Hypoxia–Reoxygenation Affects Whole-Genome Expression in the Newborn Eye

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PURPOSE. Resuscitation of newborns is one of the most frequent procedures in neonatal medicine. The use of supplementary oxygen during resuscitation of the asphyxiated newborn has been shown to be detrimental to vulnerable tissues. We wanted to assess transcriptional changes in ocular tissue after the acute use of oxygen in the delivery room in a hypoxia–reoxygenation model of the newborn mouse.

METHODS. C57BL/6 mice (n = 57), postnatal day 7, were randomized to receive either 120 minutes of hypoxia, at 8% O2, followed by 30 minutes of reoxygenation with 21, 40, 60, or 100% O2 or to normoxia followed by 30 minutes of 21% or 100% O2. Whole ocular homogenates were analyzed by Affymetrix 750k expression array, and RT-PCR was performed for validation. Bayesian analysis of variance for microarray data (BAMarray) was used to identify single significant genes, and Gene Set Enrichment Analysis (GSEA) was applied to reveal significant pathway systems.

RESULTS. In total, ~92% of the gene expression changes were altered in response to reoxygenation with 60% or 100% O2 compared to expression at the lower percentages of 21% and 40%. After 100% O2 treatment, genes involved in inflammation (Ccl12, angiogenesis (Igfr1, Stat3), and metabolism (Hk2) were upregulated. Pathway analyses after hypoxia–reoxygenation revealed significant alterations of six pathways which included apoptosis, TGF-beta signaling, oxidative phosphorylation, voltage-gated calcium channel complex, mitochondrion, and regulation of RAS protein signal transduction.

CONCLUSIONS. Hypoxia–reoxygenation can induce immediate transcriptional responses in ocular tissue involving inflammation, angiogenesis, energy failure, and Ras signaling.

Keywords: gene expression, hyperoxia, hypoxia, resuscitation

Perinatal asphyxia introduces global hypoxia-ischemia that mainly damages the central nervous system (CNS) and leads to different degrees of neurological injuries, including ischemic proliferative retinopathy (IPR).1 All parts of the CNS are very sensitive to oxygen deprivation, and in the eyes, the inner layer of retina seems to be particularly sensitive to oxygen fluctuations which can lead to various degrees of retinopathy. Retinopathy of prematurity (ROP) resulting from IPR can cause both visual impairment and blindness.2 Resuscitation of the newborn at birth with 100% oxygen is known to increase the oxidative burden, with concomitant deleterious effects.3 The latest International Liaison Committee on Resuscitation (ILCOR) guidelines state that for term and near-term babies “it is best to start with air rather than 100% oxygen” and “that supplementary oxygen should be guided by pulse oximetry.”4 The optimal fraction of inspired oxygen (FiO2) to start with for the preterm neonate is still being discussed, but it is recommended to avoid “both hyperoxemia and hypoxemia.”4

Our aim in this study was to mimic a hypoxia–reoxygenation event that could be part of perinatal asphyxia and to study possible early transcriptional changes introduced to hypoxia-immature5 ocular tissue after different oxygen therapies. Premature newborn infants exposed to hyperoxia following neonatal intensive care are susceptible to ROP.6 The mouse model of oxygen-induced retinopathy (OIR) developed by Smith et al.7 together with the rat model of fluctuating oxygen concentration (50:10 OIR) developed by Penn et al.8 are the most frequently used models for studying ROP. Several microarray gene expression studies have been conducted by other investigators to explore the profile of hyperoxic and hypoxic retinas9 and to differentiate gene expression in vulnerable regions of the retina,10 as well as between strains.11,12 We recently published data from patients with and without ROP, identifying differentially expressed genes in ROP at day 5, as well as 2 and 4 weeks after birth.13 Until now, focus has mostly been on the oxygen level delivered beyond the delivery room as the risk factor for ROP. In contrast, the importance of
exposure to hyperoxia in the delivery room influencing the development of ROP has recently been emphasized in clinical trials.14,15 In the meta-analysis and systematic review by Brown et al.,14 there was no difference in ROP regardless of whether resuscitation was started with a low or a high fraction of FiO₂. Still, there is a lack of studies aiming at the acute delivery room oxygen treatments’ effect on the newborn eye. We therefore conducted an exploratory hypoxia–reoxygenation study in order to screen vulnerable hypoxia-sensitive tissues like lung,16 brain,17 and ocular tissue of the newborn. Moreover, using the whole eye allowed us to identify signaling contributions from the retina and vitreous body and immune and glial cells. We hypothesized that reoxygenation with high FiO₂ would induce different gene expression patterns and activate different signaling pathways compared to those induced when low FiO₂ treatment was applied. This could be a more specific approach to investigate whether the oxygen concentration per se contributes to upregulating or downregulating genes and initiation of more complex signaling systems immediately after delivery room management.

METHODS

Animals

All experiments were approved by the Norwegian Animal Research Authority. The animals were cared for and handled in accordance with the European Guidelines for Use of Experimental Animals by Federation of European Laboratory Animals Science Association researchers and in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 mice were received from the Taconic Facility in Tornbjerg, Denmark. Mice were stabled and bred at 24°C on a 12L:12D cycle with access to a diet of pellets and water ad libitum. All experiments were performed on postnatal day 7 (P7). The day of discovery was counted as day 1.

Hypoxia and Reoxygenation Model

Custom-made Lucite chambers were used for hypoxia and reoxygenation experiments. Hypoxia was performed with premixed gas: 8% oxygen balanced in nitrogen (Yara, Oslo, Norway). Following hypoxia, reoxygenation with different FiO₂ levels (0.21, 0.40, 0.60, and 1.00) was performed simultaneously in separate chambers. During reoxygenation, FiO₂ was monitored with conventional oxygen mixers. Atmospheric pressure was maintained at a constant level by a continuous supply of 0.5 L/min and open outlets. CO₂ concentrations were <0.3%. The temperature in the chambers was kept constant at 35°C ± 0.5°C by floor heating, and humidity was maintained at 40% to 50% with an open water source. Pups were separated from their dams throughout the experiment. Mice were not anesthetized or restrained.

Microarray Experiment

Sixty-seven P7 mice from 10 different litters were randomized to undergo hypoxia or normoxia for 120 minutes. Eight animals died during hypoxia, and two were excluded due to technical problems. Among the 57 included mice there were no significant differences in weight (hypoxia, 3.9 ± 0.4 g, n = 39; versus normoxia 3.9 ± 0.3 g, n = 18) or sex between the groups. The hypoxia group (H) was further randomized to undergo subsequent reoxygenation for 30 minutes with FiO₂ at 0.21 (H21, n = 10), 0.40 (H40, n = 9), 0.60 (H60, n = 9), or 1.00 (H100, n = 11), whereas the normoxia group (controls [C]) was randomized to undergo either FiO₂ at 0.21 (C21, n = 9) or 1.00 (C100, n = 9; Fig. 1). Following 150 minutes of survival in air after hypoxia–reoxygenation, mice were decapitated and rapidly dissected on ice. The brain was removed from the skull, and the whole eye was dissected free before being subsequently preserved in RNA later stabilization reagent at −20°C (Qiagen, Valencia, CA). RNA extraction was performed as described previously.16

Microarray Analysis

One hundred nanograms of total RNA was used for each of the 57 microarray experiments. Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA) is composed of 750,000 unique 25-mer oligonucleotide features constituting over 28,000 gene-level probe sets, and microarray analysis was performed according to the manufacturer’s protocol (GeneChip Whole
Primer Design and Real-Time RT-PCR

RT-PCR was performed with the 37 samples (H21, n = 6; H40, n = 6; H60, n = 7; H100, n = 10; C21, n = 8) that had sufficient cDNA for the analysis to verify the differentially expressed transcripts detected by microarray. Primers were designed for each transcript (Casp1, Ccl12, Il118, M12, Osmr; and Stat3) using Primer Express 3.0 software (Applied Biosystems, Inc., Grand Island, NY; see Supplementary Table S1). Total mRNA was extracted, reverse transcribed, amplified, and analyzed as described previously. Briefly, RT-PCR was performed with 50 ng of cDNA and 400 nM of primers for the target genes and reference gene peptidylprolyl isomerase A (PPIA). Data were analyzed by the comparative cycle threshold (CT) method of relative quantification (RQ; $2^{-\Delta\Delta CT}$). Ct values of the target transcripts were normalized to Ct values of the PPIA gene in the same sample, resulting in $\Delta CT$ sample. $\Delta CT$ calibrator was calculated from the average $\Delta CT$ in the nine controls in the C21 group. RQ was defined as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ sample $- \Delta CT$ calibrator.

Statistical Analysis

Microarray data were preprocessed using R software Robust Multiarray Average (RMA; Bioconductor) as previously described. In RMA, raw intensity values are background corrected, log2 transformed, and then quantile normalized before an additive linear model is applied to the normalized data. The result is an expression measure for each probe set on each array. Arrays were preprocessed together to ensure comparability across conditions.

The Bayesian analysis of variance for microarray data (BAMarray) method was used to identify differentially expressed genes as previously described. This method balances the number of falsely detected genes and falsely nondetected genes, that is, it controls both the false discovery rate (FDR) and the false nondiscovery rate. The cut-off value that results in an optimal balance is determined automatically from the data, contrary to most other software that requires the users to specify a cut-off value for the test statistic. In BAMarray, the expression values for all probe sets are taken into account, resulting in a list of differentially expressed probe sets or genes as indicated by their known gene symbol. To reduce the number of genes to be further studied, cut-off criteria were, as previously, set at a fold-change (FC) $\geq$ 1.2 or $<0.8$. To compare the number of significantly differentially expressed genes across various group comparisons, we used the binominal test (function binom.test) in R software (available in the public domain at http://www.r-project.org), in which the number of genes on the array was treated as the number of trials and the number of significant genes as the number of successes ($P$ values for each comparison can be found in Supplementary Table S2).

In the cluster analysis, genes were clustered according to similarity in expression values. The genes significantly expressed in at least one of the four hypoxia groups versus controls (C21) were clustered by their average FC by using Pearson correlation as the distance measure.

As previously, Gene Set Enrichment Analysis (GSEA) was applied. GSEA is a method for determining whether a set of a priori defined gene sets is significantly enriched in one group compared to another. GSEA analysis was performed for the probe sets with known gene symbols only, and the median of the probe set intensities was used for probes sharing gene symbols. The gene sets were selected from among the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases of gene sets available in the Molecular Signatures Database (MSigDB version 2.5). A weighted Kolmogorov-Smirnov-like statistic is calculated for each gene set, and statistical significance is assessed by permutation tests. To adjust for multiple hypotheses testing when several gene sets are considered, the FDR is controlled using the FDR $q$ value of $<0.15$ as the cut-off criterion.

Pearson correlation was used to examine the correlation between RT-PCR and microarray results, using FC values relative to the mean values of the C21 group for each individual. Prism version 6.01 software (GraphPad, San Diego, CA) was used for calculations and designing graphs.

All microarray data are minimum-information-about-a-microarray-experiment-compliant, and the following link was created to allow review of the data in Gene Expression Omnibus (GSE32500).

RESULTS

Microarray Analysis

In whole-ocular homogenates, 1374 of the 34,670 probe sets on the microarray chip were found to be differentially expressed in the four hypoxia–reoxygenation groups (H21, H40, H60, H100) compared to controls (C21). Among the 24,674 probe sets with known gene symbols, 916 genes were differentially expressed compared to controls (C21). With a further cut-off FC limit of $\geq$1.2 or $\leq$0.8, the number was reduced to 60, and genes were distributed as follows in the different groups: H21, 8%; H40, 0%; H60, 42%; and H100, 50% (Fig. 2a). $P$ values for the different comparisons and a complete list of genes with FC $\geq$ 1.2 or $\leq$0.8 are listed in Supplementary Table S2 and Table S3, respectively.

Compared to reoxygenation with air (H21), the three groups receiving supplemental oxygen had differential expression of 1353 of 34,670 probe sets. A total of 823 of the probe sets had known gene symbols, and with the cut-off described above, 70 genes remained with the following distribution: H40, 5.8%; H60, 55.1%; and H100, 39.1% (Fig. 2b, and see Supplementary Tables S2 and S4). Hypoxia induced $\sim$5.5 times more genes with differential expression when followed by supplemental oxygen (H100 versus C100, respectively) than by air (H21 versus C21, respectively; Fig. 2c, and see Supplementary Tables S2 and S5).

Hypoxia per se (C100 versus C21) induced $\sim$5 times more differentially expressed genes than those seen after 120 minutes of hypoxia (H21 versus C21) in this model (Fig. 2d, and see Supplementary Tables S2 and S6).

Identification of Unique and Jointly Expressed Genes

Comprehensive analyses of the differentially expressed genes compared to control revealed overlapping results between the hypoxia–reoxygenation groups H60 and H100 and the hypoxia-per-se group C100. Genes of interest were selected based on their pivotal functions and FC $\geq$1.2 or $\leq$0.8; Fig. 3). The number of genes that were significant in two or more comparisons ranged between 20% and 28% of the number of genes significant in each comparison separately. The H21 and H40 groups did not have any significant genes in common with each other or with the other hypoxia–reoxygenation groups (data not shown).
Cluster Analysis

The expression patterns of transcripts that showed significant changes in at least one of the hypoxic groups compared to controls (C21) were hierarchically clustered. The heat-map shows two major clusters consisting of the up- and downregulated genes. The hypoxia–reoxygenation groups H60 and H100 revealed several smaller clusters together in the heat-map (Fig. 4).

Pathways and Functional Categories

GSEA was performed with the preprocessed and normalized data independently of the BAMarray analysis and revealed six significantly altered pathways on a priori-selected gene sets with a chosen cut-off FDR q value of <0.15 in six different comparisons (Table). The genes of each of the significant pathways are listed in Supplementary Tables S7 through S12.

<table>
<thead>
<tr>
<th>Database</th>
<th>Pathway</th>
<th>Statistical Comparison</th>
<th>FDR q Value†</th>
<th>NES‡</th>
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<td>KEGG</td>
<td>Apoptosis</td>
<td>H21 vs. C21</td>
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<td>Oxidative phosphorylation</td>
<td>H60 vs. C21</td>
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<td>TGFbeta signaling pathway</td>
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<td></td>
<td>Voltage-gated calcium channel complex</td>
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<tr>
<td></td>
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<tr>
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<td></td>
<td>H100 vs. C21</td>
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<td>1.52</td>
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</tbody>
</table>

Results in boldface type are close to significant limitations of FDR q values < 0.15.

* Statistical comparison of group pairs.
† False discovery rate q value.
‡ NES, normalized enrichment score.
Validation of Microarray Results

To validate the microarray data, six transcripts were analyzed with RT-PCR in 37 of the samples as those used in the microarray study (Fig. 5). The Pearson correlation coefficient \( r \) for the transcripts and corresponding \( P \) values ranged between 0.38 and 0.94 \( r \) and from \(< 0.001\) to 0.04 \( P \).

DISCUSSION

Our aim was to mimic a hypoxia–reoxygenation event that could be part of a perinatal asphyxia and to study early transcriptional changes introduced to hypoxic ocular tissue after different FiO\(_2\) oxygen therapies. For this purpose, we performed microarray analysis in whole ocular homogenates to determine transcription activation or repression in a mouse model of hypoxia–reoxygenation at P7, approximating the late preterm in brain development\(^{18}\) and immature eye development.\(^5\) The present hypoxia model resulted in physiological stress, revealed by significant metabolic acidosis\(^{16}\) and cardiac and respiratory changes which were in line with the definition of intrapartum fetal asphyxia.\(^9\) The main finding of this study is that, in the whole eye, a considerably higher number of expressed genes were induced when hypoxia was followed by 30 minutes of hyperoxic reoxygenation with 60% or 100% O\(_2\) than when lower percentages of O\(_2\), 21% and 40%, were used during reoxygenation. Indeed, \(~92\%\) of the gene expression changes were observed in the two high-FiO\(_2\)-oxygenation treatments. Specifically, pathway analyses after hypoxia–reoxygenation revealed significant alterations in six pathways involved in apoptosis, differentiation and proliferation (TGF-beta signaling), energy failure (oxidative phosphorylation), membrane Ca\(^{2+}\) transport, and mitochondrial and RAS-signaling. Validation of the microarray results showed significant correlation with results obtained by RT-PCR, which supports the method for studying gene expression in the current model.

A number of studies have shown that exposure to hyperoxia in a resuscitation situation during the first hour of life may lead not only to increased mortality\(^3\) but also to long-term morbidity of susceptible organs like brain\(^{20}\) and lungs\(^{21,22}\) and even malignancies in childhood.\(^{23,24}\) However, no increased risk of ROP has so far been found after exposure to hyperoxia during resuscitation post partum.\(^14\) Different experimental perinatal asphyxia models have shown both features of ROP\(^1\) and no ROP\(^2\) post partum. However, there are both clinical and experimental studies which indicate a strong genetic susceptibility to ROP.\(^26\) The nature of the present study was mainly explorative, and gene expression in the whole eye was analyzed. Whereas different parts of the eye have different metabolic demands and susceptibility to oxygen supply (hypoxia–hyperoxia),\(^27,28\) the retina is at particular risk to hypoxic damage. Exposure to hypoxia will increase the burst of reactive oxygen species (ROS) in the newborn, and even higher levels are seen after hyperoxia during resuscitation.\(^29\)

Oxidative stress plays an important role in the onset and progression of damage to different eye structures. ROS is the most relevant free radical in eye disease and affects the cell integrity because DNA, proteins, and lipids are damaged during this process.\(^30\) The ocular tissue has a protective function against oxidative damage,\(^30\) but the immature antioxidant state of the premature newborns make them especially vulnerable.\(^51\) Oxidative stress has long been implicated in the development of ROP by generating excessive amounts of ROS due to a fluctuating oxygen tension, unstable metabolism, and high
concentration of polyunsaturated fatty acids in the photoreceptors. Signaling pathways like apoptosis and angiogenesis are triggered by oxidative stress, possibly explaining the pathologic features of vascular development in ROP. Several studies of hypoxia and hypoxia-reoxygenation also show tight connections to accumulation of oxidative stress, thereby initiating both gene and protein expression as well as metabolites in different eye structures.

**Effect of Hypoxia–Reoxygenation on the Genome**

Ocular tissue is capable of inducing protective mechanisms such as glycolysis, vasodilation, angiogenesis, and induced erythropoietin transcription early after a hypoxic-ischemic condition, but despite early damage-limiting mechanisms, cell death and tissue damage can still occur later. In the current study, the BAMarray analysis provided a gene profiling pattern 150 minutes after hypoxia–reoxygenation, showing that ~92% of the significantly differentially expressed genes within the groups reoxygenated with 60% and 100% O2. Various soluble factors enter under hypoxic conditions the vitreous cavity by secretion, including cytokines, chemokines, and growth factors. The low number of transcriptional changes after 21% and 40% reoxygenation can be explained by the rather modest hypoxia applied in the present model. The use of 100% oxygen after hypoxia can exacerbate the damage in the CNS. Herein, both chemokine (C-C motif) ligand 12 (Ccl12; inflammation), hexokinase 2 (Hk2; glycolysis), the NMDA2A glutamate receptor (Grin2a; excitotoxicity), the signal transducer and activator of transcription 3 (Stat3), and insulin-like growth factor I receptor (Igfr1; angiogenesis) were upregulated after 100% O2 during reoxygenation, in line with other studies.

**Pathway Analysis**

Pathway analyses performed by using GSEA independently of the BAMarray analyses found several interesting pathway systems. Apoptosis and voltage-gated calcium channel complex were both initiated by hypoxia when followed by room air. Apoptosis is a physiological process in the developing CNS but can increase after ROS attack, causing a delayed vascular development in the retina. Activation of glutamate receptors after hypoxia depolarizes the membranes and increases intracellular calcium levels. Ca2+ overload can play an important role in neuronal death because it stimulates the activation of proteases, nucleases, and lipases, all of which harm cellular constituencies, further generating ROS and causing mitochondrial failure, which secondarily leads to energy failure. Ca2+ influx via voltage-dependent Ca2+ channels is also of great importance in this process, as reviewed by Kaur et al.

After reoxygenation with 60% O2, both mitochondrion and oxidative phosphorylation pathways were repressed. Both hypoxia and ischemia may lead to inadequate supplies of glucose and oxygen, followed by dose-dependent depletions of high-energy phosphates. Our group has shown that high

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**FIGURE 4.** Cluster analysis is shown. Significantly, differentially expressed transcripts were clustered according to similarity in FC expression values in the four hypoxia groups versus controls (C21). The color key indicates the degree of regulation in the range of the given values: red, upregulation; and blue, downregulation. Major clusters of up- and downregulated genes are clearly observed. Several minor common clusters are revealed in the two hyperoxic groups H60 and H100, indicating a similar gene expression pattern in these groups compared to H21 and H40.
FIGURE 5. Validation of microarray results by RT-PCR is shown. Relative gene expression (RQ) signal levels are shown of six selected transcripts determined by microarray analysis (black bars) compared to levels determined by RT-PCR (white bars). Data are mean FC, and error bars are SEM for each hypoxia group (H21, H40, H60, H100) compared to controls (C21, dotted line). Pearson correlation coefficient (r) and corresponding P values are calculated for each gene *Ccl12* (r = 0.70, P < 0.0001), *Mt2* (r = 0.94, P < 0.0001), *Stat3* (r = 0.38, P = 0.04), *Osmr* (r = 0.43, P = 0.019), *Casp1* (r = 0.73, P < 0.0001), and *Il18* (r = 0.79, P < 0.0001).
percentages of supplemental oxygen during resuscitation impairs the energy production through suppression of the oxidative phosphorylation observed after a hypoxic insult. \(^{15,44}\) The Ras/Raf/MEK pathway is a key regulator for cell growth in all eukaryotic cells and has a central role in signal transduction responding to extracellular stimuli like growth factors and cytokines. \(^{35}\) After reoxygenation with both 60% and 100% \(\text{O}_2\), an upregulation of Ras protein signal transduction was observed. Ras signaling pathway can promote both cell death and cell survival pathways, whereas the detrimental effects of downstream targets of Ras are involved in the initiation and/or progression of human malignancies. \(^{45}\) The benzoquinoid antibiotic 17-allylamino geldanamycin (17-AAG) used in cancer treatment inhibits the Ras/Raf/MEK and PI3-kinase signaling pathways and downregulates VEGF expression. In an ROP treatment inhibits the Ras/Raf/MEK and PI3-kinase signaling pathways and downregulates VEGF expression. In an ROP model, 17-AAG reduced angioproliferative retinopathy without changing mRNA levels of VEGF, probably by inhibiting tyrosine kinases through the Ras/Raf/MEK pathway. \(^{46}\)

**Effect of Supplemental Oxygen on the Genome**

When we compared the three groups receiving supplemental \(\text{O}_2\) with the group reoxygenated with room air, as much as 94% of the differentially expressed genes were in the groups reoxygenated with 60% or 100% \(\text{O}_2\). Pathway analyses revealed that only one pathway in these comparisons, namely transforming growth factor-beta (TGF-beta) signaling pathway, was upregulated after using 60% \(\text{O}_2\). This pathway is involved in a variety of cellular processes such as apoptosis, differentiation, and proliferation, mostly through activation of transcription factors like Smad2 and Smad3. \(^{47}\)

**Limitations**

Herein we studied the whole eye, thereby allowing us to identify signaling contributions deriving both from the vitreous body and retina, as well as from immune and glial cells. This will, of course, not provide the fraction of contribution from the specific parts of the eye in gene expression. Knowledge of gene expression from the whole eye provides hypothesis generation for further functional analyses of specific ocular parts after hypoxia–reoxygenation. Moreover, a temporal profile of the gene expression pattern will also add information about long-term consequences of this acute stimulus.

**CONCLUSIONS**

A global profile of gene expression after hypoxia–reoxygenation in the ocular tissue of the newborn mouse indicates an increased activation of the transcriptome when supplemental oxygen is applied after hypoxia. Exposure to 100% \(\text{O}_2\) during reoxygenation uniquely upregulates genes involved in inflammation, angiogenesis, and glycolysis. In general, the hypoxia–reoxygenation model induces several pathways connected to the development of ROP like apoptosis, energy failure, and Ras signaling, raising questions as to what extent the oxygen treatment in the delivery room can affect the ocular tissues’ susceptibility to develop ROP in the long-term. Further studies are needed to address both the expression in specific parts of ocular tissue and the temporal profile of the gene expression patterns after hypoxia–reoxygenation.

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