Profiling molecular changes induced by hydrogen treatment of lung allografts prior to procurement

Yugo Tanaka [a,b], Norihisa Shigemura [a], Tomohiro Kawamura [a,c], Kentaro Noda [a], Kumiko Isse [d], Donna Beer Stolz [e,f], Timothy R. Billiar [g], Yoshiya Toyoda [a], Christian A. Bermudez [a], James Lyons-Weiler [h], Atsunori Nakao [a,g,*]

[a] Department of Cardiothoracic Surgery, University of Pittsburgh Medical Center, United States
[b] Division of Thoracic Surgery, Kobe University Graduate School of Medicine, Kobe, Japan
[c] Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, Osaka, Japan
[d] Department of Pathology, Division of Transplantation, and Thomas E. Starzl Transplantation Institute, University of Pittsburgh Medical Center, United States
[e] Center for Biologic Imaging, University of Pittsburgh, United States
[f] Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, United States
[g] Department of Surgery, University of Pittsburgh Medical Center, United States
[h] Genomics and Proteomics Core Laboratories, Bioinformatics Analysis Core, University of Pittsburgh, United States

ARTICLE INFO

ABSTRACT

Background: We previously demonstrated that donor treatment with inhaled hydrogen protects lung grafts from cold ischemia/reperfusion (I/R) injury during lung transplantation. To elucidate the mechanisms underlying hydrogen's protective effects, we conducted a gene array analysis to identify changes in gene expression associated with hydrogen treatment.

Methods: Donor rats were exposed to mechanical ventilation with 98% oxygen and 2% nitrogen or 2% hydrogen for 3 h before harvest; lung grafts were stored for 4 h in cold Perfadex. Affymetrix gene array analysis of mRNA transcripts was performed on the lung tissue prior to implantation.

Results: Pretreatment of donor lungs with hydrogen altered the expression of 229 genes represented on the array (182 upregulated; 47 downregulated). Hydrogen treatment induced several lung surfactant-related genes, ATP synthase genes and stress-response genes. The intracellular surfactant pool, tissue adenosine triphosphate (ATP) levels and heat shock protein 70 (HSP70) expression increased in the hydrogen-treated grafts. Hydrogen treatment also induced the transcription factors C/EBPα and C/EBPβ, which are known regulators of surfactant-related genes.

Conclusion: Donor ventilation with hydrogen significantly increases expression of surfactant-related molecules, ATP synthases and stress-response molecules in lung grafts. The induction of these molecules may underlie hydrogen's protective effects against I/R injury during transplantation.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Ischemia/reperfusion (I/R) injury, which affects 10–20% of lung transplant recipients, is a major complication of lung transplantation and can lead to primary graft dysfunction, the main cause of early posttransplantation morbidity and mortality [1]. Furthermore, I/R injury increases the risk of bronchiolitis obliterans syndrome and contributes to late mortality.

Inhaled therapeutic gas therapy may be a straightforward and reasonable approach for lung disease [2–4], and hydrogen is a promising therapeutic gaseous agent. Hydrogen can reduce cellular oxidation and has potent anti-inflammatory and antiapoptotic properties [5]. Recently, our group demonstrated that preloading hydrogen gas into donor lungs during ventilation prior to organ procurement effectively protected lung grafts from I/R-induced injury in a rat lung transplantation model [6]. One possible explanation was the ability of hydrogen to induce heme oxygenase-1 (HO-1), an antioxidant enzyme, in the lung grafts prior to implantation [6]. However, the mechanisms underlying hydrogen's protective effects against lung I/R injury remain largely unknown.

In this study, we conducted a gene array analysis to begin elucidating the mechanisms underlying the protective effects of preloading hydrogen into lung grafts prior to procurement. We then examined the expression of several of the upregulated genes and the transcription factors regulating their expression in our established rat orthotopic lung transplant model.

2. Materials and methods

2.1. Animals

Inbred male Lewis (LEW, RT1^l) rats and Brown Norway (BN, RT1^n) rats weighing 250–300 g were purchased from Harlan Sprague Dawley, Inc., (Indianapolis, IN) and maintained in laminar flow cages in a specific pathogen-free animal facility at the University of Pittsburgh. Animals were fed a standard diet and provided water *ad libitum*. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Research Council's Guide for the Humane Care and Use of Laboratory Animals.

2.2. Donor treatment

Donor rats underwent tracheotomy and were mechanically ventilated for 3 h with isoflurane (Aerrane; Baxter, Deerfield, IL) and either mixed gas with 98% oxygen and 2% nitrogen (N_2), or mixed gas with 98% oxygen and 2% hydrogen (H_2) with a tidal volume of 10 ml/kg and a respiratory rate of 60 breaths/min and a positive end expiratory pressure (PEEP) of 3.0 cm H_2O. The lungs were flushed through the main pulmonary artery with 20 ml of cold (4 °C) low potassium dextran (Perfadex®, Vitrolife, Gothenburg, Sweden) and excised. After excision of the heart–lung block, cuffs were attached to the pulmonary artery, pulmonary vein, and bronchus; then, the graft was placed in low potassium dextran and stored at 4 °C for 4 h. Sham-operated animals underwent anesthesia, tracheotomy, and mechanical ventilation for 5 min with 100% oxygen. The lungs of the sham animals were then immediately removed by thoracotomy for analysis.

2.3. Gene array analysis

We studied the differential gene-expression pattern of allografts from LEW donors. Graft tissue was collected for analysis after mechanical ventilation of the donor for 3 h with either 2% hydrogen or 2% nitrogen and 4 h of cold storage, but prior to implantation into a recipient. Changes in gene expression in the allografts exposed to hydrogen were compared with changes in allografts exposed to nitrogen using Affymetrix arrays (Illumina RatRef-12; Affymetrix, Inc., Santa Clara, CA). Optimization of perfect-match probe set data was conducted using an efficiency analysis (EA) [7]. In the EA, technical and biological replicates were subsampled repeatedly to create data sets that were then analyzed independently. Various methods for data transformation, normalization and testing for differentially expressed genes were applied in combination to the two independent subsamples. Combinations of methods were identified that exhibited the highest internal consistency (reproducibility). In this study, the optimal combination was z-transformation (within array/sample) followed by the use of the J5 test [8]. The J5 test is the ratio of the between-group difference (δ) in average expression for a given gene to the average absolute between-group δ of all of the genes on the array. Standard data quality measures, such as box-and-whiskers plots, global correlation, coefficient of variation, and confounding index, were also used to rule out undesired effects of data transformation; no such indicators were found. A threshold of 13.72 was selected for the J5 test using the EA criterion.

2.4. Orthotopic left lung transplantation in rats

Orthotopic left lung transplantation was performed (allogeneic: LEW to BN) utilizing a cuff technique as previously described [9,10]. After 4 h of cold storage, the lung grafts were transplanted into the recipients. The recipients were evaluated 2 h after reperfusion. No animals exhibited overinflation of the native lung or macroscopic atelectasis of the transplanted lung.

2.5. Immunohistochemical staining

Prior to implantation, lung graft tissues were fixed in 10% formalin, embedded in paraffin, and sectioned to 4-μm thickness. Anti-clara cell secretory protein antibodies (cat. no. 07–623; Millipore, Billerica, MA; dilution, 1:2000) were used, as described previously [6].

2.6. Western blot analysis

Western blot analysis was performed on 30 μg of whole cell protein from each lung graft, as described previously [10]. The following primary antibodies were used: anti-phospho-p38 (Thr180/Tyr182); anti-total p38; anti-phospho-extracellular signal-regulated protein kinase (ERK)1/2 (Thr202/Tyr204); anti-total-ERK1/2; anti-phospho-c-Jun N-terminal kinase (JNK) (Thr183/Tyr185); anti-total-JNK (all from Cell Signaling Technology, Beverly, MA); anti-clara cell protein 16 (CC16); anti-palate, lung, nasal epithelium clone (PLUNC); anti-CCAAT/enhancer binding protein (C/EBP) α; anti-C/EBP β (all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-heat shock protein (HSP)70 (Abcam, Cambridge, MA); and anti-β-actin (Sigma–Aldrich, St. Louis, MO). Expression of CC16, PLUNC, CEBP α, CEBP β, HSP70 and β-actin was examined in lung graft tissues were taken prior to implantation. Expression and phosphorylation of p38, JNK and ERK were examined in lung graft tissues taken 2 h after reperfusion.

2.7. SYBR Green real-time RT-PCR

The mRNAs for CC16; surfactant protein A (SP-A); dual specificity phosphatase 1 (DUSP1); secretoglobin, family 3A, member 2 (SCGB3A2); hydroxysteroid 11-β dehydrogenase 1 (HSD11β1); heat shock protein 5 (HSPA5); PLUNC; adenosine triphosphate (ATP) synthase H+ transporting mitochondrial F1 complex beta polypeptide (ATP5b); ATP synthase H+ transporting mitochondrial F1 complex O subunit (ATP5o); ATP synthase H+ transporting mitochondrial F0 complex subunit F6 (ATP5j); ATP synthase H+ transporting mitochondrial F0 complex subunit G (ATP5 l); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified in duplicate using SYBR Green two-step, real-time reverse transcriptase (RT-PCR), as described previously [10], on frozen lung graft tissues taken prior to implantation.

2.8. Measurement of tissue ATP levels

ATP level in the lung graft tissue prior to implantation was quantified using the ENLITEN ATP luciferin/luciferase bioluminescence assay system (Promega, Madison, WI) according to manufacturer's instructions. Luminescence was measured at a set lag time of 1 s using a 1420 VICTOR multi-label counter (PerkinElmer Life Sciences, Waltham, MA).

2.9. Transmission electron microscopy

Prior to implantation, lung grafts were harvested and immersion-fixed in 2.5% glutaraldehyde overnight at 4 °C. Following fixation, the tissue was dehydrated through a graded series of 30–100% ethanol, 100% propylene oxide, and then infiltrated with a 1:1 mixture of propylene oxide: Polybed 812 epoxy resin. After several changes of 100% resin, the tissue was embedded and cured. Ultrathin (70 nm) sections were collected on 200 mesh copper grids and stained with 2% uranyl acetate and 1% lead citrate. Sections were

visualized using a JEOL JEM 1210 transmission electron microscope at 80 kV.

2.10. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The data were analyzed with one-way analysis of variance followed by *post hoc* analysis with the Bonferroni correction. A probability level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Gene array analysis

We previously demonstrated that preloading hydrogen gas into donor lungs during ventilation prior to organ procurement effectively protected lung grafts from I/R-induced injury in a rat lung transplantation model [6]. To begin elucidating the mechanisms underlying the protective effects of preloading hydrogen into lung grafts prior to procurement, we employed a global approach to quantify changes in gene expression associated with H_2 inhalation prior to organ harvest. To this end, we compared the lung tran-

scriptomes of rats treated with 2% hydrogen with those of rats treated with 2% nitrogen (control) using Affymetrix arrays with 45,101 unique probe sets representing 36,512 target genes. Of these, 229 genes were differentially expressed. Hydrogen treatment reduced the expression of 47 genes and increased the expression of 182 genes, as compared with N_2-treated lungs (Table 1, Supplemental Tables 1 and 2).

3.2. Surfactant-related genes and C/EBPs in the lung grafts prior to implantation

Clara cell protein 16 (CC16) was the most upregulated gene after hydrogen pretreatment of the donor lungs (Table 1, Fig. 1A). CC16, 15.8-kDa homodimeric protein, is a secretoglobin family member (also called CC10 or uteroglobin). CC16 is one of the major proteins produced by the nonciliated cells of the tracheobronchial epithelial tree and has anti-inflammatory and antioxidant properties. (7) Preloading hydrogen by mechanical ventilation also significantly increased other surfactant-related mRNAs including SP-A, SCGB3A2 and HSD11β1 (Fig. 1B–D). Interestingly, the mRNA most dramatically downregulated by hydrogen pretreatment also encoded a surfactant-related protein, PLUNC, a secretory product of the epithelium with airway surfactant properties (Table 1, Supple-

Table 1
The top 25 genes upregulated by hydrogen pretreatment of lung grafts and other interesting genes regulated by hydrogen treatment.

Rank	Gene name	Accession number	J5 score
1[a]	Secretoglobin family 1A member 1 (uteroglobin) (Scgb1a1)	NM_013051.1	112.181
2	P:myosin light polypeptide 7 regulatory (Myl7_)	XM_214074.4	82.586
3	Collagen type I alpha 2 (Col1a2)	NM_053356.1	58.645
4[a]	Surfactant pulmonary-associated protein A1 (Sftpa1)	NM_017329.1	58.545
5	Hemoglobin alpha adult chain 2 (Hba-a2)	NM_013096.1	57.481
6	Niemann-Pick disease type C2 (Npc2)	NM_173118.1	54.093
7	Peptidylprolyl isomerase A (cyclophilin A) (Ppia)	NM_017101.1	53.375
8	S100 calcium binding protein A11 (calizzarin) (S100a11)	NM_001004095.1	52.942
9	Myosin heavy polypeptide 6 cardiac muscle alpha (Myh6)	NM_017239.1	51.154
10	P:RGD1561181 (RGD1561181_)	XM_001058739.1	50.147
11	P:ribosomal protein L23a transcript variant 2 (Rpl23a)	XM_001060002.1	49.884
12	P:actin gamma cytoplasmic (Actg_)	XM_213540.3	48.852
13	P:similar to RIKEN cDNA 2410116I05 (LOC363377)	XM_343712.2	48.235
14[a]	Dual specificity phosphatase 1 (Dusp1)	NM_053769.2	47.277
15	Ribosomal protein S4 X-linked (Rps4x)	NM_001007600.1	46.703
16	Lysosomal-associated protein transmembrane 4A (Laptm4a)	NM_199384.1	46.299
17	Insulin-like growth factor binding protein 7 (Igfbp7)	NM_001013048.1	45.933
18	Keratin 19 (Krt19)	NM_199498.1	44.734
19	S100 calcium binding protein A6 (S100a6)	NM_053485.2	43.624
20	Ribosomal protein L32 (Rpl32)	NM_013226.2	43.613
21	Tumor protein translationally-controlled 1 (Tpt1)	NM_053867.1	43.531
22	Defensin beta 4 (Defb4)	NM_022544.2	41.889
23	Calmodulin 2 (Calm2)	NM_017326.1	41.262
24	Hemoglobin beta chain complex (Hbb)	NM_033234.1	40.688
25	ATPase Ca++ transporting cardiac muscle slow twitch 2 (Atp2a2)_GI_8392934-S	NM_017290.1	40.471
	Other surfactant-related genes		
27[a]	Secretoglobin family 3A member 2 (Scgb3a2)	NM_001024283.1	39.612
50[a]	Hydroxysteroid 11-beta dehydrogenase 1 (Hsd11b1)	NM_017080.2	31.318
68	Secretoglobin family 3A member 1 (Scgb3a1)_GI_61557201-S	NM_001013180.1	24.925
−1[a,b]	Palate lung and nasal epithelium associated (Plunc)_GI_62645787-A	NM_172031.1	−324.0
	ATP synthase subunits		
32[a]	ATP synthase H+ transporting mitochondrial F1 complex beta polypeptide (Atp5b) nuclear gene encoding mitochondrial protein	NM_134364.1	37.291
74[a]	ATP synthase H+ transporting mitochondrial F1 complex O subunit (Atp5o) nuclear gene encoding mitochondrial protein_GI_20302060	NM_138883.1	23.893
157[a]	ATP synthase H+ transporting mitochondrial F0 complex subunit F6 (Atp5j) nuclear gene encoding mitochondrial protein_GI_16758387-S	NM_053602.1	15.428
165[a]	ATP synthase H+ transporting mitochondrial F0 complex subunit G (Atp5 l) nuclear gene encoding mitochondrial protein_GI_47058993-S	NM_212516.1	15.18
	Stress-response gene		
66[a]	Heat shock protein 5 (Hspa5)_GI_25742762-S	NM_013083.1	25.314

[a] Indicates genes chosen for further analysis.
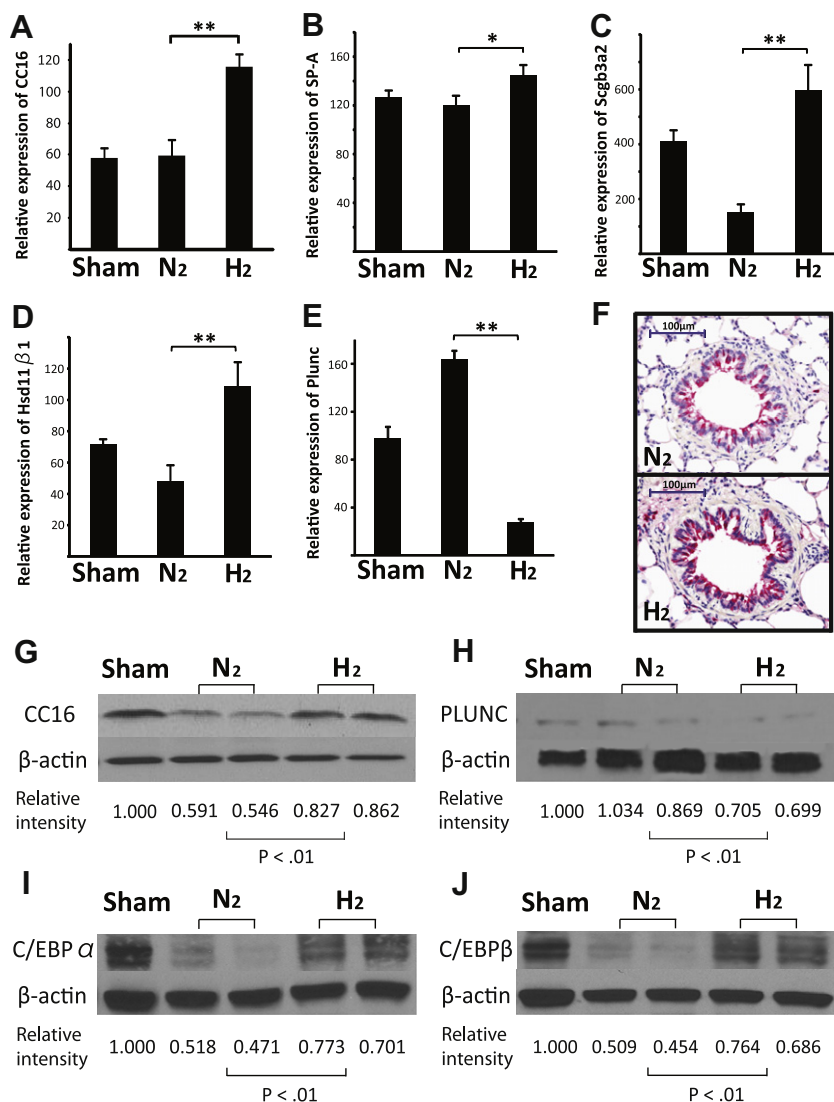[b] Downregulated by hydrogen treatment.

Fig. 1. Quantitative RT-PCR for (A) CC16, (B) SP-A, (C) SCGB3A2, (D) HSD11β1 and (E) PLUNC ($n = 4$ for each group; ∗∗$p < 0.01$ and ∗$p < 0.05$). (F) Representative immunohistochemistry for CC16 in the lung grafts before transplantation. Western blots for (G) CC16, (H) PLUNC, (I) C/EBPα, (J) C/EBP β, and β-actin on protein extracts from lung grafts taken after 3 h of mechanical ventilation and 4 h cold storage. The images are representative of 3 independent experiments.

mental Table 2, Fig. 1E). Consistent with mRNA expression, CC16 protein levels were increased and PLUNC protein levels were decreased in the allografts prior to implantation. Immunohistochemical analysis revealed more prominent CC16-positive epithelial cells in the hydrogen-preloaded allografts than in control allografts, although there were no obvious histopathological changes (Fig. 1F–H).

The CC16, SP-A, SCGB3A2 and HSD11β1 genes are regulated by same transcription factor family, the C/EBPs. Although C/EBPs were not identified in the microarray as hydrogen-regulated mRNAs, preloading hydrogen increased C/EBPα and β protein levels in the lung allografts prior to implantation as compared with control allografts (Fig. 1I and J).

Morphological analysis of alveolar surfactant in the grafts prior to transplant was performed using transmission electron microscopy. The intracellular surfactant pool, indicated by the number of lamellar bodies, was increased in H_2-treated grafts (Fig. 2). However, no obvious changes in the morphology of the alveolar epithelial type II cells were observed between H_2-treated grafts and N_2-treated grafts.

3.3. Expression of ATP synthase genes in the lung grafts

The gene array analysis indicated that four ATP synthase genes (ATP5b ATP5o ATP5j and ATP5l) were upregulated by hydrogen treatment of the lung grafts prior to harvest (Table 1). Because maintenance of tissue ATP is associated with reduced I/R in lung grafts [11], we chose to further investigate these genes. Significant increases in the mRNA levels for these 4 ATP synthase genes after hydrogen treatment were confirmed with real-time RT-PCR (Fig. 3A–D). The lung grafts pretreated with hydrogen prior to harvest also had significantly higher tissue ATP levels after cold storage than those treated with nitrogen (Fig. 3E).

3.4. Expression of stress-response genes in the lung grafts

Changes in the expression of several stress-response proteins were noted in the gene-expression array. Specifically, the mRNA for HSPA5, which encodes a member of the HSP70 protein family, and the mRNA for DUSP1, a dual specificity phosphatase that decreases mitogen-activated protein kinase (MAPK) phosphoryla-
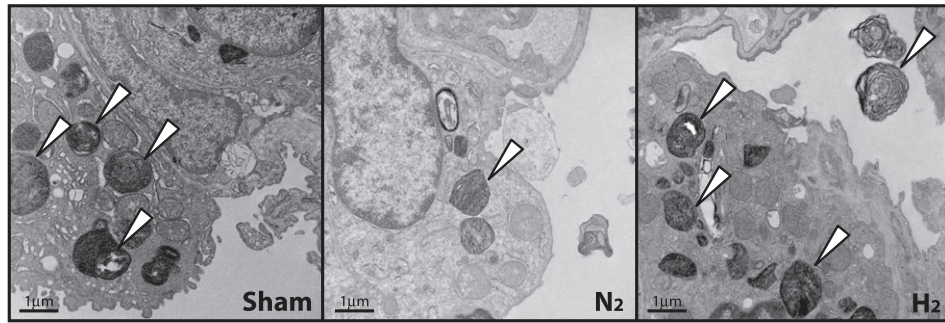
Fig. 2. Transmission electron microscopy of lung grafts. Cell structure of alveolar epithelial type II cells in hydrogen-treated and nitrogen-treated grafts. Lamellar bodies (white arrowheads) contain intracellular surfactant.
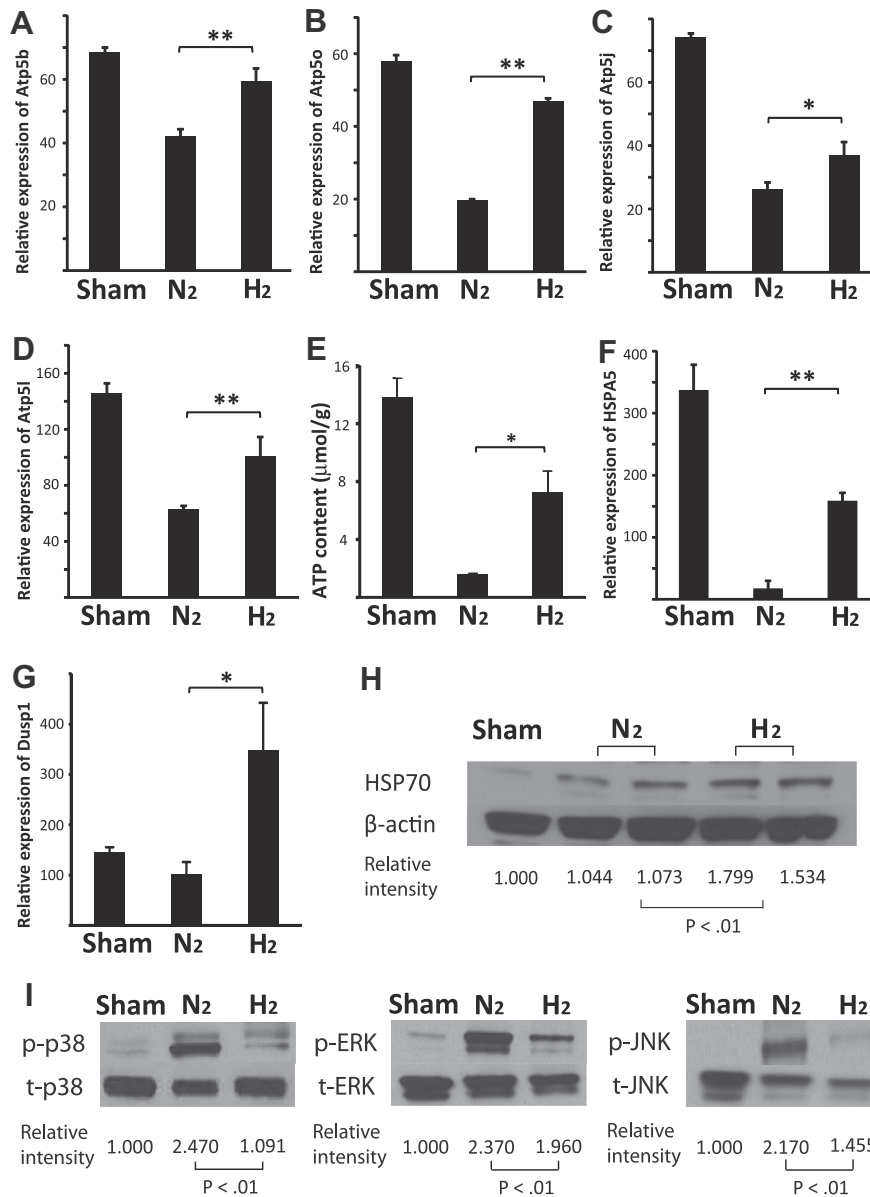

Fig. 3. Quantitative RT-PCR for (A) ATP5b, (B) ATP5o, (C) ATP5j and (D) ATP5 l ($n = 4$ for each group; $**p < 0.01$ and $*p < 0.05$). (E) Lung ATP content prior to implantation. ($n = 4$ per group $*p < 0.05$). Quantitative RT-PCR for (F) HSPA5 and (G) DUSP1 ($n = 4$ for each group; $**p < 0.01$ and $*p < 0.05$). (H) Western blots for HSP70 and β-actin on protein extracts from lung grafts taken after 3 h of mechanical ventilation and 4 h of cold storage. (I) Western blots for phosphorylated (p) and total (t) p38, ERK1/2 and JNK on protein extracts from lung grafts taken 2 h after reperfusion. The images are representative of 3 independent experiments ($n = 3$ for each group).

tion/ activation [12], were upregulated after hydrogen inhalation. We confirmed these results using real-time RT-PCR (Fig. 3F and G). We continued our analysis by examining the expression of the stress-response proteins encoded by these mRNAs in hydrogen pretreated lung grafts prior to implantation. Consistent with mRNA expression, HSP70 protein levels increased in the allografts (Fig. 3H). To assess DUSP1 expression, activation of the 3 major MAPKs (p38, ERK1/2 and JNK), as indicated by phosphorylation, was examined in the lung tissue 2 h after reperfusion. Preloading hydrogen into the lung significantly reduced activation of p38 MAPK, ERK 1/2 and JNK in the grafts as compared with nitrogen-treated controls (Fig. 3I).

4. Discussion

In this study, we demonstrated that preloading hydrogen into lung allografts prior to harvest was associated with upregulation of lung surfactant-related molecules, ATP synthases and stress-response molecules. Hydrogen is an important physiologic regulatory gas molecule with antioxidant, anti-inflammatory and antiapoptotic effects on cells and organs that underlie its potential therapeutic benefits for the prevention of lung injuries [5]. Our previous studies demonstrated that treating donor lungs with hydrogen prior to harvest and cold storage ameliorated lung cold I/R injury during transplantation [6]. We expand on this observation in the current study, revealing previously unknown molecular changes that may underlie the beneficial effects of inhaled hydrogen therapy for lung disease.

Extracellular surfactants play important roles in innate host defense by binding and clearing infectious pathogens and regulating immune cell activity through multiple cellular pathways [13–16]. Likewise, there is increasing evidence that the pulmonary surfactant system is altered after lung transplantation. Lung perfusion with preservation solution or prolonged storage of donor lungs impairs the integrity of the surfactant system, and these modifications are associated with early primary graft dysfunction. Recent work in a rat lung transplantation model showed that pulmonary surfactant continued to be deficient 1 week after transplantation [17]. Exogenous surfactant treatment not only increases the quantity of surfactant but also enhances recovery from reperfusion injury after lung transplantation [15,18]. Exogenous administration of surfactant prior to reperfusion improved oxygenation and dynamic compliance in grafts stored for 20 h and was beneficial for endogenous surfactant metabolism [17]. A clinical retrospective review also revealed that bronchoscopic instillation of surfactant improved oxygenation and prognosis in lung transplant recipients after severe I/R injury [19]. Thus, it is likely that maintenance of surfactant in the pulmonary graft is critical for successful outcomes after lung transplantation.

In our gene array analysis, CC16 was the most upregulated gene in response to preloading hydrogen in lung grafts. CC16 (also known as CC10, CCSP and uteroglobin) is one of the major proteins secreted by the respiratory epithelium. It modulates the production and activity of various inflammatory mediators including phospholipase A2 (PLA2), interferon-γ and TNF-α. CC16 also increases lung surfactant protein levels and prevents lung surfactant degradation by PLA2 inhibition [20,21]. In addition to the prominent upregulation of CC16 mRNA, the mRNAs for SP-A, SCGB3A2 and HSD11β1 were upregulated by preloading hydrogen in the lung grafts. SP-A is a surfactant protein and has a significant role in surfactant homeostasis and protection of the alveoli against inflammation [16,22]. SCGB3A2 (also known as uteroglobin-related protein 1) is a novel cytokine-like molecule. It belongs to the secretoglobin family, as does CC16, and exhibits anti-inflammatory effects in the lung [23,24]. HSD11β1 is highly expressed in liver and lung and increases intracellular glucocorticoid activity by converting glucocorticoids from inactive to active forms. HSD11β1 has been implicated in xenobiotic detoxification and surfactant production in lung [25]. Thus, it is likely that these proteins contribute to mitigation of I/R injury after lung transplantation and expand the possible mechanisms underlying inhaled hydrogen therapy. PLUNC was the only surfactant-related gene with decreased expression after hydrogen treatment. PLUNC is a secretory product of the epithelium and has airway surfactant properties that may interfere with biofilm formation by the airway pathogen *Pseudomonas aeruginosa* [26]. Further study will be needed to determine whether downregulation of PLUNC contributes to the attenuation of I/R injury.

ATP synthase synthesizes ATP from adenosine diphosphate (ADP) and inorganic phosphate (P) using the energy of proton gradients [27]. Decreasing adenine nucleotides caused edema in the isolated, perfused rat lung and adding ATP to the preservation solution mitigated cold I/R injury in rats [11]. In our gene array analysis, 4 ATP synthase subunits genes were upregulated by hydrogen and higher ATP levels were observed in the hydrogen pretreated lung grafts. This upregulation of ATP might contribute to mitigation of I/R injury after lung transplantation.

Our gene array analysis also showed upregulation of stress-response molecules. DUSP1 can dephosphorylate and inactivate mitogen-activated protein kinases (MAPKs) and it may play an important role in suppressing the inflammatory response [12]. HSPA5 encodes an HSP70 protein that is induced by various types of cellular stress and has anti-inflammatory and antiapoptotic properties [28]. Upregulation of DUSP1 and HSPA5 by hydrogen treatment may explain the hydrogen-dependent decreases in MAPK phosphorylation/activation seen after reperfusion and increases in HSP70 expression prior to implantation in vivo.

CC16, SP-A, SCGB3A2, DUSP1 and HSD11β1 are all induced by the C/EBP transcription factor family [12,22,23,25]. There are 6 C/EBP family members (C/EBPα-ζ) that modulate numerous cellular responses including differentiation, inflammation, liver regeneration, and metabolism [22,29]. C/EBPα, -β, and -δ are expressed in the lung epithelium and regulate proliferation, differentiation and response to acute inflammation or injury. In our study, treatment with 2% hydrogen by mechanical ventilation prior to harvest significantly increased C/EBPα and C/EBPβ protein expression in the donor lungs. These observations suggest that amelioration of I/R injury with inhaled hydrogen is conveyed, at least partially, by increasing C/EBP expression, which results in the transcriptional upregulation of CC16, SP-A, SCGB3A2, DUSP1 and HSD11β1. Other C/EBP-regulated genes may also contribute to the protective effects of hydrogen treatment and require further investigation.

In conclusion, donor treatment with inhaled hydrogen was associated with the upregulation of pulmonary surfactant-related molecules, ATP synthases and stress-response molecules in lung allografts. These molecules may play pivotal roles in hydrogen-induced cytoprotective mechanisms. Although the therapeutic efficacies of hydrogen have been extensively studied, there is very limited information on the pathways and processes regulated in vivo by the hydrogen molecule. Considering the potential for wide clinical application of hydrogen treatment, it will become important to understand the mechanisms underlying the protective effects of hydrogen. To our knowledge, our study is the first gene-array analysis on hydrogen-treated lung tissue and the first demonstration of induction of lung surfactant-related genes by hydrogen treatment. In the future, further studies on hydrogen's protective effects including the role of the C/EBPs will be essential. This study paves the way for understanding the mechanisms underlying the protective effects of hydrogen treatment during lung transplantation.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.005.

References

[1] J.D. Christie, L.B. Edwards, P. Aurora, F. Dobbels, R. Kirk, A.O. Rahmel, D.O. Taylor, A.Y. Kucheryavaya, M.I. Hertz, Registry of the international society for heart and lung transplantation: twenty-fifth official adult lung and heart/lung transplantation report–2008, Journal of Heart and Lung Transplantation 27 (2008) 957–969.

[2] C.S. Huang, T. Kawamura, S. Lee, N. Tochigi, N. Shigemura, B.M. Buchholz, J.D. Kloke, T.R. Billiar, Y. Toyoda, A. Nakao, Hydrogen inhalation ameliorates ventilator-induced lung injury, Critical Care 14 (2010) R234.

[3] T. Kawamura, C.S. Huang, N. Tochigi, S. Lee, N. Shigemura, T.R. Billiar, M. Okumura, A. Nakao, Y. Toyoda, Inhaled hydrogen gas therapy for prevention of lung transplant-induced ischemia/reperfusion injury in rats, Transplantation 90 (2010) 1344–1351.

[4] A. Nakao, R. Sugimoto, T.R. Billiar, K.R. McCurry, Therapeutic antioxidant medical gas, Journal of Clinical Biochemistry and Nutrition 44 (2009) 1–13.

[5] C.S. Huang, T. Kawamura, Y. Toyoda, A. Nakao, Recent advances in hydrogen research as a therapeutic medical gas, Free Radical Research 44 (2010) 971–982.

[6] T. Kawamura, C.S. Huang, X. Peng, K. Masutani, N. Shigemura, T.R. Billiar, M. Okumura, Y. Toyoda, A. Nakao, The effect of donor treatment with hydrogen on lung allograft function in rats, Surgery 150 (2011) 240–249.

[7] R. Jordan, S. Patel, H. Hu, J. Lyons-Weiler, Efficiency analysis of competing tests for finding differentially expressed genes in lung adenocarcinoma, Cancer Information 6 (2008) 389–421.

[8] S. Patel, J. Lyons-Weiler, CaGEDA: a web application for the integrated analysis of global gene expression patterns in cancer, Applied Bioinformatics 3 (2004) 49–62.

[9] R. Sugimoto, A. Nakao, I. Nagahiro, J. Kohmoto, S. Sugimoto, M. Okazaki, M. Yamane, H. Inokawa, T. Oto, K. Tahara, J. Zhan, Y. Sano, K.R. McCurry, Experimental orthotopic lung transplantation model in rats with cold storage, Surgery Today 39 (2009) 641–645.

[10] J. Kohmoto, A. Nakao, T. Kaizu, A. Tsung, A. Ikeda, K. Tomiyama, T.R. Billiar, A.M. Choi, N. Murase, K.R. McCurry, Low-dose carbon monoxide inhalation prevents ischemia/reperfusion injury of transplanted rat lung grafts, Surgery 140 (2006) 179–185.

[11] T. Hirata, T. Fukuse, S. Ishikawa, R. Miyahara, H. Wada, Addition of ATP and MgCl2 to the preservation solution attenuates lung reperfusion injury following cold ischemia, Respiration; International Review of Thoracic Diseases 68 (2001) 292–298.

[12] K. Johansson-Haque, E. Palanichamy, S. Okret, Stimulation of MAPK-phosphatase 1 gene expression by glucocorticoids occurs through a tethering mechanism involving C/EBP, Journal of Molecular Endocrinology 41 (2008) 239–249.

[13] J.R. Wright, Pulmonary surfactant: a front line of lung host defense, Journal of Clinical Investigation 111 (2003) 1453–1455.

[14] J. Goerke, Pulmonary surfactant: functions and molecular composition, Biochimica et Biophysica Acta 1408 (1998) 79–89.

[15] M. Struber, S. Fischer, J. Niedermeyer, G. Warnecke, B. Gohrbandt, A. Gorler, A.R. Simon, A. Haverich, J.M. Hohlfeld, Effects of exogenous surfactant instillation in clinical lung transplantation: a prospective, randomized trial, Journal of Thoracic and Cardiovascular Surgery 133 (2007) 1620–1625.

[16] P.S. Kingma, J.A. Whitsett, In defense of the lung: surfactant protein A and surfactant protein D, Current Opinion in Pharmacology 6 (2006) 277–283.

[17] M.E. Erasmus, G.J. Hofstede, A.H. Petersen, H.P. Haagsman, S.B. Oetomo, J. Prop, Effects of early surfactant treatment persisting for one week after lung transplantation in rats, American Journal of Respiratory and Critical Care Medicine 156 (1997) 567–572.

[18] J.M. Hohlfeld, E. Tiryaki, H. Hamm, H.G. Hoymann, N. Krug, A. Haverich, H. Fabel, Pulmonary surfactant activity is impaired in lung transplant recipients, American Journal of Respiratory and Critical Care Medicine 158 (1998) 706–712.

[19] F.D. Kermeen, K.D. McNeil, J.F. Fraser, J. McCarthy, M.D. Ziegenfuss, D. Mullany, J. Dunning, P.M. Hopkins, Resolution of severe ischemia-reperfusion injury post-lung transplantation after administration of endobronchial surfactant, Journal of Heart and Lung Transplantation 26 (2007) 850–856.

[20] F. Broeckaert, A. Bernard, Clara cell secretory protein (CC16): characteristics and perspectives as lung peripheral biomarker, Clinical and Experimental Allergy 30 (2000) 469–475.

[21] M.R. Wolfson, V.L. Funanage, S.M. Kirwin, A.L. Pilon, B.N. Shashikant, T.L. Miller, T.H. Shaffer, Recombinant human Clara cell secretory protein treatment increases lung mRNA expression of surfactant proteins and vascular endothelial growth factor in a premature lamb model of respiratory distress syndrome, American Journal of Perinatology 25 (2008) 637–645.

[22] T.N. Cassel, M. Nord, C/EBP transcription factors in the lung epithelium, American Journal of Physiology. Lung Cellular and Molecular Physiology 285 (2003) L773–781.

[23] T. Kido, T. Tomita, M. Okamoto, Y. Cai, Y. Matsumoto, C. Vinson, Y. Maru, S. Kimura, FOXA1 plays a role in regulating secretoglobin 1a1 expression in the absence of CCAAT/enhancer binding protein activities in lung in vivo, American Journal of Physiology. Lung Cellular and Molecular Physiology 300 (2011) L441–452.

[24] Y. Chiba, R. Kurotani, T. Kusakabe, T. Miura, B.W. Link, M. Misawa, S. Kimura, Uteroglobin-related protein 1 expression suppresses allergic airway inflammation in mice, American Journal of Respiratory and Critical Care Medicine 173 (2006) 958–964.

[25] C. Bruley, V. Lyons, A.G. Worsley, M.D. Wilde, G.D. Darlington, N.M. Morton, J.R. Seckl, K.E. Chapman, A novel promoter for the 11beta-hydroxysteroid dehydrogenase type 1 gene is active in lung and is C/EBPalpha independent, Endocrinology 147 (2006) 2879–2885.

[26] L. Gakhar, J.A. Bartlett, J. Penterman, D. Mizrachi, P.K. Singh, R.K. Mallampalli, S. Ramaswamy, P.B. McCray Jr., PLUNC is a novel airway surfactant protein with anti-biofilm activity, PLoS ONE 5 (2010) e9098.

[27] T. Elston, H. Wang, G. Oster, Energy transduction in ATP synthase, Nature 391 (1998) 510–513.

[28] M.A. Yenari, J. Liu, Z. Zheng, Z.S. Vexler, J.E. Lee, R.G. Giffard, Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection, Annals of the New York Academy of Sciences 1053 (2005) 74–83.

[29] D.P. Ramji, P. Foka, CCAAT/enhancer-binding proteins: structure, function and regulation, Biochemical Journal 365 (2002) 561–575.