Differential effect of mild and severe pulmonary embolism on the rat lung transcriptome

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Abstract

Background: Pulmonary thromboembolism (PTE) is a common diagnosis and a leading cause of cardiovascular morbidity and mortality. A growing literature has associated PE with systemic inflammation, and global hyper-coagulability, which contribute to lung remodeling and clot recurrence. The source and mechanism of inflammation remains unstudied. In humans, inhibition of cholesterol synthesis with statins decreases biomarkers of inflammation. We test the differential effect of pulmonary vascular occlusion during mild and severe pulmonary embolism on the lung transcriptome.

Methods: Experimental PE was induced in adult male rats by injection of 25 micron polystyrene microspheres into the jugular vein. The effect of Mild PE, (2-h right ventricular systolic pressure [RVSP] normal, 18-h RVSP 44 mmHg) and Severe PE (2-h RVSP > 50 mmHg; 18-h RVSP 44 mmHg) on lungs was assessed by measuring transcriptome-wide changes in gene expression by DNA microarrays.

Results: Severe PE was associated with a large change in lung gene expression and in the expression of KEGG pathways and other gene functional annotation groups. Mild PE was also associated with a large number of significant changes in gene expression and in the expression of KEGG pathways and gene functional annotation groups, even after only 2 h of PE. Up-regulated pathways included increased adipocytokine, chemokine and cytokine signaling as well as cholesterol synthesis.

Conclusions: Mild PE without acute pulmonary hypertension (PH) increased lung gene expression of inflammatory pathways, including increased cholesterol synthesis. These data indicate that even mild persistent pulmonary vascular occlusion is capable of inciting an inflammatory response from the lung. These data imply the detrimental effect of unresolved pulmonary obstruction from PE.

Keywords: Lung, Pulmonary hypertension, Inflammation, Microarray, GeneSifter, DAVID, Fibrinolysis, Thrombolysis
inflammatory response [7–11]. Increased lung inflammation has been implicated as a mechanism of reduced angiogenesis, and for increased hyper-coagulability, leading to recurrent PE [10, 12, 13]. Recurrent PE is a major risk factor for CTEPH development [8, 14]. Potential causes of inflammation include cells and molecules liberated by clots themselves [15], direct interaction of fibrin and the vessel wall [16], and the effect of shear on the vessel wall and platelets resulting in microparticle formation [9, 17]. Pulmonary vascular occlusion, with deprivation of blood flow to the lung also triggers a brisk inflammatory response [18].

We have previously described a rat model of PE induced by injection of 25 micron polystyrene microspheres into the right jugular vein [18–25]. Although microsphere PE lacks many of the characteristics of PTE such as platelet activation and thrombosis, it does faithfully produce the desired features of pulmonary ischemia and, at high doses, pulmonary hypertension. Rat lungs receiving doses of microspheres that produced acute PH (Severe PE, right ventricular systolic pressure [RVSP] >50 mmHg and 10% animal mortality) had a >5-fold increase in recoverable bronchoalveolar lavage (BAL) neutrophils compared to control rats, indicating a neutrophilic inflammation [18]. Strong neutrophil chemotactic activity was measured in isolated alveolar lavage fluid and this activity was inhibited greater >50% by treatment with anti-rat CXCL1 antibody. Rat lungs embolized with a lower dose of microspheres (Mild PE, normal RVSP 2-h post-PE and zero mortality), did not show neutrophil or protein accumulation in alveoli, but did show elevated expression of the chemokine genes CXCL1, CXCL2, CXCL3 and CCL2 [18]. These latter data led to a realization that Mild PE was sufficient to induce a pro-inflammatory environment within lung tissues, at least at the level of gene expression, and that lungs might be more sensitive to Mild PE than hearts. In this present study, the transcriptome-wide effect of Mild PE and Severe PE on rat lungs has been examined using DNA microarrays. The primary question was to answer the question whether Mild PE, which has been observed to be benign to RV dysfunction and inflammation [20–23], had a disproportionately worse effect on lungs.

Methods

Animal care

Experiments were done on male Sprague–Dawley rats weighing between 375 and 400 g. All experiments were conducted in accordance with the NIH Guide For the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Carolinas Medical Center, Charlotte NC (4-02-01A and 11-00-01A). Prior to use, rats had ad libitum access to food and water.

Pulmonary embolism model

PE was induced in rats by intra-jugular vein injection of 25-micron polystyrene microsphere beads (Duke Scientific #7525A, Palo Alto CA) as previously published [18–25]. Anesthetized rats were injected with either 1.3 or 2.0 million microspheres/100 g body weight to produce Mild PE and Severe PE, respectively. Control rats were injected with 0.15 ml/100 g of 0.01% Tween 20 (the resuspension vehicle for microspheres), which was equivalent in volume to the Severe PE dose of microspheres. These treatment groups are referred to as “Vehicle” or “Veh”.

Microarray analyses

Lung tissue samples from whole right lung lobes used for this study are the same as those collected for a previously published study [18]. In that study, Mild PE produced 2-h RVSP that was not statistically significant from RVSP in control rats (mean 39 mmHg for Mild PE verses mean 32 mmHg for controls, p > 0.05) while 2-h Severe PE caused elevation of RVSP to >50 mmHg [18]. Lungs were also collected from rats after 18-h of PE but RVSP was not measured. In subsequent studies, Mild PE was shown to cause a rise in 18-h RVSP to mean 44+/-1.3 mmHg (p < 0.05 relative to vehicle) while the RVSP in 18-h Severe PE was shown to fall from a peak of >50 mmHg to 44+/-0.9 mmHg [20, 22]. We have consistently concluded that Severe PE is associated with PH for the full time course of 2–18 h while Mild PE consistently shows PH only at the 18-h time. RNA was prepared from crushed whole right lung tissue which had been stored at -80 °C using the acid-phenol guanidinium isothiocyanate method of Chomczynski and Sacchi [26] followed by a second round of purification on RNeasy columns (Qiagen, Germantown, MD). Total lung RNA was prepared for microarray hybridization by standard Affymetrix procedures as previously described and checked for RNA integrity on agarose gels prior to use [23, 24]. Fragmented cRNAs were then hybridized to Affymetrix Rat Genome 230 v2.0 microarrays, washed and fluorescently stained in the Affymetrix Fluidics Station 400 using Affymetrix procedures. Each array was scanned twice by an Agilent Gene Array Scanner G2500A (Agilent Technologies, Palo Alto, CA).

Microarray data were initially analyzed with GeneSifter web-based software (Geospiza, Seattle, WA; (http://www.genesifter.net)). Affymetrix “.cel” files were uploaded to the GeneSifter web site using GC-RMA normalization into “Pair-wise” and “Project” folders for access to t-test and ANOVA statistical methods, respectively. A 2-way ANOVA was used to initially
compare the six treatment groups using time as the first factor (2-h and 18-h) and microsphere dose as the second factor (Vehicle, Mild PE, Severe PE). A 1.5-fold expression difference threshold and Benjamini and Hochberg correction for false discovery ($p < 0.05$) was used as “pass” criteria. Genes that passed any one of the criteria of time, dose or interaction were accepted. The 6 sample groups in the 2-way ANOVA were then subjected to hierarchical clustering to determine the similarities of the groups using the GeneSifter “Cluster” function.

Separate 1-way ANOVAs were used to compare the three 2-h treatment groups and three 18-h treatment groups for genes with related expression patterns based on the factor of microsphere dose (1.5-fold expression difference threshold relative to 2-h vehicle and 18-h vehicle groups as controls, respectively, Benjamini and Hochberg correction for false discovery, $p < 0.05$). Clustering of genes within the 2-h and 18-h ANOVAs were done using the GeneSifter PAM function (Partitioning Around Medoids) with a user-defined 12-cluster output. PAM searches a gene list for groups of genes (clusters) within the list that have a characteristic that is shared by all genes within that group but different from genes within other groups (hence, “clustering”). The characteristic used by GeneSifter was the pattern of expression each gene showed for the Vehicle, Mild PE and Severe PE treatments. GeneSifter allowed the user to specify the number of clusters that the entire gene list would be sorted into. For the analyses in Figs. 2 and 3, the 2-h and 18-h gene lists were sorted into 12 clusters. This number of clusters was determined empirically as the fewest number of clusters which yielded Mild PE-selective and Severe PE-selective expression patterns for the 2-h ANOVA data. These patterns were defined as expression patterns in which the change in expression from control was maximal between Vehcile and Mild PE and between Mild PE and Severe PE, respectively.

Pairwise comparison of treatment groups was done with GeneSifter using 2-sided unpaired t-tests with a 1.5-fold expression difference threshold relative to vehicle groups and with Benjamini and Hochberg correction for false discovery, $p < 0.05$.

Excel spreadsheet exports of 2-h and 18-h GeneSifter t-tests were used to prepare gene lists for further analysis using DAVID (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov; [27, 28]). Lists of official gene symbols were then separately pasted into the search input in DAVID. DAVID analyzes gene lists and identifies all of the annotations that are attributed to each gene in the list. Gene annotations are categories of gene function, protein structural features, biochemical pathways and other shared gene or protein properties that are manually given by database curators to all genes and proteins. DAVID then produces several types of output that group the members of the input gene list into these annotations and determines statistical significance by comparing the number of genes from the submitted gene list that are present in each annotation with the total number of genes that are in the annotation (this is an “enrichment calculation”). Functional annotation charts were generated using DAVID default stringency settings (“moderate”). Each downloaded chart was a spreadsheet containing a list of all known annotations that contained at least one gene from the input list of PE genes from the t-test and the probability that the input list of PE genes was enriched in genes of that annotation. Final annotation charts were then assembled by merging the UP- and DOWN-enriched annotations for each combination of PE dose and time. These charts were then reduced in complexity by discarding annotation terms that were not significantly enriched in the input PE gene list (t-test, Benjamini and Hochberg values >0.05) and by discarding all Gene Ontology (GO) terms in the DAVID output regardless of statistical significance. GO term annotations greatly increased the length of the annotation lists but have limited investigative value in the opinion of the authors. All microarray data have been deposited in the NIH/NCBI “GEO” database (http://www.ncbi.nlm.nih.gov/projects/geo; GEO accession number GSE13535).

**Results**

**Six-group ANOVA, gene clustering, and pair-wise t-tests of treatment groups**

A comprehensive summary of all microarray data was first generated by comparing the six treatment groups (Mild PE, Severe PE and Vehicle each at 2-h and 18-h) in this study using a 2-way ANOVA, using the GeneSifter software suite (1.5-fold expression change minimum, Benjamini and Hochberg correction for false discovery, $p < 0.05$). The data is contained in Additional file 1 and shows that 8075 Affymetrix probesets passed the ANOVA for at least one of the tests (Factor 1, time; Factor 2, dose; interaction). The data from the ANOVA was then subjected to hierarchical clustering using GeneSifter to define the relationships between the six treatment groups. This output is summarized in the dendrogram shown in Fig. 1. The three 2-h groups were closely associated, as were the 18-h Mild PE and 18-h Severe PE groups while the 18-h vehicle clustered with the 2-h vehicle.

The six groups were also compared pair-wise (t-test, 1.5-fold expression change minimum, Benjamini and
Separate analyses of 2-h and 18-h treatment groups

The responses of rats to mild PE and Severe PE were compared using separate 1-way ANOVAs of the 2-h and 18-h treatments with GeneSifter (expression difference threshold >1.5-fold, t-test <0.05, Benjamini and Hochberg). For the three 2-h treatment groups 775 probesets were altered in expression relative to the 2-h vehicle control, while 4360 probesets were altered in the 18-h groups relative to the 18-h vehicle control (1.5-fold expression difference threshold, t-test <0.05, Benjamini and Hochberg). The altered probesets in the 2-h and 18-h ANOVAs were then clustered into similar expression patterns with GeneSifter using the PAM function (Partitioning Around Medoids) with a user-designated output of 12 clusters for each ANOVA. These data are presented in Figs. 2 and 3 for 2-h and 18-h treatment groups, respectively.

The PAM-clustering of the 2-h ANOVA data is shown in Fig. 2, and clearly indicates three distinct patterns. 5 of 12 clusters had a “Mild PE-selective” pattern (clusters 3, 6, 8, 10 and 12). Probesets in these clusters had greater changes in expression between the vehicle and Mild PE samples than between the Mild PE and Severe PE samples. 2 of 12 clusters had a “Severe PE-selective” pattern (clusters 4 and 5). Probesets in these clusters had greater changes in expression between the Mild PE and Severe PE samples but minimal change in expression between the Mild PE and Mild PE treatments. The magnitudes of the Mild PE expression changes in two of these clusters were within a single log2 increment of the vehicle and Severe PE groups (clusters 1 and 2) while the magnitudes of the Mild PE expression changes in the other two clusters (9 and 11) reached 4 log2 increments of the vehicle and Severe PE groups. These four clusters, and clusters 9 and 11 in particular, suggest that there were opposing signals for up- and down-regulation of gene expression in the Mild PE and Severe PE groups.

The PAM-clustering of the 18-h ANOVA data is shown in Fig. 3. Clustering of the 18-h treatment groups resulted in fewer clusters with obvious Mild PE-selective and Severe PE-selective gene expression than observed for the 2-h ANOVA. A plurality of clusters had “non-selective” patterns of gene expression (2, 3, 7, 8 and 10). Probesets in these clusters had nearly identical changes in expression between the vehicle and Mild PE samples and Mild PE and Severe PE samples. 4 of 12 clusters had a Mild PE-selective pattern (clusters 4, 5, 11 and 12) while two clusters (1 and 9) were intermediate between Mild PE-selective and non-selective. In contrast to the 2-h treatments, none of the clusters were indicative of Severe PE-selective expression.

Enrichment of PE genes in functional annotations

Gene expression data from the pairwise t-tests of Mild PE and Severe PE treatment groups (Additional file 2A – F) were examined for significant enrichment of PE-altered probesets present in database annotations using DAVID.
web-based software (KEGG pathways, INTERPRO, SMART and SP_PIR database terms; GO terms excluded). Tables 1 and 2 summarize the functional annotations enriched in the Mild PE and Severe PE treatment groups, respectively.

Functional annotations enriched in the Mild PE treatment groups relative to time-matched vehicle controls are shown in Table 1. Annotations unique to Mild PE treatment groups are highlighted with bold font and italics while annotations common to Low- and Severe PE are in standard font. Blank cells in the data indicate that an annotation was not significantly over-represented at a particular time and treatment combination. At the 2-h time, the majority of annotations (10 of 21 total annotations) were unique to Mild PE, while a minority (11 of 21) were shared with the Severe PE 2-h group (Table 2). Conversely, at 18-h only 5 of 22 total annotations were unique to Mild PE (23 %) while 17 of 22 (77 %) were shared with the Severe PE group (Table 2). These data support a distinct mechanistic difference between Mild and Severe PE. If Mild PE were merely a less robust manifestation of Severe PE, all annotations present during Mild PE would be expected to be present during Severe PE.

Functional annotations enriched in the Severe PE treatment groups relative to time-matched vehicle controls are shown in Table 2. Annotations unique to Severe PE treatment groups are highlighted with bold font and italics while annotations common to Low PE and Severe PE are
in standard font. It is noteworthy that the data in Table 2 showed a distinct separation of annotations between the two times. Of the 57 total annotations listed in the table only one, “SP-PIR chemotaxis”, was present at both times. The remaining 56 annotations were present at only one of the time points. This pattern was not seen with the Low PE data in Table 1 in which a greater proportion of the total annotations were present at both times. Together, these data suggest a rapid “progression” of the Severe PE condition at the transcriptional level compared to a more gradual transcriptional “progression” for Mild PE.

**Annotations unique to mild PE**

Further examination of the Mild PE expression data in Table 2 revealed that 13 of 35 total annotations were unique to Mild PE. Several of these unique annotations involved pro-inflammatory KEGG pathways or protein families. The KEGG pathways were: rno04920 “adipocytokine signaling”, rno04062 “chemokine signaling”, rno04060 “cytokine-cytokine receptor”, and rno04620 “Toll-like receptor signaling”. Rno04010 “MAPK signaling pathway” was also present, but this pathway intersects with diverse cellular processes beside inflammation. The Interpro annotation IPR000827 “small cytokine C-C” and Protein Information Resource annotation PIRSF001950 “small inducible chemokine” were also present. The presence of gene annotations unique to the 2-h Mild PE treatment, which shares little physiological similarity with Severe PE, continue to support a conclusion that Mild PE and Severe PE may be mechanistically dissimilar at the transcriptional level.

**Are mild PE and severe PE related?**

The possible similarities between the 18-h Mild PE and 2-h Severe PE found in Tables 1 and 2 prompted a direct
comparison between these two treatment groups. These data are presented in Table 3. However, only 6 of 36 annotations present in either of the two treatments were shared by both: SP-PIR chemotaxis, SP-PIR cytokine, SP-PIR “inflammatory response”, rno04621 “NOD-like receptor signaling pathway”, SM00199 “SCY” and IPR001811 “small chemokine interleukin-8-like”. The remaining 31 annotations which were enriched in either

Table 1 DAVID Functional Annotation Charts, 2-h and 18-h Mild PE verses Vehicle

| Category | Term                                | 2-h Mild-PE vs. Vehicle | 18-h Mild-PE vs. Vehicle |
|----------|-------------------------------------|-------------------------|--------------------------|
|          | Ct | % | Dir | Fold | B&H | Ct | % | Dir | Fold | B&H |
| SP-PIR   | acetylation                         |                          |                          |
| rno04920 | Adipocytokine signaling pathway     | 6 | 2.93 | UP | 7.47 | 0.015 |                          |                          |
| rno00970 | Aminoacyl-tRNA biosynthesis         |                          |                          |
| rno04210 | Apoptosis                           | 6 | 2.93 | UP | 5.89 | 0.028 |                          |                          |
| rno00330 | Arginine and proline metabolism    |                          |                          |
| rno05217 | Basal cell carcinoma                |                          |                          |
| IPR004827| Basic-leucine zipper (bZIP) transcrip| 7 | 3.41 | UP | 11.97 | 0.003 |                          |                          |
| SM00338  | BRLZ                                | 7 | 3.41 | UP | 10.46 | 0.001 |                          |                          |
| rno04062 | Chemokine signaling pathway         | 12 | 5.85 | UP | 5.85 | 0.000 | 20 | 3.60 | UP | 3.25 | 0.001 |
| SP-PIR   | chemotaxis                          | 9 | 4.39 | UP | 22.67 | 0.000 | 10 | 1.80 | UP | 3.97 | 0.019 |
| rno04610 | Complement and coagulation cascades |                          |                          |
| SP-PIR   | cytokine                            | 11 | 5.37 | UP | 9.02 | 0.000 | 16 | 2.88 | UP | 4.88 | 0.000 |
| rno04060 | Cytokine-cytokine receptor interaction | 10 | 4.88 | UP | 4.24 | 0.008 | 23 | 4.14 | UP | 3.25 | 0.000 |
| rno04623 | Cytosolic DNA-sensing pathway       | 5 | 2.44 | UP | 9.07 | 0.024 |                          |                          |
| SP-PIR   | disulfide bond                      | 76 | 13.67 | UP | 1.43 | 0.044 |                          |                          |
| SP-PIR   | dna-binding                         | 20 | 9.76 | UP | 2.43 | 0.023 |                          |                          |
| rno00982 | Drug metabolism                     | 13 | 1.45 | D | 3.94 | 0.004 |                          |                          |
| rno04512 | ECM-receptor interaction            | 14 | 1.56 | D | 3.77 | 0.005 |                          |                          |
| PIRS001719| fos transforming protein            | 4 | 1.95 | UP | 59.84 | 0.001 |                          |                          |
| IPR000837| Fos transforming protein            | 4 | 1.95 | UP | 40.08 | 0.010 |                          |                          |
| rno00480 | Glutathione metabolism              | 9 | 1.00 | D | 3.93 | 0.042 |                          |                          |
| rno04640 | Hematopoietic cell lineage          | 6 | 2.93 | UP | 6.42 | 0.022 |                          |                          |
| rno00340 | Histidine metabolism               | 7 | 0.78 | D | 6.37 | 0.017 |                          |                          |
| SP-PIR   | inflammatory response               | 8 | 3.90 | UP | 16.01 | 0.000 | 11 | 1.98 | UP | 8.18 | 0.000 |
| rno04010 | MAPK signaling pathway              | 10 | 4.88 | UP | 3.14 | 0.029 |                          |                          |
| rno00980 | Metabolism of xenobiotics by cytochrome P450 | 13 | 1.45 | D | 4.73 | 0.002 |                          |                          |
| rno04621 | NOD-like receptor signaling pathway | 9 | 4.39 | UP | 12.11 | 0.000 | 10 | 1.80 | UP | 4.49 | 0.016 |
| SP-PIR   | oxidoreductase                      | 42 | 4.68 | D | 1.89 | 0.034 |                          |                          |
| rno05020 | Prion diseases                      | 7 | 1.26 | UP | 5.56 | 0.028 |                          |                          |
| SP-PIR   | ribosome biogenesis                 | 7 | 1.26 | UP | 8.85 | 0.007 |                          |                          |
| SM00199  | SCY                                 | 8 | 3.90 | UP | 16.89 | 0.000 | 10 | 1.80 | UP | 8.76 | 0.000 |
| IPR000827| Small chemokine, C-C group, conserved site | 4 | 1.95 | UP | 25.50 | 0.033 |                          |                          |
| IPR001811| Small chemokine, interleukin-8-like | 8 | 3.90 | UP | 19.35 | 0.000 | 10 | 1.80 | UP | 8.70 | 0.001 |
| PIRS001950| small inducible chemokine, C/CC types | 5 | 2.44 | UP | 24.93 | 0.001 |                          |                          |
| rno04620 | Toll-like receptor signaling pathway | 9 | 4.39 | UP | 8.34 | 0.000 |                          |                          |

Functional annotations significantly over-represented in the lists of up-regulated and down-regulated genes during 2-h and 18-h Low-PE. Annotations unique to Low-PE are highlighted in bold italics; annotations common to Low-PE and High-PE (Table 2) are in standard font. Key: “Ct.”, number genes from a GeneSifter pairwise t-test result (Additional file 3A-C) that were present in the functional annotation indicated; “%”, percent of genes contained within a list that were present in an annotation; "Dir", UP, annotations that were identified by DAVID when up-regulated genes were used as the search query; “Dir, D”, annotations that were identified by DAVID when down-regulated genes were used as the search query. “Fold”, expression relative to vehicle group; B&H, value of Benjamini and Hochberg adjustment for false discovery following t-test. "No 2-h DOWN annotations meet B&H < 0.05"
### Table 2 DAVID Functional Annotation Charts, 2-h and 18-h Severe PE verses Vehicle

| Category | Term | 2-h Severe PE verses Vehicle | 18-h Severe PE verses Vehicle |
|----------|------|-----------------------------|------------------------------|
|          |      | Ct  | %     | Dir | Fold | B&H | Ct  | %     | Dir | Fold | B&H |
| **SP_PIR** acetylation | | | | | | | | | | | |
| **SP_PIR** activator | | 7  | 7.29  | UP  | 5.58  | 0.025  | 183 | 17.72  | UP  | 1.86  | 0.000  |
| **SP_PIR** acute phase | | | | | | | | | | | |
| rno00520 Amino sugar and nucleotide sugar metabolism | | 12  | 1.16  | UP  | 4.37  | 0.002  |
| mo00970 Aminoacyl-tRNA biosynthesis | | 12  | 1.16  | UP  | 4.70  | 0.002  |
| **SP_PIR** Aminoacyl-tRNA synthetase | | 10  | 0.97  | UP  | 5.11  | 0.003  |
| mo00330 Arginine and proline metabolism | | 11  | 1.06  | UP  | 3.25  | 0.046  |
| rno05412 Arrhythmogenic right ventricular cardiomyopathy (ARVC) | | 15  | 0.96  | D   | 2.56  | 0.040  |
| mo05217 Basal cell carcinoma | | 12  | 0.77  | D   | 2.97  | 0.036  |
| IPR011700 Basic leucine zipper | | 4  | 4.17  | UP  | 41.80  | 0.004  |
| IPR004827 Basic-leucine zipper (bZIP) transcription factor | | 8  | 8.33  | UP  | 28.54  | 0.000  |
| SM00338 BRLZ | | 8  | 8.33  | UP  | 20.66  | 0.000  |
| IPR011616 bZIP transcription factor, bZIP-1 | | 4  | 4.17  | UP  | 25.44  | 0.015  |
| **SP_PIR** Chaperone | | 18  | 1.74  | UP  | 2.78  | 0.006  |
| **SP_PIR** chemotaxis | | 4  | 4.17  | UP  | 20.78  | 0.025  |
| **SP_PIR** Cholesterol biosynthesis | | 9  | 0.87  | UP  | 4.43  | 0.016  |
| rno04610 Complement and coagulation cascades | | 17  | 1.65  | UP  | 3.80  | 0.000  |
| **SP_PIR** cytokine | | 9  | 9.38  | UP  | 15.21  | 0.000  |
| **SP_PIR** DNA binding | | 6  | 6.25  | UP  | 7.38  | 0.028  |
| **SP_PIR** dna-binding | | 15  | 15.63  | UP  | 3.75  | 0.002  |
| **UP_SEQ** DNA-binding region: Basic motif | | 10  | 10.42  | UP  | 15.44  | 0.000  |
| **UP_SEQ** domain: Leucine-zipper | | 9  | 9.38  | UP  | 19.96  | 0.000  |
| rno00982 Drug metabolism | | 20  | 1.28  | D   | 3.51  | 0.000  |
| rno04512 ECM-receptor interaction | | 17  | 1.09  | D   | 2.65  | 0.020  |
| SM00180 EGF_Lam | | 9  | 0.58  | D   | 5.44  | 0.020  |
| IPR002049 EGF-like, laminin | | 9  | 0.58  | D   | 5.98  | 0.026  |
| **SP_PIR** endoplasmic reticulum | | 63  | 6.10  | UP  | 2.04  | 0.000  |
| PI0S001719 fos transforming protein | | 4  | 4.17  | UP  | 109.2  | 0.000  |
| IPR000837 Fos transforming protein | | 4  | 4.17  | UP  | 83.59  | 0.001  |
| mo00480 Glutathione metabolism | | 12  | 0.77  | D   | 3.03  | 0.041  |
| mo00340 Histidine metabolism | | 9  | 0.58  | D   | 4.73  | 0.019  |
| **SP_PIR** inflammatory response | | 4  | 4.17  | UP  | 16.51  | 0.027  |
| **SP_PIR** Initiation factor | | 12  | 1.16  | UP  | 4.55  | 0.002  |
| **SP_PIR** Isomerase | | 17  | 1.65  | UP  | 3.14  | 0.003  |
| **SP_PIR** lipid synthesis | | 19  | 1.84  | UP  | 3.82  | 0.000  |
| rno00980 Metabolism of xenobiotics by cyt. P450 | | 18  | 1.15  | D   | 3.79  | 0.000  |
| **UP_SEQ** mutagenesis site | | 59  | 3.77  | D   | 1.75  | 0.042  |
| rno04621 NOD-like receptor signaling pathway | | 5  | 5.21  | UP  | 12.52  | 0.034  |
| **SP_PIR** nucleotide-binding | | 99  | 9.58  | UP  | 1.36  | 0.024  |
| **SP_PIR** nucleus | | 22  | 22.92  | UP  | 2.01  | 0.026  |
| **SP_PIR** phosphoprotein | | #  | #     | #    | #    | #    |
| IPR011993 Pleckstrin homology-type | | 32  | 2.05  | D   | 2.27  | 0.027  |
the 2-h Severe PE or 18-h Mild PE groups were confined to one or the other group. These data refute a similarity between early Severe PE and late Mild PE.

Steroid synthesis during 18-h severe PE

Three annotations were detected in the 18-h Severe PE gene lists which were associated with steroid and/or sterol biosynthesis. A total of 16 unique genes were contained within these annotations. These data are presented in Table 4. Most of these genes were contained on the KEGG pathway rno00100 (R. norvegicus Steroid Biosynthesis; http://www.kegg.jp/kegg-bin/show_pathway?rno00100). This pathway terminates with several branches but all of the genes induced by Severe PE were located on the branch terminating with cholesterol.

HMGCR, HMGCS1 and IDI1 function upstream of rno00100 (R. norvegicus Steroid Biosynthesis; http://www.kegg.jp/kegg-bin/show_pathway?rno00100). This pathway begins with acetyl-CoA, proceeds through 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), mevalonate, isopentenyl-diphosphate and farnesyl-diphosphate, which is the metabolic intermediate that feeds KEGG Pathway rno00100.

Discussion

Microsphere-induced PE caused profound changes in gene expression in rat lungs even when RVSP was lower than typically considered clinically relevant. These data contrast sharply with previous results on the effects of this same PE model on transcriptional changes in hearts [23]. In this latter study, Zagorski et al. demonstrated an almost obligatory requirement for PH to cause altered gene expression in RV tissues [23]. In particular, 2-h Mild PE resulted in no statistically significant transcriptional changes in RVs and few changes after 18-h. The effect of Mild PE on gene expression in lung tissue reported here was much more dramatic. 2-h of Mild PE with minimal PH was sufficient to cause numerous changes in gene expression and statistically significant alteration in at least 21 expression pathways or other gene group annotations. Examination of the annotations enriched in the 2-h Mild PE "UP" gene expression data revealed several pro-inflammatory annotation terms. The abundance of pro-inflammatory annotations enriched in 2-h Mild PE samples indicates that lungs exposed to even mild PE with minimal PH rapidly enter into a pro-inflammatory state. This data supports a conclusion that even mild PE has the potential to initiate damage to lung tissues. This is in stark contrast to the total lack of right ventricular inflammation seen in the microsphere model of Mild PE [19–25].

Mild and Severe PE are hemodynamically distinct at 2-h post-PE, with only the latter having PH. This brings into question the stimuli imparted on lungs with 2-h Mild PE which are responsible for the large changes in gene expression. The most likely explanation is ischemia, which is often considered synonymous with hypoxia. However, ischemia resulting from occlusion of the pulmonary vasculature is unique, since the pulmonary artery circulates oxygen-poor and nutrient-depleted blood derived from the venous circulation. In essence, even normal circulation through the pulmonary artery

Table 2 DAVID Functional Annotation Charts, 2-h and 18-h Severe PE verses Vehicle (Continued)

| Annotation | Functional Description | 2-h | 18-h | Fold Change (2-h) | Fold Change (18-h) | B&H adjusted p-value |
|------------|------------------------|-----|-----|------------------|-------------------|---------------------|
| SP_PIR protein biosynthesis | 22 | 2.13 | UP | 2.04 | 0.049 |
| SP_PIR protein transport | 34 | 3.29 | UP | 1.87 | 0.016 |
| SP_PIR Redox-active center | 10 | 0.97 | UP | 4.74 | 0.005 |
| SP_PIR ribosome biogenesis | 8 | 0.77 | UP | 5.31 | 0.014 |
| SP_PIR rna-binding | 33 | 3.19 | UP | 2.16 | 0.002 |
| SM00360 RRM | | | | | |
| SM00199 SCY | 5 | 5.21 | UP | 18.26 | 0.003 |
| IPR001811 Small chemokine, interleukin-8-like | 5 | 5.21 | UP | 25.22 | 0.002 |
| SP_PIR Steroid biosynthesis | 13 | 1.26 | UP | 5.08 | 0.000 |
| rno00100 Steroid biosynthesis | 9 | 0.87 | UP | 8.29 | 0.001 |
| SP_PIR sterol biosynthesis | 11 | 1.06 | UP | 7.31 | 0.000 |
| SP_PIR Transcription | 14 | 14.58 | UP | 3.23 | 0.009 |
| SP_PIR transcription regulation | 14 | 14.58 | UP | 3.48 | 0.006 |
| SP_PIR translocation | 11 | 1.06 | UP | 3.75 | 0.014 |
| rno00350 Tyrosine metabolism | 10 | 0.64 | D | 3.71 | 0.032 |

Functional annotations significantly over-represented in the lists of up-regulated and down-regulated genes during 2-h and 18-h High-PE. Annotations unique to High-PE are highlighted in bold italics; annotations common to High-PE and Low-PE (Table 1) are in standard font. All other keys are the same as in Table 1. *No 2-h DOWN annotations meet B&H < 0.05
has no effect on oxygenation of lung tissue, which is dependent on the separate bronchial circulation. Furthermore, lung tissue is continuously exposed to atmospheric oxygen via the airways. It seems unlikely that hypoxia accounts for the early lung transcriptional response to Mild PE. Supporting evidence for this conclusion is contained in this study. Expression of the hypoxia marker genes Hif1 (hypoxia-inducible factor-1) and Hyou1 (hypoxia up-regulated 1) were up-regulated in the 18-h Severe PE treatment group by modest 1.6851-

| Category | Term                                         | 2-h Severe-PE verses Vehicle | 18-h Mild-PE verses Vehicle |
|----------|----------------------------------------------|-------------------------------|----------------------------|
|          |                                              | Ct   | %   | Dir | Fold | B&H   | Ct   | %   | Dir | Fold | B&H   |
| SP_PIR   | acetylation                                   | 7    | 7.29 | UP  | 5.58 | 0.025 | 78   | 14.03 | UP  | 1.51 | 0.009 |
| SP_PIR   | activator                                     | 8    | 1.44 | UP  | 5.56 | 0.017 | 9    | 1.62  | UP  | 4.72 | 0.015 |
| mro00970 | Aminoacyl-tRNA biosynthesis                   | 10   | 1.11 | D   | 4.28 | 0.016 |      |       |     |      |       |
| mro00330 | Arginine and proline metabolism               |      |     |     |      |       | 8    | 8.33  | UP  | 20.66 | 0.000 |
| mro05217 | Basal cell carcinoma                          |      |     |     |      |       |      |       |     |      |       |
| IPRO11700| Basic leucine zipper                          | 4    | 4.17 | UP  | 41.80 | 0.004 |      |       |     |      |       |
| IPRO04827| Basic-leucine zipper (bZIP) transcription factor | 8    | 8.33 | UP  | 28.54 | 0.000 |      |       |     |      |       |
| SM00338  | BRLZ                                         | 8    | 8.33 | UP  | 20.66 | 0.000 |      |       |     |      |       |
| IPRO11616| bZIP transcription factor, bZIP-1             | 4    | 4.17 | UP  | 25.44 | 0.015 |      |       |     |      |       |
| mro04062 | Chemokine signaling pathway                   |      |     |     |      |       |      |       |     |      |       |
|          |                                              | 20   | 3.60 | UP  | 3.25 | 0.001 |      |       |     |      |       |
| SP_PIR   | chemotaxis                                    | 4    | 4.17 | UP  | 20.78 | 0.025 | 11   | 1.98  | UP  | 10.30 | 0.000 |
| mro04610 | Complement and coagulation cascades           | 10   | 1.80 | UP  | 3.97 | 0.019 |      |       |     |      |       |
| SP_PIR   | cytokine                                      | 9    | 9.38 | UP  | 15.21 | 0.000 | 16   | 2.88  | UP  | 4.88  | 0.000 |
| mro04060 | Cytokine-cytokine receptor interaction        | 23   | 4.14 | UP  | 3.25 | 0.000 |      |       |     |      |       |
| SP_PIR   | disulfide bond                                | 76   | 13.67 | UP  | 1.43 | 0.044 |      |       |     |      |       |
| SP_PIR   | DNA binding                                   | 6    | 6.25 | UP  | 7.38 | 0.028 |      |       |     |      |       |
| SP_PIR   | dna-binding                                   | 15   | 15.63 | UP  | 3.75 | 0.002 |      |       |     |      |       |
| UP_SEQ   | DNA-binding region: Basic motif               | 10   | 10.42 | UP  | 15.44 | 0.000 |      |       |     |      |       |
| UP_SEQ   | domain: Leucine-zipper                        | 9    | 9.38 | UP  | 19.96 | 0.000 |      |       |     |      |       |
| mro00982 | Drug metabolism                               |      |     |     |      |       | 13   | 1.45  | D   | 3.94  | 0.004 |
| mro04512 | ECM-receptor interaction                      |      |     |     |      |       | 14   | 1.56  | D   | 3.77  | 0.005 |
| PIRSF00179| fos transforming protein                      | 4    | 4.17 | UP  | 109.20 | 0.000 |      |       |     |      |       |
| IPRO00837| Fos transforming protein                      | 4    | 4.17 | UP  | 83.59 | 0.001 |      |       |     |      |       |
| mro00480 | Glutathione metabolism                        | 9    | 1.00 | D   | 3.93 | 0.042 |      |       |     |      |       |
| mro00340 | Histidine metabolism                          | 7    | 0.78 | D   | 6.37 | 0.017 |      |       |     |      |       |
| SP_PIR   | inflammatory response                         | 4    | 4.17 | UP  | 16.51 | 0.027 | 11   | 1.98  | UP  | 8.18  | 0.000 |
| mro00980 | Metabolism of xenobiotics by cytochrome P450  |      |     |     |      |       |      |       |     |      |       |
| mro04621 | NOD-like receptor signaling pathway           | 5    | 5.21 | UP  | 12.52 | 0.034 | 10   | 1.80  | UP  | 4.49  | 0.016 |
| SP_PIR   | nucleus                                       | 22   | 22.92 | UP  | 2.01 | 0.026 |      |       |     |      |       |
| SP_PIR   | oxidoreductase                                | 42   | 4.68 | D   | 1.89 | 0.034 |      |       |     |      |       |
| mro05200 | Prion diseases                                | 7    | 1.26 | UP  | 5.56 | 0.028 |      |       |     |      |       |
| SP_PIR   | ribosome biogenesis                           | 7    | 1.26 | UP  | 8.85 | 0.007 |      |       |     |      |       |
| SM001999 | SCY                                          | 5    | 5.21 | UP  | 18.26 | 0.003 | 10   | 1.80  | UP  | 8.76  | 0.000 |
| IPRO01811| Small chemokine, interleukin-8-like            | 5    | 5.21 | UP  | 25.22 | 0.002 | 10   | 1.80  | UP  | 8.70  | 0.001 |
| SP_PIR   | Transcription                                 | 14   | 14.58 | UP  | 3.23 | 0.009 |      |       |     |      |       |
| SP_PIR   | transcription regulation                      | 14   | 14.58 | UP  | 3.48 | 0.006 |      |       |     |      |       |

Functional annotations significantly over-represented in the lists of up-regulated and down-regulated genes during 2-h Severe PE and 18-h Mild PE. Annotations unique to both treatments are highlighted in bold Italic; unique annotations are in standard font. Keys are the same as in Table 1. *No 2-h DOWN Annotations meet B&H < 0.05
fold and 3.3772-fold levels, respectively (Additional file 2E; lines 6823 and 4267) but neither was up-regulated by the Mild PE treatment. These results discount a role for lung hypoxia following PE, at least at the Mild PE dose of microspheres.

It seems reasonable that some hemostatic disruption related to ischemia, but not based on transfer of oxygen and/or nutrients to lung tissue, was responsible for the effects on gene expression seen in this study. An intriguing possibility is “stop-of-flow”, a phenomenon introduced by Fisher and co-workers [29–34]. Pulmonary endothelial cells are adapted to conditions of flow in vivo, with cell membranes and cytoskeletons aligning along the axis of blood flow. This can also be mimicked in vitro by applying flow over cells which were initially cultured in the absence of flow [33]. Endothelial cells grown in the absence of flow show a random organization of membranes and cytoskeletal structures, but when a laminar flow is applied to these cultures the cells adopt a flow-axial organization similar to that seen in vivo. Importantly, when flow is discontinued on cultures of flow-adapted cells, several signaling pathways are activated, including a signaling cascade mediated by NADPH oxidase 2-dependent ROS production (reactive oxygen species; 32). This also occurs in vivo in isolated perfused lungs subjected to ischemia. Pulmonary endothelial membrane depolarization, H2O2 production and increased intracellular Ca2+ have been observed within 10–15 s after the onset of non-hypoxic ischemia [34]. This rapid response can easily accommodate the increased gene expression seen in rats with 2-h Mild PE. Several comprehensive reviews have been published on the proposed general applicability of the “stop-of-flow” mechanism to explain the pathophysiology of tissue ischemia, including the ischemia associated with PE [35–37].

Finally, 18-h Severe PE resulted in the over-expression of genes present in several gene annotations related to steroid, lipid, and/or cholesterol biosynthesis. These results are consistent with a recent report that bile acids accumulate in lung tissues suffering from pulmonary artery hypertension; bile acids are downstream of cholesterol biosynthesis [38]. They are also consistent with numerous studies that have demonstrated the efficacy of statin drugs on reducing serum cholesterol, have been proposed to explain the efficacy of statins for PH but a clear explanation is premature.

Clinical relevance includes the potential hypothesis that PE without PH may produce inflammatory changes in the lung, leading to lung-initiated, systemic inflammation, and increased risk of ongoing hypercoagulability and clot recurrence [13, 14]. Humans with PE have 4–7 fold increases in circulating biomarkers of inflammation.
Conclusion
This is the first report to show that mild pulmonary embolism produces profound alteration in gene transcription in lungs, primarily in terms of increased expression of genes encoding inflammatory chemokines and cytokines and cholesterol synthesis. These data show that unresolved pulmonary vascular occlusion produces ongoing lung inflammation even in the absence of elevated pulmonary arterial pressures. Translational implications include the adverse effects of ongoing inflammation from unresolved pulmonary vascular occlusions, and conversely, possible benefit of treating PE to an endpoint of complete clot resolution.

Additional files

**Additional file 1:** Rat lung 6-group 2-way ANOVA. (XLS 3757 kb)

**Additional file 2:** Pairwise t-test of PE treatments. (XLS 2775 kb)

**Additional file 3:** ANOVAs of 2-hour and 18-hour PE treatments. (XLS 1660 kb)

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Availability of data and materials
All microarray data have been deposited in the NIH/NCBI “GEO” database (http://www.ncbi.nlm.nih.gov/projects/geo; GEO accession number GSE13535).

Authors’ contributions
JZ conceived the study, carried out the molecular and Affymetrix studies, data analysis and drafted the manuscript. JK participated in the conception and design of the study, developed and supervised the animal model, participated in drafting and editing the manuscript. Both authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Ethics approval and consent to participate
All experiments were conducted in accordance with the NIH Guide For the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Carolinas Medical Center, Charlotte NC (Protocols 4-02-01A and 11-00-01A).

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