Original Article
Hemoxygenase-1 deficiency exacerbates methicillin resistant Staphylococcus aureus (MRSA) mediated pleural mesothelial barrier dysfunction via miR-26a induction

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Abstract: Methicillin Resistant *Staphylococcus aureus* (MRSA) causes necrotizing pneumonia and empyema with high morbidity and mortality. Hemoxygenase-1 (HO-1) is an anti-inflammatory cytoprotective molecule that is highly expressed in the pleural fluids during inflammation. A defect in HO-1 expression increased susceptibility to pneumonia. Since microRNAs (miRs) regulate gene expression at post-translational level, we hypothesize that MRSA induces exaggerated expression of miR-26a in HO-1$^{-/-}$ Pleural mesothelial cells (PMCs) and affects pleural permeability. PMCs isolated from HO-1$^{+/+}$ and HO-1$^{-/-}$ mice were infected with MRSA (MOI 1:10). The expression profile of cellular miRNAs during MRSA infection was analyzed using microarray and quantitative RT-PCR. PMC permeability was measured by Electrical Cell Substrate Impedance Sensing (ECIS) system. In MRSA infected HO-1$^{-/-}$ PMCs miR-26a expression was several folds higher than MRSA infected HO-1$^{+/+}$ PMCs. During MRSA infection in HO-1$^{-/-}$ PMCs we also noticed concomitant decreases in E-cadherin expression along with increased permeability. Treatment with CORM2 significantly blocked MRSA induced miR-26a expression and permeability in HO-1$^{-/-}$ PMCs. Whereas, Biliverdin treatment failed to inhibit MRSA induced miR-26a expression in HO-1$^{-/-}$ PMCs. Inhibition of miR-26a with 2'O-methyl oligonucleotide (2'OMO) blunted MSRA induced pleural hyper-permeability. In addition, we also noticed CORM2 treatment blocked MRSA mediated pleural hyper-permeability in HO-1$^{-/-}$ PMCs, whereas Biliverdin treatment failed to block MRSA induced pleural hyper-permeability. Our data suggests that MRSA infection up regulate miR-26a in PMCs. Absence of HO-1 leads to a further increase in MRSA mediated pleural hyper permeability and increases disease severity due to loss of pleural mesothelial integrity in empyema.

Keywords: MRSA, miR-26a, pleural mesothelial cells, HO-1, CORM2, biliverdin, permeability

Introduction

Pneumonia is the most common cause of infection related mortality and MRSA is a leading cause of bacterial pneumonia [1]. MRSA now accounts for 20-40% of all hospital-acquired pneumonia [2]. Recent data suggests that an estimated 1.5% of population (~4.1 million persons) is colonized with MRSA in the United States [3]. During the past ten years, outbreaks of more virulent strains of MRSA have been noticed among certain athletes, prisoners and military recruits [4-7]. The First Multicenter Intrapleural Sepsis Trial (MIST1) demonstrated that hospital acquired pleural infections include more Staphylococcal infections (including MRSA), whereas, the community acquired pleural infections include more Streptococcus infections [8]. MRSA is now found more frequently than sensitive strains in hospitals [9]. Besides, hospital acquired pleural infections had significantly higher mortality than community acquired pleural infections [10].

MRSA causes pneumonia and empyema thoraces. Empyema is characterized by inflamed pleura with exudative effusion in response to infection [11, 12]. Pleural mesothelium is a dynamic monolayer of cells and its integrity plays a key role in pleural barrier maintenance.

Pleural mesothelial cell (PMC) tight junctions, adherin junctions, cell-cell connecting, hemophilic proteins are responsible for maintaining pleural mesothelial barrier function [13]. Dysregulation of these proteins leads to increases in pleural permeability and formation of pleural effusion. Formation of exudative pleural effusion is the early-phase of response associated with localized inflammation, loss of mesothelial integrity, and pleural injury [14]. In earlier studies we reported S. aureus induces pleural mesothelial permeability via induction of VEGF [15]. However the mechanisms were not clear.

Heme Oxygenases (HOs) are responsible for the oxidative degradation of heme to Biliverdin, free iron and carbon monoxide (CO). Three isoforms HO have been described: HO-1, HO-2 and HO-3 which are encoded with separate genes [16]. HO-1 is a 32-kDa, an inducible, anti-inflammatory, cytoprotective molecule found in all cell types during stress. It catalyzes the first rate-limiting step in the degradation of heme and is highly expressed in the pleural fluids during inflammation. HO-1 induction is recognized as important defense mechanisms during acute and chronic lung diseases [17, 18]. Defective HO-1 expression due to polymorphism was associated with increased susceptibility to pneumonia [19]. However, if HO-1 plays any role in the regulation of permeability of the pleura during MRSA infection is not known.

Micro-RNAs (miRs) are small non-coding RNAs that regulate gene expression at post translational level. MiRs are linked to many biological functions including membrane permeability [20, 21]. MiRs negatively regulate the expression of gene by complementary binding into the 3'UTR region of target messenger RNA [22]. Emerging evidence indicate that host response to bacterial infection is mediated via dysregulated expression of miRs [23]. MiRs regulate epithelial and endothelial barrier functions [24]. MiR21 and miR29a over expression enhanced intestinal epithelial permeability [25, 26]. A search in MicroCosm Targets data-base revealed that miR-26a targets E-cadherin [a cell-cell junction protein] which maintains epithelial barrier function and membrane integrity. However, if miR-26a has any role in PMC permeability in MRSA infection has not been explored. Therefore in this study we have investigated the role of HO-1 in MRSA induced PMC permeability. We have demonstrated HO-1 absence contributed to loss of barrier functions during MRSA infection. Upregulation of miR-26a contributed to PMC hyper permeability and addition of HO-1 by-product, the CO reversed MRSA-mediated PMC barrier function. MiR-26a targets E-cadherin which is critical for maintaining cell-cell adhesions and for pleural mesothelial integrity and induced hyper-permeability in MRSA infected PMC.

Materials and methods

PMC culture and MRSA infection

PMCs were isolated from HO-1 wild type (HO-1$^{+/+}$) and HO-1 knock out (HO-1$^{-/-}$) mice as reported earlier [14]. The PMCs were infected with USA-300 strain of MRSA (MOI 1; 10; and 100 of MRSA). In order to evaluate the role of HO-1, PMCs were infected with MRSA in presence and absence of HO-1 metabolic products such as Biliverdin hydrochloride (20 and 40 µM, from Frontier Scientific, Inc., UT) or a CO generating compound of Tricarbonyldichlororuthenium (II) dimer, (CORM-2; 50-100µM), from Sigma Aldrich, MO; and 2' O'-methyl oligonucleotide to miR-26a (QIAGEN, Maryland).

RNA isolation and quantitative reverse-transcriptase polymerase chain reaction (q-PCR)

The total cellular mRNA was isolated from resting and MRSA infected PMCs by using RNeasy kit (QIAGEN, Maryland) according to manufacturer's recommendation as reported earlier [27]. 100 ng/µl, of total RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase and oligo (dT). The SYBR Green JumpStart Taq Ready Mix™ was used to perform PCR as reported earlier [28]. After reverse transcription, 10 µl of diluted cDNA product was mixed with 25 µl of SYBR Green JumpStart Taq ReadyMix™, 0.5 µl of Internal Reference Dye, and 14.5 µl of specific oligonucleotide primers (80 nM final concentration) to a total of 50 µl volume for quantification real-time PCR. The primer sequences used for miRs were purchased from SA biosciences. PCR amplification consisted of 40 cycles (95°C for 15 s, 60°C for 1 minute and 72°C for 45 s) after the initial denaturation step (95°C for 2 min). Expression levels of the genes were based on the amount of the target message relative to the b-actin as
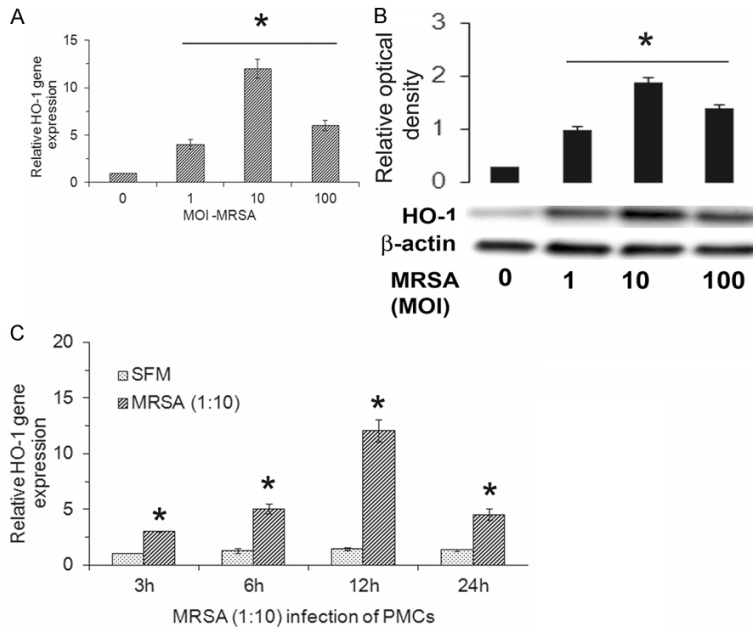
Figure 1. MRSA induced HO-1 expression PMCs. A: PMCs infected with various MOI of MRSA (1:1, 1:10, and 1:100), for 12 hours total RNA was isolated and HO-1 mRNA was detected by quantitative RT-PCR analysis. Data presented is mean of three experiments. *P<0.001 vs control. B: The PMCs infected with 10 MOI of MRSA for 18 hours HO-1 expression was also determined by Western blot analysis. β-actin was probed to show equal loading of protein samples. Data presented is mean of three similar observations. *P<0.001 vs control. C: PMCs were infected with 10 MOI and HO-1 expression was determined by quantitative RT-PCR analysis over time. Data presented is mean of three experiments. *P<0.001 vs respective time matched controls.

control to normalize the initial input of total RNA. E-cadherin Forward 5'-GCTGCACAGGGG-CCTGGATG-3' and E-cadherin Reverse 5'-GCC-ACACGGGGGAGACTTGC-3'.

Microarray analysis

The expression profile of cellular miRNAs during MRSA infection was analyzed in HO-1$^{-/-}$ and HO-1$^{+/+}$ PMCs by using RT2 miRNA PCR array from QIAGEN following manufacturer's instructions. In brief, the cellular miRNA was isolated using RT2 quantitative PCR miRNA isolation kit (QIAGEN, Valencia, CA). The expression of miR-26a was confirmed by using RT2 miRNA qPCR assay kit according to manufacturer's protocol.

Luciferase reporter assay

The luciferase reporter plasmids (pEZX-MT01) were purchased from GeneCopia™, (Rockville, MD). Each luciferase reporter construct, including Luc + miR-26a, Luc + E-cadherin 3'UTR and negative control plasmids was co-transfected

into HO-1$^{-/-}$ and HO-1$^{+/+}$ PMCs in 6-well plate using Lipofectamin-2000 (Invitrogen, CA) as reported earlier [29]. The cultures were incubated for 18 hours, the cells were transferred into 96-well plate and Firefly and Renilla luciferase activities were determined after 24 hours, using Luc-Pair™ miR Luciferase Assay kits (GeneCopia™) according to the manufacturer's instructions.

Western Blot analysis

HO-1$^{-/-}$ and HO-1$^{+/+}$ PMCs were cultured in 60 mm culture dishes (Corning, Tewksbury, MA), and infected with MRSA for 24 hours. E-cadherin expression in MRSA infected PMCs was determined by Western blot analysis as reported earlier [30]. In brief, PMCs were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0 with 150 mM sodium chloride, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate from Sigma Aldrich, St. Luis, MO). Protein concentration was measured using Pierce's BCA™ Protein Assay Kit obtained from PIERCE, Rockford, IL. Typically, 20 µg of total protein was resolved on 4%-20% Tris glycine SDS-PAGE gels (Bio-Rad), and transferred to PVDF membranes. The blots were blocked and were incubated with the primary antibody (Rabbit anti-E-cadherin) at a dilution of 1:1000 (BD Transduction Labs, Franklin Lakes, NJ) for overnight at 4°C.The immuno-complex was washed three times, then incubated with goat-anti rabbit secondary antibody at a dilution of 1:5000 for one hour at room temperature. Proteins were detected by enhanced Immuno-Star™ HRP Chemiluminescent Kit (BIO-RAD).

PMC permeability

PMC permeability was measured by Electrical Cell Substrate Impedance Sensing [(ECIS), Applied Biophysics, Inc. NY]. We have determined MRSA induced permeability changes in HO-1$^{-/-}$ and HO-1$^{+/+}$ PMCs monolayers by mea-
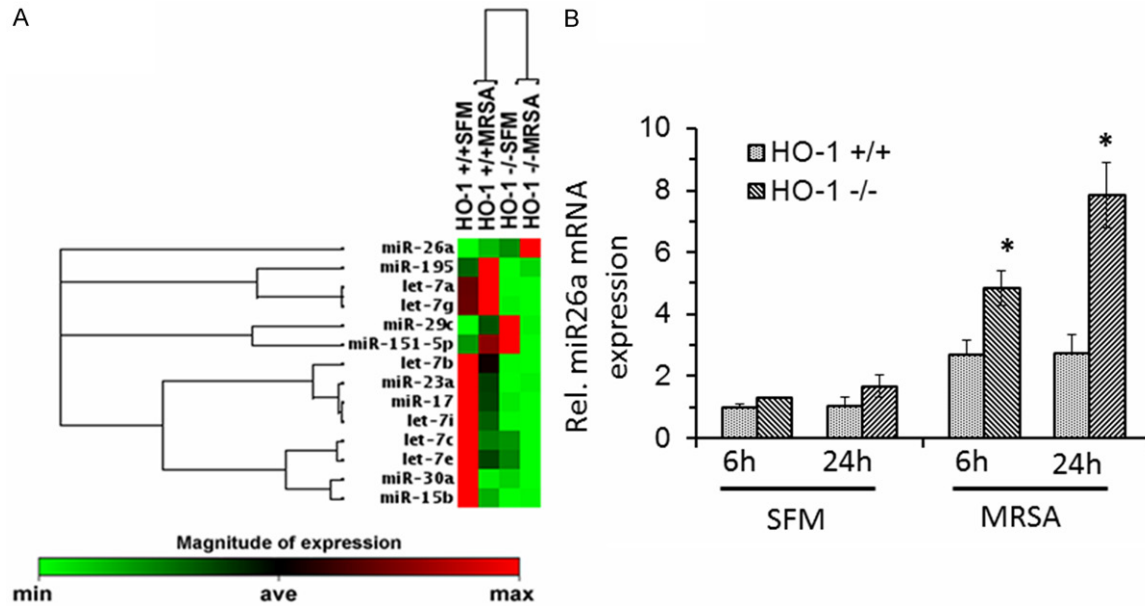
Figure 2. MiRNA expression in MRSA infected HO-1$^{-/-}$ and HO-1$^{+/+}$ PMC. A: Clustergram showing miRs expression in HO-1$^{+/+}$ and HO-1$^{-/-}$ PMCs. PMCs were infected with 10 MOI of MRSA and incubated for 24 hours, total mRNA extracted following manufacturer's guidelines and miRs expression was detected by miR finder gene array analysis (QIAGEN, Frederick, MD). B: MRSA induced miR-26a expression over time in HO-1$^{+/+}$ and HO-1$^{-/-}$ PMC. Total mRNA was isolated and miR-26a was detected by quantitative PCR analysis. Data presented is mean of three experiments. Statistical significance *P<0.001 vs respective time matched controls.

suring electrical resistance by ECIS system in real-time as described earlier [27, 31]. Briefly, HO-1$^{-/-}$ and HO-1$^{+/+}$ PMCs were cultured on ECIS 8 well electrode arrays that consists of approximately 1 cm^2 chambers (8W1E) with a maximum volume of 600 μl. When PMC reached confluency, they were treated with either CORM2 or Biliverdin 1 hour prior to MRSA infection. Trans-mesothelial electrical resistance was measured over time.

Statistical analysis

The statistical analyses were performed by using SigmaStat 3.5 (SYSTAT Software, Inc. San Jose, CA). Results are expressed as mean ± SEM. To assess the overall significance for the experiments with more than one treatment group was determined by ANOVA, we used the Kruskal-Wallis and the Mann-Whitney *U* tests. Differences were considered significant if *p*-values were <0.05.

Results

*MRSA induces HO-1 expression in PMCs*

HO-1$^{+/+}$ PMCs were exposed to MRSA with varying concentrations (MOI of 1, 10, and 100) for varying times (up to 24 hours) and the expres-

sion of HO-1 was evaluated by RT-PCR. The MOI-1:10 showed 12 fold increases in the expression of HO-1 mRNA compared to untransfected PMCs (**Figure 1A**). The expression of HO-1 was significantly low at MOI 1:1 and 1:100. Whereas maximum HO-1 expression was noticed at 1:10 MOI of MRSA compared to uninfected PMCs. We then performed the MRSA infection of PMCs choosing MOI 1:10 for further experiments. This was also confirmed by Western Blot analysis **Figure 1B**. HO-1 expression over time 3 h, 6 h, 12 h, and 24 h was determined upon MRSA infection in PMCs **Figure 1C**. PMCs infection with MRSA induced HO-1 mRNA expression in a time dependent manner. HO-1 mRNA expression significantly increased at 3 h and 6 h post MRSA infection, and a maximum induction in HO-1 mRNA was noticed after 12 hours of MRSA infection than compared to uninfected PMCs. Taken together these data suggest that MRSA induced HO-1 expression in PMCs as a protective response against infection.

MRSA induces differential expression of miR-NAs in PMCs

The expression of miRNA profile in MRSA infected HO-1$^{-/-}$ and HO-1$^{+/+}$ PMCs was determined by performing PCR microarray (**Figure 2A**). MRSA
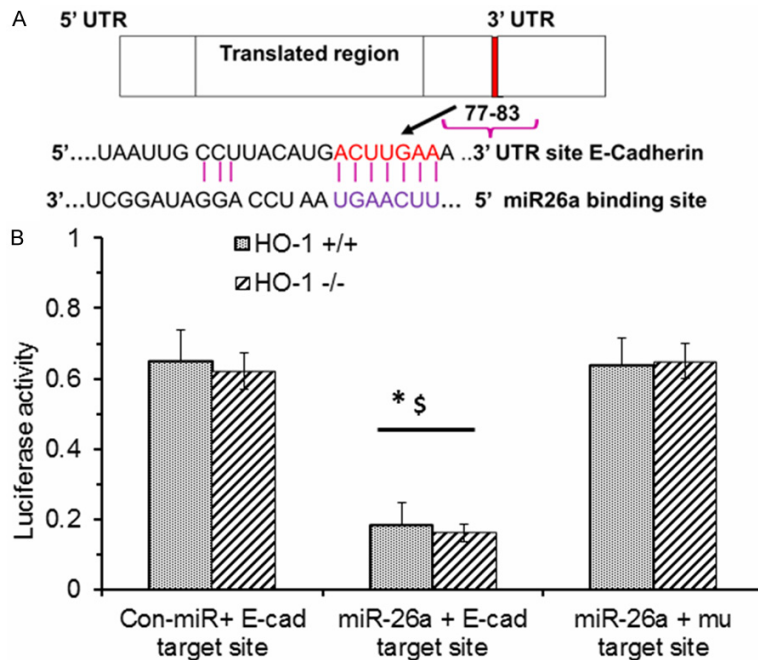
Figures 3. Miranda-target scan and Luciferase activity demonstrates Mir26 targets E-cadherin. A: Schematic diagram of predicted miR-26a target site. A search on target scan miRanda site predicts the binding site of E-cadherin with miR-26a. The 3'UTR region (length-1166) of E-cadherin contains complementary site for miR-26a (Position 77-83) which restricts reporter gene expression of E-cadherin in a miR-26a dependent manner. B: Luciferase assay was performed to determine the effect to miR26 on E-cadherin expression in both HO-1^{-/-} and HO-1^{+/+} PMCs. Statistical significance *P<0.001 is PMCs co-transfected with miR-26a specific site + E-cadherin plasmid Vs respective control (con) miR plasmid; and $P<0.001 indicates PMCs co-transfected with miR-26a plasmid + mutated (mu) E-cadherin plasmid Vs respective control miR plasmid. Data presented is mean ± SE of three independent experiments.

HO-1^{-/-} PMCs to determine the target effect of miR-26a using the miR-26a plasmid and a control plasmid following the recommendations of manufacturer. Co-transfection of PMCs with miR-26a plasmid and E-cadherin specific plasmid induced a significantly low luciferase activity indicating that miR-26a binds to the complementary site of E-cadherin and represses its expression (**Figure 3B**). A significant decrease in the luciferase activity was observed in PMCs transfected with miR-26a plasmid and E-cadherin target site plasmid than compared to control-miRs containing plasmids transfected PMCs. In addition the plasmid with mutated target site of E-cadherin did not show any effect on luciferase activity. This data suggests that miR-26a targets E-cadherin and represses its expression in PMCs.

E-cadherin expression decreases in MRSA infected PMCs

induced the maximum expression of miR-26a in HO-1^{-/-} PMCs when compared to MRSA infected HO-1^{+/+} PMCs. This was also confirmed by quantitative RT- PCR analysis (**Figure 2B**). Several folds higher expression of miR-26a was observed in HO-1^{-/-} PMC infected with MRSA when compared to HO-1^{+/+} PMCs. In MRSA infected HO-1^{-/-} PMCs a 8 fold increase in miR-26a expression was noticed. On the other hand, in MRSA infected HO-1^{+/+} PMCs, the miR-26a expression was several folds lower. This data suggests that HO-1 gene plays a key role in the modulation of miR-26a levels during MRSA infection in PMCs.

MiR-26a suppresses E-cadherin expression in PMCs

A target scan of MicroCosm Targets data-base indicated complementary binding site of E-Cadherin with miR-26a (**Figure 3A**). The luciferase essay was performed on the HO-1^{+/+} and

HO-1^{+/+} and HO-1^{-/-} PMCs were infected with MRSA and E-cadherin expression was analyzed by Western blot analysis. MRSA infection significantly inhibited the expression of E-cadherin in PMCs (**Figure 4A** and **4B**). Whereas the knock down of miR-26a with anti-sense 2' *O*-methyl oligonucleotide (2'OMO) for miR-26a blunted MSRA mediated down regulation of E-cadherin expression in both HO-1^{+/+} and HO-1^{-/-} PMCs. A remarkable repression in the E-cadherin was noticed in HO-1^{-/-} PMCs compared to the HO-1^{+/+} PMCs suggesting higher levels of miR-26a expression was responsible for the repression of E-cadherin in MRSA infected PMCs.

MRSA induces hyper-permeability across PMC monolayer

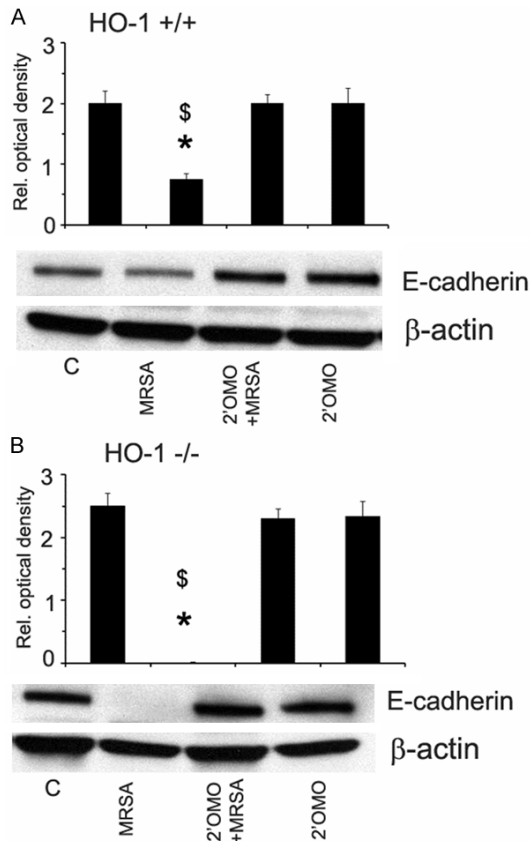MRSA infection increased permeability across both HO-1^{-/-} and HO-1^{+/+} PMCs monolayers (**Fig-**

Figure 4. E-Cadherin expression in HO-1$^{-/-}$ and HO-1$^{+/+}$ PMC. In both Panels (A) and (B), the upper panels are relative optical densities; the middle panel are autoradiographs probed for E-cadherin and lower panel probed for β-actin to demonstrate equal protein loading. Statistical significance *$P<0.001$ MRSA Vs control; and $^{\$}P<0.001$ MRSA + 2'OMO Vs 2'OMO. Data presented is representation of three similar observations. Control = HO-1$^{+/+}$ or HO-1$^{-/-}$ PMCs were left un-infected; MRSA = PMCs infected with 10 MOI of MRSA; 2'OMO + MRSA = PMCs treated with *2' O'*-methyl oligonucleotide miRNA-26a and infected with 10 MOI of MRSA; 2'OMO = PMCs treated with *2' O'*-methyl oligonucleotide miRNA-26a only.

Figure 5. Inhibition of miR-26a with MiR-26a anti-sense oligo attenuates MRSA induced pleural mesothelial permeability. A and B shows the electrical resistance measured in HO-1$^{-/-}$ and HO-1$^{+/+}$ PMCs respectively. Inhibition of miR-26a completely restored MRSA induced permeability in HO-1$^{-/-}$ as well as HO-1$^{+/+}$ PMC. Cells only = HO-1$^{+/+}$ or HO-1$^{-/-}$ PMCs were left untreated; Scrambled oligo + MRSA = PMCs treated with scrambled *2' O'*-methyl oligonucleotide miRNA-26a and infected with 10 MOI of MRSA; miR-26a anti-sense OMO = PMCs treated with *2' O'*-methyl oligonucleotide miRNA-26a and infected with 10 MOI of MRSA; MRSA = PMCs infected with 10 MOI of MRSA. Data presented is a single representative of three similar observations each measured at different times.

ure **5A**, **5B**). Whereas, inhibition of miR-26a with 2'OMO blunted MSRA induced pleural hyper-permeability when compared to MRSA infected PMCs. However, the recovery response in HO-1$^{-/-}$ PMCs was poor. This data indicate that miR-26a plays a key role in MRSA induced barrier dysfunction in PMCs and HO-1 influence MRSA induced pleural permeability.

CO blocked MRSA induced miR-26a expression and permeability in PMCs

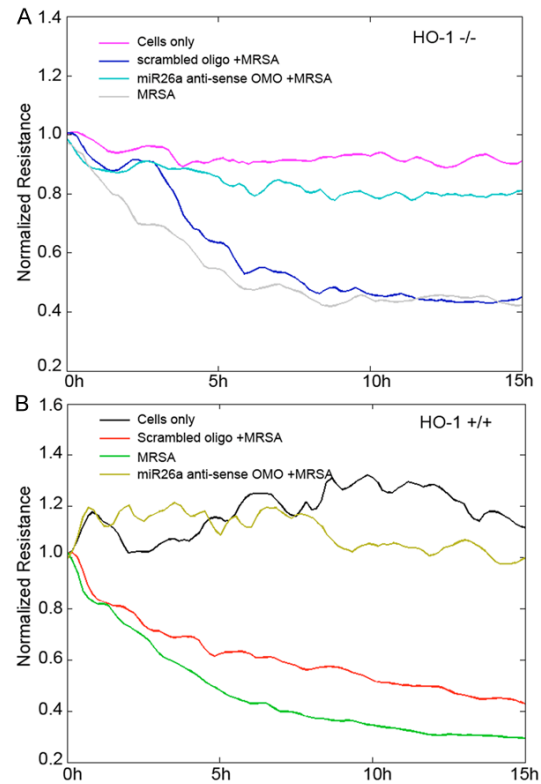In order to determine if the byproducts of HO-1 that is carbon monoxide (CO) and Biliverdin reg-

ulate miR-26a expression in PMCs, modulate MRSA induced permeability increases in PMCs, HO-1$^{-/-}$ PMCs were treated with 50 µM CORM2, and Biliverdin and determined the miR-26a expression by RT PCR analysis. CORM2 significantly blocked MRSA induced miR-26a expression in HO-1$^{-/-}$ PMCs. However, Biliverdin treatment failed to inhibit MRSA induced miR-26a expression (**Figure 6A**). In addition, HO-1$^{-/-}$ PMCs were treated with pharmacological by products of HO-1 with and without MRSA and permeability across the HO-1$^{-/-}$ PMCs monolayer was determined by measuring electrical resistance by ECIS system. CORM2 treatment
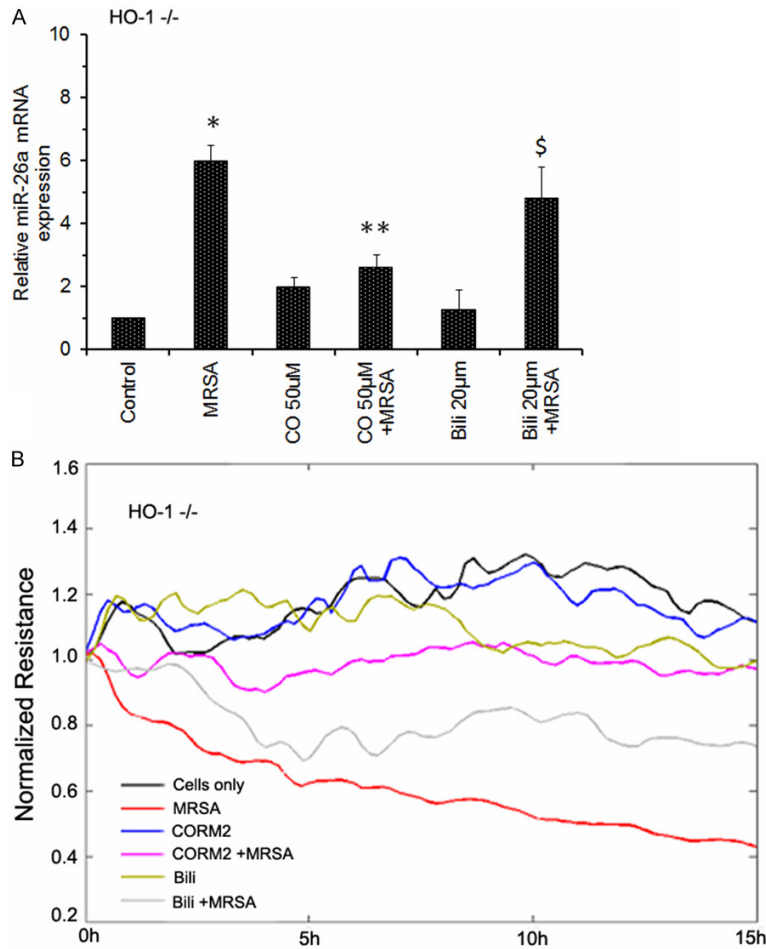
Figure 6. CORM2 blunted MRSA induced permeability in HO-1$^{-/-}$ PMC. A. MiR-26a mRNA expression in PMC treated with pharmacological agents either CORM2 or Biliverdin along with MRSA infection for 24 hours. Data presented is mean ±SEM of three separate observations. Statistical significance *$P<0.01$ control vs MRSA infected PMC; **$P<0.01$ CORM2 + MRSA vs MRSA infected PMCs; and $^{$}P<0.01$ Biliverdin + MRSA Vs MRSA activated PMC. B. Electrical resistance measured in HO-1$^{-/-}$ and PMCs. CORM2 (50 mm) treatment significantly restored MRSA induced electrical resistance in HO-1$^{-/-}$ PMCs. Biliverdin (20 mm) treatment showed minimal restoration of MRSA induced electrical resistance in HO-1$^{-/-}$ PMCs. Data presented is a representative of three similar observations each measured at different times.

restored the MRSA induced increases of permeability in PMCs when compared to MRSA infected cells. However, pretreatment with Biliverdin failed to restore the permeability changes. These data indicate that HO-1 regulated pleural permeability and the CO may have a therapeutic significance in patients with MRSA empyema.

Discussion

MRSA infection is the leading cause of pneumonia and empyema. MRSA empyema is char-acterized by pleural inflammation with exudative effusions. MRSA empyema often leads to localized inflammation associated with pleural injury. Pleural mesothelium consists of dynamic monolayer of cells which maintains barrier function and regulates influx of leukocytes and proteins to the pleural space [12, 32]. In addition, the volume of pleural fluid and its composition is tightly regulated, and any imbalance of this regulation leads to the accumulation of fluid in the pleural space called effusion [33]. Therefore, pleural mesothelial integrity is critical for maintaining the pleural homeostasis and barrier functions. Earlier we have reported that *S. aureus* causes pleural mesothelial permeability by inducing VEGF, and in a recent study others have reported the importance of tight junction proteins in barrier function and pleural inflammation [34]. However, the mechanisms involved are not well known. A growing body of evidence indicates that HO-1 plays a key role in maintaining barrier functions [35, 36]. In this study we have determined the role of HO-1, a cytoprotective molecule, in MRSA induced pleural barrier dysfunction. We report that MRSA induced miR-26a expression in PMCs and HO-1 plays a critical role as modulator of miR-26a expression in PMCs and in regulating the pleural integrity during MRSA infection.

MRSA induced HO-1 expression in PMCs in a time and MOI dependent manner. MRSA infection also induced exaggerated expression of miR-26a in HO-1$^{-/-}$ PMCs than compared to HO-1$^{+/+}$ PMCs. In addition, in MRSA infected HO-1$^{-/-}$ PMCs the E-cadherin expression was significantly decreased. Transfection of HO-1$^{-/-}$ PMCs with miR-26a anti-sense 2'OMO signifi-

cantly blocked the MRSA induced decreases of E-cadherin in PMCs indicating that miR-26a play a key role in barrier function during MRSA infection. The miRNA-microarray analysis data revealed MRSA induced exaggerated expression of miR-26a in HO-1^{-/-} PMCs. In addition, we confirmed the expression of miR-26a in both HO-1^{-/-} and HO-1^{+/+} PMCs infected with MRSA. We also demonstrated that MRSA infection downregulates the E-cadherin expression in PMCs. Absence of HO-1 contributed to a remarkable down regulation of E-cadherin in HO-1^{-/-} PMCs; whereas, transfection of PMCs with anti-sense 2'OMO of miR-26a prior MRSA infection significantly restored the E-cadherin expression in HO-1^{-/-} PMCs. In earlier studies we reported that miR-26a targets E-cadherin by binding to its 3'UTR region of mRNA and represses its expression thereby promoting epithelial mesenchymal transition (EMT) in PMCs [29]. Taken together, these data demonstrate that miR-26a regulate the expression of E-cadherin in PMCs during MRSA infection.

HO-1 is cytoprotective anti-inflammatory molecule which catalyzes the first rate-limiting step in the oxidative degradation of heme to Biliverdin, free iron and CO. HO-1 plays crucial role in maintaining the cellular homeostasis and integrity against stress and infection. HO-1 induction is recognized as important defense mechanisms during acute and chronic lung diseases [17, 18]. Defective HO-1 expression due to polymorphism was found to be associated with increased susceptibility to pneumonia [19]. We noticed in the absence of HO-1, MRSA exacerbated PMC permeability and transfection of PMCs with anti-sense 2'OMO of miR-26a protected the barrier function compared to PMCs infected with MRSA. In HO-1^{+/+} PMCs MRSA induced drop in membrane electrical resistance was restored close to control cells when miR-26a was blocked indicating the protective role of HO-1 in PMCs. In addition, we also determined the effect of HO-1 byproducts CO and Biliverdin on expression of miR-26a in HO-1^{-/-} PMCs. Furthermore, the significance of CO and Biliverdin was also studied in the HO-1^{-/-} PMCs permeability. The CO, and Biliverdin have been shown to possess endogenous anti-inflammatory activities and provide protection against injury [37]. In addition, accumulating evidence indicates that induction of HO-1 is a fundamental response to acute infla-

mmation [38, 39]. Treatment of HO-1^{-/-} PMCs with CORM2 prior to MRSA infection showed a significant decrease in the miR-26a expression when compared to MRSA alone. In addition, the MRSA induced permeability was reversed in the presence of CORM2 in HO-1^{-/-} PMCs. Whereas, Biliverdin failed to inhibit MRSA induced miR-26a mRNA expression, and the permeability changes in Biliverdin treated HO-1^{-/-} PMCs remained close to MRSA infected PMCs. Besides, CORM2 has been found to protect LPS induced permeability in intestinal epithelia cells [40]. Taken together these data suggest HO-1 metabolic byproduct the carbon monoxide prevents pleural permeability during MRSA infection and HO-1 absence exacerbates MRSA induced pleural permeability in empyema.

In conclusion, we have demonstrated that HO-1 plays a regulatory role in miR-26a expression during MRSA infection. Targeting miR-26a expression by anti-sense 2'OMO restored the adherin junction protein E-cadherin and protected the PMC barrier function. Our data suggests that during MRSA infection, absence of HO-1 results in over expression of miR-26a in PMCs which targets PMC E-cadherin and contributes to pleural hyper-permeability facilitating pleural effusion formation in empyema. Since CORM2 attenuates MRSA-induced increases in miR-26a expression and PMC hyper-permeability, it may have therapeutic significance in treating patients with MRSA empyema.

Disclosure of conflict of interest

None.

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