The Biochemical Mechanism of Dimethyloxalylglycine in the Prevention of Retinopathy of Prematurity

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The Biochemical Mechanism of Dimethylxalylglycine in the Prevention of Retinopathy of Prematurity

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Abstract

Retinopathy of prematurity (ROP), a retinal disease characterized by neovascularization and vasoobliteration, is becoming more prevalent in premature infants as technological advances succeed in better sustaining the life of infants that are the result of premature birth. Dimethyloxalylglycine (DMOG) has been proven to lessen the severity of the symptoms of ROP in mice through the inhibition of prolyl hydroxylase, an enzyme crucial to the degradation of hypoxia inducible growth factor (HIF). The identification of DMOG as a potential novel therapeutic treatment for ROP has brought the mechanism by which DMOG functions into question. To further define this mechanism, wild-type (WT) and liver HIF1α knockout mice (LHKO) were injected with DMOG and the subsequent expression of target genes in the liver, kidney, and retina were measured at varying time points. Notable results include the upregulation of erythropoietin (EPO) and angiopoietin-like 3 (ANGPTL3). Such upregulation proves that EPO and ANGPTL3 should be considered a key target in future efforts to prevent the formation of ROP. Furthermore, these results suggest that EPO and ANGPTL3 play a role in protecting the retina from damaging hyperoxic conditions. It is unclear, however, how EPO and ANGPTL3 directly affect the retina, making it worthy of being studied further. This work serves as a solid foundation for future examination of the biochemical mechanism of DMOG in the treatment of ROP.

Introduction

Dr. Theodore L. Terry first reported retinopathy of prematurity in 1942 in the American Journal of Ophthalmology as retrolental fibroplasia. Since that time, the scientific community...
has expounded on this disease, coining it as retinopathy of prematurity (ROP) while continuing to define the expansive pathology behind this disease. With significant advances having been made in the understanding of preserving premature life, ROP is becoming more prevalent as more premature infants survive. To understand the reasoning behind the prevalence of ROP, it is important to understand the progression of the disease itself.

ROP is defined by two stages characterized by the presence of oxygen and the quality of the vasculature of the retina. Stage one occurs during the period of time in which a premature infant is placed into a hyperoxic environment to sustain his or her life. During this time the excess of oxygen signals still developing tissue, e.g., retina, to cease the production of growth factors previously present in the hypoxic uterus of the mother. Without necessary growth factors, the vasculature of the retina halts in growth, which does not pose any immediate danger. When the infant reaches a stage in growth in which supplemental oxygen is no longer necessary, relative hypoxic conditions develop in the retinal tissue due to the lack of blood circulation. It is important to note that “relative hypoxia” refers to the comparison between the oxygenation in the mother’s uterus and the premature infant, not to the oxygenation levels in a fully developed retina. In the presence of relative hypoxia, the stunted growth of vessels poses a problem. Not enough oxygen is reaching the retina, so the body responds by reactivating pro-angiogenic growth factors. These growth factors cause neovascularization to occur in the retina. Neovascularization produces weakened vessels incapable of sustaining sufficient blood flow. The subsequent weakening of vessels ultimately leads to the formation of scaring and in extreme cases a detached retina and blindness. In short, stage one is characterized by stunted growth in relative hyperoxic conditions, whereas stage II is characterized by excessive, unorganized growth in relative hypoxic conditions.
Experimentation in ROP research has answered many questions about the mechanisms of this disease, but still has failed to define a definitive cure. Early studies attempted to regulate and lower oxygen concentrations within the oxygen hoods of premature infants to mimic prenatal hyperoxic conditions, to great success in preventing ROP\textsuperscript{3}. However, physicians worried that such oxygen levels contributed to the mortality of premature infants, and such an approach was abandoned. Since that time, treatment has been focused on post-diagnostic methods, or the management of the disease after it has developed. Such treatments are invasive and do not guarantee full functionality of the retina. Those infants surviving with eyesight intact do not survive without consequences, as corrective lenses will still be required later on in life\textsuperscript{5}. Within the past few years, novel treatments have arisen, each promising more than the past. One study suggest that exposure to 670 nm light prevents the vasoobliteration common to this disease\textsuperscript{6}.

The most promising treatments are those that target the inhibition of prolyl hydroxylase, a molecule that tags hypoxia inducible growth factor (HIF) for degradation in the hyperoxic stage of the disease. Under normal circumstances, HIF places a crucial role in promoting normal retinal growth and angiogenesis. In hyperoxic conditions with excess oxygen, prolyl hydroxylase is active and hydroxylates HIF rendering HIF inactive. When HIF is inactive, blood vessels cease growth causing a cascade of many negative effects ultimately leading to the loss of vision (Figure 1). Thus, inhibiting prolyl hydroxylase helps to maintain higher levels of HIF and prevents the cascade leading to loss of vision. One such inhibitor is dimethyloxalylglycine (DMOG), which actively inhibits prolyl hydroxylase and “tricks” the body into maintaining hypoxic processes, allowing for continued growth of retinal blood vessels, similar to those conditions found in the uterus of the mother. This maintenance of HIF thereby prevents the degradation of blood vessels (vasoobliteration) and the subsequent occurrence of the rapid
unorganized growth of vessels (neovascularization), traits that characterize ROP. This approach is beneficial because it allows for the prevention of the disease and the maintenance of standard care for premature infants.

Figure 1: HIF Cascade Under Normal Gestation and Premature Conditions

It has already been established that DMOG is effective in the prevention of neovascularization and vasoobliteration. In the mouse model of ROP, commonly referred to as oxygen-induced retinopathy (OIR), WT mice exposed OIR-inducing conditions exhibited neovascularization and vasoobliteration (Figure 2B). However, WT mice exposed to OIR-inducing conditions and treated with DMOG injections exhibited healthy vascularization (Figure 2C). Figure 2 also shows the importance of HIF in protecting the retina from the destructive effects of OIR. Scientific literature suggests that DMOG functions primarily through the liver allowing for many routes of delivery. Thus HIF1α was removed from nearly 80% of the hepatocytes in the liver to create the LHKO mouse. As a result, DMOG lost its ability to protect the retina from OIR-inducing conditions as neovascularization and vasoobliteration occurred (Figure 2F).
Figure 2: Retinal flat mounts of WT and LHKO mice exposed to room air (RA) [A, D] or OIR-inducing conditions [B, C, E, F].

Scientific literature has already established the importance of HIF in the maintenance and normal growth of the undeveloped retina. However, it currently not understood which genes or molecules HIF activates or deactivates in order to have vasoprotective effects of the retina. Thus the aim of this research is to establish which genes were upregulated in response to the injection of DMOG in both the WT and LHKO mouse. Scientific literature suggests that erythropoietin (EPO) plays a crucial role during hypoxic conditions in the body and has the potential to promote angiogenesis\(^2\). This expression during hypoxic conditions contributes to the physiological response of increasing erythrocytes to better circulate oxygen throughout the body. Considering that DMOG inhibits prolyl hydroxylase, thereby mimicking hypoxic conditions in the body it is possible to predict that EPO is crucial to the biochemical mechanism of DMOG.
Other genes of interest included: Chemokine Ligand 16 (CXCL16), Vascular Endothelial Growth Factor B (VEGFB), Mannose Receptor C – Type 1 (MRC1), Vascular Endothelial Growth Factor A (VEGFA), Angiopoietin Like 3 (ANGPTL 3), Insulin-like Growth Factor-1 (IGF-1). The previously mentioned genes were identified as genes of interest based on a previously conducted Genechip microarray. A Genechip microarray measured the differentially expressed genes within the liver 6 hours after the administration of DMOG. CXCL16 is thought to be chemokine that influences angiogenesis. VEGFA promotes vascular growth through increasing endothelial cell permeability. VEGFB works by maintaining the newly formed blood vessels created by VEGFA. MRC1 was indicated as upregulated by the gene chip for unknown reasons. Scientific literature suggests that MRC1 is crucial in immune responses. If MRC1 is actually upregulated in response to DMOG injections, it may suggest a possible immunological mechanism. IGF-1 plays a role in childhood growth. Finally ANGPTL3 contains a c-terminal that has been known to function in angiogenesis.

Using reverse transcriptase polymerase chain reactions (RT-PCR), it is possible to determine which of the previously mentioned genes are upregulated in the WT mouse and which genes are unaffected in the LHKO mouse. This information will help to define a mechanism by which DMOG functions to protect the retina from developing ROP.

Methods

Mice Preparation for Retinal Flatmounts. To induce OIR in mice, a protocol previously established by Smith, et. al was followed. The OIR mouse model mimics the formation of ROP in humans. Unlike the fully developed retina of a full-term human baby, the mouse retina in healthy pup is underdeveloped. This allows for the undeveloped retina to be studied without
having to induce early labor in the mother. Additionally, the most notable difference in the mouse model is that vasoobliteration occurs in the central portion of the retina. In ROP, vasoocessation occurs in the peripheral retina. Otherwise, the OIR mouse model is suitable for studying ROP, especially because of its reproducibility and affordability. To develop OIR in mice, C57BL/6J mice pups were exposed to hyperoxic environments (75% oxygen) over the span of time ranging from 7 to 12 days after birth in a Plexiglas incubator with an adjustable oxygen sensor and feedback system (Pro-Ox)\textsuperscript{4}. Mouse pups were injected with DMOG or PBS on postnatal day 6 (P6), P8, and P10. On P12 the pups were placed in room air until P17, when the retinas were extracted for analysis. The retinal flat mounts and lectin stains were completed in accordance with a protocol previously established by Connor \textit{et al}\textsuperscript{9}.

\textbf{Mice Preparation for Gene Expression Measurement by RT-PCR.}

Mice included in the study were those of C57BL/6J wild-type (WT) genotype and mice with the Liver HIF1\(\alpha\) gene removed from nearly 80\% of the hepatocytes (LHKO). LHKO mice were bred at the Jackson Laboratory. P8 mice were injected intraperitoneally with 200 micrograms/gram mouse weight of DMOG.

\textbf{Organ Processing and RT-PCR.} After the designated time of 3, 6, or 24 hours after injections, the kidney, liver, and retina were dissected and preserved in Qiagen RNAlater reagent overnight. Organs collected at the 0 hour time point served as a control group and did not receive any injections. Afterward, organs were stored in at -80°C until the organs could be further processed. The collected organs were processed using Qiagen QiaShredder and Qiagen RNeasy MiniKit to isolate RNA from the organs. The RNA was further converted into cDNA utilizing Qiagen
QuantiTect Reverse Transcription Kit. The expression of particular genes was measured using Qiagen QuantiTect SYBR Green PCR kit and Qiagen QuantiTect Primer Assays for respective genes of interest. The genes of interest analyzed included: CXCL16, VEGFB, MRC1, EPO, VEGFA, ANGPTL 3, IGF-1, and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) as an endogenous control. Results were measured and quantified using RQ Manager Software. Statistical significance was measured in excel using the t-test, non-paired, equal variance.

**Results**

Conducting RT-PCR using EPO as the target gene in the liver yielded results show a marked increases in expression levels at all time points in the WT mouse. Similar but muted results were demonstrated in the liver of the KO mouse (Figure 3A).
The gene expression of EPO in the liver of the DMOG-injected WT mice yielded a nearly 200-fold increase at 3 hours. This level of EPO gradually decreased with each successive time point, but still maintained elevated levels compared to those injected for 0 hours. KO mice expressed lower levels of EPO expression than the WT, but still shared a similar gene expression pattern with a peak expression at 3 hours and a successive decrease (Figure 3A). This gene expression in the liver of KO mice compared to WT mice was statistically different at 3, 6, and 24 hours (t-test, non-paired, equal variance at 3-hours: p-value = 0.04, 6-hours: p-value = 0.003, 24-hours: p-value = 0.02. The kidney expression of EPO was less in comparison with the liver (Figure 3B). Additionally, KO mice exhibited similar levels of EPO expression at each time point compared to the WT mice. Maximum expression occurred 5-fold at 3 hours in the WT and KO mice. The retina had the lowest levels of induced expression of EPO (Figure 3C). No difference is
detectable between the WT and KO mice, with peak expression occurring by 1.75-fold at 3 hours post-injection.

When ANGPTL3 was used as the target gene for RT-PCR results indicated that the gene expression in the liver gradually increased in both the WT and KO mice as time progressed (Figure 4a). The levels of expression at 3-hours were not statistically different from the levels of expression at 0 hours (p-value = 0.08). However, both the 6 (p-value = 0.03) and 24-hour time point (p-value = 0.00004) were statistically significant. The p-value was based on the t-test, non-paired, equal variance. This suggests that the gene expression of ANGPTL3 in response to DMOG is not immediate like the expression of EPO. Furthermore, a 5-fold induction occurred at 24 hours in WT and a 4-fold induction occurred in KO mice. The expression of ANGPTL3 in KO mice is muted in comparison to WT. Experimentation concerning the kidney and retina had not been completed by the time of this publication.

![Figure 4](image.png)

**Figure 4:** The levels of ANGPTL3 gene expression in response to DMOG injections in the liver of both WT and KO mice after 0, 3, 6, and 24-hour intervals.

The measured gene expression of CXCL16 in response to DMOG yielded results that
demonstrated that the expression levels appeared to be unaffected by DMOG administration (Figure 5A). Given that the levels of gene expression did not vary after injection with DMOG, KO mice were not examined for CXCL16 gene expression.

![Graph](image.png)

**Figure 5:** The levels of CXCL16 gene expression in response to DMOG injections in the liver of only WT mice after 0, 3, 6, and 24-hour intervals.

The measured gene expression of VEGFB in response to DMOG yielded results that demonstrated that the expression levels appeared to be unaffected by DMOG administration (Figure 6A). Given that the levels of gene expression did not vary after injection with DMOG, KO mice were not examined for VEGFB gene expression. A similar conclusion was reached for both IGF-1 and MRC1 (Figures 7A and 8A, respectively).
Figure 6: The levels of VEGFB gene expression in response to DMOG injections in the liver of only WT mice after 0, 3, 6, and 24-hour intervals.

Figure 7: The levels of IGF-1 gene expression in response to DMOG injections in the liver of only WT mice after 0, 3, 6, and 24-hour intervals.
Figure 8: The levels of MRC1 gene expression in response to DMOG injections in the liver of only WT mice after 0, 3, 6, and 24-hour intervals.

The gene expression of VEGFA in response to DMOG in the liver markedly increased between the 0 and 3 hour time point in WT mice, but gradually decreased as time progressed (Figure 9A). A 3-fold maximal induction occurred at 3 hours. While the expression in both WT and KO mice was evaluated, the gene expression between KO and WT mice at each time point did not differ.
Figure 9: The levels of VEGFA gene expression in response to DMOG injections in the liver of WT and KO mice after 0, 3, 6, and 24-hour intervals.

Discussion

The results of RT-PCR indicated an upregulation of EPO, ANGPTL3, and VEGFA in response to DMOG injections. However, contrary to what was expected, MRC1, IGF-1, VEGFB, and CXCL16 did not have altered expression levels after the injection of DMOG. Even still, such information plays a crucial role in the establishment of the biochemical mechanism of DMOG.

Like humans, EPO expression normally occurs in the kidney of mice. However, experimental results indicated that the levels of expression from the liver were more elevated than the kidney. Using imaging techniques in previous studies, however, it is possible to observe that DMOG functions primarily through the liver, which could serve as an explanation as to why induction was so exponential in the liver. As previously mentioned in the introduction, it is not surprising that EPO expression was markedly increased after the injection of DMOG. This is because EPO is expressed in the body as a response to hypoxic conditions\(^1\). This expression during hypoxic conditions contributes to the physiological response of increasing erythrocytes to better circulate oxygen throughout the body. Considering that DMOG inhibits prolyl hydroxylase, thereby mimicking hypoxic conditions in the body, it serves as no surprise that EPO is released as a response.

EPO gene expression was also upregulated in the KO mice. This raises the question: how is it that EPO expression is still upregulated in the liver even though KO mice retinas cannot be protected by DMOG from the formation of ROP (refer to Figure 1)? While EPO expression is upregulated in the KO mouse, it is not upregulated to the same extent as in WT mice. This difference between the WT and KO mice suggest these varying levels of expression are enough
to make a difference between a healthy retina and one that is plagued by ROP. It is also important to remember that muted levels of gene expression from the KO mice are expected. This is because HIF1α was only removed from 80% of the hepatocytes of the liver. The hepatocytes only account for some of the cells contained in the liver. This means that other liver cells are still capable of producing genes that would function to preserve the retina in WT mice, however the levels in the KO mice are muted enough to be deemed ineffective.

This research establishes the role that EPO contributes after injections of DMOG; however, it is also known that EPO cannot function alone to save the retina from ROP. In previous experiments, mice exposed to OIR and given injections of only EPO still developed ROP. Thus, it is believed that while DMOG induces heightened expression of EPO, other molecules are crucial in the preservation of the retina. This research helped to establish that IGF-1, CXCL16, MRC1, VEGFA, and VEGFB do not work alongside DMOG to achieve a vasoprotective effect. However, the effect of ANGPTL3 is worth being investigated further.

ANGPTL3 is known for being expressed mainly in the liver. Thus, the expression of this gene from the liver is expected. Additionally, scientific literature suggests that ANGPTL3 functions to promote angiogenesis. Given the increase of ANGPTL3 in response to DMOG it may be possible that ANGPTL3 works alongside EPO, in promoting angiogenesis thereby preventing the progression of ROP in WT mice. Furthermore, the muted response from KO mice may suggest that lower levels of ANGPTL3 compared to the WT may account for the lost ability of the KO mice to preserve the retina. To further establish the role that ANGPTL3 may play in biochemical mechanism of DMOG, it is important to complete the RT-PCR results for both the kidney and retina.
While these results suggest that EPO and ANGPTL3 play a role in protecting the retina from damaging hyperoxic conditions, it is still unclear, however, how EPO and ANGPTL3 directly affect the retina, making it worthy of being studied further. This work serves as a solid foundation for future examination of the biochemical mechanism of DMOG in the treatment of ROP.

**References**


7. G. Hoppe & J. Sears, unpublished data
