O₂ or an equal volume of H₂O at 0 min. Total RNA was extracted and 8-oxo-G levels were analyzed as described in Materials and methods. Pulse treatment was carried out by exposing the cultures to H₂O₂ for 10 min. Cells were pelleted and grown in fresh H₂O₂-free medium for the rest of the time course. (B) Increase of 8-oxo-G levels in RNA depends on the dose of H₂O₂. Exponentially grown cultures were treated with indicated concentrations of H₂O₂ for 15 min. Total RNA was isolated and 8-oxo-G level was measured.

Figure 2.

H₂O₂ treatment causes a higher elevation of 8-oxo-G in RNA than that of 8-oxo-dG in DNA.

Exponential phase (OD₅₅₀=0.5) cell culture was treated with and without 5 mM H₂O₂ for 15 min. RNA and DNA were isolated together, then 8-oxo-G in RNA and 8-oxo-dG in DNA were measured as described (Wu et al., 2009). Treatment with lower concentrations of H₂O₂ does not cause a detectable increase of 8-oxo-dG in DNA under these conditions (data not shown).

Figure 3.

The levels of 8-oxo-G in various cellular RNA species under normal conditions and in response to H₂O₂ treatment.

(A) 8-oxo-G levels in long and short RNA fractions of total RNA isolated from cultures that were treated with and without 3 mM H₂O₂. (B) 8-oxo-G levels in RNA from ribosome, non-ribosome and long and short non-ribosome fractions. Exponentially grown cells were treated with 0.5 or 1 mM H₂O₂ for 15 min. Various RNA fractions were prepared and 8-oxo-G level was measured as described in Materials and methods. The mean and standard error of triplicates were plotted.

Figure 4.

Native RNA structures do not protect RNA from H_2O_2 -mediated oxidation *in vitro*.

(A) tRNA was isolated from total RNA by isopropanol differential precipitation (see Figure 3). Native and denatured tRNA samples were incubated with indicated concentrations of H_2O_2 *in vitro* and 8-oxo-G levels were determined as described in the Materials and methods section. (B) rRNA was prepared from ribosomes as shown in Figure 3. Native and denatured rRNAs were treated with indicated concentrations of H_2O_2 *in vitro* and 8-oxo-G level was determined. The mean and standard error of at least three replicates were plotted.

Figure 5.

RNA fragmentation induced by oxidative stress.

Left panel, H_2O_2 treatment causes a slight increase in the amount of RNA products that are shorter than 23S or 16S rRNAs. Total RNA was prepared using TRI Reagent from exponential cultures treated with and without 1 mM H_2O_2 for 15 min and was separated by electrophoresis on a 1.5 % agarose gel. The major RNA species are marked on the left.

Middle panel, Northern blot of 23S rRNA showed a slightly darker smear under the full-length 23S upon H_2O_2 treatment, representing a slight increase in 23S rRNA fragments.

Right panel, Northern blot of 16S rRNA showing increased levels of 16S fragments in response to H_2O_2 treatment.

Figure 6.

H_2O_2 treatment causes a dose dependent growth reduction of *E. coli*.

(A) H_2O_2 was added into exponential-phase cultures ($OD_{550}=0.5$) at 0 min to final concentrations indicated in the Figure. The cultures were then immediately dispensed into the wells of a 96-well plate. The plate was incubated with shaking and the OD_{550} was recorded by a plate reader in a time course. Four replicates were read and the mean and standard error were plotted. Note the actual OD_{550} shown is lower than 0.5 initially due to the thinner cultures in the wells than in a regular cuvette. (B) Colony forming unit in $OD_{550}=0.5$ cultures in a time course after addition of H_2O_2 to the indicated final concentrations at 0 min. Colony forming units were determined as described in Materials and methods. The mean and standard error of triplicates were plotted. (C) Colony forming unit in $OD_{550}=0.01$ cultures in a time course after treatment with indicated concentrations of H_2O_2 . The mean and standard error of triplicates were plotted.

Table 1Cell death upon H₂O₂ insult.

H ₂ O ₂ (mM)	% of CFU $\pm$ standard error	
	30 min	60 min
0	100 $\pm$ 1.2	100 $\pm$ 1.0
1	91.8 $\pm$ 0.2	83.5 $\pm$ 2.5
2	82.0 $\pm$ 0.9	70.6 $\pm$ 1.1
5	57.8 $\pm$ 0.2	42.4 $\pm$ 0.4
10	0.8 $\pm$ 0.6	0.04 $\pm$ 0.04

Cultures of OD₅₅₀ = 0.5 were treated with the indicated concentration of H₂O₂. The CFU of control cultures (0 mM H₂O₂) was set at 100 %.

Table 2

Steady-state levels of RNA oxidative damage in *E. coli* in response to H₂O₂ treatment.

Growth condition	8-oxoG/10 ⁵ G ^a	8-oxo-G RNA ^b	Damaged RNA ^c	Cell viability (CFU) ^a (set to 100 %)
Normal aeration	1.0 ± 0.1	0.25 %	2.4 %	
Plus 1 mM H ₂ O ₂	3.9 ± 0.1	0.98 %	9.3 %	83 %
Plus 5 mM H ₂ O ₂	10.9 ± 0.5	2.7 %	24 %	42 %

^a8-oxo-G levels were determined 15 min after addition of H₂O₂ to exponentially growing cultures. CFU was determined after treatment with H₂O₂ for 60 min.

^bBased on the assumption that the length of RNA is 1 kb on average, the GC content is 50 % and 8-oxo-G is randomly distributed so that the percentage of 8-oxo-G containing RNA can be described by Poisson's distribution.

^cBased on the assumption that total damage occurrences can be 10 times of 8-oxo-G content.