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p47phox and reactive oxygen species production modulate expression of microRNA-451 in macrophages

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Abstract

The production of microRNAs (miRNA) is influenced by various stimuli, including environmental stresses. We hypothesized that reactive oxygen species (ROS)-associated stress could regulate macrophage miRNA synthesis. miRNAs undergo unique steps of maturation processing through either one of two pathways of cytoplasmic processing. Unlike the canonical pathway, the regulation of alternative cytoplasmic processing of miRNA has not been fully elucidated yet. We cultured bone marrow derived macrophages (BMDM) from wild type (WT) and p47^{phox}^{-/-} mice and profiled miRNA expression using microarrays. We analyzed 375 miRNAs including four endogenous controls to normalize the data. At resting state, p47^{phox}^{-/-} BMDM has the markedly reduced expression of miR-451 compared to WT BMDM, without other significant differences. Unlike majority of miRNAs, miR-451 goes through the unique alternative processing pathway, in which Ago2 plays a key role. In spite of significant reduction of mature miR-451, however, its precursor form, pre-miR-451, was similar in both BMDMs, suggesting that the processing of pre-miR-451 is impaired in p47^{phox}^{-/-} BMDM. Moreover, p47^{phox}^{-/-} BMDM expressed significantly reduced level of Ago2. In contrast, Ago2 mRNA levels were similar in WT and p47^{phox}^{-/-} BMDM, suggesting a post-transcriptional defect of Ago2 production in p47^{phox}^{-/-} macrophages, which resulted in impaired processing of pre-miR-451. In order to examine the functional significance of miR-451 in macrophages, we cultured BMDMs from miR-451 knock-out mice. Of interest, miR-451-deficient BMDM exhibited reduced ROS generation upon zymosan stimulation,

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Supplementary material available online

Supplementary Figures 1–3 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10715762.2014.974037>.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

compared to WT BMDM. Our studies suggest functional crosstalk between ROS and miR-451 in the regulation of macrophage oxidant stress.

Keywords

macrophages; reactive oxygen species; stress; microRNA; NADPH oxidase; p47phox

Introduction

MicroRNAs (miRNAs) are important regulators of gene expression via RNA interference mechanisms including target mRNA degradation and translation inhibition [1]. miRNAs regulate gene expression in response to environmental stresses in a vast array of diverse species from plants to mammals and are implicated to be involved in the pathogenesis of genetic and acquired human diseases [2,3]. Various types of cellular stresses including hypoxia, temperature shock, pain, cigarette smoke, light, and nutrients alter miRNA expression [4–8]. miRNAs are encoded in diverse locations of the genome in coding and non-coding regions. Similar to protein coding genes, the expression of miRNAs is regulated at multiple levels, both transcriptionally and post-transcriptionally, and many transcription factors are involved in transcriptional regulation of miRNAs. However, miRNA biogenesis has unique steps of processing of maturation [9]. In order to become a functional mature form of miRNA, the premature forms are processed via multiple endonucleases activity in the nucleus and the cytoplasm. In the nucleus, the primary miRNA transcript (pri-miRNA) is cleaved into pre-miRNA by Drosha, a member of ribonuclease III (RNase III) family, and DGCR8/Pasha [10]. The resulting pre-miRNA hairpins are transported into the cytoplasm, where they are cleaved on their terminal loop ends by a cytoplasmic processing step, which has emerged as an important mechanism in defining the spatiotemporal pattern of miRNA expression [11]. Two different pathways of cytoplasmic processing have been discovered. The “canonical pathway” for miRNA biogenesis is broadly conserved amongst vertebrates and invertebrates and is catalyzed by Dicer, a RNase III family member. Dicer is viewed as a central processing enzyme in the maturation of miRNAs [12,13]. It has been reported that Dicer activity is regulated by inflammation and stresses [14]. Recently, an “alternative pathway” of miRNA biogenesis has been discovered. This pathway bypasses Dicer function and is dependent on the catalytic activity of Ago2 which has robust RNaseH-like endonuclease activity and regulates the expression of some miRNAs including miR-451 [15–17]. The biological significance of this Ago2-dependent pathway has not been fully elucidated yet.

Macrophages have capacities to do a robust production of ROS by enzymatic activity of NADPH oxidase during inflammatory process and to infiltrate into the tissue site where oxygen tension is usually low. By using macrophages with NADPH oxidase deficiency, we investigated how ROS regulates miRNA expression in macrophages.

Here, we report that macrophages respond to oxidant stress by altering the expression of miR-451 through post-transcriptional modulation of Ago2 synthesis. Conversely, miR-451 also affects ROS generation in macrophages.

Materials and methods

Animals and genotyping

p47^{phox} knock-out mice (p47^{phox}^{-/-}) have a targeted disruption of the p47^{phox}, which results in a defective NADPH oxidase activity, rendering macrophages incapable of generating measurable superoxide [18]. Wild type mice with same background (C57BL/6J) were used for the control. MiR-144/451^{-/-} (referred to as miR-451^{-/-}) mice were defective in expressing miR-451 as previously described [19]. The deletion of 388 base pair segment of genomic DNA containing both the pre-miR144 and pre-miR451 region was confirmed by PCR genotyping. The mice were obtained from University of Pennsylvania, and housed at the animal facility of the University of Illinois at Chicago in a standard pathogen free environment. The experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois at Chicago.

Cell culture of bone marrow-derived macrophages and RAW 264.7 cells

Bone marrow derived macrophages (BMDM) were isolated from mice (WT, p47^{phox}^{-/-}, and miR-451^{-/-}), as previously described [20]. Mouse bone marrow cells were flushed from femurs and tibias with Ca²⁺/Mg²⁺-free Hanks' balanced salt solution (HBSS, Mediatech Inc). Bone marrow progenitor cells were cultured in DMEM (Mediatech Inc) containing 10% heat inactivated FBS (HyClone), 100 U of Penicillin–Streptomycin (Mediatech Inc) and supplemented with 10% L929 Cell Culture Media (LCCM). After 7d, BMDM (~99% macrophages based on flow cytometry using anti-F4/80) were collected for the experiments.

RAW 264.7, murine macrophage/monocyte cell line, was cultured in 1 × DMEM media containing 10% FBS (Hyclone) supplemented with penicillin (100 U/mL)/ streptomycin (100 µg/mL), and was maintained at 37°C in a humidified air containing 5% CO₂.

Western-blot and quantitative real-time PCR

BMDMs were lysed with 1 × RIPA buffer containing 1 × protease inhibitor cocktail (Roche Diagnostics) and used for western blot. The antibodies used are Ago2 (ab32381, Abcam), p47-phox (DAM1821146, Milipore), α-tubulin (sc-8035, Santa Cruz Biotechnology, Inc), and β-actin (Sigma). The experiments were performed in triplicate.

For measuring mRNA expression, the total RNA was isolated by using the RNeasy Plus Mini kit (Qiagen) according to the manufacturers' protocol, and quantitated by Nanodrop spectrophotometry (Thermo Scientific). Quantitative RT-PCR was performed by LightCycler 480 real-time PCR instrument (Roche Diagnostics). Primers for Ago2 and β-actin were obtained from Applied Biosystems (Grand Island, NY). The relative expression was normalized to the expression of β-actin. Relative fold changes of gene expression were calculated by the CT method and the values are expressed as 2^{-CT} (22).

Flowcytometry

Single-cell suspensions of BMDM (1 × 10⁴/sample) were washed and incubated on ice for 30 min with the anti-mouse CD-206 Alexa Fluor (AbD Serotec). The cells were analyzed on

FACS Vantage flow cytometer (BD Biosciences) where gating was based on respective unstained cell population and isotype matching control antibodies. The data were analyzed with CellQuest software (BD Biosciences).

Micro-RNA microarray analysis

BMDM were cultured from WT and p47^{phox}^{-/-} mice and total microRNA was isolated by using *mirVana* miRNA isolation kit (Ambion), and converted using the specific cDNAs from mature miRNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and the cDNAs were loaded onto TaqMan Rodent MicroRNA array-A kit chips (Applied Biosystems). This kit contained 375 lyophilized rodent TaqMan miRNA sequences plus 4 small nuclear RNAs that are endogenous controls. Quantitative real-time PCR was performed using the 7900HT Fast Real-Time PCR instrument (Applied Biosystems) and the data were analyzed on RQ manager software (Applied Biosystems). The data have been submitted to NCBI GEO database and can be accessed with the accession number GSE55189.

ROS detection by chemiluminescence assay

The isoluminol-enhanced chemiluminescent assay for ROS detection were performed as described previously [21]. Primary cultured BMDM were cultured from three WT and miR-451 mice. The matured BMDM were seeded into 96-well culture plate at 5×10^4 /well in duplicate and primed with 100 ng/ml LPS or left un-treated for 16 h prior to ROS measurement. The BMDM were washed once with phenol red and serum-free DMEM (Mediatech Inc), and stimulated with 200 µg/ml of Zymosan A (Sigma-Aldrich). The measurement of ROS was initiated by immediately adding the mixture of 100 µM lucigenin (Molecular Probes) and 100 µM NADPH (Sigma-Aldrich) in DMEM (phenol red and serum-free). Chemiluminescence signals of each well were continuously recorded for 2 h at 37°C post-stimulation in a BioTek Synergy 2 multi-detection microplate reader (BioTek, Winooski, VT), and the averaged change in the chemiluminescence signals was used to quantify the ROS production in each sample.

Measurement of NO production

Primary cultured BMDM (5×10^5 cells/well) were stimulated with increasing dose of LPS (upto 1000 ng/ml) for 24 h. The concentration of nitrite in culture supernatants was measured by a Griess assay (1% sulfanilamide and 0.1%N-[1-naphthyl]-ethylenediamine dihydrochloride in 5%phosphoric acid) as we reported previously [22].

Northern blot analysis

Northern blot was performed by using a sensitive non-radioactive system with slight modification as described [23]. Total RNA was extracted using *mirVana* miRNA isolation kit (Ambion). The quantity and quality of RNA was evaluated by nano-drop spectrophotometer (Thermo Scientific) and agarose gel electrophoresis. A biotin-labeled LNA probe for mmu-mir-451 was used. Five micrograms of the total RNA was resolved on 15% denaturing polyacrylamide gel using the SequaGel UreaGel System (National Diagnostics) and the electrophoresis was done in $1 \times$ Tris-Borate-EDTA buffer (Sigma), and

tRNA was visualized using ethidium bromide staining to ensure the quality and relative amount of the RNA. The resolved RNA was transferred to positively charged nylon membrane (Hybond-N⁺, Amersham Pharmacia Biotech) using a semidry Transblot electrophoresis apparatus (BioRad) and the RNA was chemically cross-linked to the membrane by freshly prepared EDC cross-linking solution [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 1-Methylimidazole (Sigma)] at 65°C for 1–2 h. The biotin-nylated products were detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) on the Gel Imaging System (BioRad).

In-vitro processing of precursor of miR-451 oligo in BMDM

We performed *in-vitro* processing assay of the precursor microRNA as described in previous report [16]. A 42-nt oligomer pre-mir-451 (5'-biotin-AAA CCG UUA CCA UUA CUG AGU UUA GUA AUG GUA ACG GUU CUG – 3') was synthesized from (Integrated DNA Technologies, Inc). BMDMs were seeded into 60 mm dishes, and were transfected with 100 nM of the oligos using RNAimax (Invitrogen). After 72 h, cells were lysed and total RNA was isolated using TriZol (Invitrogen) according to manufacturer's protocol. Five micrograms of the total RNA was resolved on 20% denaturing polyacrylamide gel, and northern blot protocol was carried out as described above.

Expression and processing of Ago2 protein

The expression of Ago2 protein was analyzed in the WT and p47^{phox}^{-/-} BMDM. To examine the involvement of proteosomal pathway, the p47^{phox}^{-/-} BMDMs were treated with 0.5 μM MG-132 (Calbiochem # 474791) for 24 h. To check the effect of oxidative stress on the expression of Ago2, we used MnTMPyP, a cell-permeant SOD mimetic, to reduce oxidative stress in WT BMDM. The cells were treated with 2.5 mM or 50 mM of MnTMPyP (Enzo Lifesciences Inc.) for 24 h. Since p47^{phox}-deficient cells are defective in generating oxidative stress, we treated the p47^{phox}^{-/-} BMDM with 0.2 mM 1-Phenyl-2-thiourea (PTU, Sigma-Aldrich) for 16 h, and 24 h. PTU contains a free thiol group capable of electron capture, and induces oxidant stress in several experimental systems [19,24]. Following the treatments, the BMDMs were probed for Ago2 by western blot.

Results

miR-451 is markedly reduced in the p47^{phox}^{-/-} macrophages

We cultured BMDMs from p47^{phox}^{-/-} mice and confirmed the knock out of the p47^{phox} gene by immunoblot analysis (Figure 1A). CD206, the macrophage mannose receptor, was similarly expressed in both p47^{phox}^{-/-} and WT BMDMs after LPS (100ng/ml) treatment (Figure 1B). LPS treatment induced the similar expression of COX-2, iNOS and Arg-1 genes in both BMDMs as well (Figure 1C).

Next, we examined the pattern of miRNA expression of both p47^{phox}^{-/-} and WT macrophages by performing an unbiased comprehensive analysis. miRNAs were isolated from WT and p47^{phox}^{-/-} BMDM, converted into specific cDNAs and quantified on a commercial array that interrogates 375 miRNAs. Four small nuclear RNAs (RU6, RU87, Sno RNA135 and Sno RNA202) were used as endogenous controls for normalizing the data.

More than one hundred miRNAs were detected in macrophages, most of which were expressed similarly in WT and p47^{phox}-deficient mice (Figure 2A and B) (Supplemental Figure 1 to be found at online <http://informahealthcare.com/doi/abs/10.3109/10715762.2014.974037>). However, one specific miRNA, miR-451, was markedly reduced in p47^{phox}-deficient macrophages compared to its WT counterpart. Specifically, miR-451 was decreased ~28.5 fold in p47^{phox}-deficient macrophages (GEO accession number GSE55189). We confirmed this result by quantitative RT-qPCR using specific primers for miR-451 (data not shown). The absolute levels and difference in expression ratios of miR-451 between WT and p47^{phox}-deficient macrophages were not changed by the stimulation with LPS alone (GEO accession number GSE55189). The miR-451 gene is part of a bicistronic locus that also encodes mir-144, and both miRNAs are transcribed as a single precursor, however they undergo different processing pathways (15, 16). The mir-144 pre-miRNA is processed by the canonical Dicer-dependent pathway, while mir-451 pre-miRNA is processed by Ago2 [15]. In spite of the large difference in miR-451 expression, miR-144 level was similar in WT and p47^{phox}-deficient BMDM (Supplemental Figure 2 to be found at online <http://informahealthcare.com/doi/abs/10.3109/10715762.2014.974037>), suggesting that the differences in miR-451 levels occur through post-transcriptional effects.

miR-451-deficient macrophages exhibit reduced ROS production

Since miR-451 has a function to control sensitivity to oxidative stress in erythroid cells [19], we investigated the functional aspect of miR-451 ablated macrophages. We quantified their generation of ROS after treatment with zymosan, a potent generator of ROS in macrophages [25,26], with or without LPS priming. The BMDMs were cultured from WT and miR-451^{-/-} mice and their genotypes were confirmed by PCR (Figure 3A). The miR-451^{-/-} BMDMs were capable to produce NO in response to LPS stimulation (Figure 3A). Next, we measured ROS production from these cells. The LPS priming before zymosan treatment induced higher luciferase activity than zymosan alone in both strains of BMDMs (Figure 3B). Despite similar expressions of COX-2, iNOS and Arg-1 in WT BMDMs (Figure 1C), however, the miR-451^{-/-} BMDMs exhibited significantly reduced ROS generation under both conditions (Figure 3B) (Supplemental Figure 3 to be found at online <http://informahealthcare.com/doi/abs/10.3109/10715762.2014.974037>), indicating the interactive role between miR-451 and oxidant stress in macrophages.

Defective post-transcriptional processing of the precursor form of miR-451 in p47^{phox}^{-/-} macrophages

In order to investigate the mechanism of reduced expression of miR-451 in p47^{phox}-deficient macrophages, we measured the primary transcript of miR-451, which reflects the transcriptional activity of the miRNA gene. MicroRNAs 451 and 144 are encoded ~100 bp apart and are evolutionally highly conserved. Mature miR-451 is initially transcribed as pre-miR-451-144 and then cleaved into the two separate miRNAs [27,28]. Northern blot analysis using a biotin-labeled LNA probe for a precursor form of miR-451 was performed. We detected two bands ~140 bp and ~75 bp, corresponding to pri-miR-451-144 and pre-miR-451 [15]. Although the mature form of miR-451 was markedly decreased in p47^{phox}-deficient macrophages (Figure 2), surprisingly, its unprocessed precursor forms (pri-miR-451-144, and pre-miR-451) were not reduced compared to WT macrophages, indicating

that the difference in mature miR-451 level is not due to differences in the rate of transcription (Figure 4A). In order to examine post-transcriptional processing of pre-mir 451 to its mature form, a 5'-biotin-labeled 42-nucleotide oligonucleotide precursor of miR-451 was synthesized and transfected into both WT and p47^{phox}-deficient BMDM to analyze its *in-vitro* processing as described in Methods [16]. The processing of the precursor to the mature form was detectable in BMDM of both genotypes, but was reduced in the p47^{phox}-deficient cells relative to WT (right column of Figure 4B), even though equal amounts of unprocessed miRNA were analyzed (lower panel of Figure 4B). These data indicate that p47^{phox} deficiency contributes to post-transcriptional processing of the precursor form of miR-451.

Reduced expression of Ago2 in p47^{phox}-deficient macrophages due to altered post-transcriptional regulation

Since miR-451 is one of the few microRNAs which undergoes dicer-independent, Ago2-dependent alternate pathway of cytoplasmic processing, we investigated the post-transcriptional processing of miR-451 [15,16]. First, we compared the expression of Ago2 protein in the WT and p47^{phox}-/- BMDM by Western blots, which revealed that p47^{phox}-deficient macrophages have markedly decreased Ago2 protein, compared to WT BMDM (Figure 5A). To determine whether the reduced Ago2 protein in p47^{phox}-/- BMDM is due to decreased transcription, we examined the Ago2 mRNA level at steady state. The data of RT-qPCR showed that the Ago2 mRNA level of p47^{phox}-/- BMDM was higher than that of WT BMDM, despite its lower protein level, suggesting altered post-transcriptional processing in the p47^{phox}-deficient BMDMs (Figure 5B).

Ago2 protein is modulated by proteosomal degradation and oxidative stress

Since the Ago2 protein was reduced in p47^{phox}-/- BMDM, we investigated whether the stability of Ago2 protein is increased in NADPH oxidase defective macrophages. It has been reported that proteosomal pathway regulates the stability of Ago2 protein [29]. We treated p47^{phox}-/- BMDM with MG-132, a proteosomal inhibitor, which resulted in significant increase of the Ago2 protein (Figure 6A). To further investigate whether Ago2 in macrophages is modulated by oxidative stress, the WT and the p47^{phox}-/- BMDMs were treated with MnTMPyP and 1-phenyl-2-thiourea (PTU), respectively, as described in Method. MnTMPyP scavenges ROS by acting as a mimetic of superoxide dismutase (SOD) and catalase, and is freely membrane permeable and non-toxic at the concentrations that used in this experiment. When WT BMDMs were treated with increasing doses of MnTMPyP, the Ago2 protein level was decreased in dose-dependent manner, indicating that a basal level oxidative stress is necessary to maintain Ago2 protein levels (Figure 6B). For increasing oxidative stress in p47^{phox}-/- BMDM, we used PTU as a non-enzymatic sustainable source of oxidative stress [19,30,31]. PTU contains a free thiol group capable of electron capture, and induces oxidant stress in several experimental systems [19,24]. On the contrary, the treatment of p47^{phox}-/- BMDM with PTU resulted in increased Ago2 protein at 16 and 24 h (Figure 6B), indicating that increased oxidative stress in NADPH oxidase-deficient macrophages results in an increase in Ago2 protein levels. Together, these experiments indicate that a basal level of ROS is necessary for maintaining Ago2 levels in macrophages.

In order to replicate the micro-environmental condition of an inflammatory exudate where tissue infiltrating macrophages are exposed to severe hypoxic conditions, we cultured macrophages (WT BMDMs and RAW 264.7 cells) under severe hypoxic conditions of 2% of oxygen. Because ROS production requires the presence of some oxygen, severe hypoxia can partially inhibit the respiratory burst [32,33]. Culture under severe hypoxia resulted in reduced Ago2 protein level without changing its mRNA level, which is similar to our findings in p47^{phox}-deficient macrophages (Figure 6C and D). Accordingly, miR-451 level was also markedly reduced in macrophages exposed to severe hypoxic conditions (right column of Figure 6D).

Discussion

MicroRNAs are a class of ~22 nucleotide short endogenous non-coding RNAs that regulate gene expression at post-transcriptional level by imprecisely binding to complementary sequences in the 3' untranslated regions (UTRs) of their target mRNAs [34]. Recent emphases on miRNAs have led to their enormous potential role in cellular machinery and gene regulation of the cells, and their involvement in almost every biological process [2,3,35]. Mir-451 is a unique microRNA which undergoes Ago2-dependent cytoplasmic biogenesis pathway, independent from Dicer activity [15,16]. The biologic significance of this pathway is unclear, but the miR-451 plays an important role in regulating oxygen carrying capacity in a knock-out mouse model [19]. The reports have shown the disruption of miR-451/144 cluster locus in mice leads to a mild anemia, suggesting its critical role for homeostasis and oxygen carrying capacity [36]. As the other functions, miR-451 has been shown to be up regulated in whole blood-derived microRNA in mice exposed to LPS [37], however, this study needs to be proved in a cell specific experiment. In contrast, the level of mir-451 was not increased in classical activation of macrophages, although it has been shown that the differential expression of microRNAs involves in polarizing the macrophages to either the classically or the alternatively activated form [38]. However, despite its important function in regulating oxygen carrying capacity in a mouse model, there is very little known about the regulation of miR-451 in context to oxidant stress. Macrophages are unique phagocytic cells which produce ROS robustly through NADPH oxidase. We reported that deficiency of the p47^{phox} component of NADPH oxidase in macrophages is associated with a paradoxical accentuation of inflammation in a whole animal model of noninfectious sepsis; however the p47^{phox}-deficient macrophages have reduced generation of ROS in the mouse model of ARDS [39]. While we were characterizing the phenotype of p47^{phox}-deficient macrophages, surprisingly, we found that that miR-451 is significantly reduced in these macrophages without alteration of its precursor form, suggesting that there is defective processing of mir-451 in p47^{phox}-deficient macrophages. The biogenesis of miRNA is complex and has numerous tightly controlled steps. Initially, the “canonical pathway” was only known as cytoplasmic pathway involving Dicer [40], but recently, an “alternative pathway” has been discovered. This pathway bypasses the Dicer function, but is dependent on Ago2 catalytic activity and regulates the expression of miR-451 [15–17]. It has not been fully elucidated how alternative cytoplasmic miRNA processing is regulated. The mammalian Argonaute subfamily comprises of Ago1, Ago2, Ago3 and Ago4, out of which only Ago2 has retained catalytic activity [41]. As described in the results, Ago2 protein

which is needed to process miR-451 was markedly reduced in macrophages that lack NADPH oxidase activity. We also showed that intentional scavenging of ROS in wild-type macrophage resulted in a reduction in Ago2 protein, whereas generation of ROS via a non-enzymatic system resulted in an increase in Ago2 protein in macrophages from p47^{phox}-/- mice. Furthermore, exposure of wild-type macrophages to near anoxic conditions resulted in a decrease in Ago2 protein and miR-451 levels. Because ROS production requires the presence of some O₂, near-anoxic condition could cause partial inhibition of the respiratory burst [32,33]. Thus, our data indicates that ROS is important for maintaining normal Ago2 protein levels that are involved in maintaining miR-451 levels in macrophages. The reports have shown that the activity and stability of Ago2 is altered in the “stress” condition of cells [2,42–44]. Ago2 protein has been known to undergo multiple types of post-translational modifications which can affect the protein stability and subsequent miRNA silencing activity. The type I collagen prolyl-4-hydroxylase(C-P4H(I)) hydroxylases Ago2 at the proline residue, and depletion of either of its subunits resulted in reduced stability of Ago2 and impaired short interfering RNA programmed RNA-induced silencing complex (RISC). The phosphorylation of the Ago2 is also mediated by p38/mitogen-activated protein kinase (MAPK) signaling pathway and inhibiting the phosphorylation of specific amino acids residues of Ago2 is also implicated in its efficient catalytic activity [44,45]. Recent reports have shown that the activity of the human Ago2 is linked to MAPK signaling which is activated by cellular stress [46]. Genetic ablation or inactivation of Argonaute protein in mice has revealed that it plays an important role in the maturation of erythrocytes which is basically oxygen carrier, suggesting that Ago2 might play a role in regulating oxygen stress [15]. It is also reported that cellular stress causes the sub-cellular localization of the Argonaute proteins to the stress granules (SGs) or the processing bodies (PBs), and this causes the decreased RNA interference [47]. Here, we showed that blocking proteosomal degradation in p47^{phox}-deficient macrophages resulted in reconstitution of Ago2 levels indicating that the ubiquitin-proteosomal process is involved in Ago2 protein degradation, which is related with basal ROS production in macrophages. It is evident that post-translational modifications such as phosphorylation and ubiquitination modulate Ago2 protein stability and function which causes the downstream effects [29,48]. Despite the decreased level of the miR-451 in p47^{phox}-deficient macrophages, these macrophages showed normal expression of the COX-2, iNOS and Arg-1 genes in response to LPS, compared to the WT macrophages. However, p47^{phox}-deficient macrophages are not quite normal in a certain circumstance. We have reported that the p47^{phox} deficiency in macrophages resulted in decreased production of IL-10 in response to treatment with LPS through attenuation of the Akt-GSK3-β signal pathway [39], which might be connected to what we presented here, the reduced Ago2 and subsequent reduced expression of miRNAs due to defective cytoplasmic alternative processing.

Conclusion

Our data indicate that ROS is a key factor for basal Ago2 protein levels that is involved in maintaining miR-451 levels and the deficiency of miR-451 results in defective ROS generation in macrophages, suggesting that miR-451 and ROS generation are interactive in macrophages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Normal LPS responsiveness in p47^{phox}-deficient macrophages. (A) Western blot confirmed the knock-out of p47^{phox} in the p47^{phox}^{-/-} BMDMs. (B, C) After being incubated overnight with medium containing only 1% fetal bovine serum, the cells were treated with LPS (100ng/mL) for 24 h. The wild type and p47^{phox}-deficient BMDMs showed similar expressions of surface receptor CD206, and COX-2, iNOS and Arg-1 genes, compared to the wild type BMDMs. The experiments were independently repeated three times with similar results. The flow cytometry data of CD206 is representative of three independent experiments which showed similar results.

Figure 2.

MicroRNA microarray analysis in WT and p47^{phox-/-} BMDMs. (A, B) Total RNA was isolated from both BMDMs and ran for low density microRNA array in order to compare the microRNA expression pattern. Amongst them, miR-451 showed the highest difference between two groups, where p47^{phox-/-} BMDMs showed ~28.5 fold decreased expression of miR-451. Raw data as well as the normalized dataset were deposited in the NCBI Gene Expression Omnibus (GEO) repository (accession number 55189).

Figure 3.

BMDM deficient in miR-451 has decreased ROS generation. (A) The deletion of miR-451 in miR-451^{-/-} mice were confirmed by genotyping the mouse tails, as described in previous report [19]. The miR-451^{-/-} mice were identified by 156 bp band, and WT (290 bp band) mice were used as control. The BMDMs of miR-451^{-/-} and WT mice were stimulated with increasing dose of LPS for 24 hrs. Supernatants were collected, and the NO concentrations in the supernatants were determined by using Griess reagent. (B) Primary cultured BMDM were either left un-stimulated or primed (with 100 ng/ml LPS, for 16 h, and then treated with Zymosan (200 µg/ml) for detection of ROS using the chemiluminescence assay. The LPS priming before zymosan treatment induced higher chemiluminescence activity than zymosan alone. The WT BMDMs expressed significant higher chemiluminescence units in both conditions, indicating more ROS production than the miR-451^{-/-} (KO) BMDM. The experiment was done using three mice from each group ($n = 3$). The average of three different measurements for each group is shown here. The details of the measurement are reported in the Supplementary Figure 3 to be found at online <http://informahealthcare.com/doi/abs/10.3109/10715762.2014.974037>.

Figure 4.

Impaired *in-vitro* processing of the mir-451 oligo. (A) BMDMs were cultured from WT and p47^{phox-/-} and total RNA was isolated. Northern blot was performed using the specific probe for pri-mir-451. Two bands are visualized at ~140 and 75 bp, which correspond to the miR-451 precursor forms [16]. p47^{phox-/-} BMDMs expressed similar amount of precursor for miR-451 in spite of marked decrease in its mature form. The equal loading of total RNA was confirmed by 5.8S, 5S, and tRNA bands on UREA PAGE (lower panel). (B). WT and p47^{phox-/-} BMDMs were transfected with biotin-labeled 42-nt oligomer pre-mir-451 as described in Methods. Total RNA was isolated at 72 h after transfection, and resolved on 20% UREA PAGE, the RNA was transferred onto positively charged nylon membrane, and the bands were detected using chemiluminescent substrate. The shorter length of oligos represents the processed forms [16]. WT BMDMs showed more processed oligos than the

p47^{phox-/-} BMDMs. Ethidium bromide stained urea PAGE confirmed equal loading in both lanes (lower panel).

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Figure 5.

Expression of protein and RNA expression of Ago2 gene in $p47^{phox-/-}$ BMDMs. (A) BMDMs were cultured from WT and $p47^{phox-/-}$ mice, and the protein lysates were immunoblotted for Ago2. Ago2 protein levels were markedly decreased in $p47^{phox-/-}$ BMDMs. (B) Total RNA was isolated from WT and $p47^{phox-/-}$ BMDMs and RT-qPCR was performed for Ago2 mRNA. Unlike the protein level, Ago2 mRNA level was paradoxically higher in the $p47^{phox-/-}$ BMDMs, suggesting that Ago2 protein stability might be altered by the $p47^{phox}$ deficiency. The expression level was normalized to β -actin, and expressed as the fold change ($n = 3$).

Figure 6.

Ago2 protein is regulated by proteosomal degradation and oxidative stress. (A) When the p47^{phox}^{-/-} BMDMs were treated with either MG-132 (500 mM) or vehicle alone (DMSO) for 24 h, Ago2 protein was significantly increased by treatment with MG-132, compared to vehicle alone, indicating proteosomal degradation pathway is involved in regulating Ago2 protein level. (B) when WT BMDMs were treated with increasing dose of MnTMPyP, a ROS scavenger, the Ago2 protein was decreased at 24 h in a dose dependent manner. In contrast, the treatment with PTU, an oxidative stress generator, increased Ago2 protein in p47^{phox}^{-/-} BMDMs at 16 and 24 h. (C) we examined the Ago2 protein level of macrophages in severe hypoxic condition. RAW 264.7 cells, a murine macrophage cell line, and WT BMDM were placed in 2% O₂ hypoxic chamber for 24 h and measured the protein level of Ago2. Compared to normoxic condition, Ago2 was markedly reduced in both RAW 264.7 cells and WT BMDMs, when the cells were cultured in severe hypoxic condition. (D) However, the Ago2 mRNA level of RAW 264.7 cells cultured in severe hypoxic condition remained at a similar level to that of normoxic condition, suggesting that this is a post-transcriptional event. Similar to p47^{phox}^{-/-} BMDMs, miR-451 was markedly reduced in RAW 264.7 cells cultured in severe hypoxic condition, compared to the cells cultured at normoxic conditions. The mRNA of β -actin and U6 snRNA were used as an endogenous control for both measurements.