

Increased expression of inflammatory genes in the neonatal mouse brain after hyperoxic reoxygenation

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BACKGROUND: Hyperoxic reoxygenation following hypoxia increases the expression of inflammatory genes in the neonatal mouse brain. We have therefore compared the temporal profile of 44 *a priori* selected genes after hypoxia and hyperoxic or normoxic reoxygenation.

METHODS: Postnatal day 7 mice were subjected to 2 h of hypoxia (8% O₂) and 30 min reoxygenation with 60% or 21% O₂. After 0 to 72 h observation, mRNA and protein were examined in the hippocampus and striatum.

RESULTS: There were significantly higher gene expression changes in six genes after hyperoxic compared to normoxic reoxygenation. Three genes had a generally higher expression throughout the observation period: the inflammatory genes *Hmox1* (mean difference: 0.52, 95% confidence interval (CI): 0.15–1.01) and *Tgfb1* (mean difference: 0.099, CI: 0.003–0.194), and the transcription factor *Nfkb1* (mean difference: 0.049, CI: 0.011–0.087). The inflammatory genes *Cxcl10* and *Il1b*, and the DNA repair gene *Neil3*, had a higher gene expression change after hyperoxic reoxygenation at one time point only. Nineteen genes involved in inflammation, transcription regulation, apoptosis, angiogenesis, and glucose transport had significantly different gene expression changes with time in all intervention animals.

CONCLUSION: We confirm that hyperoxic reoxygenation induces a stronger inflammatory gene response than reoxygenation with air.

Neonatal asphyxia is still one of the leading causes of mortality in children under 5 y of age worldwide (1). Hypoxia–reoxygenation is an important mechanism involved in the pathogenesis of injury caused by neonatal asphyxia (2,3). Hypoxia–reoxygenation leads to inflammation and augmented oxidative stress through energy failure, increased production of reactive oxygen and nitrogen species, and decreased antioxidant activity. This may subsequently induce lipid peroxidation, increased cellular permeability, glutamate excitotoxicity, and damage to proteins and DNA, ultimately resulting in necrosis and apoptosis (3–5). The neonatal brain is particularly vulnerable to oxidative stress (5,6).

Until recently, hyperoxic reoxygenation was recommended when initiating neonatal resuscitation (7). However, many studies on hyperoxic reoxygenation after asphyxia, hypoxia–ischemia (HI), and hypoxia have been conducted both in humans and animals (reviewed in refs. (8,9)), concluding that substantial evidence supports that starting with air is better than 100% O₂ when resuscitating term neonates, and hyperoxia should be avoided (9). Hyperoxia alone has also been shown to have detrimental effects (10). However, there is still insufficient evidence to give recommendations on which strategy of supplemental oxygen to use in moderately and late premature neonates (7).

Although meta-analyses show that resuscitation with air after neonatal asphyxia in term neonates gives lower mortality than resuscitation with 100% O₂ (11), the mechanisms have not been fully elucidated. One avenue of research is gene expression profiling. Hedtjörn *et al.* (12,13) have studied gene expression profiling after HI in neonatal mice. However, to the best of our knowledge, no other groups have studied gene expression profiling after hyperoxic reoxygenation. We have established a model subjecting postnatal day seven (P7) mice to a global hypoxic event followed by reoxygenation with different fractions of inspired oxygen (14). P7 mice have a brain development comparable to moderate and late premature humans at GA 32–36 wk (15). Moderate and late preterm neonates have a higher risk of neonatal mortality and morbidity and also a higher risk of later sequelae than term neonates, and there has been a call for research into this large group of neonates (16). In a recent study from our group, we presented the transcriptome profile in the forebrain of P7 mice 2.5 h after hypoxia and reoxygenation (17). Hyperoxic reoxygenation gave increased expression of inflammatory genes and decreased expression of genes of the oxidative phosphorylation pathway.

To explore further the gene expression changes of the brain after hypoxia and hyperoxic reoxygenation, we made a temporal profile of genes in the hippocampus and striatum. Hazelton *et al.* (18) found increased inflammation and neuronal cell death in the hippocampus, and decreased performance on the Morris Water Maze, after hyperoxic reoxygenation in adult rats. Problems with cognition and memory are also common among

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moderate and late preterm neonates (16). The striatum is one of the first regions to show damage on magnetic resonance imaging after mild to moderate asphyxia (19). In our transcriptome profiling study, there were many differentially expressed genes after reoxygenation with 60% O₂ (17). 100% O₂ is less clinically relevant after the International Liaison Committee on Resuscitation guidelines changed in 2010 (7); thus we decided on 60% O₂ as hyperoxia. Forty-four target genes were selected (Table 1) based on our transcriptome profiling studies (14,17), a pilot study and literature search. The objective of the present study was to compare the gene expression changes in the hippocampus and striatum of P7 mice after hypoxia followed by reoxygenation with 60% O₂ or air (21% O₂), and to make a temporal profile of the genes up to 3 d after the intervention.

RESULTS

P7 mice were randomized to hypoxia in 8% O₂ for 2 h, followed by reoxygenation for 30 min with either 60% O₂ (H60) or air (H21), or controls (C) kept in air for the duration of the experiment (Figure 1). mRNA and protein were examined in hippocampus and striatum. There were no statistical differences between main groups in weight or sex distribution in either the mRNA or protein samples (data not shown).

Blood glucose was significantly lower in the period of separation from the dams in both hypoxia and control mice compared to baseline (Supplementary Figure S1 online). Blood glucose was also significantly lower in the hypoxia mice compared to control mice right after hypoxia and at 0 h after reoxygenation, but at 2.5 h after reoxygenation, the blood glucose of hypoxia mice had returned to the level of controls.

Gene Expression After Hypoxia–Reoxygenation

There was a statistically higher gene expression change in six genes related to inflammation, transcription regulation, and DNA repair after reoxygenation with 60% O₂ (H60) compared to air (H21) (Figure 2). Three genes had an overall higher expression in the H60 group in the general linear model: The inflammatory genes *Hmox1* (for gene name see Table 1, H60 vs. H21 mean difference: 0.52, 95% confidence interval (CI): 0.15–1.01, $P = 0.009$) and *Tgfb1* (mean difference: 0.099, CI: 0.003–0.194, $P = 0.044$), and the transcription factor *Nfkb1* (mean difference: 0.049, CI: 0.011–0.087, $P = 0.012$). At single time points, we found a higher gene expression change after hyperoxic reoxygenation at 0 h of the inflammatory gene *Cxcl10* (Mann–Whitney U -test, $P = 0.009$) and the DNA-repair gene *Neil3* (mean difference: 0.17, CI: 0.001–0.34, $P = 0.048$), and at 5 h of the inflammatory gene *Il1b* (Mann–Whitney U -test, $P = 0.021$).

Patterns of Gene Expression Temporal Profiles

Nineteen genes involved in inflammation, transcription regulation, apoptosis, angiogenesis, and glucose transport, had significantly different gene expression changes with time after hypoxia and reoxygenation in the general linear model in all intervention animals compared to controls. We identified three patterns of gene expression temporal profiles: (i) Genes

that were highly increased in expression right after reoxygenation at 0 h, and then decreased (*Bnip3*, *Ccl2*, *Edn1*, *Il6*, *Mt1*, *Slc2a1*, *Vegfa*, gene names and main functions in Table 1) (Figures 3 and 4a); (ii) Genes that had the highest increase in expression between 2.5 and 5 h after completed reoxygenation (*Ccl12*, *Ccl5*, *Cxcl10*, *Hmox1*, *Il1b*, *Lcn2*, *Stat3*, *Tnf*) (Figures 2, 3, and 4b); (iii) Genes where the expression fluctuated around fold change of 1, but had significant differences between time points (*Apaf1*, *Ccnd1*, *Neil3*, and *Nfkb1*) (Figures 2 and 4c).

Protein Analysis

For seven of the genes with significant changes in mRNA expression, protein concentration was measured by enzyme-linked immunosorbent assay in tissue homogenate from the hippocampus and striatum (Gene symbol^b and gene names in Table 1). The proinflammatory chemokine CCL12 had peak mRNA expression after 2.5 h, while the peak protein concentration was after 12 h (Figure 3). CCL12 had a higher concentration in the H60 group than the H21 group in the general linear model (mean difference 1.2 pg/mg protein, CI: 0.11–2.3, $P = 0.032$), although no such difference could be detected in mRNA after hyperoxic reoxygenation. The increased CCL12 concentration after hyperoxic reoxygenation started already at 0 h after reoxygenation, before the mRNA peak. Vascular endothelial growth factor A, important in angiogenesis, had a significant increase in protein concentration in all intervention animals compared to controls at 0 h (mean difference: H21–C 44.3 pg/mg prot, CI: 28.5–60.1; mean difference: H60–C 49.4, CI: 33.9–64.9), concurring with the peak mRNA expression. LCN2 is an iron chelator involved in the innate immune and stress response. It showed a significant change in protein concentration with time in the hypoxia groups with a peak expression after 12 h, but there were no significant differences between hypoxia and controls at any time points. Four proteins had no significant differences at the time points analyzed: IL1B, MT1, NEIL3, and STAT3.

DISCUSSION

In this study, we have used a P7 mouse model of hypoxia followed by reoxygenation with 60% O₂ or air, to examine the gene expression changes of 44 *a priori* selected genes in the hippocampus and striatum, and to make a temporal profile up to 3 d after the intervention. Our main result is that hyperoxic reoxygenation gave a significantly higher gene expression change in six genes involved in inflammation (*Cxcl10*, *Il1b*, *Tgfb1*, *Hmox1*), transcription regulation (*Nfkb1*), and DNA repair (*Neil3*). The augmented inflammatory gene response was still evident at 72 h after completed reoxygenation. Other studies have also shown increased inflammatory and oxidative stress response after hyperoxic reoxygenation, although, most have used 100% O₂ in either term or immature animal models, while in this study we have used 60% O₂ for 30 min in a moderate to late preterm model.

In our transcriptome profiling study, we have recently shown that hyperoxic reoxygenation induced increased expression of inflammatory genes in the neonatal mice brain (17). Previously, we have reported a dose dependent increase in markers of

Table 1. Target and reference genes

Gene symbol	Gene name	Main function(s)
Apaf1^a	Apoptotic peptidase activating factor 1	Apoptosis
<i>Apex1</i>	Apurinic/aprimidinic endonuclease 1	DNA repair, oxidative stress
<i>Atm</i>	Ataxia telangiectasia mutated homolog (human)	Cell cycle arrest
<i>Bax</i>	BCL2-associated X protein	Apoptosis
<i>Bcl2</i>	B cell leukemia/lymphoma 2	Apoptosis
<i>Bcl2l1</i>	BCL2-like 1	Apoptosis
Bnip3	BCL2/adenovirus E1B interacting protein 3	Autophagy
<i>Casp3</i>	Caspase3	Apoptosis
Ccl12^b	Chemokine (C-C motif) ligand 12	Inflammation
Ccl2^b	Chemokine (C-C motif) ligand 2	Inflammation
Ccl5	Chemokine (C-C motif) ligand 5	Inflammation
<i>Ccnb1</i>	Cyclin B1	Cell cycle arrest
Ccnd1	Cyclin D1	Cell cycle arrest
<i>Chek1</i>	Checkpoint kinase 1	Cell cycle arrest
Cxcl10^b	Chemokine (C-X-C motif) ligand 10	Inflammation
Edn1	Endothelin 1	Angiogenesis
<i>FasL</i>	fas ligand (TNF superfamily, member 6)	Apoptosis
<i>Gadd45g</i>	Growth arrest and DNA-damage inducible 45γ	Cell cycle arrest
<i>Grin2b</i>	Glutamate receptor, ionotropic, NMDA2B (epsilon 2)	Oxidative stress
<i>Hif1a</i>	Hypoxia inducible factor 1, α-subunit	Response to hypoxia, transcription factor
Hmox1	Heme oxygenase (decycling) 1	Inflammation, oxidative stress
<i>Igf1r</i>	Insulin-like growth factor I receptor	Glucose regulation
<i>Ikbkb</i>	Inhibitor of κB kinase-β	Apoptosis
<i>Il10</i>	Interleukin-10	Inflammation
Il1b^b	Interleukin-1β	Inflammation, apoptosis
Il6	Interleukin-6	Inflammation
<i>Jun</i>	jun proto-oncogene	Transcription factor
Lcn2^b	Lipocalin 2	Inflammation
<i>Mapk14</i>	Mitogen-activated protein kinase 14	Inflammation
Mt1^b	Metallothionein 1	Oxidative stress
<i>Mutyh</i>	mutY homolog (<i>Escherichia coli</i>)	DNA repair
<i>Neil1</i>	nei endonuclease VIII-like 1 (<i>E. coli</i>)	DNA repair
Neil3^b	nei like 3 (<i>E. coli</i>)	DNA repair
Nfkb1	Nuclear factor of κ light polypeptide gene enhancer in B cells 1, p105	Transcription factor, inflammation
<i>Ogg1</i>	8-oxoguanine DNA-glycosylase 1	DNA repair
<i>Prkaa1</i>	Protein kinase, AMP-activated, α1 catalytic subunit	Glucose regulation
Slc2a1	Solute carrier family 2 (facilitated glucose transporter), member 1	Glucose transport
<i>Sod1</i>	Superoxide dismutase 1, soluble	Oxidative stress
Stat3^b	Signal transducer and activator of transcription 3	Transcription factor
Tgfb1	Transforming growth factor, β1	Inflammation, growth factor
<i>Tlr4</i>	Toll-like receptor 4	Inflammation
Tnf^a	Tumor necrosis factor	Apoptosis, inflammation
<i>Trp53</i>	Transformation related protein 53	Transcription factor
Vegfa^b	Vascular endothelial growth factor A	Angiogenesis
<i>Actb</i>	Actin, β	Reference gene, lung
Polr2a	Polymerase (RNA) II (DNA directed) polypeptide A	Reference gene, brain and lung
Ubc	Ubiquitin C	Reference gene, brain
18S		Manufacturer control

^aBold: Genes mentioned in the manuscript. ^bAlso attempted analyzed for protein.

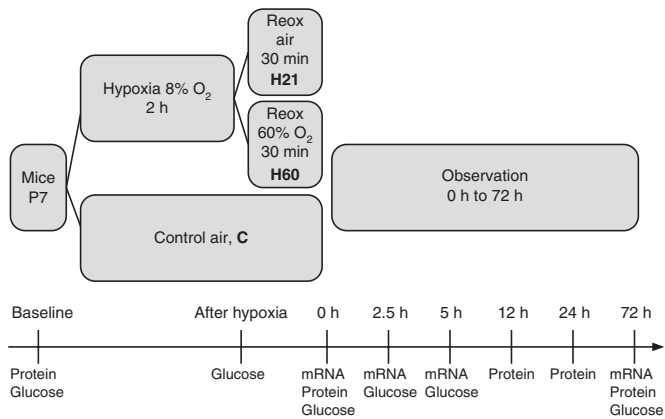


Figure 1. Experimental design. P7 mice were randomized to either hypoxia for 2 h or controls kept in air. The hypoxia group was further randomized to 30 min reoxygenation with either 60% O₂ (H60) or air (H21). The hippocampus and striatum were harvested right after reoxygenation (0 h), or 2.5, 5, or 72 h after reoxygenation in the mRNA experiment ($n = 21$ at each time point); at baseline and 0, 12, 24, or 72 h after reoxygenation in the protein experiments ($n = 7, 22, 21, 21, 21$, respectively); and blood glucose was measured at baseline, after hypoxia, and 0, 2.5, 5, or 72 h after reoxygenation ($n = 12, 17, 48, 39, 16, 13$, respectively).

oxidative stress with increasing oxygen concentration during reoxygenation in neonatal pigs (20). In NT2-N neurons Almaas *et al.* (21) found increased CXCL10 after oxidative stress, and we have earlier reported increased CXCL10 after hypoxia, but with no augmented response after hyperoxic reoxygenation (22). In concordance with our study, increased expression of *Il1b* has been found after hyperoxic reoxygenation in fetal sheep (23), and after hyperoxia alone in neonatal rats (10). Perez-Polo and coworkers found a stronger inflammatory response after hyperoxic reoxygenation following HI in rats (24,25). We also found increased protein concentration of CCL12, a proinflammatory chemokine, after hyperoxic reoxygenation, consistent with our recent reports of increased *Ccl12* expression in the eye and brain of neonatal mice after hyperoxic reoxygenation (17,26). Mojsilovic-Petrovic *et al.* (27) found increased expression of *Ccl12* in astrocyte cultures from P7 mice after hypoxia alone.

Hmox1 expression is responsive to both hypoxia and hyperoxia (28), and we found that hyperoxic reoxygenation gave a stronger induction of *Hmox1* gene expression than reoxygenation with air. In contrast to our findings, Bergeron *et al.* (29) only found increased expression of *Hmox1* after HI in neonatal

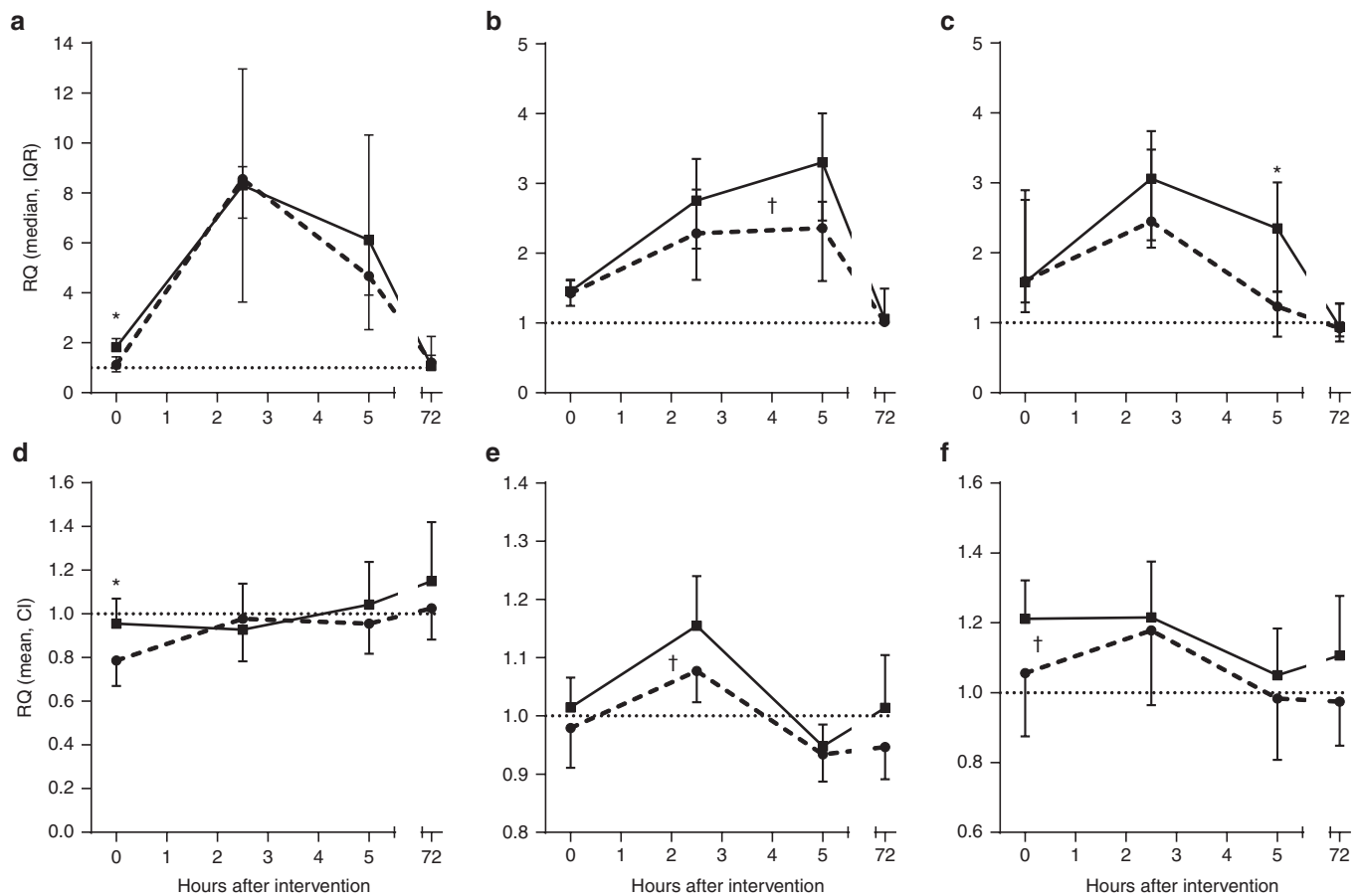


Figure 2. Genes with significantly higher expression after hyperoxic reoxygenation. Gene expression change, relative quantification (fold change compared to controls), of animals subjected to hypoxia and reoxygenation, and then observed for 0, 2.5, 5, or 72 h after the intervention. In six genes, there was significantly higher expression after hyperoxic than normoxic reoxygenation either overall in a general linear model or at a single time point. Data expressed as mean, confidence interval, or median, interquartile range (IQR), depending on the distribution of the data. Panel a, *Cxcl10*; panel b, *Hmox1*; panel c, *Il1b*; panel d, *Neil3*; panel e, *Nfkb1*; panel f, *Tgfb1*. Gene names are shown in Table 1. Solid line: Group subjected to hypoxia and reoxygenation with 60% O₂ (H60). Dashed line: Group subjected to hypoxia and reoxygenation with air (H21). Dotted line: Average fold change of 1 for controls. * $P < 0.05$ H60-H21 at a single time point. † $P < 0.05$ H60-H21 across all time points in the general linear model.

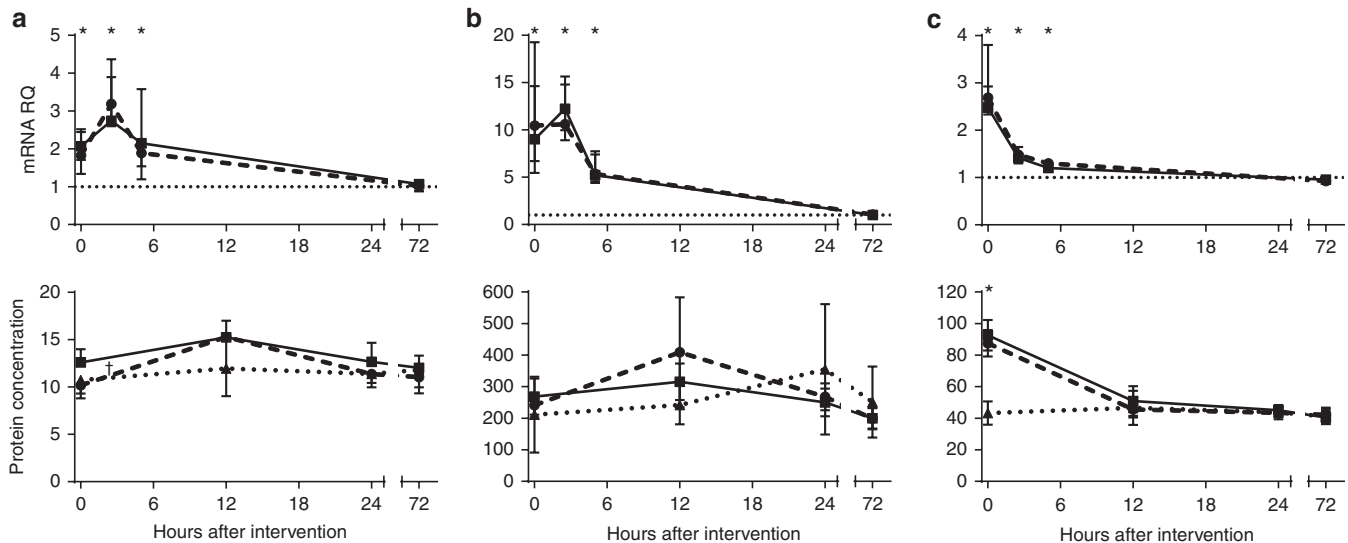


Figure 3. Genes with significant changes in both mRNA expression and protein concentration. mRNA expression (relative quantification, fold change compared to controls, median, IQR, top three panels) and protein concentration (protein concentration, pg/mg protein, bottom three panels) for the same gene with time after hypoxia and reoxygenation. Panel **a**: CCL12 had an overall higher protein concentration after hyperoxic reoxygenation. The peak mRNA expression was after 2.5 h and the peak protein concentration was after 12 h. Panel **b**: LCN2 had significant differences with time in both protein concentration and gene expression. Panel **c**: vascular endothelial growth factor A had peak both protein concentration and mRNA expression right after reoxygenation at 0 h. Gene names are shown in Table 1. Observation time after the intervention: mRNA 0, 2.5, 5, or 72 h; protein 0, 12, 24, or 72 h. Solid line: Group subjected to hypoxia and reoxygenation with 60% O₂ (H60). Dashed line: Group subjected to hypoxia and reoxygenation with air (H21). Dotted line: Controls kept in air, average fold change of 1 in mRNA group. [†]*p* < 0.05 H60-H21 in the general linear model; **p* < 0.05 compared to controls.

rats, but not after hypoxia alone. The growth factor *Tgfb1* is known to be involved in hyperoxia-induced injury in the lung (30), but to our knowledge, increased expression has not been reported earlier in relation to hyperoxia in the brain.

Hyperoxic reoxygenation also gave increased expression of the pluripotent transcription factor *Nfkb1*, which is known to be involved in hypoxia–reoxygenation injury (3). We have earlier reported increased *Nfkb1* after hyperoxic reoxygenation in P5 mice (31), in concordance with the present study. The DNA glycosylase *Neil3* is important in the repair of oxidative DNA-damage. Dalen *et al.* (32) reported decreased expression of *Neil3* in neonatal pigs after post hypoxic hypothermia, but with no statistically significant difference between hyperoxic or normoxic reoxygenation. In this study, *Neil3* was significantly less suppressed in animals reoxygenated with 60% O₂ compared to air, possibly suggesting increased need of DNA repair after hyperoxic reoxygenation.

We identified three patterns of gene expression temporal profiles in intervention animals compared to controls: Firstly, high expression right after the intervention and rapidly decreasing; secondly, highest expression from 2.5–5 h after the intervention; and thirdly, fluctuating expression with fold change around one. Among genes induced within 5 h after the intervention, there were several genes involved in inflammation and oxidative stress (*Ccl12*, *Ccl2*, *Ccl5*, *Cxcl10*, *Hmox1*, *Il1b*, *Il6*, *Lcn2*, *Mt1*, *Stat3*, *Tnf*), and this study confirms that hypoxia–reoxygenation induces inflammatory and oxidative stress responsive genes. Other groups have found an increased expression of inflammatory genes after HI (13) and hypoxia alone (33). Genes involved in angiogenesis (*Edn1*, *Vegfa*), and the facilitative glucose transporter *Slc2a1* (homolog to GLUT1) were also

induced within 5 h after the intervention. *Vegfa* is known to be induced by hypoxia (34), but to our knowledge, increased expression of *Edn1* has not previously been described after hypoxia–reoxygenation or HI in the brain. Upregulation of glucose transport after HI has been described previously in both brain hemispheres applying the Vannucci model (5,35). In this study, the hypoxia mice had lower blood glucose right after hypoxia–reoxygenation, and this might contribute to the induction of *Slc2a1* that we found.

Inflammation is considered an important cause of damage after hypoxia–reoxygenation injury (2,6,13,36,37), and there is evidence that increased inflammation aggravates such injury (36). Several groups have found increased brain lesions in neonatal rats after hyperoxic reoxygenation following HI (25,38,39). The increased inflammatory gene response after hyperoxic reoxygenation we found in the present study, supports the evidence that increased inflammation might be one of the contributing factors to the increased brain damage after hyperoxic reoxygenation. In our previous transcriptome profiling study, we found that hypoxia–reoxygenation led to increased microglial activation, as a sign of increased inflammation, but with no difference between normoxic and hyperoxic reoxygenation (17), in contrast to the findings of Hazelton *et al.* (18). Large treatment groups would probably be needed to show the subtle pathological differences expected when comparing groups where the only difference is 30 min air or 60% O₂ during reoxygenation. However, it has been hypothesized that there is a continuous specter of insults and outcomes, thus small differences in an insult may give differences in the outcome (40).

Some limitations of this study are that only 44 target genes were examined, and not the whole genome, and there was

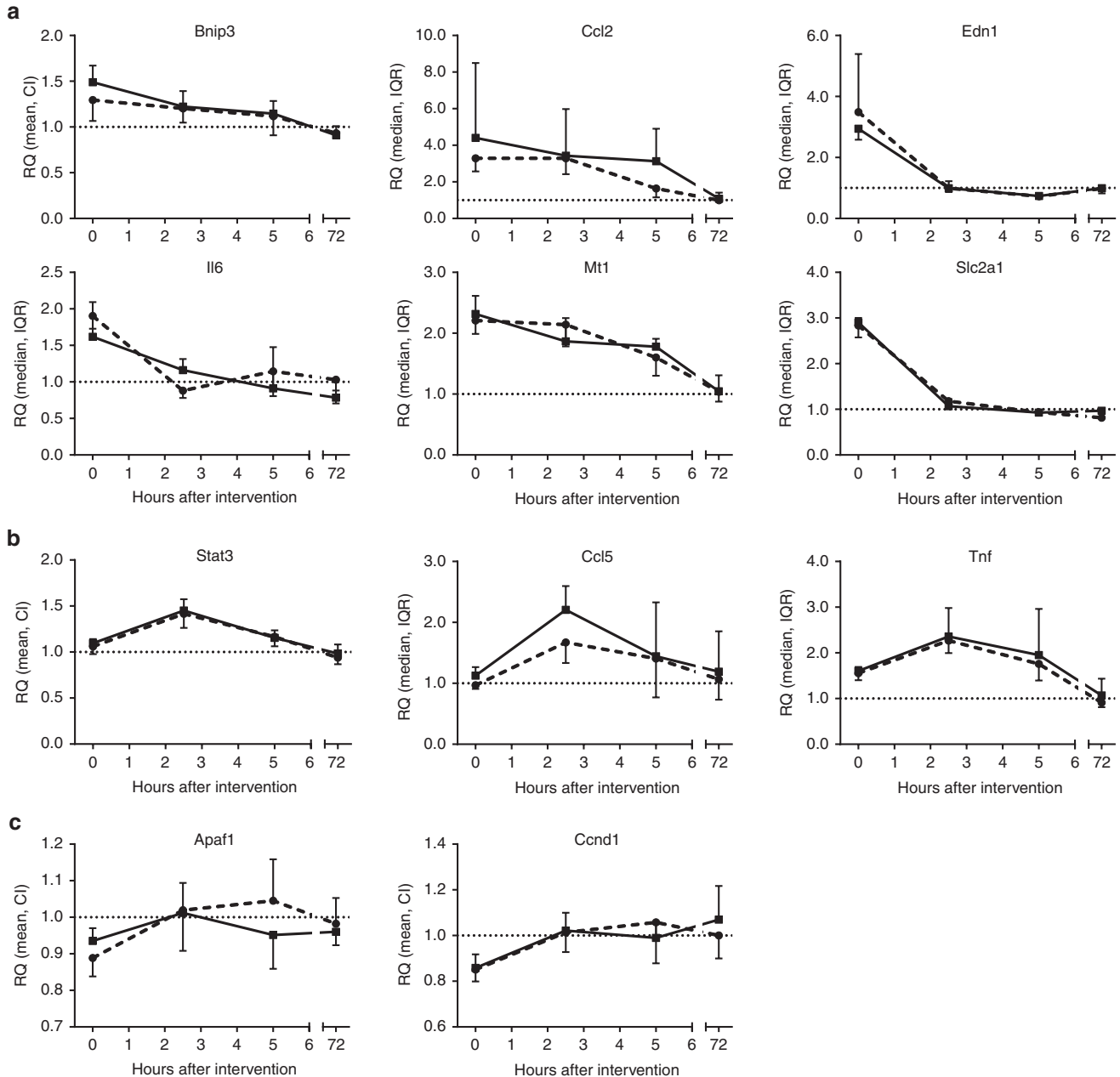


Figure 4. Temporal profile patterns of gene expression changes. We identified three temporal patterns of gene expression changes after hypoxia and reoxygenation. Panel **a**: Genes that were highest in expression right after reoxygenation and then decreased. Panel **b**: Genes that had the highest expression between 2.5 and 5 h after the intervention. Panel **c**: Genes with fold change fluctuating around 1. Data expressed as relative quantification, fold change compared to controls, in mean, confidence interval, or median, interquartile range (IQR), depending on the distribution of the data. Gene names are shown in Table 1. Solid line: Animals given hypoxia and reoxygenation with 60% O_2 (H60). Dashed line: Animals given hypoxia and reoxygenation with air (H21). Dotted line: Control animals with average fold change of 1.

unfortunately not enough tissue to study both the mRNA expression and protein concentration in each mouse. Neither was there enough tissue to analyze the protein concentration for all 19 genes with significant changes in mRNA. The absolute differences in gene expression, although statistically significant, are not very large, and this study cannot conclude on the biological significance of the observations. However, the changes in mRNA expression and protein concentrations were mainly consistent, and earlier studies have reported biologically different phenotypes with a 20% change in gene expression (41).

In conclusion, we have shown an increased inflammatory gene response after hyperoxic reoxygenation following a hypoxic episode in neonatal mice. This could contribute to the increased morbidity and mortality after hyperoxic reoxygenation seen in humans (11).

METHODS

Animals

The Norwegian Animal Research Authority approved all experiments (FOTS ID 3112 and 4543), and animals were cared for and handled in accordance with Norwegian legislation and Directive 2010/63/EU.

Mice of strain C57BL/6J BomTac were bought in December 2008 (Taconic, Ejby, Denmark), and inbred locally. The mice were stabled in ventilated Green-line cages (Techniplast, Varese, Italy) with standard bedding at 24 °C on a 12:12 h light/dark cycle with access to standard rodent pellets and water *ad libitum*. Dams nursed their own pups. In total, 333 pups were used in the experiments, including pilots and testing, of which 19 died during the experiment and were excluded before analysis (mortality: 5.7%).

Experimental Model

Mice pups on P7 of both sexes were used in the experiments (day of discovery = P1). The pups were randomized to either hypoxia in 8% O₂ for 2 h or controls (C) kept in air for the duration of the experiment. This hypoxic event has previously been shown to give the pups a respiratory compensated metabolic acidosis fulfilling the BE-criteria for neonatal asphyxia (14). The hypoxia group was randomized further to reoxygenation with either 60% O₂ (H60) or air (H21) (Figure 1). After the designated observation period, the mice were decapitated and rapidly dissected on ice. The hippocampus and striatum were harvested, snap frozen in liquid nitrogen, and stored at -79 °C. For all experiments, hippocampus and striatum were pooled from the same animal and analyzed together. Blood glucose was measured (Freestyle Light, Abbott, Abbott Park, IL) right after decapitation at baseline (right after separation from the mother, *n* = 12), after hypoxia before reoxygenation (*n* = 17), right after completed reoxygenation (0 h, *n* = 48), or at 2.5, 5, and 72 h after completed reoxygenation (*n* = 39, 16, and 13, respectively) (Figure 1). Due to the small size of the animals, there was no repeated sampling.

Custom made Lucite chambers with floor heating were used for the experiments. Hypoxia was induced with premixed gas 8% O₂ balanced with nitrogen (Yara, Oslo, Norway, from 2012 Aga, Munich, Germany). Reoxygenation was performed in separate chambers with either air, or 60% O₂ achieved with a conventional oxygen mixer. Atmospheric pressure was maintained through a continuous flow of inlet gas of minimum 0.5 l/min and open outlets, CO₂ concentration was less than 0.3%. An open water source kept air humidity at 40–50%. The skin temperature of the mice was kept close to nesting temperature at 34–36 °C through monitoring with IR-thermometer (Fluke 62 mini, Fluke, Everett, WA). The mice were not anesthetized or restrained. For the intervention and observation up to 2.5 h, the pups were separated from their dams, but pups with longer designated observation were returned.

Real-Time Reverse-Transcriptase PCR

Pooled samples of hippocampus and striatum from the same animal were prepared and total RNA was extracted, reverse transcribed to cDNA, and amplified as described previously (14). TaqMan array microfluidic cards (Life Technologies, Carlsbad, CA) were used to study gene expression changes. 250 ng cDNA was used in each fill reservoir and the cards were prepared and analyzed in Applied biosystems 7900HT Fast Real-time PCR system (Life Technologies) with 40 thermal cycles according to the manufacturer's instructions. All samples were run in duplicates. Eighteen possible mouse reference genes were tested in 16 samples in triplicates (TaqMan Array Mouse Endogenous Control Panel). The results were analyzed with Qbase+ (Biogazelle, Zwijnaarde, Belgium), and Pol2ra and Ubc were chosen as reference genes in the brain samples. In a pilot study, TaqMan Mouse immune panel was analyzed in 20 samples. For the main study, a Custom TaqMan array micro fluidic card (Life Technologies) was designed with 44 target genes suitable for either brain or lung tissue (List of assays in Supplementary Table S1 online), three reference genes, and one manufacturer control, in eight replicates on the 384 well card. Lung results will be presented in a separate article. Gene expression changes were evaluated using the comparative C_T method of relative quantification (2^{-ΔΔCT}). Eighty-four samples were analyzed with the Custom TaqMan array. The mice were sacrificed either right after completed reoxygenation (0 h), or 2.5, 5, or 72 h after completed reoxygenation (Figure 1, C *n* = 5, H21 *n* = 8, and H60 *n* = 8 at all time points). The 2.5 and 5 h time points were chosen to examine the gene expression changes in the window between first and second energy failure (42). PCR evaluation determined one extreme outlier in the H60 group at 5 h, which excluded this mouse from statistical analysis.

Protein Analysis

The frozen hippocampus and striatum were homogenized in lysis-buffer (Tris-HCL) containing 1% NP-40 and a protease inhibitor cocktail (Roche diagnostics, Basel, Switzerland) using Omni tip (OMNI International, Kennesaw, GA), and centrifuged twice at 12,000 × *g*, 4 °C, for 15 min. The supernatant was stored in -79 °C until analysis. Protein concentration was measured using BioRad Protein Assay and Multiskan Ascent spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Ten proteins were chosen for analysis (Gene name^b in Table 1) based on the mRNA results. We conducted a pilot of LCN2, and chose time points of organ harvest at baseline, or at 0, 12, 24, or 72 h after completed reoxygenation (*n* = 7, 22, 21, 21, and 21, respectively). The commercially available enzyme-linked immunosorbent assay kits Mouse CCL12 (MCC120, dilution 1:1), Mouse CCL2 (MJE00, dilution 1:1), Mouse CXCL10 (MCX10, dilution 1:1), Mouse IL1B (MLB00C, dilution 1:1), Mouse LCN2 (DY1857, dilution 1:10), Mouse TNF-α (MTA00B, dilution 1:1), and Mouse/rat vascular endothelial growth factor (MMV00, dilution 1:2) from R&D (R&D, Minneapolis, MN) and MT1 (E91119Mu, dilution 1:5), NEIL3 (E82291Mu, dilution 1:5), and STAT3 (E91743Mu, dilution 1:5) from USCN Life sciences (Wuhan, People's Republic of China) were bought and samples analyzed according to the manufacturer's instructions. Three proteins (TNFA, CXCL10, and CCL2) had values below minimum when testing the enzyme-linked immunosorbent assay kits, and were not analyzed on the complete set of samples.

Statistical Analysis

All statistical analyses were performed with PASW statistics 18 (IBM, Armonk, NY). A general linear model was developed with time and reoxygenation as fixed factors, and multiple comparisons were performed with Bonferroni *post hoc* correction. If the interaction between time and reoxygenation was not significant, the interaction was excluded from the final model. Based on line plots of the data, we determined that for some genes, analysis at each time point separately was more suitable. This method was also used if the interaction was significant in the general linear model. Single time point comparisons with three or more groups were performed with One-way ANOVA with *post hoc* Bonferroni correction for normally distributed data, or Kruskal-Wallis one-way ANOVA with *post hoc* Mann-Whitney *U*-test for non-normally distributed data. For two groups, single time point comparisons were performed with Student's *t*-test, or Mann-Whitney *U*-test if the data were not normally distributed. A 95% CI was chosen. We did not correct for analyzing 44 genes. Equal variances were assumed unless Levene's test was significant. All graphs were made in GraphPad Prism 6 (GraphPad, La Jolla, CA).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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