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Temporal Patterns of Gene Expression Profiles in the Neonatal Mouse Lung after Hypoxia-Reoxygenation

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Key Words

Hypoxia · Reoxygenation · Hyperoxia · Neonatal lung disease · Gene expression · Inflammation · Apoptosis · DNA repair

Abstract

Background: One out of four children with neonatal asphyxia has lung involvement. Still, there has been little research on injury mechanisms of hypoxia-reoxygenation in the neonatal lung. ***Objectives:*** To make a temporal profile of the gene expression changes of 44 a priori selected genes after hypoxia-reoxygenation in the newborn mouse lung, and to compare the changes after hyperoxic and normoxic reoxygenation. ***Methods:*** Postnatal day 7 mice were randomized to 2-hour hypoxia (8% O_2) and 30-min reoxygenation in either 60% O_2 or air. After 0–72 h of observation, gene expression changes and protein concentrations in whole lung homogenates were examined. ***Results:*** Immediately after completed reoxygenation, 7 genes of mediators of inflammation were downregulated, and there was an antiapoptotic gene expression pattern. Three DNA glycosylases were downregulated, while genes involved in cell cycle renewal indicated both increased and decreased cell cycle arrest. *Sod1* (T2.5h median H60: 1.01, H21: 0.88, $p = 0.005$; T5h median H60: 1.04, H21: 0.85, $p = 0.038$) and *Il1b* (T0h median H60: 0.86, H21: 1.08, $p = 0.021$) were significantly differentially expressed when comparing hyperoxic and normoxic reoxygenation. ***Conclusion:*** In this newborn mouse lung hypoxia-reoxygenation model, we found downregulation of genes of mediators of inflammation, an antiapoptotic gene expression pattern, and downregulation of DNA glycosylases. *Sod1* and *Il1b* were significantly differentially expressed when comparing reoxygenation using 60% O_2 with air.
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Introduction

One out of four asphyxiated newborns has lung involvement ranging from mild respiratory distress to pulmonary hemorrhage and severe respiratory failure, and lung involvement is often present in multiorgan affection [1]. Hypoxia-reoxygenation increases oxidative stress in the neonatal rat lung [2], even short exposures to hypoxia and hyperoxia influence alveolar development [3], and hyperoxia increases pulmonary artery contractility [4]. Prolonged hyperoxic exposure has det-

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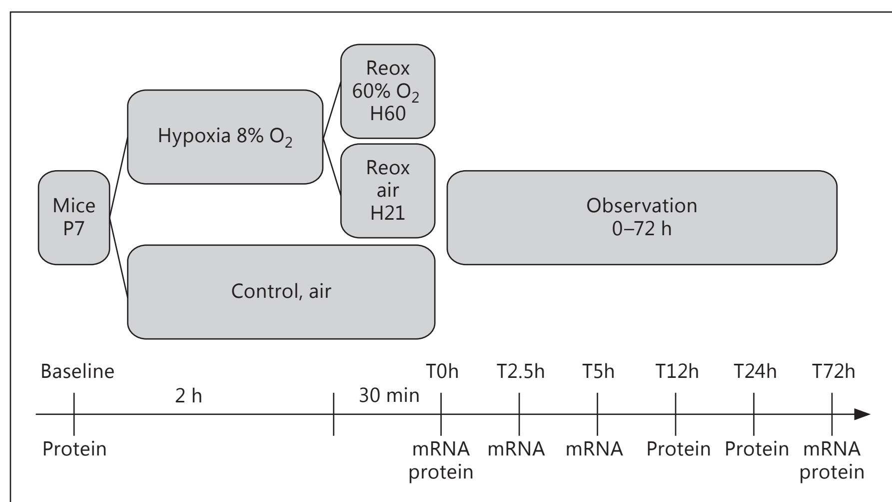

Fig. 1. Experimental design. P7 mice were randomized to either hypoxia for 2 h or controls kept in air. The hypoxia group was further randomized to 30-min reoxygenation with either 60% O_2 (H60) or air (H21). Lungs were harvested 0, 2.5, 5, or 72 h after reoxygenation (T0h, T2.5h, T5h, and T72h, respectively) in the mRNA experiment (H60 n = 8, H21 n = 8, controls n = 5 at each time point) and at baseline and 0, 12, 24, or 72 h after reoxygenation (T0h, T12h, T24h, and T72h, respectively) in the protein experiments (n = 7, 22, 21, 21, and 21 at each time point, respectively).

rimental effects on the neonatal lung, and hyperoxia has been implicated in the pathogenesis of both respiratory distress syndrome [5] and bronchopulmonary dysplasia [6]. However, there has been limited research into the effect on the neonatal pulmonary lung of hyperoxic reoxygenation in a resuscitation setting. Our group has previously reported that hyperoxic reoxygenation of piglets gave an augmented inflammatory response in the neonatal lung [7, 8], and decreased total antioxidant capacity [7]. Furthermore, hypoxia-reoxygenation in mice induced HIF-1 targets independent of the reoxygenation regime applied, while hyperoxic reoxygenation especially affected cell growth and survival and DNA-damage-responsive genes [9].

In neonatal asphyxia, the whole body is affected by the hypoxemia. Most established models today use hilar clamping to produce a local hypoxic/ischemic lung insult [10, 11]. However, we wanted to study hypoxia-reoxygenation in several organs in the same mice without focal clamping of the blood flow. Thus, we established a neonatal mouse model where mice on postnatal day 7 (P7) are subjected to whole-body hypoxia (8% O_2) followed by reoxygenation for 30 min in 60% O_2 or air, resulting in a respiratory compensated metabolic acidosis fulfilling the criteria for newborn asphyxia [9]. Gene expression changes in the lung can be linked to pathophysiological changes [12, 13]. To date, we have found no studies that have made an extensive temporal profile of gene expression after a whole body hypoxia-reoxygenation event. Previously, we have reported the transcriptome profile at one time point in the same model [9]. The objective of the present study was to make a temporal profile up to 3 days after hypoxia-reoxygenation in 44 a priori selected genes in the neonatal mouse lung, and to compare the gene expression changes after reoxygenation in 60% O_2 versus air across the different time points. The selected genes have important functions related to mediators of inflammation and injury, apoptosis, cell cycle renewal, DNA repair and vascular development; processes involved in hypoxia-reoxygenation injury.

Materials and Methods

Animals

C57BL/6J mice from Taconic (Tornbjerg, Denmark) were bred and harbored under the conditions described [14]. Experiments were approved by the Norwegian Animal Research Authority, and the animals were handled in accordance with Norwegian legislation and Directive 2010/63/EU. Two hundred and thirty-seven pups were used in the experiments [mRNA studies n = 84, protein studies n = 92, pilot and testing n = 49, mortality n = 12 (5.1%)].

Experimental Model

The experimental model has previously been described in detail [9, 14], and the rationale behind it is further described in online supplementary methods (for all online suppl. material, see www.karger.com/doi/10.1159/000447322). P7 mice pups of both sexes (day of discovery = P1) were randomized to hypoxia (8% O_2) for 2 h or controls kept in air (fig. 1). During hypoxia, the average O_2 saturation of the mice was 42% [9]. The hypoxia group was randomized to reoxygenation with either 60% O_2 (H60) or air (H21). The combination of hypoxia and reoxygenation is referred to as 'the intervention'. mRNA was studied at 0, 2.5, 5, or 72 h after completed reoxygenation (T0h, T2.5h, T5h, and T72h, respectively), and proteins at baseline and 0, 12, 24, or 72 h (T0h, T12h, T24h, and T72h, respectively). Lungs were harvested, snap frozen in liquid nitrogen, and stored at –79°C.

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Table 1. Target and reference genes

Gene symbol	Gene name	Other function	Assay ID[a]
Mediators of inflammation			
Ccl12	chemokine (C-C motif) ligand 12		Mm01617100_m1
Ccl2	chemokine (C-C motif) ligand 2		Mm00441242_m1
Ccl5	chemokine (C-C motif) ligand 5		Mm01302428_m1
Cxcl10[b]	chemokine (C-X-C motif) ligand 10		Mm00445235_m1
Hmox1	heme oxygenase (decycling) 1	stress response	Mm00516004_m1
Ikbkb	inhibitor of kappaB kinase beta		Mm00833995_m1
Il10	interleukin 10		Mm00439616_m1
Il6	interleukin 6		Mm00446190_m1
Lcn2[b]	lipocalin 2		Mm01324470_m1
Mapk14	mitogen-activated protein kinase 14		Mm01301009_m1
Nfkb1	nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105	transcription factor	Mm00476361_m1
Stat3	signal transducer and activator of transcription 3	transcription factor	Mm00456961_m1
Tgfb1	transforming growth factor, beta 1	growth factor	Mm00441724_m1
Tlr4	toll-like receptor 4		Mm00445273_m1
Response to injury			
Edn1	endothelin 1	vasoconstriction	Mm00438656_m1
Grin2b	glutamate receptor, ionotropic, NMDA2B (epsilon 2)		Mm00433820_m1
Hif1a	hypoxia inducible factor 1, alpha subunit	response to hypoxia	Mm00468869_m1
Mt1[b]	metallothionein 1		Mm00496660_g1
Prkaa1	protein kinase, AMP-activated, alpha 1 catalytic subunit		Mm01296700_m1
Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1		Mm00441473_m1
Sod1	superoxide dismutase 1, soluble		Mm01700393_g1
Apoptosis			
Apaf1	apoptotic peptidase activating factor 1		Mm01223702_m1
Bax	BCL2-associated X protein		Mm00432050_m1
Bcl2[b]	B cell leukemia/lymphoma 2		Mm00477631_m1
Bcl2l1	BCL2-like 1		Mm00437783_m1
Bnip3	BCL2/adenovirus E1B interacting protein 3	autophagy	Mm01275600_g1
Casp3[b]	caspase 3		Mm01195085_m1
Fasl	fas ligand (TNF superfamily, member 6)		Mm00438864_m1
Igf1r	insulin-like growth factor I receptor	growth factor	Mm00802831_m1
Il1b	interleukin 1 beta	inflammation	Mm00434228_m1
Tnf[b]	tumor necrosis factor	inflammation	Mm00443258_m1
Trp53	transformation related protein 53	transcription factor, cell cycle arrest	Mm01731287_m1
Cell cycle renewal			
Atm	ataxia telangiectasia mutated homolog (human)		Mm01177457_m1
Ccnb1	cyclin B1		Mm00838401_g1
Ccnd1[b]	cyclin D1		Mm00432359_m1
Chek1[b]	checkpoint kinase 1		Mm01176757_m1
Gadd45g[b]	growth arrest and DNA damage-inducible 45 gamma		Mm01352550_g1
Jun	jun proto-oncogene		Mm00495062_s1
DNA repair			
Apex1	apurinic/apyrimidinic endonuclease 1	transcription co-factor, stress response	Mm00507805_g1
Mutyh	mutY homolog *(E. coli)*		Mm00504731_m1
Neil1	nei endonuclease VIII-like 1 *(E. coli)*		Mm00452910_m1
Neil3	nei like 3 *(E. coli)*		Mm00467596_m1
Ogg1	8-oxoguanine DNA-glycosylase 1		Mm00501781_m1

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Table 1 (continued)

Gene symbol	Gene name	Other function	Assay ID[a]
Other			
Vegfa	vascular endothelial growth factor A	angiogenesis	Mm00437304_m1
Actb	actin, beta	reference gene lung	Mm00607939_s1
Polr2a	polymerase (RNA) II (DNA directed) polypeptide A	ref. brain and lung	Mm00839493_m1
Ubc	ubiquitin C	reference gene brain	Mm01201237_m1
18S		manufacturer control	Hs9999901_s1

[a] Gene expression assay ID at Life Technologies used when designing Custom TaqMan low density array card. [b] Also analyzed for protein concentration.

Real Time RT-PCR

Total RNA was extracted from whole lung homogenates, reverse transcribed to cDNA and amplified as described previously [9]. *Actb* and *Pol2ra* were chosen as reference genes in the lung samples based on an endogenous control pilot (TaqMan Array Mouse Endogenous Control Panel, Life Technologies, Carlsbad, Calif., USA) and evaluation with Qbase+ (Biogazelle, Zwijnaarde, Belgium). Custom TaqMan array microfluidic cards (Life Technologies) were designed with 44 target genes suitable for lung and brain tissue (list of assays in table 1), 3 reference genes, and one manufacturer control. The 44 target genes were chosen based on a pilot study (TaqMan Mouse immune panel, Life Technologies) and previous studies [9, 15] (see online suppl. methods for details). Gene expression changes were evaluated using the comparative C_T method of relative quantification ($2^{-\Delta\Delta CT}$). PCR evaluation determined one general failure to amplify in the H60 group after 72 h, excluding this mouse from statistical analysis.

Protein Analysis

Protein was extracted from frozen lung homogenates as previously described [14]. Ten proteins were chosen for analysis (superscript [b] in table 1) based on the mRNA results. Commercially available ELISA kits for CASP3 TOTAL, Cleaved CASP3, CXCL10, LCN2, TNFα, BCL2, CCND1, CHEK1, GADD45G, and MT1 were used (details in online suppl. methods). TNFα had values below lower threshold in the test, and was not analyzed further.

Statistical Analysis

All statistical analyses were performed with PASW statistics 19 (IBM, Armonk, N.Y., USA). A general linear model was developed with time and reoxygenation as fixed factors and Bonferroni post hoc correction according to the procedure outlined in Rognlien et al. [14]. When there was no significant difference between H60 and H21, all intervention animals were analyzed together. One-way ANOVA/Student's t test or Kruskal-Wallis/Mann-Whitney U test was used for single time point comparisons according to the distribution of the data. Two-sided p values with $p < 0.05$ were considered statistically significant. Data are given as mean difference (confidence interval), unless otherwise stated. All graphs were made with GraphPad Prism 6 (GraphPad, La Jolla, Calif., USA).

Results

There were no significant differences in weight or sex distribution in the different experimental groups (data not shown). Hypoxia-reoxygenation resulted in statistically significant changes in gene expression with time in 36 of 44 genes using a general linear model (online suppl. table 1). Thirty-one of 44 genes had significant changes in gene expression in animals subjected to hypoxia-reoxygenation compared to controls at one or more time points; the expression of 23 genes was downregulated, 10 genes were upregulated, and 2 of the genes were up- and downregulated at different time points (fig. 2a).

Temporal Profiles of mRNA Expression

The most pronounced changes in gene expression occurred immediately after completed reoxygenation at T0h; 18 genes were downregulated and 7 were upregulated (fig. 2a). At that time point, the expression of 7 genes characterized as mediators of inflammation were downregulated and only 1 was upregulated (fig. 2b), while the injury-responsive genes *Mt1* and *Slc2a1* (gene names in table 1) were also upregulated (online suppl. table 1). There was an upregulation of antiapoptotic genes and downregulation of proapoptotic genes, with the exception of the proapoptotic gene *Bnip3* which was upregulated (fig. 2c). Four DNA glycosylases were downregulated (fig. 3). Four genes involved in cell cycle renewal were also changed right after the intervention at T0h; the inducer of cell cycle arrest *Gadd45g* was upregulated, while *Atm* and *Chek1* were downregulated. *Ccnd1*, which induces cell cycle arrest when suppressed, was also downregulated (online suppl. table 1).

With increasing time after hypoxia-reoxygenation, the expression of most of the examined genes tended to nor-

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malize towards the gene expression of controls (fig. 2a). The genes of mediators of inflammation had few significant changes at later time points; at T2.5h, only *Il10* was downregulated, and *Lcn2* was upregulated, and only *Tlr4* was downregulated at T72h (online suppl. table 1). The injury-responsive genes *Grin2b* and *Hif1a* were downregulated at both T2.5h and T72h, while *Edn1* was upregulated at T2.5h (online suppl. table 1). The antiapoptotic gene *Bcl2* changed from being upregulated at T0h to downregulated at T2.5h and T5h. Some of the proapoptotic genes continued to be downregulated at T2.5h *(Bax, Casp3, Trp53)*, but there were no significant differences at the last two observation times (online suppl. table 1). The DNA glycosylases *Mutyh* and *Ogg1* were still downregulated at T2.5h, while *Ogg1* changed to upregulation at T5h (fig. 3). Also the cell cycle renewal influencing genes *Atm*, *Ccnd1*, and *Chek1* continued to be downregulated at T2.5h and *Ccnd1* and *Chek1* also at T5h, while no genes involved in cell cycle renewal had changes at T72h (online suppl. table 1).

In conclusion, the genes of mediators of inflammation were mostly downregulated. There was an antiapoptotic gene expression pattern immediately after the intervention at T0h, while at T2.5h, all pro- and antiapoptotic genes were downregulated. Genes involved in DNA repair were all downregulated immediately after the intervention, while the genes involved in cell cycle renewal had a diverging gene expression pattern. There were few changes in the genes involved in vascular development. Most gene expression changes had leveled off by 72 h after completed reoxygenation.

Effect of Reoxygenation with 60% O_2 Compared to Air

In two genes, there were significant differences in gene expression after hyperoxic compared to normoxic reoxygenation. The expression of *Sod1* was higher in the group reoxygenated with 60% O_2 (H60) than the group reoxygenated with air (H21) at T2.5h and T5h [T2.5h (median H60: 1.01, H21: 0.88, p = 0.005) and T5h (median H60: 1.04, H21: 0.85, p = 0.038); fig. 4]. The expression of *Il1b* was higher after normoxic compared to hyperoxic reoxygenation at T0h (median H60: 0.86, H21: 1.08, p = 0.021).

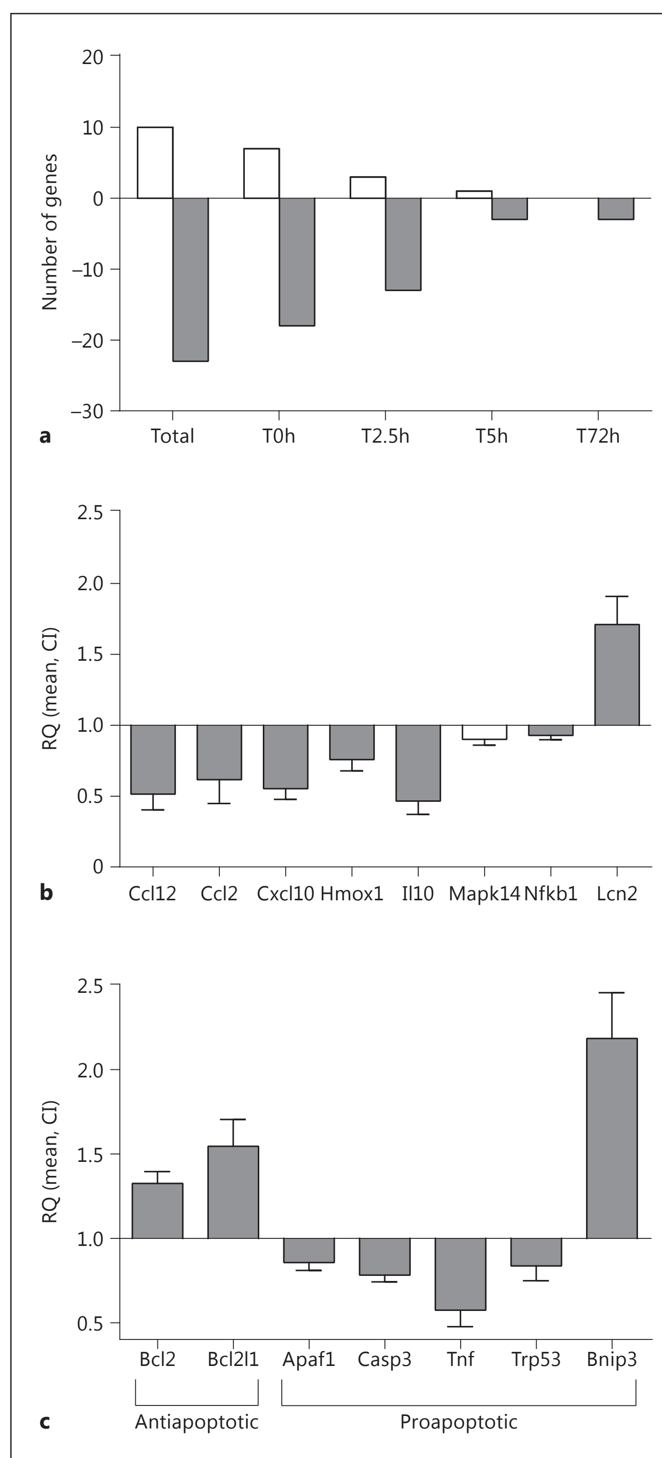

Fig. 2. Number of genes up- and downregulated, and gene expression changes at T0h. **a** The number of genes upregulated (positive white bars) or downregulated (negative grey bars) in the intervention animals compared to controls in total and 0, 2.5, 5, and 72 h after reoxygenation (T0h, T2.5h, T5h, and T72h, respectively). Immediately after completed reoxygenation, at T0h, the inflammatory genes were all downregulated except *Lcn2* (**b**), while the antiapoptotic genes *Bcl2* and *Bcl2l1* were upregulated, and the proapoptotic genes were all downregulated except *Bnip3* (**c**). Gene expression change [relative quantification (RQ) compared to controls] of animals subjected to hypoxia-reoxygenation. For gene names, see table 1.

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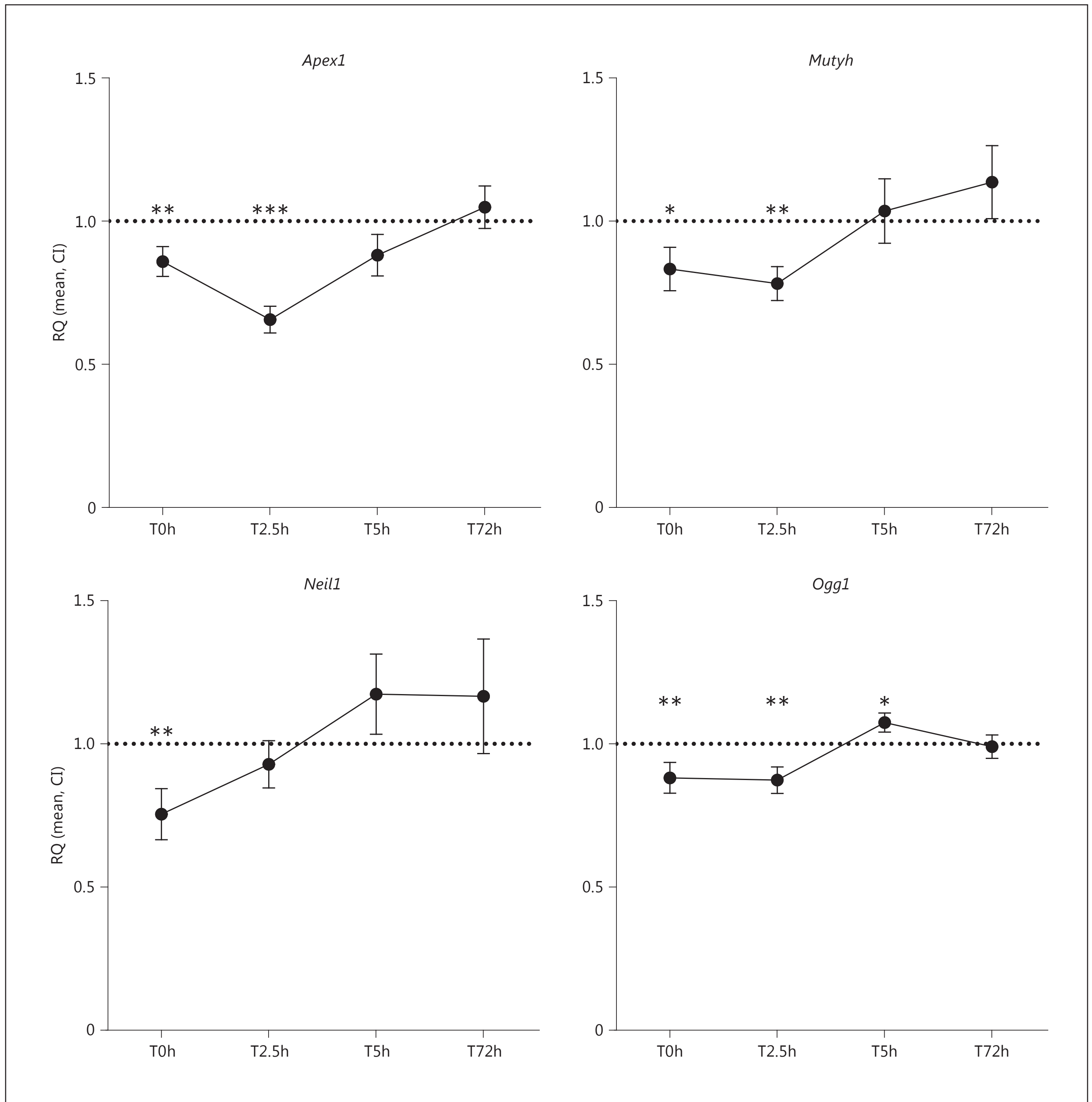

Fig. 3. Genes involved in DNA repair. The DNA repair genes were all downregulated in intervention animals compared to controls immediately after completed reoxygenation, but *Ogg1* became upregulated after 5 h of observation at T5h. Gene expression change [relative quantification (RQ) compared to controls] of animals subjected to hypoxia-reoxygenation. For gene names, see table 1. Dotted line indicates average fold change of 1 for controls. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, intervention animals vs. controls.

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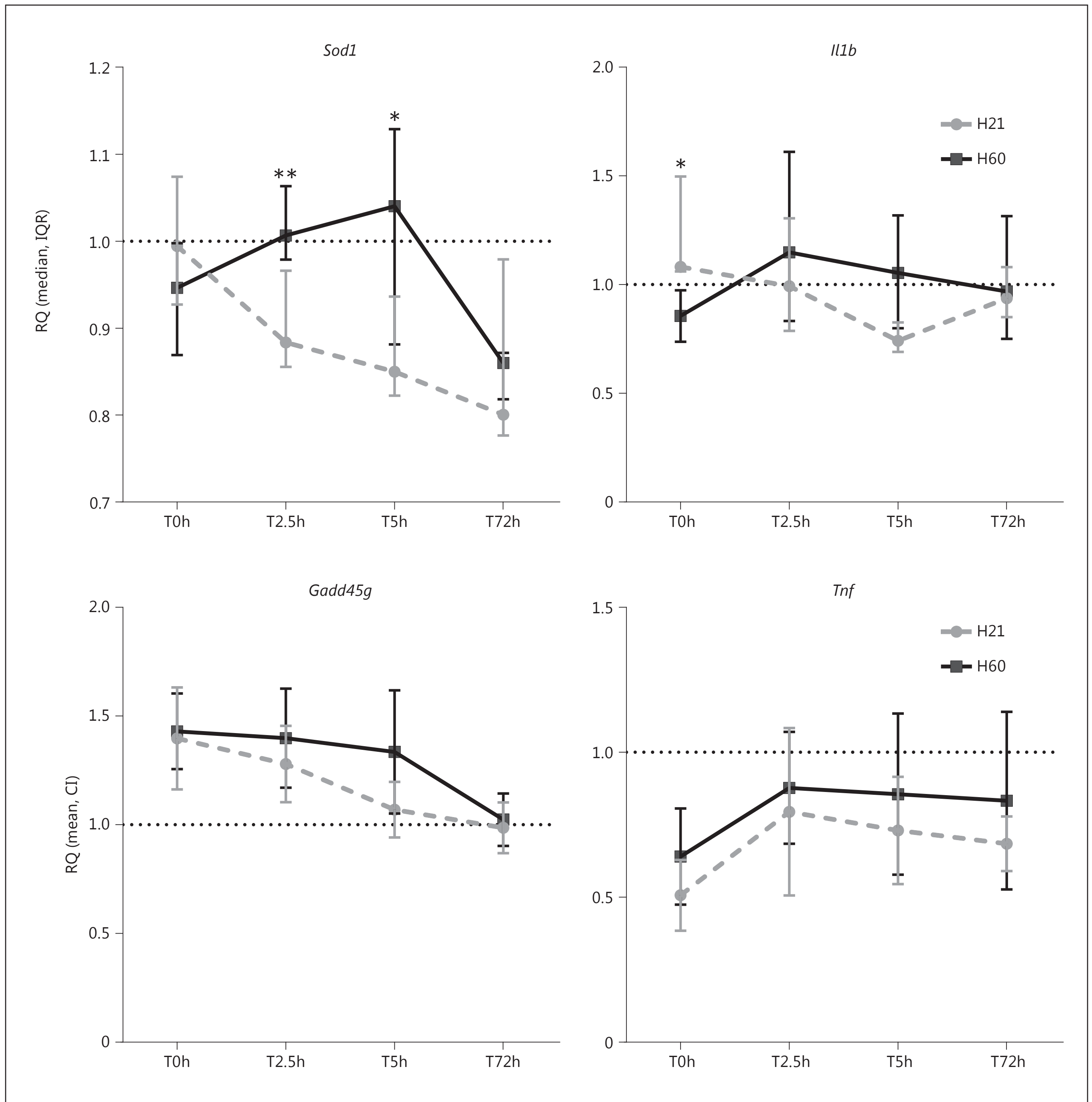

Fig. 4. Genes with differential expression after reoxygenation with 60% O_2 or air. *Sod1* and *Il1b* had a statistically significant differential gene expression change in the H60 and H21 groups. *Gadd45g* and *Tnf* had borderline statistically significantly different gene expression changes. Gene expression change [relative quantification (RQ) compared to controls] of animals subjected to hypoxia-reoxygenation. Data expressed as mean and CI, or median and IQR, depending on the distribution of the data. For gene names, see table 1. Solid line indicates the group subjected to hypoxia-reoxygenation with 60% O_2 (H60). Dashed line indicates the group subjected to hypoxia-reoxygenation with air (H21). Dotted line indicates average fold change of 1 for controls. * $p < 0.05$, ** $p < 0.01$ comparing the H60 and the H21 group at a single time point.

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The expression of *Gadd45g* and *Tnf* was borderline significantly higher in the hyperoxic group [*Gadd45g* 0.115 (–0.001, 0.230), p = 0.051, and *Tnf* 0.122 (–0.002, 0.246), p = 0.054, fig. 4].

Protein Concentration

There were significant differences in 3 out of 10 tested proteins (fig. 5). The protein concentration of BCL2 significantly increased with time in the general linear model, but the pattern was the same in all animals. The protein concentration of CXCL10 was lower in intervention animals compared to controls at T0h [–2.84 (–4.99, –0.70), p = 0.012] and was significantly lower in the H21 group than the H60 group in the general linear model [1.336 (0.11, 2.56), p = 0.034]. The protein concentration of LCN2 was higher in intervention animals compared to controls at T0h [1.57×10^4 (1.13×10^4, 2.01×10^4), p < 0.001]. There were no significant differences in control animals compared to baseline (data not shown).

Discussion

In this study, we present the temporal profile of 44 a priori selected genes, involved in inflammation and response to injury, apoptosis, cell cycle renewal, DNA repair, and vascular development, after hypoxia-reoxygenation in the lungs of neonatal mice. The main results were that immediately after completed reoxygenation, there was a downregulation of genes of mediators of inflammation and a decrease in the protein concentration of the proinflammatory chemokine CXCL10, increased expression of antiapoptotic and decreased expression of proapoptotic genes and downregulation of DNA glycosylases. Additionally, Sod1 had higher expression after hyperoxic than normoxic reoxygenation.

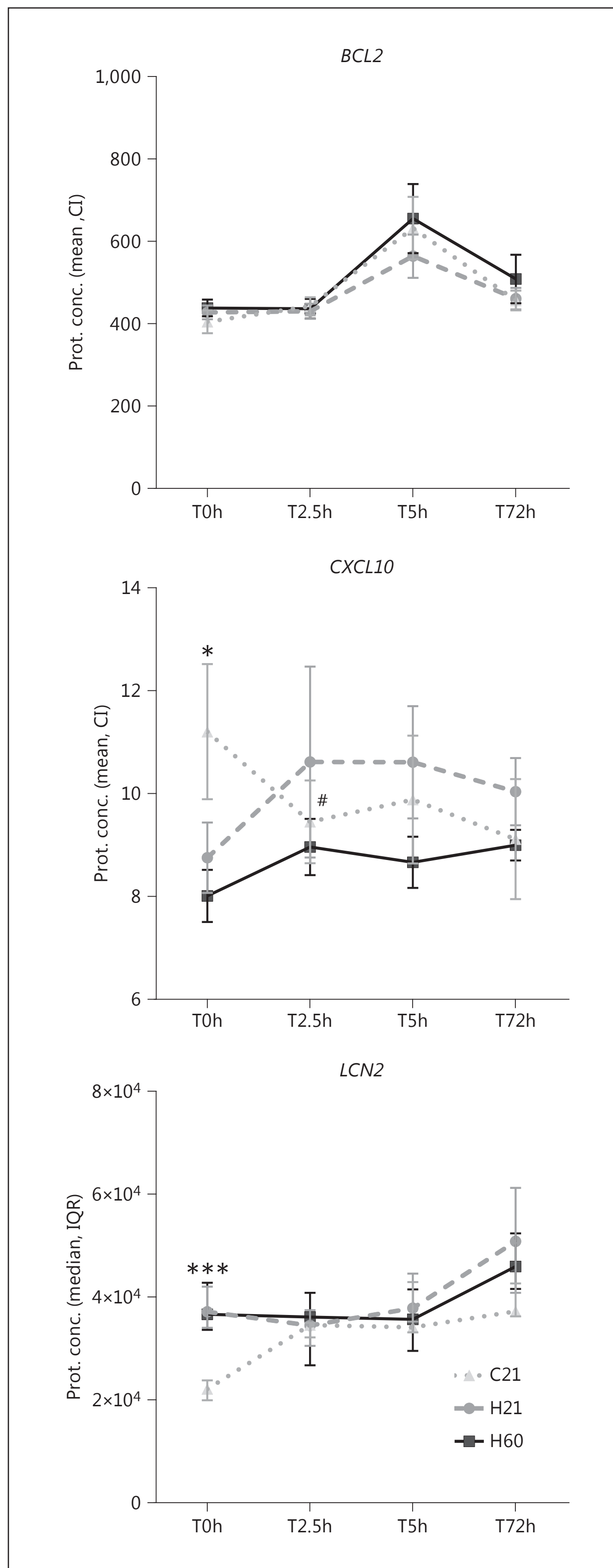

Fig. 5. Protein concentration. Protein concentration (pg/mg protein) after hypoxia-reoxygenation and observation for 0, 12, 24, or 72 h (T0h, T12h, T24h, and T72h, respectively). For gene names, see table 1. *BCL2* had a statistically significant increase in protein concentration with time in all animal groups. *CXCL10* was downregulated in all intervention animals at T0h, and *CXCL10* had a statistically significantly lower protein concentration in the H21 than the H60 group in the general linear model. *LCN2* was upregulated at T0h in all intervention animals compared to controls. Solid line indicates the group subjected to hypoxia-reoxygenation with 60% O_2 (H60). Dashed line indicates the group subjected to hypoxia-reoxygenation with air (H21). Dotted line indicates controls kept in air (C21). * p < 0.05, *** p < 0.001 in intervention animals compared to controls; # p < 0.05 between the H60 and the H21 group in the general linear model.

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Among the inflammatory genes, only *Lcn2* was upregulated, while the rest were downregulated. This was confirmed at the protein level with an increase in LCN2 and a decrease in CXCL10 in mice subjected to hypoxia-reoxygenation. In concordance with these results, downregulation of inflammatory genes has been reported after ischemia-reperfusion in adult rats [16], while another study reported increased expression of inflammatory genes [11]. Previously, we have reported both unaltered [8] and increased inflammatory markers [7] after hypoxia-reoxygenation in neonatal pigs, and upregulated *Lcn2* after hypoxia-reoxygenation in mice [9]. *Lcn2* is a part of the innate immune system [17] and has also been reported to be increased after RDS in the neonatal lung [18]. Three injury-responsive genes were upregulated: *Mt1,* the inducible glucose transporter *Slc2a1* (also known as GLUT1), and the vasoconstrictor *Edn1*. This suggests that the intervention induced the expression of injury-responsive genes, although the downregulation of *Hif1a* and *Grin2b* speaks to the contrary.

We found an antiapoptotic gene response right after the intervention. Contrary to this, we found upregulation of *Bnip3*. *Bnip3* is induced by hypoxia [19] and can overcome *Bcl2* suppression and induce autophagy [20], and possibly increases as a response to hypoxia and secondary to the *Bcl2* increase. Others have reported increased apoptosis after hypoxic/ischemic insults in the lungs [10, 11, 21], while one study reported no increase in apoptosis [22].

Previously, we have reported a downregulation of the DNA repair pathway after hypoxia-reoxygenation [9], and in this study we found that four of the DNA glycosylases involved in the repair of oxidative DNA damage were downregulated right after the intervention. We have previously reported that hypoxia-reoxygenation did not increase the content of the oxidized DNA lesion 8-oxoguanine in the lungs [23], and this may explain why the DNA glycosylases are not induced after this intervention. The expression of genes involved in cell cycle renewal had more diverging changes. Some gene expression changes suggested increased cell cycle arrest (*Ccnd1* and *Gadd45g*), others suggested decreased cell cycle arrest *(Atm, Chek1, Trp53)*. We have previously reported downregulation of *Ccnd1* after hypoxia-reoxygenation, suggesting increased cell cycle arrest [9]. Northway et al. [24] showed that hyperoxia decreased DNA synthesis in the neonatal mouse lung, and our study suggests that also hypoxia-reoxygenation influences cell cycle renewal. Others have reported increased *p53* indicating increased cell cycle arrest and apoptosis after ischemia and reperfusion in adult rats [11].

Sod1, an important enzyme in the defense against superoxide radicals, had higher expression after hyperoxic reoxygenation, suggesting a greater need for antioxidant defenses. However, the proinflammatory chemokine CXCL10 had a lower protein concentration and *Il1b* a lower gene expression change after hyperoxic reoxygenation, suggesting less inflammation in the hyperoxic groups. Previously, we have reported that hyperoxic reoxygenation gave an augmented inflammation in neonatal pig lungs [7, 8], but a downregulation of inflammatory genes in mice (online suppl. data [9]).

Because of differences in studied time points, we could not study gene expression changes and protein concentration in the same animals. We chose to evaluate the protein concentration of the genes with the most significant results in mRNA and not all the 44 genes. The strength of the study is that we have examined changes in the hippocampus/striatum [14] and lungs in the same animals. The biological significance of the reported gene expression changes cannot be determined from this study alone, but the changes in mediators of inflammation were also confirmed at the protein level. In addition, it has been reported previously that even a 20% difference in gene expression change might give a difference in disease phenotype [25]. We wanted to study the response in vivo, and thus had to study the whole lung as a homogenate instead of one singular cell type. It was not within the scope of this study to do long-term follow-up of the animals beyond 72 h after the intervention.

In conclusion, we present in this article the first prolonged temporal profile of gene expression changes after a hypoxia-reoxygenation event without hilar clamping in the neonatal mouse lung. We found a downregulation of the genes of mediators of inflammation and DNA glycosylases, an early increased antiapoptotic gene expression pattern, and an increased expression of *Sod1* after hyperoxic compared to normoxic reoxygenation. This study shows that hypoxia-reoxygenation induces changes in gene expression in the neonatal lung, which may lead to long-term consequences.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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