THESIS APPROVED BY

4/30/2020
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RESPIRATORY BIOMARKERS IN Kv1.1 KNOCKOUT MICE, A MODEL FOR TEMPORAL LOBE EPILEPSY

By

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A THESIS

Submitted to the faculty of the Graduate School of Creighton University in Partial Fulfillment of the Requirements for the degree of Master of Science in Neuroscience.

Omaha, NE

May 16, 2020

ABSTRACT

Rationale: Approximately 1:26 people have epilepsy and around 30% of those cannot effectively treat their seizures with medications, putting them at risk for Sudden Unexpected Death in Epilepsy (SUDEP). We recently demonstrated the progressive nature of epilepsy and mortality in Kv1.1 knockout (KO) mice, a model of temporal lobe epilepsy and SUDEP. We reported respiratory abnormalities in KO mice using the methacholine (MCh) challenge, indicating that increased breathing rate, apnea and respiratory failure is associated with SUDEP. We have also found that the metabolic therapy, ketogenic diet (KD), significantly reduces seizures and prolongs lifespan in KO mice. Markers of inflammation activated in toll-like receptor 4 (TLR4) pathway are associated with apnea and respiratory failure, therefore we determined whether an inflammatory pathology is apparent in lung tissue in older, KO mice that are at high-risk for SUDEP and wildtype (WT) control littermates treated with either control diet or ketogenic diet (KD).

Hypothesis: Levels of inflammatory markers are apparent in respiratory tissue of high-risk KO mice, and this is attenuated in KO mice treated with the KD.

Methods: Lung tissue was obtained from KO and WT littermates that had been treated with either KD or control standard diet. Immunohistochemistry was performed on alveoli and bronchiole focused regions in the left lobe of 50-μm sections from paraformaldehyde-fixed, 1:1 OTC and 1XPBS inflated mouse lung tissue. Tissue was incubated with either polyclonal primary antibodies against iNOS, MyD88, or NF-kB p65, then with secondary fluorescent antibody, and imaged. Relative arbitrary fluorescent units (RAFU) were quantified using ImageJ software. Differences in fluorescent density and cell number were analyzed using GraphPad Prism software.

Results: KO mice experienced a significant increase in relative florescent units of MyD88, NF-kB p65, and iNOS expression in the bronchiole focused regions. KO showed no difference in MyD88 or iNOS levels in the alveoli focused sections, but NF-kB p65 was elevated. Data from KD groups indicate no differences between WT on a standard diet and WT on a KD. KO on a KD show reduced levels of MyD88, NF-kB p65, and iNOS in the bronchial focused region. In alveolar focused regions, MyD88 and iNOS had a decreased presence in KO and NF-kB p65 values were restored to the WT level.

Conclusion: Data indicates respiratory pathology in KO mice. Increased expression of MyD88, NF-kB p65, and iNOS in bronchiole

focused regions of lung tissue from high risk KO may suggest a greater susceptibility to inflammatory mediated damage and promote respiratory failure when challenged by severe seizures. Treatment with the KD protects against inflammation with reduction in inflammatory markers. Reduced inflammation may contribute to the increased longevity in the KD treated KO mice.

Acknowledgments

I would like to express my sincere appreciation to my advisor, Dr. Kristina Simeone, who guided me and encouraged me to explore various techniques and fields of study. Without her help, I would not have become the scientist that I am today.

I am also extremely thankful for my mother and father. It is whole-heartedly appreciated that your great advice proved monumental towards the success of this study.

Finally, I would like to acknowledge the support of my thesis committee members, Dr. Annemarie Shibata, Dr. Dimitrios Katsavelis, and my second advisor, Dr. Jo Shattuck, as I pursued a novel study for a second thesis that was unable to be completed due to COVID-19: *Kinesthetic Awareness Training and its Role in Movement Pattern Correction*. This second study would not have been possible without the love and support of Justin Wick, who inspired the study.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

Epilepsy is a neurological disorder of seizures, characterized by sudden, recurrent episodes of sensory disturbance, loss of consciousness, or convulsions (Minor & Murray, 1992). Approximately 1:26 people, 50 million globally, have epilepsy (Fisher et al., 2014). Around 30% of epileptic patients cannot effectively treat their seizures with medications, putting them at risk for Sudden Unexpected Death in Epilepsy (SUDEP). Risk for sudden death among refractory patients is 1:150 each year (Thurman et al., 2014). Not fully understood, risk factors for SUDEP include uncontrolled seizures, generalized tonic-clonic seizures, age, and cardiac arrhythmias (Thurman et al., 2014; Auerbach et al., 2013; Ryvlin et al., 2013; Goldman et al., 2009; Forsgren et al., 2005; Naritoku et al., 2003; Walczak et al., 2001). Generalized tonic-clonic seizures, most directly linked to SUDEP, are associated with dysfunctional respiratory response reflexes, lower blood oxygen saturation followed by a period of increased breathing rate, cardiac arrhythmia/bradycardia, apnea, respiratory arrest, and asystole (Ryvlin et al., 2013). We have previously reported that respiratory dysfunction and apnea worsen in high-risk Kv1.1 knockout (KO) mice (Simeone et al., 2018). Here, we tested the hypothesis that pathophysiological markers of lung

inflammation increase as risk for sudden death increases in a pre-clinical SUDEP model. Pathophysiological biomarkers that influence measurable respiratory dysfunction, such as apnea, would allow for refractory epileptic patient status to be monitored and provide an opportunity to intervene with therapies that may postpone or prevent SUDEP.

In this study, we used a mouse model that knocks out the *Kcna1* gene. This gene encodes for the Kv1.1 alpha subunit of the delayed rectifier potassium channel and affects repolarization of neuronal membrane potential. These KO mice create a model for both temporal lobe epilepsy and SUDEP. Onset of epilepsy in this model occurs at postnatal 22-25 days, where mice exhibit spontaneous, recurrent limbic seizures for the duration of their life, with severity and pathology worsening with age (Smart et al., 1998; Moore et al., 2014). During their life KO mice display risk factors for SUDEP, such as generalized tonic-clonic seizures and are the only model with a predictable life span leading to a 100% early mortality rate (Figure 1, from Iyer et al., 2020) (Simeone et al., 2016). The probability of death in this model dramatically increases from 45-65 days postnatal (Figure 1, from Iyer et al., 2020), allowing for the monitoring of physiological biomarkers through the progression of the disorder.

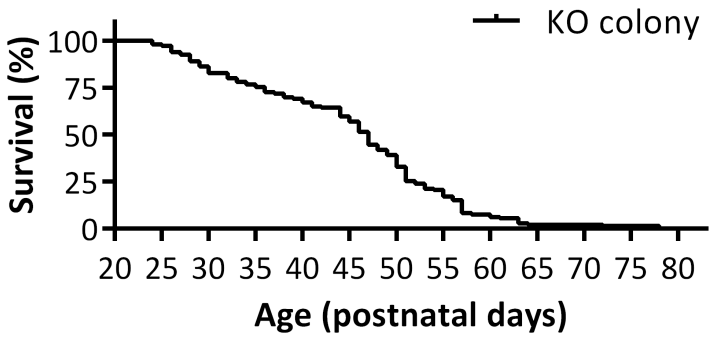

Figure 1. Probability of survival within the KO colony. An updated survival curve of the KO colony indicates the mean age of sudden death occurs at postnatal 45 ± 1 days. There is also a dramatic increase in probability of mortality between postnatal 45-60.

We previously reported respiratory abnormalities in KO mice using the methacholine (MCh) challenge, where mice breathe in nebulized MCh that causes mild narrowing of the lung airways. Our studies indicated that increased breathing rate, apnea and respiratory failure is associated with SUDEP (Simeone et al., 2018). We determined whether an inflammatory pathology is apparent in lung tissue in older, KO mice that are at high-risk for SUDEP and age-matched, wildtype (WT) control littermates treated with either control diet or ketogenic diet (KD). Our first hypothesis examined whether markers of inflammation increased in the lungs of high-risk KO mice. We report that high-risk KO mice have greater levels of markers of inflammation.

In another cohort, we implemented the ketogenic diet (KD), a high fat, low carbohydrate, and average protein metabolic therapy (Figure 3.2A). The KD is the most effective non-surgical treatment for refractory seizures, reducing central inflammation and increasing longevity in the epileptic KO mouse model, and has been found to restore physiologic abnormalities when differences occur between KO to WT littermates (Simeone et al., 2016). Our second hypothesis investigated whether the KD reduces markers of inflammation in high-risk KO lungs. The KD was found to protect against markers of inflammation in high-risk KO mice and did not change the basal presence of inflammatory markers in WT controls.

CHAPTER 2

METHODS

2.1 ANIMALS

Kv1.1 KO mice on a C3HeB/FeJ congenic background were bred and housed in the Animal Resource Facilities at Creighton University School of Medicine (Simeone et al., 2018). The mice were provided food and water ad libitum and kept on a regulated 12-hr light/dark cycle in a pathogen-free environment. The temperature and humidity were controlled to 25°C and 50-60% respectively. Genotype was determined (Transnetyx Inc., Cordova, TN, U.S.A.) and both male and female mice were used in this study. No sex differences were identified. Procedures followed the guidelines of the National Institute of Health, the EU Directive 2010/63/EU and were approved by the Institutional Animal Care and Use Committee at Creighton University School of Medicine.

2.2 KD TREATMENT

KO and WT mice were randomly weaned to either a standard diet or KD at 21 days postnatal. The KD is a 6:3:1 ratio of fat to carbohydrates plus proteins (Bio-Serv F3666, Frenchtown, NJ, U.S.A.). Mice remained on the treatment until 51-55 days postnatal, an age when 70% of the KO colony had succumbed to death

(SD_{70}), when mice were sacrificed and the lung tissue evaluated (Simeone et al., 2016; Simeone et al., 2014; Fenoglio-Simeone et al., 2009; Kim et al., 2015).

2.3 LUNG INFLATION AND SECTIONING

When mice reached SD_{70} (51-55 days postnatal), mice were anesthetized with isoflurane (Patterson Veterinary Supply Inc., Devens, MA, U.S.A.). Then, lungs were inflated using a 1:1 OTC (Neg-50 Thermo Fisher Scientific Ref: 6502) and 1XPBS solution, followed with a flash freeze protocol. The left lobe was separated and sectioned into 50-μm thick sections with the cryostat (Leica Biosystems, Buffalo Grove, IL, U.S.A.) set to -14°C and -16°C for the object temperature and chamber temperature respectively. The first forty sections of the lower bronchial region were designated as “alveolar” focused sections, the middle forty to sixty sections were discarded from use in this study, and the final forty sections of the upper bronchial region were designated as “bronchial” focused sections (Figure 2.3).

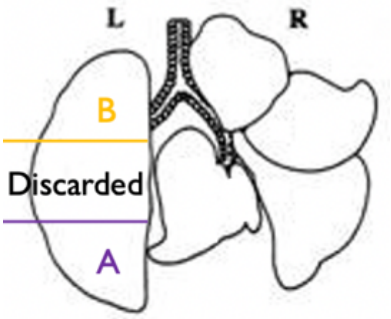

Figure 2.3. Sectioning the left lobe of mouse lung tissue allowed for identification of regional specific pathologies. When sectioning the left lobe of mice lungs, the first forty sections were designated as “alveolar” (A) focused sections, the middle forty to sixty sections were discarded from use in this study, and the final forty sections were designated as “bronchial” (B) focused sections.

2.4 IMMUNOHISTOCHEMISTRY (IHC)

IHC was performed on alveolar and bronchial focused paraformaldehyde-fixed lung tissue. In order to ensure specific binding, Normal Goat Serum (NGS) (Ref: PCN5000, Life Technologies Corp., Frederick, MD, U.S.A.) was used as a blocking reagent for MyD88 (diluted at 5% NGS), NF-kB p65 (diluted at 5% NGS and 1% NGS for the listed primary antibodies respectively), and iNOS (diluted at 1% NGS). To reduce background fluorescence, tissue was then incubated overnight with polyclonal antibodies against MyD88 (diluted at 1:100, ab2064, Abcam, Cambridge, UK),

NF-kB p65 (diluted at 1:50, ab16502, Abcam; diluted at 1:100, NB100-2176, Novus Biologicals, Littleton, CO, U.S.A.), and iNOS (diluted at 1:500, ab15323, Abcam, Cambridge, UK). Tissue was incubated with secondary antibody tagged with Green Fluorescence Protein (diluted at 1:500, A11008, Thermo Fischer Scientific, Waltham, MA, U.S.A.), and double-labeled with DAPI (Ref: H-1500, Vector Laboratories, Burlingame, CA, U.S.A.) as a control.

2.5 FLUORESCENT IMAGING AND STATISTICAL ANALYSIS

Samples were imaged with an Evos fluorescent microscope (Fisher Scientific, Waltham, MA). Relative arbitrary fluorescent units (RAFU), with the background subtracted, were quantified using ImageJ software. Differences in florescent density and cell number were analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, U.S.A.) and florescent values were normalized to cell number. KO samples were normalized to WT mice (SD_{70} age matched). Values are expressed as means ± SEM. Unpaired, nonparametric, Mann Whitney *t*-tests were used to assess statistical significance because KO data sets which do not follow Gaussian distribution.

CHAPTER 3

RESULTS

3.1 AIM 1: KO MICE SHOW INCREASED LEVELS OF PRO-INFLAMMATORY PROTEINS DOWNSTREAM OF TLR4 ACTIVATION

When the toll-like receptor 4 (TLR4) pathway is activated by lipopolysaccharides (LPS), MyD88 functions to stabilize the receptor that leads to the upregulation of transcription factor, NF-kB, and subsequent gene transcription and activation of pro-inflammatory cytokines (such as iNOS), causing inflammation (Figure 3.1A). Here, we determined whether MyD88 and downstream markers of inflammation, NF-kB and iNOS, are increased in either bronchial or alveoli-focused regions of KO lungs at older ages high-risk KO mice (SD_{70}).

Between-group analyses of RAFU indicate SD_{70} KO mice on a standard diet, in comparison to age-matched WT mice, have a significantly higher presence of MyD88 ($p < 0.01$, Mann-Whitney test of Standard Deviation) in bronchiole focused regions (Figure 3.1B). There was no difference found in MyD88 presence ($p = 0.9797$, Mann-Whitney test of Standard Deviation) between SD_{70} KO mice and WT in the alveoli focused region (Figure 3.1C). Similarly, SD_{70} KO also demonstrated an upregulation of NF-kB p65 ($p < 0.001$, Mann-Whitney test of Standard Deviation) in bronchiole focused regions (Figure 3.1B), however, there was

also significantly more NF-kB p65 ($p < 0.001$, Mann-Whitney test of Standard Deviation) in alveoli focused regions (Figure 3.1C). NF-kB p65 is a known activator of cytokines that lead to inflammation. In the alveolar focused regions, an up-regulation of NF-kB signifies greater induction of pro-inflammatory agents (Villapol, 2017; Wieland et al., 2005). The same trend within the bronchiole focused regions continued as downstream iNOS ($p < 0.01$, Mann-Whitney test of Standard Deviation, Figure 3.1B) was also present in increased quantities in the SD_{70} KO mice. Levels of iNOS in SD_{70} KO mice were not increased in alveolar focused regions ($p = 0.1675$, Mann-Whitney test of Standard Deviation, Figure 3.1C. These data suggest that KO mice have greater levels of inflammation in their bronchioles and alveoli when compared to WT controls.

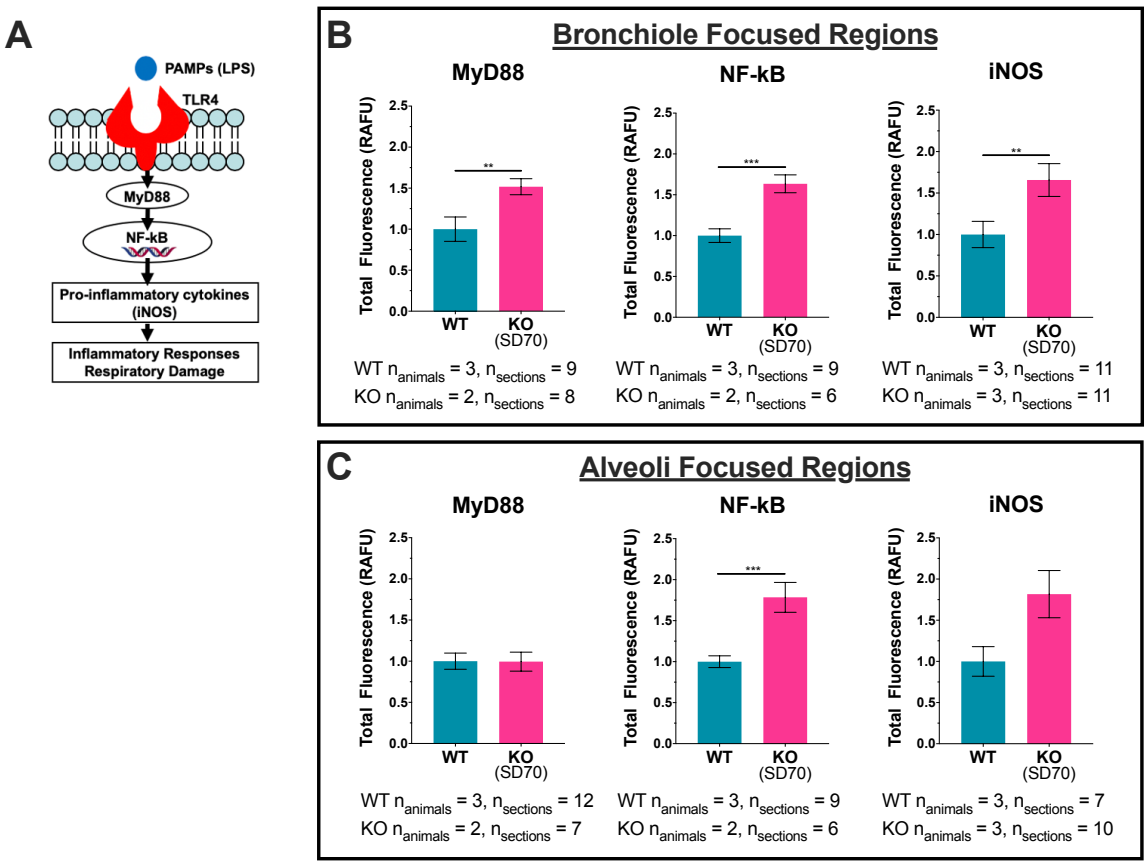

Figure 3.1. Pathological presentation in Kv1.1 KO mice of pro-inflammatory mediators in the TLR4 pathway. (A) LPS, part of the pathogen-associated molecular pattern molecule (PAMP) family, activate the TLR4 pathway. MyD88 stabilizes the receptor and an upregulation of NF-kB occurs. Gene transcription leads to activation of pro-inflammatory cytokines, such as iNOS, resulting in an inflammatory response. (B) Data indicate that SD_{70} KO mice on a standard diet have significantly more presence of MyD88 ($p < 0.01$, Mann-Whitney test of Standard Deviation), NF-kB p65 ($p < 0.001$, Mann-Whitney test of Standard Deviation), and iNOS ($p < 0.01$, Mann-Whitney test of Standard Deviation) in bronchiole

focused regions of the lung than do age-matched WT mice. (C) SD_{70} KO mice on a standard diet do not display differences from WT in presence of MyD88 or iNOS in alveoli focused lung tissue. However, KO mice show increased levels of transcription factor NF-kB p65 in alveoli focused lung tissue, when compared to WT. Data are expressed as mean ± SEM. Black '*' indicates significantly differs from WT * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2 AIM 2: KD PROTECTS AGAINST INFLAMMATION

We determined that the KD reduces MyD88 NF-kB and iNOS in the bronchial focused regions and NF-kB in the alveoli-focused regions of SD_{70} KO lungs. Data were pooled for WT mice on a standard diet and KD as no difference was found between them for either MyD88, NF-kB p65, or iNOS. In bronchiole focused regions, SD_{70} KO on KD (KOKD) displayed a significantly decreased presence of pro-inflammatory proteins: MyD88 ($p < 0.0001$, Mann-Whitney test of Standard Deviation, Figure 3.2B), NF-kB p65 ($p < 0.001$, Mann-Whitney test of Standard Deviation, Figure 3.2B), and iNOS ($p < 0.05$, Mann-Whitney test of Standard Deviation, Figure 3.2B) when compared to WT controls. In the alveolar focused regions, MyD88 was also significantly reduced in KOKD ($p < 0.0001$, Mann-Whitney test of Standard Deviation, Figure 3.2C). KOKD treatment showed a normalization of NF-kB p65 to WT levels in the alveolar region

($p = 0.0631$, Mann-Whitney test of Standard Deviation, Figure 3.2C). KOKD also reduced iNOS expression in the alveoli focused regions ($p < 0.0001$, Mann-Whitney test of Standard Deviation, Figure 3.2C). These data suggest that KD protects against inflammatory mediated damage with reduction of pro-inflammatory protein markers in the lung tissue of high-risk KO mice.

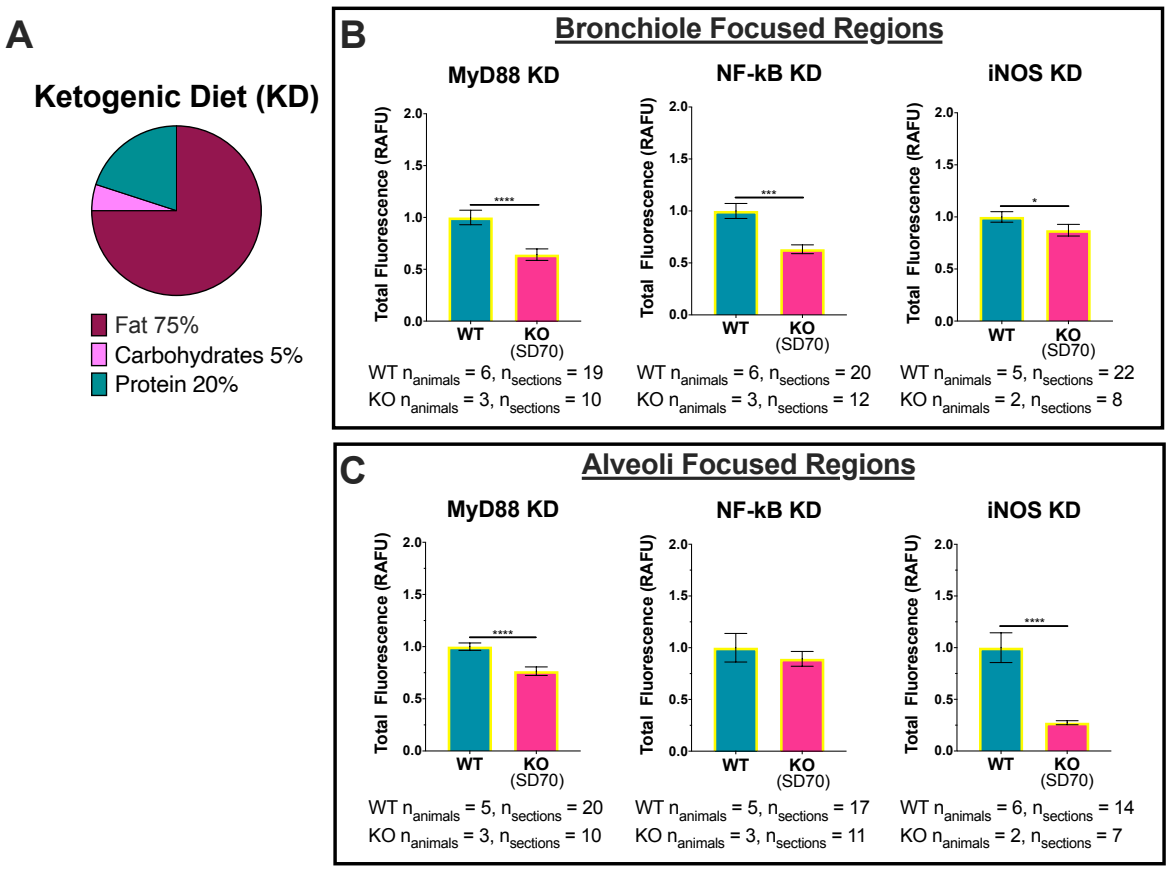

Figure 3.2. KD attenuates pathological presentation of pro-inflammatory mediators. (A) The KD consists of 75% fat, 5% carbohydrates, and 20% protein intake that is administered on a ratio of fats to carbohydrates plus protein. (B) Data are pooled for WT mice on a standard diet and ketogenic diet as no difference was found between them for either MyD88, NF-kB p65, or iNOS. Data indicate that SD_{70} KO mice on KD have significantly less presence of MyD88 ($p < 0.0001$, Mann-Whitney test of Standard Deviation), NF-kB p65 ($p < 0.001$, Mann-Whitney test of Standard Deviation), and iNOS ($p < 0.05$, Mann-Whitney test of Standard Deviation) in bronchiole focused regions of the lung than do age-matched WT mice

on KD. (C) SD_{70} KO mice on KD also display decreased MyD88 ($p < 0.0001$, Mann-Whitney test of Standard Deviation) and iNOS ($p < 0.0001$, Mann-Whitney test of Standard Deviation) when compared to age-matched WT mice in alveoli focused regions of the lung. KO mice on KD show a normalization of NF-kB p65 to the level of WT mice in alveoli focused lung tissue. Data are expressed as mean ± SEM. Black '*' indicates significantly differs from WT * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

CHAPTER 4

DISCUSSION

This study examines the pathophysiological changes that occur in the lungs of aged KO mice that are high-risk for SUDEP. Our results suggest that there is a connection between pro-inflammatory proteins and SUDEP probability, and that the KD protects against pro-inflammatory markers in the lungs.

Lung tissue from KO mice display markers of inflammation associated with the TLR4 pathway (MyD88, NF-kB-p65, and iNOS). Greater levels of inflammation increase the susceptibility of lung tissue to inflammatory mediated damage (Jiang et al., 2015). Through the TLR4 pathway, MyD88 is also linked respiratory disorders outside the field of epilepsy including sleep apnea and an increase in respiratory drive, which is also associated with asthma, fibrosis, COPD, and respiratory failure (Piggott, 2004; Thorburn et al., 2016; Wieland et al., 2005; Yang et al., 2018).

Literature has shown that diets high in fat and carbohydrates are detrimental to lung function, whereas lower carbohydrate diets aid in normal lung function. Diets composed of high fat and high carbohydrate caloric intake have been found to negatively impact airway hyperresponsiveness, a characteristic feature of asthma (Singh et al., 2015). Increased respiratory quotients, hypothesized to increase the work of breathing and

potentially lead to respiratory failure in patients with underlying pulmonary disease, have been shown to decrease with a lower carbohydrate (37% of caloric intake) diet (Suteerojntrakool et al., 1998). Likewise, a low carbohydrate (14% of caloric intake) diet has also demonstrated a reduction in carbon dioxide stores, benefiting patients with increased carbon dioxide arterial pressure due to respiratory failure (Alessandro et al., 2015).

Treatment with the KD differentially influences pro-inflammatory protein markers in KO lung tissue, but not WT control tissue. These data support previous studies which multiple studies indicate the KD reduces inflammation in other models of injury, and lacks effects in control conditions (Masino et al., 2017).

KOKD mice experienced a reduction of pro-inflammatory proteins. Mean age of mortality in the KO mouse model is postnatal 42.8 ± 1.3 days and KD increases the lifespan of these mice by 47% (Simeone et al., 2016). The KD is effective in increasing fatty acids, which lead to an upregulation of PPARγ and subsequent decrease in inflammation (Simeone et al., 2017; Masino et al., 2017; Villapol, 2017). While there is research indicating that the KD reduces central neural NF-kB and iNOS, the effects of the KD in the lung, and more specifically reducing MyD88, NF-kB p65,

and iNOS, were previously unknown (Ju et al., 2020; Lu et al., 2018; Pinto et al., 2018).

Clinical studies provide evidence that respiratory dysfunction, and more specifically, apnea related events are risk factors for SUDEP. A retrospective study monitoring cardiorespiratory function in epileptic patients noted that terminal central apnea preceded terminal asystole in all cases of SUDEP (Ryvlin et al., 2013; Vilella et al., 2019). Following a seizure but prior to SUDEP, patients typically experience a sequence of rapid breathing (18-50 breaths/min), apnea, bradycardia, and terminal apnea (Somboon et al., 2019).

Ictal central apnea, preceding seizure onset, poses a danger when prolonged and is likely caused by seizure discharges in cortical areas of respiration (Lacuey et al., 2019; Vilella et al., 2019). However, it is the postictal apnea, obstructive or central that is associated with greater risk for sudden death (Vilella et al., 2019; Ryvlin et al., 2013; Nashef et al., 1996; Seyal et al., 2010; Lacuey et al., 2018; So et al., 2000). Obstructive sleep apnea is particularly common in adults with epilepsy, occurring twice as often when compared to age-matched control subjects. Incidence of obstructive sleep apnea in epileptic patients also increases with age, however not knowing when SUDEP will occur in humans limits the clinical interpretations as to whether apnea may be a temporal biomarker

for SUDEP (Somboon et al., 2019; Harnod et al., 2017; Hollinger et al., 2006; Chihorek et al., 2007; Foldarvy-Schaefer et al., 2012; Lin et al., 2017; Mauroussset et al., 2017). A recent pre-clinical SUDEP study found that incidence of apnea does increase in subjects who are at high risk for sudden death (Simeone et al., 2018). Progression of respiratory dysfunction and associated pathology with age may promote respiratory failure when challenged by severe seizures and could influence susceptibility of KO mice to sudden death and possibly have implications for refractory epileptic patients. This possibility is further supported by a trial utilizing CPAP therapy, which found a 50% reduction in seizures in drug-resistant epileptic patients with obstructive sleep apnea, suggesting a possible treatment to reduce risk of sudden death (Somboon et al., 2019, Malow et al., 2008).

In conclusion, older (SD_{70}) KO mice that have a greater risk for SUDEP also have a unique lung pathophysiology that, outside the field of epilepsy, is associated with apnea and respiratory failure (Yang et al., 2018). The KD attenuates the lung pathology and is associated with a reduction in the incidence of apnea (Netzel and Simeone, unpublished observations). Our results indicate that the KD acts to reduce peripheral lung inflammation by normalizing proteins downstream of the TLR4 pathway (MyD88, NF-kB, and iNOS). This mechanism may protect against SUDEP and

contribute to the increased longevity of KOKD mice. These data indicate that apnea may act as a temporal biomarker for SUDEP in this preclinical model and monitoring of apneic events may provide a biomarker of temporal SUDEP risk and an indicator for intervention.

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