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MicroRNA-mediated inflammation and coagulation effects in rats exposed to an inhaled analog of sulfur mustard

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Abstract

Exposure of rats to 2-chloroethyl ethyl sulfide (CEES), an analog of sulfur mustard, can cause acute lung injury (ALI), resulting in increased inflammation and coagulation and altered levels of plasma microRNAs (miRNAs). Rats were exposed to aerosolized CEES and euthanized 12 h later for collection of tissue and plasma. Profiling of miRNAs in plasma, using a TaqMan-based RT-PCR array, revealed 14 differentially expressed miRNAs. Target gene prediction and pathway analysis revealed miRNA-mediated regulation of organismal injury, inflammation, and respiratory diseases. miR-140-5p, a marker of ALI, was downregulated in the plasma, lung, liver, and kidney of CEES-exposed rats, with a concomitant increase in the expression of the inflammation markers IL-6 and IL-1 α and the coagulation marker tissue factor (F3). Exposure of rat airway epithelial cells (RL-65) to CEES (0.5 mM) caused cell death and a decrease in miR-140-5p both in cells and media supernatant. This was accompanied by an increase in cellular mRNA levels of IL-6, IL-1 α , and F3, as well as FGF9 and EGR2, putative targets of miR-140. Knockdown of miR-140 by specific oligos in RL-65 cells mimicked the *in vivo* CEES-mediated effects, leading to significantly increased mRNA levels of IL-6, IL-1 α , F3, FGF9, and EGR2. Our study identifies miR-140-5p as a mediator of CEES-induced ALI, which could potentially be targeted for therapy.

Keywords

sulfur mustard; CEES; miR-140-5p; inflammation; coagulation; acute lung injury

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Author contributions

Aftab Ahmad conceived the idea and designed experiments. T.R., Aamir Ahmad, I.Z., N.M., M.H., S.A., and Aftab Ahmad carried out the experiments and acquired and analyzed data. T.R., Aamir Ahmad, S.A., and Aftab Ahmad wrote the manuscript. D.S.C., T.H., and S.V. acquired and analyzed data.

Competing interests

The authors declare no competing interests.

Introduction

Sulfur mustard (SM; bis(2-chloroethyl) sulfide), a potent vesicant¹ used against humans, continues to be recognized as a threat agent.^{2,3} Stockpiles of SM and similar chemicals still exist, and the threat of intentional or accidental exposures is ever increasing.^{4,5} Of particular concern is the lack of effective therapies, which is, in part, due to gaps in our knowledge of how chemical agents, including SM, cause injuries.⁶ Our understanding of these is based on case reports of victims of exposure and animal studies.^{7–9} Lungs are one of the primary organs affected by exposure to SM, along with skin and eyes.^{10,11} Inhalation of high doses of SM or its analogs can cause acute lung injury (ALI), which has been described in a few reports as being associated with progression to acute respiratory distress syndrome,^{12–14} a condition with significant mortality.⁶ CEES (2-chloroethyl ethyl sulfide, a.k.a. half-mustard), an SM analog, also can cause ALI,^{15,16} similar to SM. Acute intense inflammation and activation of the coagulation cascade are characteristic features of these injuries.^{11,16} Mechanisms by which these pathways are activated are beginning to be understood.¹⁶ Growing evidence suggests that nucleic acids, including microRNAs (miRNAs), can influence the inflammation and coagulation pathways as well as other pathways involved in tissue injury and repair. Recent data have indicated the presence of altered expression of miRNAs in SM-exposed individuals,¹ and a possible role of miRNAs in determining sensitivity to SM.¹⁷ However, beyond preliminary reports on the differential expression of individual miRNAs in SM-induced injury, not much is known. To evaluate the role of miRNAs in pulmonary injury caused potentially by SM and related chemicals, we exposed rats to aerosolized CEES and focused on altered miRNA expression and the deregulation of inflammation and coagulation pathways, as these pathways are well-characterized clinical manifestations of exposure to CEES or SM.^{11,16,18} Our study also investigated the functional roles of the identified miRNAs in regulating CEES-induced toxicity.

Materials and methods

Animal and CEES exposure

All experiments involving animals were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Male Sprague–Dawley rats (275–300 g; Harlan, Indianapolis, IN) were kept 2–3 days for acclimatization after their arrival at the University of Alabama at Birmingham, with *ad libitum* food and water. Exposure to CEES was performed exactly as described earlier.¹⁶ Prior to exposure, rats were anesthetized using a mixture of ketamine (75 mg/kg), xylazine (7.5 mg/kg), and acepromazine (1.5 mg/kg) injected intraperitoneally. They were then loaded into polycarbonate tubes in a Jaeger nose-only inhalation system (CH Technologies, Westwood, NJ). A solution of CEES (10% CEES in 100% ethanol) was injected into a bioaerosol nebulizing generator via syringe pump (Razel Scientific, St. Albans, VT) at a rate of 12.5 milliliter per hour. Particle size was characterized by measurements from a gravimetric sampler and a seven-stage Mercer impactor. Control rats were similarly exposed to vehicle (100% ethanol). After 15 min of exposure, rats were returned to their respective cages. Rats were monitored continuously for 12 h, after which they were euthanized and

blood was collected for platelet-free plasma (PPP) preparation. There was 50% mortality in the model. Samples were taken from rats that either survived 12 h or were euthanized after meeting euthanasia criterion prior to reaching the end point but were close to 12 hours. Lungs ($n = 8$ animals/group) were perfused through the pulmonary artery and snap-frozen in liquid nitrogen. The right middle lobe was used for gene and miRNA expression studies. Tissues from the other organs (liver and kidney; $n = 8$) were also collected without separate perfusion and stored at -80°C . Separate animals were used for fixed tissues. Lungs were inflation-fixed in 4% paraformaldehyde in phosphate-buffered saline.

PPP preparation from blood

Blood was collected in citrate tubes and centrifuged at $2000 \times g$ for 10 min at room temperature. Supernatant was further centrifuged at $10,000 \times g$ for 10 min at room temperature¹⁹ to obtain PPP from the supernatant.

Isolation of RNA from plasma and tissues

RNA was isolated from PPP, lung, liver, and kidney tissues ($n = 8$) using the TRIzol[®] LS (Trizol reagent for liquid samples) method (Ambion Cat# 10296010, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The concentration and purity of purified RNA were measured by absorption at 260 and 280 nm (A260/A280).

TaqMan array and data analysis

Initially isolated RNA was reverse transcribed using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA) and Megaplex[™] RT primers (Rodent Poll Set v2.0, Applied Biosystems), as per manufacturer's protocol. MicroRNA profiling was carried out using the TaqMan[™] Array Rodent MicroRNA A+B Cards set v2.0 and v3 (Cat# 4444909; Applied Biosystems), which contained a total of 750 miRNA assays and six controls spanning human, mouse, and rat species. All PCR reactions were performed on a ViiA[™]7 system (Applied Biosystems) using conditions per manufacturer's protocol. Threshold cycle (CT) values were analyzed using the comparative CT ($-CT$) method.²⁰

Pathway analysis

To analyze the pathways and gene networks affected by miRNAs that were found differentially expressed in CEES- versus vehicle-treated rats, we used four different algorithms (IPA, Metacore, Diana, and microRNA Target Prediction Database: miRDB). Ingenuity Pathway Analysis (IPA) was used to list molecular functions, biological processes, and specific network connections, as assigned, based on the predicted target genes.

qRT-PCR

For qRT-PCR to detect miRNAs, 10 ng of RNA was used for cDNA synthesis. RNA and primers specific for miR-1894, miR-17, miR-429, miR-140-5p, and U6snRNA (Life Technologies) were reverse transcribed using the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer's protocol. qRT-PCR was performed using TaqMan Universal Master Mix (Applied Biosystems) in a CFX96 Bio-Rad system. The PCR reaction mixture (20 μL) included 1 μL RT products, 10 μL TaqMan Universal

PCR Master Mix, no UNG 2× (Applied Biosystems), and 1 µL TaqMan probe mix. The reactions were performed using 96 well plates, with conditions set at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 minute. miRNA expression levels (ETOH versus CEES) were calculated using the comparative CT (- CT) method and normalized against U6 snRNA levels. All reactions were run at least as duplicates in 96-well plates.

For qRT-PCR to assess gene expression, we used the RNeasy Mini Kit (Qiagen Co, Germantown, MD) to extract total RNA. RNA quality was assessed using the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). For qRT-PCR, first-strand cDNA was obtained by reverse transcription of 1 µg of total RNA using iScript reverse transcription super mix (Bio-Rad, Hercules, CA). cDNA (50 ng) was then amplified in a total volume of 25 µL using the Bio-Rad CFX96 RT-PCR machine (Bio-Rad). Taqman primers specific for interleukin (IL)-1α, IL-6, and F3 mRNAs were used for gene expression analysis. Results were normalized to expression of the reference housekeeping gene β-actin (*Actb*) and calculated as a ratio of gene expression to the expression of the reference gene. All primer/probe sets for cytokines/chemokines and coagulation genes were procured from Applied Biosystems.

Fibrin deposition

Fibrin staining in the lung was carried out in inflation-fixed and paraffin-embedded lung sections. Immunostaining for fibrin was performed using a polyclonal rabbit anti-human fibrinogen (DAKO; Carpinteria, CA) at a 1:2000 dilution for 60 minutes. Rabbit IgG control (DAKO) was used at the same specifications and served as a negative control. The stains were developed using the peroxidase-based Envision detection system (DAKO). The counterstaining was performed using hematoxylin.

Cell culture and CEES treatments

The RL-65 cell line (CRL-10354), an immortalized rat lung-derived airway epithelial cell line, was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were cultured using Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (DMEM/Ham F12) 1:1, supplemented with 85 nM selenium, 2.5 µg/mL bovine insulin, 5.4 µg/mL human transferrin, 30 µM ethanolamine, 100 µM phosphoethanolamine, 500 nM hydrocortisone, 5 µM forskolin, 50 nM retinoic acid, 0.15 mg/mL bovine pituitary extract, and 10% fetal bovine serum (FBS). Medium was exchanged thrice weekly, and cells were passaged when 90% confluent using a 1:20 split ratio.²¹ For experiments involving CEES exposure, RL-65 cells were seeded at a density of 1×10^5 cells per well in 6-well plates. The stock solution of CEES (1M) was prepared in 100% ethanol, and RL-65 cells were exposed to different concentrations of CEES, as indicated, or the vehicle control (100% ethanol) in 2.0 mL serum-free medium for 1 h, following which, the culture medium with vehicle/CEES was replaced with complete culture medium with FBS, but without CEES or the vehicle. At the end of the incubation (24 h), cell media as well as the cells were collected for further experiments.

Crystal violet staining

RL65 cells were exposed to CEES (500 μ M) diluted in ethanol. Cell viability was assessed in ethanol controls and CEES groups. Cells were fixed after 24 h of exposure and stained with crystal violet dye (blue staining). Cell viability was also quantified by manually counting stained cells as described earlier.²²

Transfection of specific anti-miRNA

RL-65 cells were seeded at 3×10^5 cells per well in 6-well plates and transfected with anti-miR-140 or a control anti-miRNA at a final concentration of 200 nM (Thermo Fisher Scientific, Waltham, MA) using DharmaFECT[®] Duo transfection reagent (Dharmacon, Lafayette, CO). After 3 days of transfection, cells were split and transfected repeatedly with anti-miR-140 or control every 3–4 days for a total of four times. Experiments were performed in triplicate and representative results from three independent repeats are presented.

Statistical analysis

GraphPad Prism[®] 8.0 software (GraphPad Prism, La Jolla, CA) was used for statistical analysis, with a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test, unless otherwise indicated. Data reported are mean values with standard error of the mean (SEM). A *P* value of < 0.05 was considered significant.

Results

CEES exposure results in differential expression of miRNAs in rat plasma

We first evaluated the plasma of rats for a total of 750 miRNAs using a TaqMan-based expression array containing human/mouse/rat miRNA-specific primers and probes. The expression patterns of the most significantly differentially expressed miRNAs are shown as a clustered heat map in Figure 1A. Since our array primarily consisted of human and mouse miRNAs, we carefully compared the differentially expressed miRNAs for similarities in seed sequences recognized by rat analogs. Using three different databases, we compiled a list of 14 statistically significant, differentially expressed miRNAs (Table 1), most of which were found to be downregulated. Next, we subjected these top differentially regulated miRNAs to pathway analysis using IPA. miRNA-regulated networks were generated with their putative target genes (Fig. 1B), and an analysis of physiological processes and diseases affected by the miRNAs (Fig. 1C) revealed “organismal injury and abnormalities” to be the most affected. As many as nine miRNAs were found to be directly connected to “organismal injury and abnormalities” (Fig. 1C). “Inflammatory disease” and “inflammatory response” were found to be affected by seven and six differentially expressed miRNAs, respectively (Fig. 1C). More importantly, six of the differentially expressed miRNAs were implicated in “respiratory disease,” further validating the significance of identified miRNAs in lung injury.

Predicted targets of the miRNAs identified inflammation- and coagulation-related genes. For confidence in our array, we validated a few differentially expressed RNAs that included miR-140-5p, miR-1894, miR-17-5p (miR-17), and miR-429 and found the expression pattern to be consistent with the initial array results (Fig. 2A). Given the significance of

increased inflammation and an activated coagulation system, we focused on the reported, as well as the predicted, activity of these miRNAs against inflammation and/or coagulation pathways and in ALI.^{23–25} Evidence for a role of the top two miRNAs (miR-138-1-3p and miR-196a-3p) in lung injury, inflammation, or coagulation could not be found in the literature. Based on these considerations, we focused on miR-140 (miR-140-5p), the third-highest downregulated miRNA, as a miRNA of interest. It was downregulated 25.9-fold in the plasma of CEES-exposed rats, compared with vehicle-exposed rats. Expression was confirmed in all the individual samples (four from each group). Since miRNA functions by suppressing gene expression in cells/tissue, and because exposure of animals to CEES caused significant lung injury, we evaluated miR-140 expression in the lung tissues of rats exposed to aerosolized CEES. Similar to the observation in plasma, miR-140 was found to be reduced by more than 50% in the lung tissues of rats exposed to CEES ($P < 0.01$) (Fig. 2B). Furthermore, miR-140 was slightly reduced in the liver (statistically non-significant) and significantly reduced in the kidneys (~40%, $P < 0.01$) of CEES-exposed rats (Fig. 2B).

CEES upregulates markers of inflammation and coagulation in rats

In lungs, increases in inflammatory cytokines and coagulation factors are frequently observed following exposure to CEES.²⁶ We therefore evaluated the effects of CEES exposure on inflammation and coagulation pathways. To test this, we checked the expression of the mRNAs of the inflammation markers IL-6 and IL-1 α and the coagulation marker F3 (tissue factor) in lungs, liver, and kidneys of rats exposed to inhaled CEES. IL-6 mRNA was significantly upregulated in lungs (~12.3-fold, $P < 0.01$) and liver (~2.8-fold, $P < 0.05$) of CEES-exposed rats (Fig. 3A). While the change in expression of IL-6 mRNA in kidneys was not found to be statistically significant (Fig. 3A), IL-1 α mRNA was significantly increased in all the tissues (lungs: ~9.8-fold, $P < 0.01$; liver: ~2.7-fold, $P < 0.01$; and kidneys: ~2.1-fold, $P < 0.05$) (Fig. 3A). Previously, it was shown that the coagulation factor F3 is increased in lungs of CEES-exposed rats.²⁷ In the current study, F3 mRNA was also significantly increased in lungs and liver (~14.8- and ~15.4-fold, respectively; $P < 0.01$) but not in the kidneys (Fig. 3A). Activation of the coagulation pathway together with leaky airways leads to deposition of fibrin-rich casts in the airways.²⁸ As a further confirmation of an activated coagulation pathway, we evaluated fibrin deposition in the airways by immunohistochemistry and found significant amounts of fibrin in airway casts of rats exposed to CEES, compared with the airways of control rats (Fig. 3B).

CEES upregulates genes of inflammation and coagulation and decreases miR-140 in lung epithelial cells

For mechanistic studies, we utilized an *in vitro* model comprising the immortalized rat lung airway epithelial cell line RL-65. When exposed to 0.5 mM CEES for 24 h, a significant ($P < 0.05$) decrease in cell viability was observed (Fig. 4A), as determined by crystal violet staining. Since inflammatory and coagulation markers were observed to be elevated in animal model of CEES injury, we evaluated mRNA levels of IL-6, IL-1 α , and F3 in RL-65 cells and found all of these markers to be significantly ($P < 0.01$) elevated (IL-6: 9.5-fold; IL-1 α : 6.8-fold; and F3: 9.3-fold) (Fig. 4B). miRNAs function by binding to the 3'-UTR of their target genes. Therefore, we searched for predicted targets of miR-140-5p using miRDB and TargetScan and found *Fgf9* (fibroblast growth factor 9) and *Egr2* (early growth response

2) to be the top target genes. qRT-PCR revealed significantly ($P < 0.01$) upregulated mRNA levels of both genes in CEES-treated RL-65 cells compared with the control cells (Fig. 4B). Further, exposure of RL-65 cells to CEES caused ~6-fold downregulation of miR-140 in the cells and ~1.7-fold downregulation in the medium supernatant (Fig. 4C).

Knockdown of miR-140 in rat lung epithelial cells increases inflammation and coagulation genes

Since we observed an inverse relationship between the expression of miR-140 and the markers of inflammation and coagulation in tissues of rats exposed to CEES as well as in our *in vitro* model, we questioned whether this was mechanistically linked. To test this, we first downregulated miR-140-5p by transfecting RL-65 cells with anti-miR-140. A more than four-fold downregulation of miR-140 was observed (Fig. 5A). This resulted in significantly ($P < 0.001$) increased mRNA expression of IL-6, IL-1 α , and F3 in these cells (~6.8-fold, 3.4-fold, and 7.9-fold, respectively) (Fig. 5B). Downregulation of miR-140 was also found to increase expression of its targets *Fgf9* and *Egr2* (~8.1-fold and 5.9-fold, respectively; $P < 0.01$) (Fig. 5B).

Discussion

The goal of this study was to investigate the mechanisms by which CEES, a surrogate of SM, causes injury. CEES (a.k.a. half-mustard) was used as a surrogate because of its structural and reactive group similarities to SM and because it mimics to a large extent the pathophysiology of animals and humans exposed to SM.^{11,28} While the injury caused by CEES/SM results in increased inflammation and dysregulated coagulation,^{11,18} the relevant signaling pathway has been elusive. There is a growing interest in identifying circulating markers of injury and disease progression and prognosis. Among the circulating factors, altered miRNA levels have been observed in a number of disease conditions, including in SM-exposed individuals.^{29,30}

CEES at the dose used in our study causes significant mortality.¹⁶ It is, therefore, not surprising that IPA analysis revealed “organismal injury and abnormalities” as the principal pathway that could potentially be affected by the identified miRNAs. The fact that IPA analysis also identified “inflammatory disease,” “inflammatory response,” and “respiratory disease” as the next most important pathways that could potentially be altered by the identified miRNAs is also consistent with our previous observation of increased levels of the inflammation genes IL-6 and IL-1 α in the lungs of CEES-exposed rats.¹⁶ Taken together, these findings highlight the role and relevance of the identified miRNAs in CEES-induced injury.

To validate our IPA analysis findings and to establish a functional role for the identified miRNAs, we choose miR-140-5p on the basis of its fold-change in CEES-exposed mice and its documented role in inflammation and coagulation pathways. Downregulation of miR-140-5p in the plasma of CEES-exposed rats followed a similar trend in lung tissue, indicating that plasma levels of miR-140-5p can be reliably used to gauge levels in the lungs. Furthermore, downregulation of miR-140-5p is accompanied by elevated markers of inflammation and coagulation in the lung. Downregulation of miR-140-5p was also

observed in kidneys, indicating a more systemic effect. These results were mimicked by cultured rat epithelial cells exposed to CEES, indicating a strong relationship between the decrease in miR-140 and an increase in markers of inflammation and coagulation. Mimicking such downregulation of miR-140 in cultured rat epithelial cells using knockdown approaches also results in effective upregulation of inflammation as well as coagulation markers, thus providing definitive proof of the role of miR-140 in regulating inflammation and coagulation.

ALI caused by exposure of rats to aerosolized CEES is correlated with increased gene expression of IL-6, IL-1 α , and F3. IL-6 and IL-1 α are markers of inflammation,^{30,31} while F3 (a.k.a. tissue factor/factor III) is known to initiate the coagulation pathway.³² Further confirming the increased coagulation, we observed significant buildup of fibrin in the airways of rats exposed to CEES. These observations are in agreement with elevated inflammatory markers, particularly IL-6 levels, in SM-exposed animals.^{33,34} Interestingly, recent studies showed the regulation of IL-6 and other inflammatory genes by miR-140,^{24,25,35} which further strengthens our observations, underscoring the role of miR-140 in CEES-induced injury.

Several potential targets of miR-140 were identified through database searches, as listed in Table 1 and Figure 1B. These included two putative targets, *Fgf9* and *Egr2*, that were confirmed using miR-140 knockdown studies. Both *Fgf9* and *Egr2* were also upregulated upon CEES treatment. FGF9 has been reported to play an important role in the injury-associated proinflammatory environment, albeit in a neuronal model.³⁶ In the lung, FGF9 may also be involved in response of reparative cells after injury.³⁷ The role of EGR2, however, is less clear as it has been shown to be a prosurvival factor involved in inflammation and also a downstream target of NF- κ B.^{38,39} MicroRNA-based modulation of *Egr2* seems to be involved in sepsis-induced vascular permeability.⁴⁰ EGR2 has also been shown to promote profibrotic responses in murine lung.⁴¹ It would be interesting to further characterize the exact role these genes play in lung injury in general and in CEES-induced and miR-140-mediated inflammation in particular.

Our study provides insight into the mechanism by which miRNAs can mediate the effects of lethal chemicals, thus presenting an additional step in the process, which can potentially be targeted for therapy. Our work identifies a role of miR-140 in the pathogenesis of CEES-induced injury and suggests that levels of extracellular miRNA species can be used to predict the severity of disease and/or disease outcome. We believe that miR-140 can potentially be targeted for therapy in cases of exposure to CEES or SM or in disease conditions where there is increased inflammation and coagulation. There is a need for further investigation into the functional role of the other identified miRNAs.

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Figure 1.

CEES exposure-mediated differential expression of miRNAs and pathway analysis. Rats were exposed to CEES (10% in EtOH) for 15 min by nose inhalation. After 12 h, animals were euthanized and plasma was collected. (A) Heat map showing trends in the miRNA expression levels in CEES- versus vehicle (ethanol, ETOH)-exposed rats ($n = 4$ each). Expression levels of miRNAs in individual rats are shown as color-coded cells. The vertical bar besides the heat map on the top left indicates the color code of the expression levels. The central level of the color code (white) was set as the median value over all the values used in the heat map. Red indicates increased expression, while blue indicates reduced expression, relative to mean. Labeling at the bottom indicates vehicle- or CEES-exposed animals. (B) Representative miRNA regulatory map network was constructed with miRNA candidate

target gene interactions using IPA. Additional targets were added to the network based on the literature and findings from the current study. Upregulated miRNAs are shown in red, while downregulated miRNAs are shown in gray. Construction of regulatory map network involved focusing on two specific pathways (inflammatory signaling and coagulation). (C) Representative diseases and physiological pathways regulated by differentially expressed (DE) miRNAs.

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Figure 2.

Validation of candidate miRNAs. (A) Select miRNAs (rat analogs of the ones from the array) were validated by qRT-PCR quantification in the plasma of CEES-exposed rats, relative to vehicle (ethanol, ETOH)-exposed rats ($n = 8$ each). (B) miR-140 (miR-140-5p) expression levels were further quantified using qRT-PCR in lungs, livers, and kidneys of rats exposed to CEES, relative to rats exposed to ethanol ($n = 8$ each). U6 served as the standard control. * $P < 0.05$ and ** $P < 0.01$, compared with the respective vehicle controls.

Figure 3.

CEES induces inflammation and coagulation markers. (A) RNA was isolated from the tissue of control and CEES-exposed rats and subjected to qRT-PCR for detection of the mRNA of the inflammatory markers IL-6 and IL-1 α and the coagulation marker F3 (tissue factor) in lungs, liver, and kidneys. β -Actin was used as the internal control ($n = 8$ each). (B) Coagulation was further assessed by fibrin(ogen) deposition in the lung airways of vehicle- and CEES-exposed rats. Immunostaining was performed using an anti-human fibrinogen antibody, as described in Materials and Methods. The negative control was the rabbit IgG control without primary antibody (DAKO). Red arrows indicate fibrin(ogen)-rich casts in the airways. * $P < 0.05$ and ** $P < 0.01$, compared with respective vehicle (ethanol, ETOH) controls.

Figure 4.

CEES reduces cell viability and downregulates miR-140 in rat lung epithelial cells along with upregulating inflammation and coagulation markers. (A) Rat lung epithelial cells (RL-65) were treated with vehicle control (ethanol (ETOH)) or 0.5 mM CEES for 24 h, followed by crystal violet staining. The bar graph on the right represents quantification for viable cells. Cells under control condition (ethanol) were quantified and marked as 100%, while the viable cells after exposure to CEES are presented as the fraction (%) of control cells. (B) Expression of markers of inflammation (IL-6 and IL-1 α) and coagulation (F3, tissue factor) and predicted gene targets of miR-140 (miR-140-5p) were evaluated in the cells by qRT-PCR. β -Actin was used as the internal control. (C) Total miRNA was isolated from vehicle- (ethanol) and CEES-treated RL-65 cells, and miR-140 (miR-140-5p) was quantified by qRT-PCR. Total miRNA was also isolated from the media supernatant of CEES-treated RL-65 cells, and miR-140 (miR-140-5p) was quantified by qRT-PCR. Results are means $\pm$ S.E.M. for three independent experiments performed in triplicate. * $P < 0.05$ and ** $P < 0.01$, compared with respective vehicle controls.

Figure 5.

Downregulation of miR-140 in RL-65 cells induces markers of inflammation and coagulation, and its target genes. (A) miR-140 (miR-140-5p) was downregulated in RL-65 cells by transfection with anti-miR-140-5p oligos, and the downregulated expression of miR-140 in specific anti-miR-140 oligo-versus nonspecific oligo-treated cells after four rounds of transfections was confirmed by qRT-PCR. U6 served as the normalization control. (B) Expression of markers of inflammation (IL-6 and IL-1 α) and coagulation (F3, tissue factor), as well as gene targets of miR-140, was evaluated by qRT-PCR. β -Actin was used as the normalization control. ** $P < 0.01$, compared with respective vehicle controls.

Table 1.

Differentially expressed miRNAs in plasma of CEES-exposed rats, relative to control rats

miRNA	miRBase ID from Taqman	CT	Fold change	P	MicroRNA target prediction resources			Union list	Coagulation # of gene targets	Inflammation # of gene targets
					micro-RNA.org	miRDB	Target-Scan			
rno-miR-138-1-3p	mo-miR-138-1-3p	6.04	-65.70	7.84E-03	542	83	1652	2147	7	49
rno-miR-196a-3p	mo-miR-196a-3p	6.00	-64.07	1.59E-02	360	19	473	825	5	9
rno-miR-140-5p	mo-miR-140-5p	4.69	-25.90	1.59E-02	401	165	257	691	4	19
rno-miR-1896	mmu-miR-1896	4.47	-22.18	9.80E-03	0	205	2811	2877	8	51
rno-miR-1188-5p	mo-miR-1188-5p	4.45	-21.92	5.17E-02	0	161	2458	2619	6	49
rno-miR-423-3p	mo-miR-423-3p	4.38	-20.86	1.34E-02	0	16	11	25	0	0
rno-miR-17-5p	mmu-miR-17-5p	-3.94	15.38	1.59E-02	0	556	261	711		
rno-miR-196c-5p	mo-miR-196c-5p	-3.80	13.93	4.07E-02	350	115	17	448	3	5
rno-miR-187-3p	mo-miR-187-3p	3.61	-12.20	5.57E-02	194	15	22	219	4	2
rno-miR-429	mo-miR-429	-3.17	8.99	1.55E-02	707	470	46	1068	4	26
rno-miR-337-3p	mmu-miR-337-3p	3.09	-8.51	3.41E-02	0	216	2938	2997	9	65
rno-miR-501-3p	mmu-miR-501-3p	2.81	-7.02	3.39E-02	0	118	23	135	0	3
rno-miR-140-3p	mo-miR-140-3p	2.53	-5.77	1.98E-02	401	355	336	1000	3	31
rno-miR-532-5p	mmu-miR-532-5p	2.45	-5.45	5.19E-02	0	137	57	182	0	1

Note: Expression of miRNAs relative to U6 RNA was determined by real-time qRT-PCR using TaqMan Arrays. Coagulation genes targets were listed by evaluation of genes involved in "complement and coagulation cascades (mo04610)." Inflammation genes targets were listed by the evaluation of genes involved in "chemokine signaling pathway (mo04062)" or "NF kappa beta signaling pathway (mo04064)" or "TNF signaling pathway (mo04668)."