Free Radical Research, February 2011; 45(2): 237–247

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healthcare

An assay for RNA oxidation induced abasic sites using the Aldehyde Reactive Probe

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(Received date: 4 August 2010; Accepted date: 17 October 2010)

Abstract

There have been several reports describing elevation of oxidized RNA in ageing or age-related diseases, however RNA oxidation has been assessed solely based on 8-hydroxy-guanosine levels. In this study, Aldehyde Reactive Probe (ARP), which was originally developed to detect DNA abasic sites, was used to assess RNA oxidation. It was found that ARP reacted with depurinated $tRNA^{Phe}$ or chemically synthesized RNA containing abasic sites quantitatively to as little as 10 fmoles, indicating that abasic RNA is recognized by ARP. RNA oxidized by Fenton-type reactions, γ-irradiation or peroxynitrite increased ARP reactivity dose-dependently, indicating that ARP is capable of monitoring oxidized RNA mediated by reactive oxygen species or reactive nitrogen species. Furthermore, oxidative stress increased levels of ARP reactive RNA in cultured cells. These results indicate the versatility of the assay method for biologically relevant oxidation of RNA. Thus, this study developed a sensitive assay for analysis of oxidized RNA.

Keywords: *Aldehyde Reactive Probe (ARP), RNA oxidation assay, abasic site, depurination*

Introduction

Oxidation of RNA has been shown to occur with increasing age in muscle [1] and brain [2]. In addition, it has been shown that oxidized RNA is elevated in several age-related diseases including atherosclerosis [3], dementia with Lewy bodies [4], Parkinson's disease [5], Amyotrophic lateral sclerosis [6] and Alzheimer's disease [7,8]. RNA oxidation is also reportedly elevated in mild cognitive impaired individuals who may be pre-disposed to Alzheimer's disease, suggesting that oxidized RNA is not merely a by-product of the pathological milieu, but one of the primary causes of disease pathogenesis [9–11].

In the aforementioned studies, RNA oxidation has been described solely based on monitoring 8-hydroxyguanosine (8-OH-Guo), a typical oxidized derivative. The RNA oxidation assays used methods established for the DNA counterpart, 8-hydroxy-2'-deoxyguanosine (8-OH-dGuo) using HPLC with electrochemical detection [12], immunological detection [13] or GC/MS [14]. However, it is presumed that a number of other ribonucleotide derivatives are generated by oxidation similar to those of deoxyribonucleotides, which have been extensively investigated [15]. In a survey of oxidoreduction activity of ribonucleosides from yeast RNA pools, Yanagawa et al. [16] identified 5-hydroxyuridine, 5-hydroxycytidine and 8-hydroxyadenosine, in addition to 8-OH-Guo in the hydrolysate. Their DNA counterparts have been isolated in oxidized DNA [15]. However, there have been few studies regarding biochemical analysis of RNA oxidation.

In addition to the oxidative modification of bases, loss of bases (primarily purines) has been reported to be a major oxidative modification of DNA. Generation

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2010.535529

of abasic sites can be induced chemically by DNA damaging or oxidizing agents such as alkylating agents or ionizing radiation. Also abasic sites are intermediates in the repair pathway initiated to eliminate oxidized bases by DNA *N*-glycosidases.

Ide et al. [17] have developed a probe reactive for the aldehyde group in abasic sugar moieties. The DNA abasic assay using the Aldehyde Reactive Probe (ARP), *N*'-amino-oxymethylcarbonylhydrazino D-biotin, has been further characterized and utilized as a representative DNA oxidation assay. ARP consists of an amino-oxy group which binds to abasic sites and a biotin moiety suitable for quantification. Based on an ELISA system using ARP, Swengerg et al. [18] reported that endogenously generated DNA abasic sites exist in the level of 3.0 out of 10^5 nucleotides in rat brain tissue .

In this study, we show that RNA containing abasic sites react with ARP with a detection limit of 10 fmoles. *In vitro* RNA oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) is dose-dependently reactive to ARP. In addition, we find increases in ARP reactive RNA in cells under oxidative stress conditions. Thus, our assay using ARP is sensitive and versatile for measurement of oxidized RNA under pathophysiological conditions but also shows the potential for applicability to other techniques including northern blotting.

Materials and methods

Reagents and cell culture

All the buffer solutions for RNA synthesis, isolation and oxidation were pre-treated with chelex 100 (Bio-Rad, Hercules CA) or contained 1 mM of EDTA. All the reagents used were purchased from Sigma-Aldrich (St. Louis, MO) unless specified. HeLa cells were purchased from ATCC, and maintained in DMEM plus 10% foetal bovine serum (Clonetech, Mountain View, CA) in 5% CO_2 at 37°C. One or two days after passage, sub-confluent 6 cm cultures were treated with hydrogen peroxide at indicated concentrations. Otherwise, after rinsing with PBS (×2), peroxynitrite (Cayman Chemical, Ann Arbor, MI) suspended with 0.3 N NaOH was added to the cell suspension and incubated for 15 min in a CO_2 incubator. The same volume of 0.3 N NaOH was added to the cell suspension for the negative control.

Preparation of RNA

In vitro RNA synthesis was conducted by mixing T7 RNA polymerase with 7.5 mM of ribonucleotide, salt buffer (Ambion, Austin, TX) and DNA template encoding luciferase2 gene. After incubation for 3 h at 37°C, RNA was treated with DNase I, followed by purification using a QIAGEN RNeasy kit (QIAGEN, Valencia, CA) according to the manufacture's protocol, except for 12 min centrifugation at 17 000 × g before the RNA elution step to completely exclude washing buffer in the final prepared RNA suspension. Exactly 250 μg/ml of the resulting RNA was reacted with hydrogen peroxide and 1:1 Fe(II)-ascorbate mixture in 10 mM HEPES buffer (pH7.2) at 37°C for 30 min. For peroxynitrite oxidation, peroxynitrite was diluted with 0.3 N NaOH and an equal volume of 0.3 N HCl was concomitantly mixed with the RNA suspension in 50 mM Na phosphate buffer (pH7.4) by brief vortexing, then incubating at room temperature for 30 min. Alternatively, RNA in 15 mM Na phosphate buffer (pH7.4) was irradiated using a gamma-ray generator (GammaCell 40 Exactor, MDS Nordion, Ottawa, Canada). The oxidation reaction was terminated by adding 5 mM EDTA, then quickly precipitated with cold ethanol, and stored at −80°C until use. Total RNA derived from cultured cells was extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol except for homogenization in the presence of 10 mM deferroxiamine. The final RNA preparation was suspended with 5 mM Tris-HCl (pH 7.5), 1 mM EDTA (Tris-EDTA buffer) and the concentration was determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and stored at −80°C until use.

Preparation of abasic RNA

Depurinated transfer RNA^{phe}. Based on previous reports that the post-transcriptionally modified base (wybutosine) in $tRNA^{Phe}$ is acid labile, RNA containing abasic sites were prepared [19]. Ten absorbance units (A_{260nm}) of yeast $tRNA^{Phe}$ were incubated with 0.1 M of ammonium formate (pH 2.9) at 37°C according to a previous report [20]. After RNA was precipitated with ethanol at −20°C for 30 min, the precipitated RNA was washed with 75% ethanol and resuspended with Tris-EDTA buffer. To monitor the generation of the abasic site, a decrease in fluorescence derived from the wybutine base was measured in the RNA solution using excitation at 315 nm and emission at 400 nm to 520 nm using a SpectraMax M2 multiwell plate reader (Molecular Devices, Sunnyvale, CA).

Chemically synthesized abasic RNA. Oligonucleotides (5'-(6-FAM)AGUUCCACGGUAACGCUUAGUC-3' and 5'-(6-FAM)AGUUCXACGGUAACGCXUAGUC-3') were prepared by standard automated oligonucleotide synthesis. For the synthesis, N-acetyl-2'-O-[(triisopropylsilyl)oxy]methyl (TOM) protected phosphoramidites (GlenResearch, Sterling, VA), 6-FAM-phosphoramidite (Nucleic Resources, Muskego, WI) and a C^{Ac}-CPG solid support (GlenResearch) were used. The 1-(2-nitrophenyl)

ethyl-2'-TOM protected phosphoramidite was synthesized as reported previously [21]. Synthesis followed a standard phosphoramidite protocol with 5-(ethylthio)-1H-tetrazole (0.25 M) as the activator and a coupling time of 6 min for each monomer.

Oligonucleotides were then deprotected using 10 M methylamine in EtOH/water (1:1) for 6 h at room temperature and subsequently silyl deprotected with 1 M tetrabutylammonium fluoride in THF for 16 h at room temperature, according to published protocols [22]. The oligonucleotides were desalted using NAP-10 columns and were finally precipitated from ethanol.

One OD of the oligomer suspension in Tris-EDTA buffer was illuminated with visible light to remove 1-(2-nitrophenyl)ethyl group, then precipitated by ethanol. After resuspending in Tris-EDTA buffer, 600 ng of oligomer was elongated by 2.4 kU of yeast poly(A) polymerase (USB Corporation, Cleveland, OH) in the presence of 0.6 mM ATP and the reaction buffer provided by manufacturer for 40 min at 37°C. The RNA was repurified using an RNeasy spin column (QIAGEN). The concentration of abasic sites after elongation was determined based on fluorescence of 6-carboxyfluorescein attached in the 5'-end (excitation: 495 nm, emission: 520 nm).

RNA abasic site detection

Exactly 200 μg/ml of *in vitro* synthesized RNA or cellular RNA (~5 μg) was incubated with 2 mM of *N'*-aminooxymethylcarbonylhydrazino D-biotin (Dojindo, Rockville, MD) in Tris-EDTA buffer for 1 h at 37°C. After mixing with 50 mM formaldehyde to terminate the reaction, derivatized RNA was precipitated by mixing with 0.1 volume 3 M sodium acetate and three volumes of ethanol at −20°C for 1 h, followed by washing (2×) with chilled 75% ethanol, then resuspended with Tris-EDTA buffer. Between 300 ng and 1 μg of the derivatized RNA was spotted by pipetting onto Hybond N+ membrane (GE Healthcare, Piscataway, NJ), which was previously soaked in Tris-EDTA buffer, then air-dried. After drying, the spotted membrane was irradiated using a UV cross-linker (Stratagene, Wilmington, DE) with 120 mJ/cm^2, followed by pre-incubation with blocking solution (Li-COR, Lincoln, NE) for 30 min at room temperature on a rocking platform. The membrane was then incubated with streptavidin-Horse radish peroxidase (Rockland, Gilbertsville, PA) at 1:20 000 in blocking solution at room temperature for 1 h. After washing with PBS containing 0.05% Tween20 (×4) for 6 min, the membrane was developed using the chemi-luminescent reagent ECLplus (GE Healthcare) for 5 min with gentle swirling. Chemi-luminescence was captured using a FluorChem CCD camera 1–15 min exposure dependent on the strength of spot intensity. The intensity was quantified using the manufacturer's software using auto-background subtraction mode (Alpha Innotech, San Leandro, CA).

8-OHGuo ELISA

Two micrograms of oxidized RNA was digested with 2 U of P1 nuclease in 10 mM Na acetate buffer (pH5.0) for 3 h, then with 1 U of alkaline phosphatase and 2 mU of phosphodiesterase in 100 mM Tris-HCl (pH 7.5) for 1.5 h. After centrifuging at 6 k × g for 5 min, the supernatant of the hydrolysate was diluted 10–50-times with Diluent Buffer provided by the 8-OHG Quantification kit (Cell Biolabs Inc, San Diego, CA). ELISA procedure was followed by the manufacturer's protocol. Colourimetric absorbance was measured by a SpectraMax M2 multiwell plate reader at 450 nm.

Abasic-ARP analyses by LC/MS

Twenty micrograms of RNA oxidized by 100 μM of Fe(II)/ascorbate and H_2O_2 was reacted with ARP, then with formaldehyde to terminate the reaction as described above. The RNA was hydrolysed to monoribonucleoside by P1 nuclease, phosphoadiesterase and alkaline phosphatase according to the previously published method [23]. Four micrograms of resultant hydrolysate was applied to a C-18 capillary column (Magic MS C18, 0.5 × 150 mm) and eluted using gradient conditions as described in the previous report [24]. Positive and negative controls were prepared by reacting 1 mM ARP and 10 mM of ribose or formaldehyde and incubating at 37°C for 2 h, respectively.

Results

Validation of ARP reactivity to abasic RNA

The aldehyde reactive probe (ARP), *N'*-aminooxymethylcarbonylhydrazino D-biotin has been used to detect DNA abasic sites [25]. Thereafter several modified probes for detecting DNA abasic sites have been developed mainly utilizing the property of the aminooxy group which binds preferentially to aldehyde groups in the open-ringed abasic sugar moiety [26]. However, until now the reactivity of ARP for the RNA abasic site has not been investigated. To this end, abasic RNA was prepared using yeast tRNA^{Phe}, since wybutine, a post-transcriptionally modified fluorescent base in tRNA, is known to be readily depurinated by mild acid treatment [20,27]. The tRNA^{Phe} was incubated in ammonium formate buffer (pH 2.9) at 37°C for various times. After excluding the released wybutine base by ethanol precipitation, the tRNA showed a decline in the fluorescence derived from the base depending on incubation time (Figure 1A).

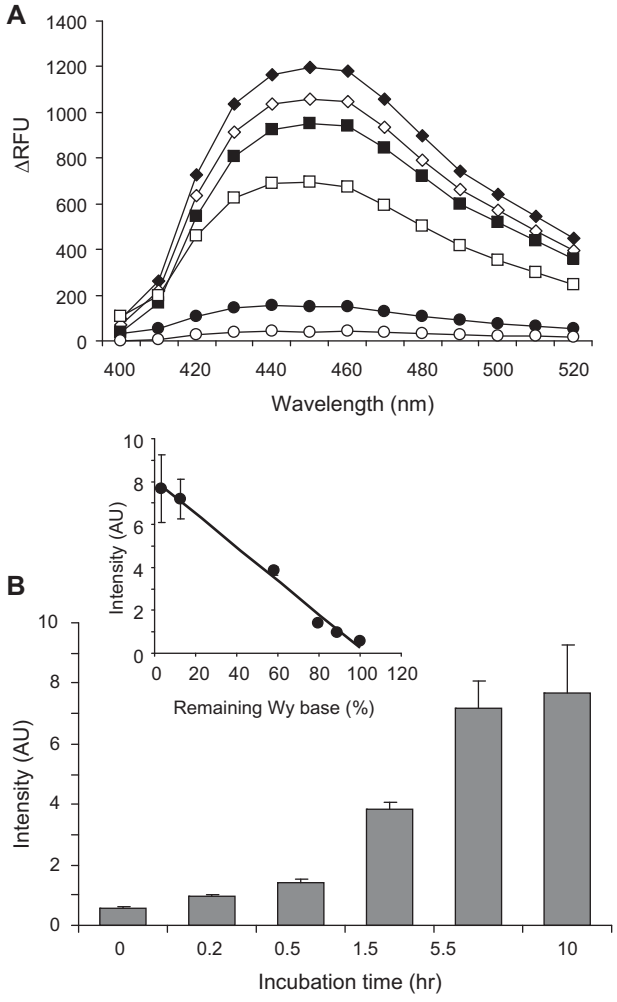

Figure 1. Depurinated tRNA^{Phe} increases ARP reactivity. (A) Fluorescence derived from the wybutosine residue remaining in yeast tRNA^{Phe} was measured, after tRNA^{Phe} was incubated in 0.1 M ammonium formate (pH2.9) at 37°C for ◆ 0 h, ◇ 0.2 h, ■ 0.5 h, □ 1.5 h, ● 5.5 h or ○ 10 h. (B) After acid treatment tRNA^{Phe} was reacted with ARP followed by incubation with streptavidin-HRP on a positively charged membrane. The chemiluminescence derived from HRP was quantified. The inset shows the correlation between the remaining wybutosine and ARP reactivity. Data represent mean ± SD (n = 4).

The depurinated tRNA was incubated with ARP at 37°C for 1 h, followed by blotting on a positive charged membrane to detect the ARP containing biotin moiety using streptavidin conjugated horseradish peroxidase (HRP) as described. Figure 1B indicates that ARP reactivity to tRNA was increased depending on the treatment time under acidic conditions. This ARP reactivity was highly correlated with the depurination of wybutine (Figure 1B, inset). Thus, depurinated tRNA^{Phe} at the wybutine base increased ARP reactivity due to the formation of an abasic sugar moiety.

To further examine the ARP reactivity to RNA abasic sites, an oligo ribonucleotide, which contains abasic sites, was prepared by chemical synthesis according to a previous report [28]. Abasic sites in the oligo ribonucleotide were deprotected from a photocleavable 1-(2-nitrophenyl)ethyl group by visible light illumination. The deprotection was confirmed by incubation with 1 M of aniline (pH 4.6), which causes strand cleavage by β-elimination at abasic sites (data not shown). The abasic oligo ribonucleotide was elongated using polyadenylate polymerase to improve the retention on the membrane. Figure 2A demonstrates the agarose gel electrophoresis of the synthetic abasic RNA before and after elongation and provides a schematic diagram of the synthesized abasic RNA.

The length of the abasic RNA was extended to ~2–3 kb (equivalent to control RNA without abasic sites). The concentration of abasic sites in the elongated RNA was determined by measuring the fluorescence of 6-carboxyfluorescein which was attached at the 5'-end. Figure 2B shows that the abasic RNA exclusively reacted with ARP compared to RNA without deprotection or control RNA without abasic sites. Using this synthetic abasic RNA, it was found that the detection limit of the ARP assay was 10^{-14} moles RNA abasic sites, which corresponds to one abasic site per 10^5 ribonucleotides. The sensitivity of ARP to abasic RNA was comparable to that of abasic DNA previously reported [25]. A positively charged membrane and UV cross-linking were necessary to ensure consistent binding of the RNA to the membrane. ARP signal intensity was decreased, when skim milk or bovine serum albumin was used for blocking or when incubated with streptavidin-HRP, possibly because of RNase contamination. Among blocking solutions tested, the blocking solution purchased from Li-COR was found to show good quality and signal intensity.

Characterization of ARP reaction to abasic RNA

The reaction propensity of ARP with abasic RNA was examined using synthetic abasic RNA. As shown in Figure 3A, the reactivity was moderately increased by increasing temperature. This was also the case for total RNA (data not shown). Figure 3B indicates that ARP reactivity increased with increasing incubation time, but the reaction was almost complete at 1 h.

It was previously reported that acidic pH tends to depurinate genomic DNA *in vitro* [29]. The pH dependency was examined together with control RNA. It was found that ARP reactivity was accelerated in acidic conditions (less than pH6) for both abasic RNA and control RNA, although the former was higher than the latter (Figure 3C). These results suggest that ribonucleotides are depurinated at acidic conditions. Figure 3D indicates that ARP reactivity is correlated with the concentration of ARP used. The reactivity increased linearly with the ARP concentration; however the ratio between abasic RNA and control RNA was not changed by the concentration of ARP.

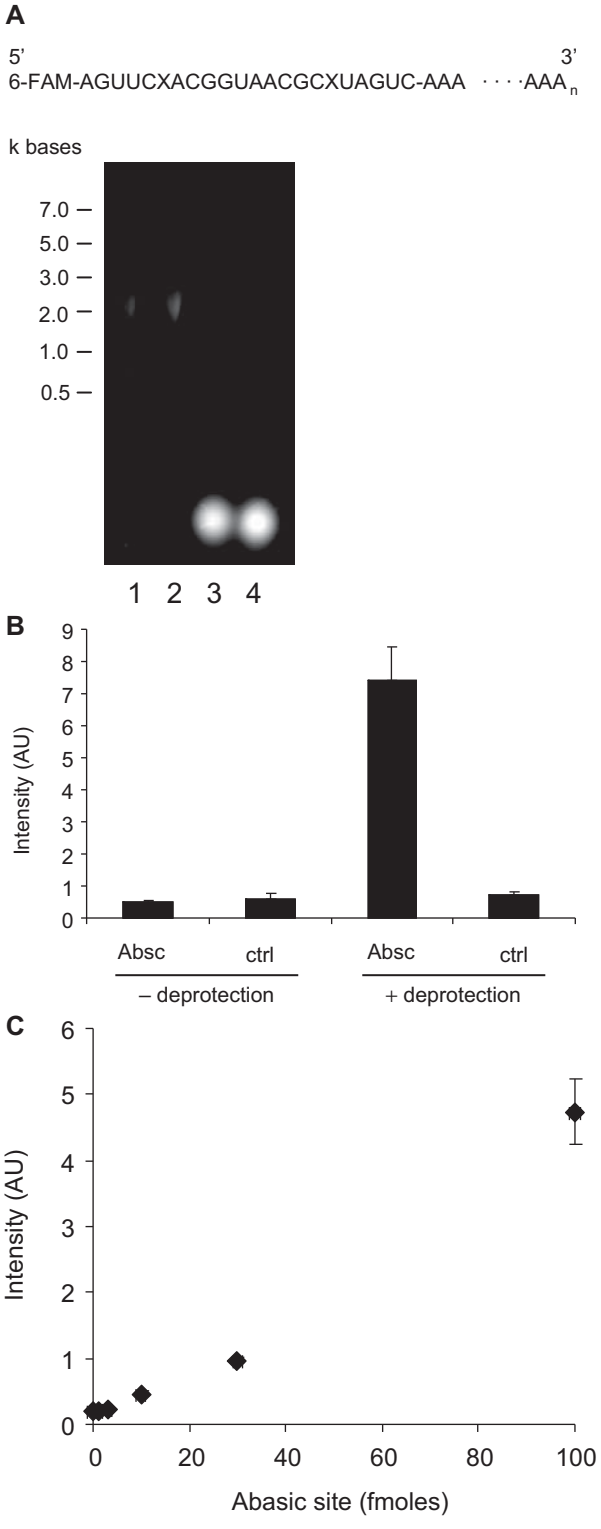

Figure 2. Abasic RNA reacts with ARP. A chemically synthesized oligo RNA with abasic sites was deprotected by visible light irradiation, followed by elongation with poly(A) polymerase. The schematic structure of the final abasic RNA is shown in (A). Abasic sites and 6-carboxyfluorescein are designated as X and 6-FAM, respectively. (A) Six hundred nanograms of RNA with abasic sites (lane 1) or control RNA (lane 2) was loaded onto agarose gels for electrophoresis, followed by staining with SYBR green. The results indicate that elongated RNA reached ~2–3 kb. The original oligo RNA with (lane 3) or without (lane 4) abasic sites was visualized using 6-FAM rather than SYBR green. (B) Elongated RNAs were examined for ARP reactivity. Abasic sites in RNA in the left lane were not deprotected by light, whereas RNAs in the right side were deprotected. Absc or ctrl denotes the RNA with or without abasic sites, respectively. (C) ARP derivatized abasic RNA (2.5–100 fmole) was spotted in the presence of 1 nmole of ribonucleotides. The concentration of abasic sites in the RNA was measured by the fluorescence derived from 6-FAM.

ARP reactivity to oxidized RNA

The formation of abasic sites has been known to be a major oxidized modification of DNA. It is known that the base-free sugar tends to be generated by cleavage of oxidative modified bases due to the weakening of the glycosidic bond. This abasic sugar moiety includes an aldehyde group at the C-1' position in the open-ring form. Otherwise, the abasic moiety is generated by DNA glycosidase to break the glycosidic bond of the oxidized base. The abasic sites generated are finally eliminated from the DNA chain by endonucleases such as apurinic/apyrimidinic endonuclease 1 (APE1) as a part of the oxidized DNA base excision repair system. It has been shown that abasic RNA can be generated enzymatically. The Ricin A chain catalyses depurination on a specific adenosine residue in 28S rRNA, leading to complete inactivation of protein synthesis [30]. Another RNA specific lyase, pokeweed antiviral protein, depurinates mRNA dependent on its cap structure [31]. The depurination of RNA by these plant-derived proteins causes loss of cellular function and cell death.

However, there have been no investigations of abasic site formation by RNA oxidation. We examined whether ARP reactivity is increased in RNA oxidized *in vitro* in response to different oxidation sources. First, RNA was subjected to γ-irradiation, which generates hydroxyl radicals.

Oxidizing RNA with less than 2.5 Gy did not show significant increases in ARP reactivity compared to non-oxidized RNA, although more than 5 Gy γ-ray irradiation significantly increased ARP reactivity dose-dependently (Figure 4). ARP reactivity increased in the presence of Fe(II), ascorbate and hydrogen peroxide (Fenton reaction) (Figure 5A).

As a comparison, we examined 8-OHGuo generation in the iron mediated oxidation. Figure 5B shows 8-OHGuo was also increased dose-dependently, similar to the ARP reactivity, indicating that ARP reactive species are increased by Fenton reaction and are correlated to other oxidative modifications such as 8-OHGuo. After derivatization with ARP the oxidized RNA was subjected to agarose gel electrophoresis, followed by transfer to membrane. In Figure 5C (lower panel), the RNA staining by methylene blue shows a slight degradation in oxidized RNA with 4 μM of Fe(II)/ascorbate/H_2O_2. The migration pattern of these derivatized RNAs was the same as those without derivatization (data not shown). Figure 5C (upper

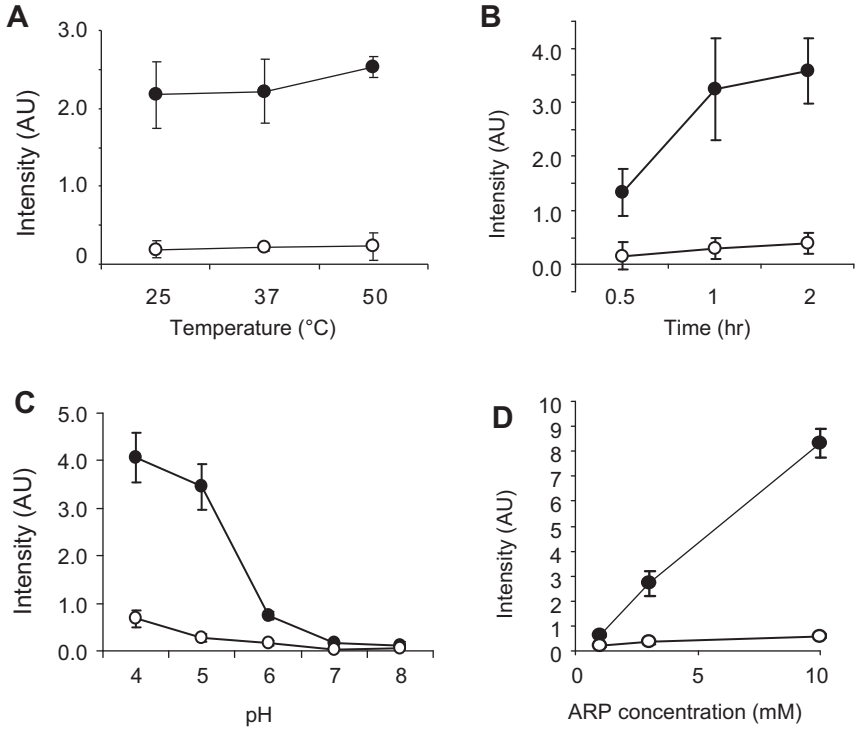

Figure 3. Characterization of ARP reactivity to abasic RNA. Exactly 100 pg of synthetic abasic RNA (closed circles) or control RNA (open circles) was reacted with ARP at different temperatures (A), incubation times (B), pH (C) or concentrations of ARP (D). The basic reaction conditions were carried out in the presence of 2 mM of ARP at 37°C for 1 h at pH 7.5. Data represent mean ± SD (n = 4).

panel) shows the chemiluminescence of ARP-derivatized RNA on the membrane using streptavidin-HRP. The oxidized full-length RNA as well as fragmented RNA reacted with ARP dose-dependently, consistent with the dot blot ARP assay result in Figure 5A. These results indicate that the RNA oxidation assay using ARP pre-derivatization method is applicable for northern blot analyses.

We investigated whether oxidation of RNA generates abasic sites that react with ARP. Oxidized RNA generated by Fe(II)/ascorbate/H_2O_2 was derivatized with ARP, then quenched using an excess of formaldehyde. The derivatized RNA was hydrolysed to mononucleoside by P1 nuclease, phosphodiesterase and alkaline phosphatase. It was presumed that the ARP-RNA abasic site after enzymatic hydrolysis is identical with a conjugate of ribose and ARP. The hydrolysate was subjected to capillary HPLC coupled with mass spectrometry.

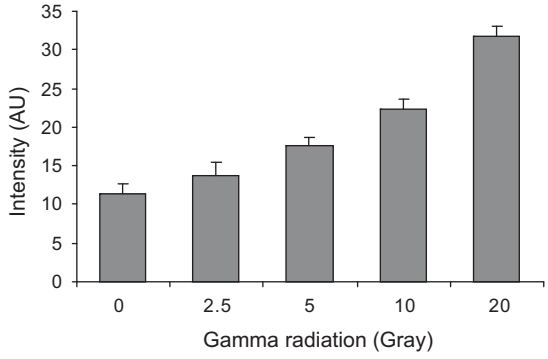

Figure 4. Gamma irradiation increased ARP reactivity. Exacty 250 μg/ml of *in vitro* synthesized RNA was irradiated at the doses indicated, then directly incubated with ARP at a final concentration 2 mM for 1 h. The derivatized RNA was analysed by streptavidin-HRP as described in Materials and methods. Data represent mean ± SD (n = 4).

In Figure 6A, a specific peak was eluted in oxidized RNA hydrolysate consistent with that of the ribose-ARP complex, but no corresponding peak was observed in non-oxidized control RNA hydrolysate. No peak was evident from ARP reacted with formaldehyde. The measured m/z of the peak in either ribose-ARP or oxidized RNA-ARP was 464.2 (Figure 6B and data not shown), which was close to the calculated mass size, 463.5. These results indicate that ARP reactive species is derived from the abasic sugar moiety.

In addition, RNA oxidized by reactive nitrogen species (RNS) was investigated. RNA was mixed with peroxinitrite in neutral pH followed by incubation at room temperature for 30 min, as the half-life is short under neutral pH.

RNA oxidized by peroxynitrite was found to increase ARP reactivity dose-dependently (Figure 7). Under the oxidation conditions tested, RNA did not show degradation based on agarose gel electrophoresis (data not shown). These results indicate that ARP reactivity correlates well with *in vitro* oxidation of RNA.

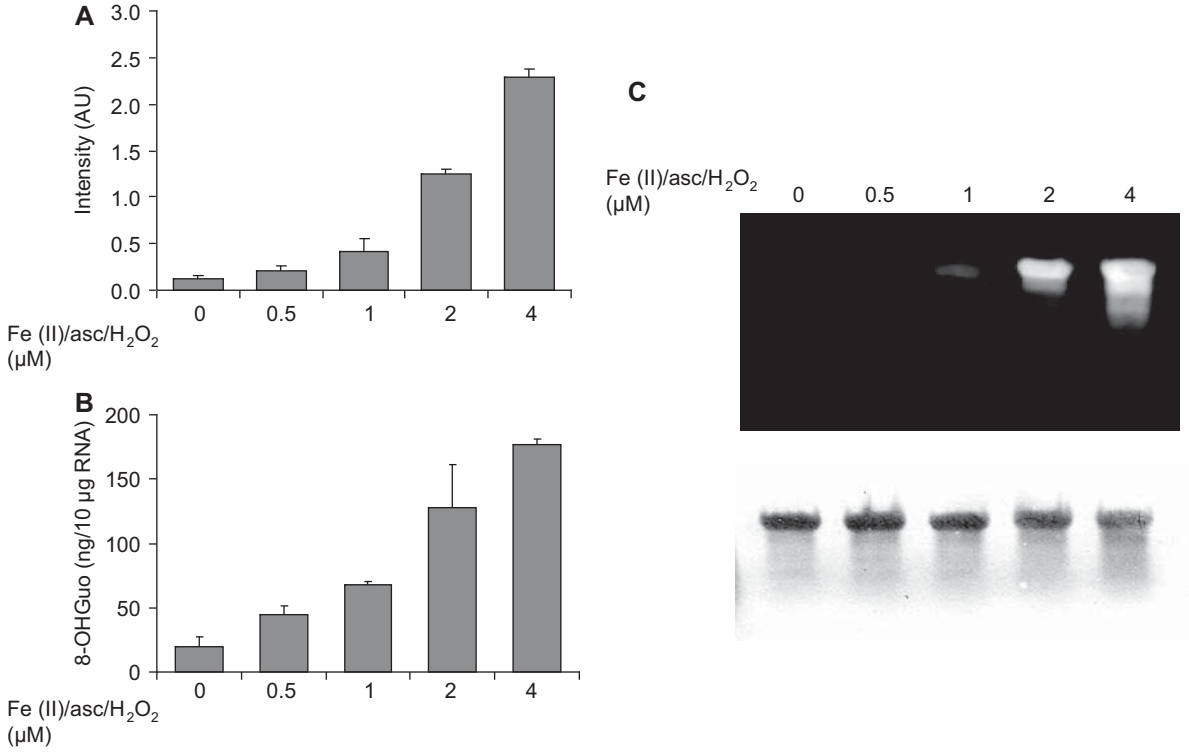

Figure 5. RNA oxidation using the Fenton reaction increased ARP reactivity. (A) *In vitro* synthesized RNA was subjected to oxidation in the presence of H_2O_2 and Fe(II)-ascorbate at the indicated concentrations at 37°C for 30 min, then precipitated with ethanol to eliminate unreacted oxidants. The oxidized RNA was examined by ARP reactivity as described in Materials and methods. (B) The oxidized RNA which was derivatized with ARP was loaded on an agarose gel and transferred to a positive charged membrane. The membrane was incubated with streptavidin-HRP and developed with chemiluminescence reagent (upper panel). Then, the membrane was stained with methylene blue to visualize RNA (lower panel).

ARP reactivity of cellular RNA under oxidative stress

Finally we examined ARP reactivity to RNA in cells under oxidative stress conditions.

Total RNA was isolated from HeLa cells after 1 h treatment with several different concentrations of H_2O_2. Figure 8A shows that ARP reactivity was increased by hydrogen peroxide challenge dose-dependently. The cell morphology was slightly altered in the cell cultures treated with the highest concentration of H_2O_2 (1000 μM), although oxidative degradation of total RNA was not observed under any oxidation condition (data not shown). In addition, ARP reactivity of total RNA was examined after peroxynitrite treatment of cell cultures (Figure 8B). After a short incubation with peroxynitrite, ARP reactive species were increased in cellular RNA dose-dependently. Thus, these results indicate that the ARP reactivity assay was able to monitor oxidative damage of intracellular RNA by either ROS or RNS.

Discussion

In this study, we identified abasic sites in oxidized RNA after derivatization with ARP, indicating that abasic RNA is reactive to ARP (Figure 6). Also it was found that *in vitro* oxidation of RNA induced depurination of the 8-oxoguanine base, suggesting that abasic sites are generated by depurination after nucleobase oxidation (M. Tanaka, unpublished data). Thus, it is likely that abasic formation is a common phenomenon in various types of oxidation of RNA similar to DNA. Supporting this concept, abasic RNA was found to be chemically more stable than its DNA counterpart under acidic or alkalinic conditions [21], implying that abasic RNA is relatively stable in physiological conditions. Thus, the stable form of the abasic site may represent one of the primary oxidized modifications in RNA oxidation.

There have been few investigations addressing the metabolism of oxidized RNA and its regulation by specific proteins. It was reported that 8-OHGuo is increased in HeLa cells by H_2O_2 within 30 min, however, after short treatment with H_2O_2, 8-OHGuo started to decrease to basal levels [32]. These results suggest that the decline in oxidized RNA was modulated by polynucleotide phosphorylase, which was previously identified as a binding protein for RNA containing 8-OHGuo [33]. It remains unknown whether this protein contributes to the repair or degradation of oxidized RNA, but alternative assays of RNA oxidation would be necessary to assess enzymatic activity to catalyse oxidized RNA.

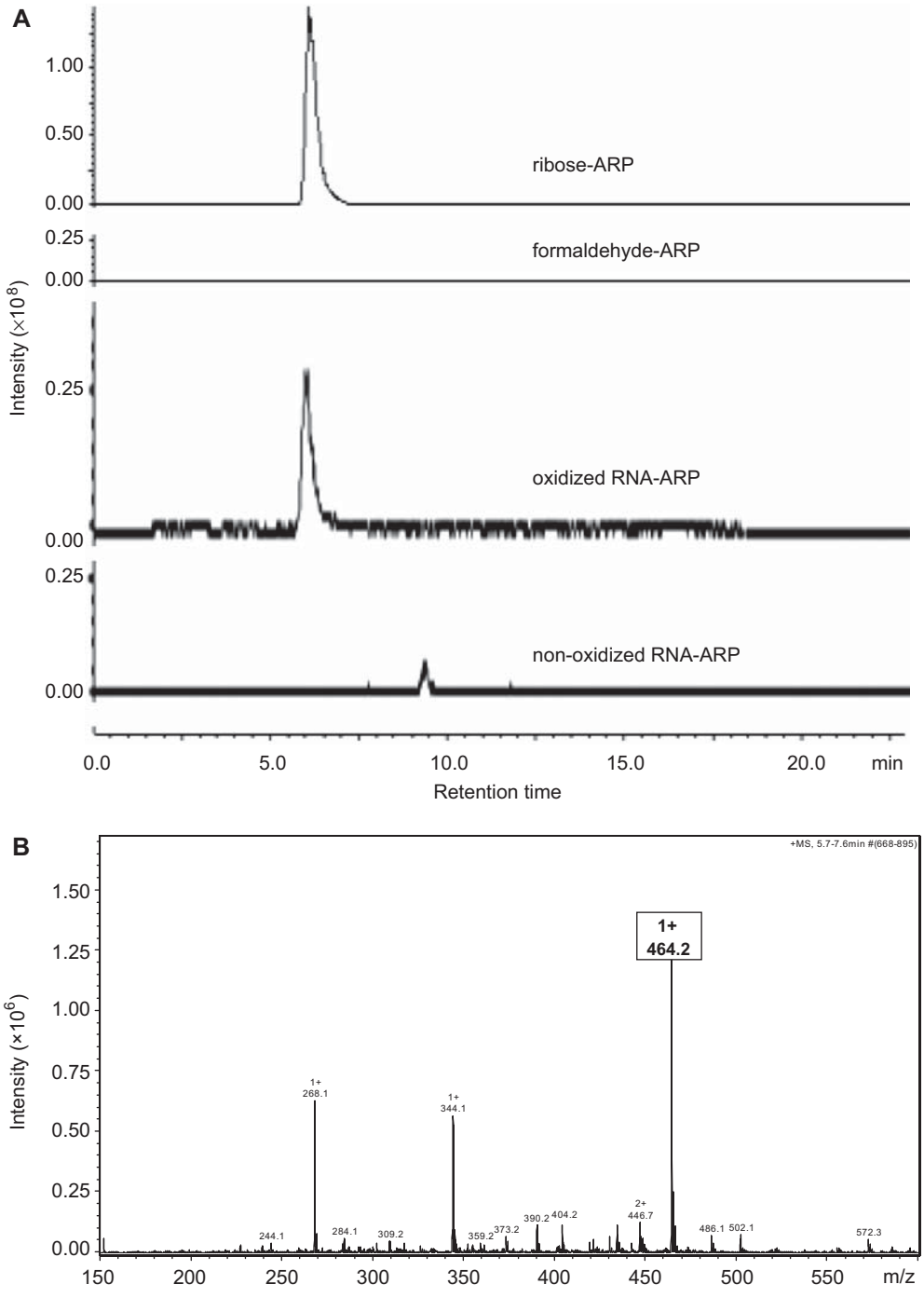

Figure 6. Abasic formation in oxidized RNA. RNA oxidized by Fe(II)/ascorbate/H_2O_2 was derivatized with ARP, then hydrolysed enzymatically. The hydrolysate was subjected to LC/MS as described in Materials and methods. (A) The extracted ion chromatograms with 464.2 ± 0.3 m/z. Ribose-ARP complex and formaldehyde-ARP complex were analysed as positive and negative controls, respectively. (B) The mass spectra of the oxidized RNA hydrolysate eluted at 6 min.

Recently, it was reported that APE1 has a crucial role in metabolism of abasic RNA in cells. APE1 has been known to be a multifunctional enzyme that is a redox-dependent regulator of transcription factors, an endonuclease incises the phosphodiester bond immediately 5' to an abasic site in DNA, interacting with several proteins. APE1 has been shown to have endonuclease activity not only on abasic DNA but also abasic RNA *in vitro* [34]. In addition, APE1 appears to degrade oxidized ribosomal RNA associating with a nuclear protein in the nucleolus. In fact, knockdown of this enzyme increased 8-OHGuo in cells and decreased translational activity [35]. These results suggest that the enzyme regulates metabolism of oxidatively damaged RNA. If abasic sites in RNA have a deleterious outcome on protein synthesis, it would be crucial to strictly regulate the abasic sites to maintain cellular homeostasis.

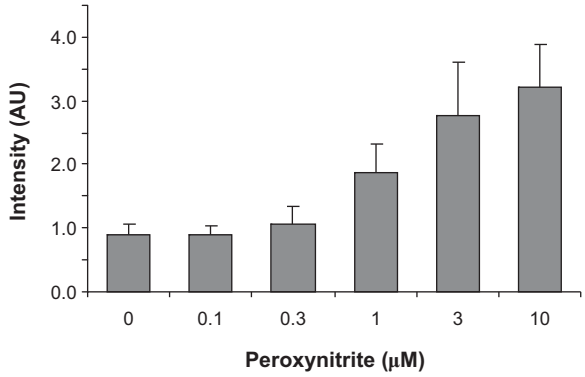

Figure 7. RNA oxidation induced by peroxynitrite increased ARP reactivity. Exactly 250 μg/ml of *in vitro* synthesized RNA was oxidized by peroxynitrite with the indicated concentrations in 50 mM potassium phosphate buffer (pH7.4) for 30 min at room temperature. After the reaction, oxidized RNA was analysed using the ARP assay. Data represent mean ± SD (n = 4).

Previously we demonstrated that in response to moderate oxidation, the oxidized mRNA was found to associate with the polysome similar to control mRNA [36] and generates dysfunctional proteins including premature polypeptides, due to translational errors. These results indicate that oxidative damage can cause abnormal protein synthesis, but the molecular mechanisms for the translational error have not been investigated. In this study, we found that RNA abasic sites are readily generated *in vitro* by biologically relevant oxidative sources. Abasic sites may have a critical impact on translation activity *in vivo*, since depurinated mRNA produced by the pokeweed anti-viral protein reportedly abolished translational activity [37].

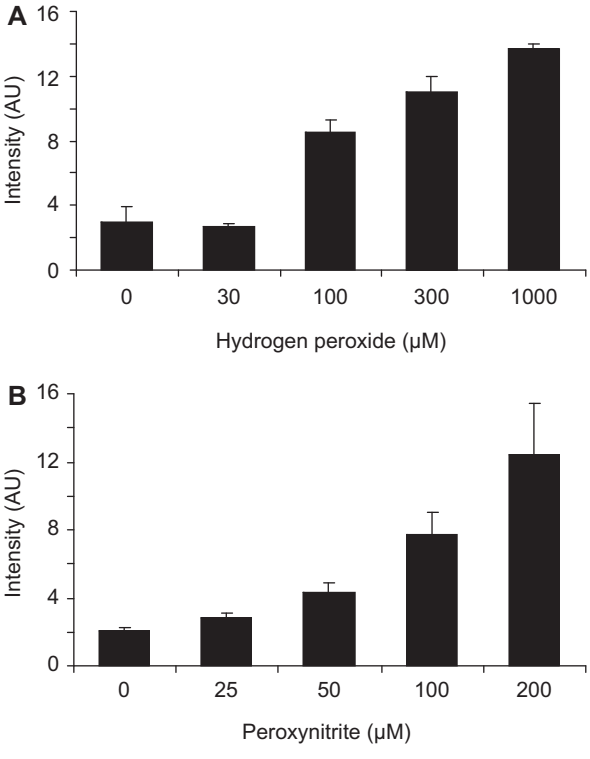

Figure 8. ARP reactivity of total cellular RNA was increased after oxidative stress. HeLa cells were treated with H_2O_2 for 1 h (A) or peroxynitrite for 15 min (B) using the indicated concentrations. Total RNA was isolated from the cells and abasic sites analysed with the ARP assay. Results indicate a dose-related rise in abasic sites in response to hydrogen peroxide or peroxynitrite. Data represent mean ± SD (n = 4).

Although 8-OHdGuo or 8-OHGuo has been generally accepted to be an oxidative marker of nucleic acid, several reports demonstrate a discrepancy between the generation of 8-OHdGuo and other DNA lesions under pathological conditions. Pang et al. [38] examined the generation of several oxidative DNA derivatives in a chronic inflammatory model and found that nitrosative stress conditions significantly increased DNA adducts derived from lipid peroxidation, such as etheno-purine, whereas levels of 8-OHdGuo did not increase in the affected organ. In a model of permanent focal cerebral ischemia, Nagayama et al. [39] observed an increase in 8-OHdGuo only in the peri-infarct region, whereas DNA single strand breaks were accumulated in the infarct regions rather than peri-infarct region. In addition, rats treated with piperonyl butoxide, a hepatocarcinogen, showed no significant increase in 8-OHdGuo production in spite of increased levels of ROS [40]. It was suggested that 8-OHdGuo could be subjected to further oxidation to an intermediate due to its low redox potential, which may, in part, cause under-estimation of DNA damage, especially in response to chronic oxidative stress conditions [41]. Thus, 8-OHdGuo is not a consistent marker of damage and therefore multiple oxidative markers are necessary to assess DNA oxidation precisely. In the case of RNA oxidation, all the previous reports were based on measuring 8-OHGuo. Although there is no direct comparison to other RNA derivatives, we observed no generation of 8-OHGuo after oxidation of RNA by cytochrome *c* and H_2O_2 in spite of significant oxidative modification of guanosine residues (unpublished data), suggesting that generation of 8-OHGuo varies depending on oxidation condition. In addition, it is unclear whether the generation of 8-OHGuo is proportional to the extent of RNA oxidative damage in any pathological setting. In order to elucidate RNA oxidative damage and assess its functional importance, an assay for an alternative oxidation marker is required. In this study, we have developed a novel RNA oxidation assay based on the abasic reactive probe. Our assay method is highly sensitive (to the femtomole range) and not only evaluates the genesis of abasic sites in response to RNA oxidation level but also provides useful insight into metabolism of abasic RNA.

Declaration of interest

This research was supported by NIH grants AG11370 and NS056218.

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This paper was first published online on Early Online on 9 November 2010.