NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF LIFE SCIENCES

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BSc in Cell and Molecular Biology

THE ROLE OF PEROXISOMAL FUNCTION IN CARBON MONOXIDE MODULATION OF NEUROINFLAMMATION

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE

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Adviser: Helena L. A. Vieira
Assistant Professor, NOVA University Lisbon

Examination Committee:

Chair: Margarida Casal Ribeiro Castro Caldas Braga,
Assistant Professor, FCT-NOVA

Rapporteurs: André R. A. Marques,
Postdoctoral Researcher, NOVA Medical School

Adviser: Helena L. A. Vieira
Assistant Professor, University

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Para a minha família e amigos

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"Porque o Senhor é quem dá a sabedoria e da sua boca procedem o saber e o entendimento." (Provérbios 2,6).

Abstract

Carbon monoxide (CO) is an endogenous gas with cytoprotective, anti-inflammatory, antioxidant and antiapoptotic properties. It is produced by the enzyme heme oxygenase (HO) in response to stress. In low concentrations, CO exerts protective effects on cells, including neuron protection, by signaling through low concentrations of reactive oxygen species (ROS). CO attenuates microglial exacerbated neuroinflammation in cases of neurodegenerative diseases. Peroxisomes are single-membrane organelles involved in various metabolic pathways, such as fatty acid oxidation, lipid synthesis, and ROS metabolism. Although the role of CO in mitochondria has been widely studied, little is known about its influence on peroxisomes, particularly in neuroinflammation. Therefore, the main objective is to understand whether the anti-inflammatory role of CO is dependent on the peroxisomal activity. To achieve this, WT BV2 cells and Acox1 KO and ABCD1/ABCD2 DKO BV2 microglial cells, which exhibit peroxisomal dysfunction, were cultured. CO was added through CO-Releasing Molecule (CORM) ALF-826 for 1 hour and 0,5 µg/mL of Lipopolysaccharide (LPS) were applied for 23 hours. In WT BV2 cells, CO increases catalase activity, which is a key peroxisomal enzyme. Moreover, cells with peroxisomal defects show a higher catalase expression that may be a compensatory effect. In KO cells, CO reverted LPS-induced NO, a molecule produced in inflammation, indicating that peroxisomes are not involved in this process. In contrast, CO did not revert LPS-induced ROS in ABCD1/ABCD2 DKO cells, indicating a potential peroxisomal role in CO's anti-inflammatory effect. The measurement of TNF-α needs to be repeated for more accuracy. In summary, peroxisomes may contribute to the anti-neuroinflammatory effects of CO in BV2 cells, but further experiments are needed to consolidate these results.

Keywords: Carbon Monoxide, Peroxisome, Microglia, Neuroinflammation

RESUMO

O monóxido de carbono (CO) é um gás endógeno com propriedades citoprotetoras, anti-inflamatórias, antioxidantes e anti-apoptóticas. É produzido pela enzima heme oxigenase (HO) em resposta ao stress. Em baixas concentrações, o CO exerce efeitos protetores nas células, incluindo a proteção de neurónios, sinalizando através de baixas concentrações de espécies reativas de oxigénio (ROS). O CO atenua a neuroinflamação exacerbada pela microglia em casos de doenças neurodegenerativas. Os peroxissomas são organelos de membrana simples envolvidos em várias vias metabólicas, como a oxidação de ácidos gordos, a síntese de lípidos e o metabolismo de ROS. Embora o papel do CO nas mitocôndrias tenha sido amplamente estudado, pouco se sabe sobre a sua influência nos peroxissomas, especialmente na neuroinflamação. Assim, o principal objetivo é perceber se o papel anti-inflamatório do CO é dependente da atividade peroxissomal. Para tal, células microgliais BV2 WT, Acox1 KO e ABCD1/ABCD2 DKO, que apresentam disfunção peroxissomal, foram cultivadas. O CO foi adicionado através da molécula libertadora de CO (CORM) ALF-826 durante 1 hora, e 0,5 µg/mL de lipopolissacárido (LPS) foram aplicados durante 23 horas. Nas células BV2 WT, o CO aumenta a atividade da catalase, que é uma enzima chave dos peroxissomas. Além disso, as células com defeitos peroxissomais apresentam uma maior expressão de catalase, o que pode resultar de um efeito compensatório. Nas células KO, o CO reverteu o NO induzido por LPS, uma molécula produzida durante a inflamação, indicando que os peroxissomas não estão envolvidos neste processo. Em contraste, o CO não reverteu o ROS induzido por LPS nas células ABCD1/ABCD2 DKO, indicando um possível papel dos peroxissomas no papel anti-inflamatório do CO. A medição de TNF-α precisa de ser repetida para maior precisão. Em resumo, os peroxissomas podem contribuir para os efeitos anti-neuroinflamatórios do CO nas células BV2, sendo ainda necessário mais estudos para consolidar estes resultados.

Palavras-chave: Monóxido de Carbono, Peroxissoma, Microglia, Neuroinflamação

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GLOSSARY

ALF-826	Novel and non-commercial molybdenum-based CORM
BV2	Microglial murine cell line
Tecan Infinite F200 PRO	Absorbance and Fluorescence Microplate reader

ACRONYMS

3-AT	3-Amino-1,2,4-triazole
ABCD1-3	ATP-binding cassette transporters
Acox	Acyl-coenzyme A oxidase
ACSL	Acyl-CoA synthetase
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
BBB	Blood-Brain Barrier
BSA	Bovine Serum Albumin
CNS	Central Nervous System
CO	Carbon Monoxide
CORM	CO-Releasing Molecule
DAO	D-amino acid oxidase
DCF	2',7'-Dichlorofluorescein
DMEM-F12	Dulbecco's Modified Eagle Medium F12
DMSO	Dimethyl sulfoxide
DPA	Docosapentaenoic Acid
ECL	Enhanced chemiluminescence
EPA	Eicosapentaenoic Acid

ETC	Electron Transport Chain
FBS	Fetal bovine serum
GSH	Glutathione
HCMV	Human Cytomegalovirus
HDL	High-density lipoprotein
HIF1α	Hypoxia-inducible factor 1-α
HO	Heme Oxygenase
IFN-γ	Interferon γ
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
IRAK-M	Interleukin 1 Receptor Associated Kinase M
KO	Knockout
LB	Loading Buffer
LBP	Lipopolysaccharide binding protein
LCFAs	Long Chain Fatty Acids
LDL	Low-density lipoproteins
LPS	Lipopolysaccharide
MD-2	Myeloid Differentiation Factor-2
MFE1/2	Multifunctional Enzyme 1 or 2
MMP	Mitochondrial Membrane Permeabilization
MnSOD	Manganese Superoxide Dismutase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric Oxide

Nrf1/2	Nuclear-Respiratory Factor-1 and -2
PABA	Para-Aminobenzoic Acid
p38 MAPK	p38 mitogen-activated protein kinases
PBS	Phosphate-buffered saline
PEX	Peroxisomal Biogenesis Factor
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator-1α
PPAR	Peroxisome Proliferator–Activated Receptors
RIPA	Radioimmunoprecipitation assay buffer
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPMI-1640	Roswell Park Memorial Institute 1640 (media)
SDS	Sodium dodecyl-sulfate
SDS PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
SHIP1	Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1
SOD	Superoxide Dismutase
STAT1	Signal transducer and activator of transcription 1
TFAM	Mitochondrial Transcription Factor
TFEB	Nuclear translocation of transcription factor EB
TGFβ	Transforming growth factor β
TLR	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor α
T-TBS	Tris-buffered saline with Tween® 20 Detergent
VLCFAs	Very Long Chain Fatty Acids
WT	Wild Type

X-ALD	X-linked Adrenoleukodystrophy

1

INTRODUCTION

1.1 Central Nervous System (CNS)

Central Nervous System (CNS) is a complex set of tissues that has the role to control most of the functions of the organism. It is composed by the brain and the spinal cord[1]. As essential functions, CNS is the responsible for the integration of sensory and cognitive information, coordination of motor responses and regulation of physiological processes[1]. The brain regulates all body functions, including respiration, temperature, hunger, touch, memory, emotion, and cognition, while spinal cord is the responsible for connecting the brain with the entire body, sending or receiving messages[1]. To function in a homeostatic way, CNS parenchyma is composed of different types of cells, as neurons and glial cells. Neurons are responsible for processing, sending and receiving messages. Glial cells maintain the homeostatic environment for the good functioning of neurons[2].

1.1.1 Neurons

Neurons are cells that reside in nervous systems, in both central and peripheral. They are composed by the body of the cell, or soma, dendrites, that usually receive impulses from other neurons, and the axon, the longest protuberance of the cell, responsible to send impulses to other cells[3]. Their main role is to process and communicate information to other neurons or to certain cell types (muscle, glands) in the body through electrochemical signals[4]. Most of the time, these signals are transmitted via neurotransmitters, small molecules that are released from the pre-synaptic neuron into the extracellular space (synaptic cleft) to act in the post-synaptic neuron[4] (**Figure 1.1**). In the synapse, neurotransmitters bind to receptors in the post-

synaptic neurons promoting or blocking the formation of an electrochemical signal[4]. In addition, a synaptic communication can also occur without the need for neurotransmitters in electrical synapses, where both neurons are directly connected by gap junctions[5].

When a synapse promotes a membrane potential excitability, there is the generation of an action potential, by the opening of the sodium channels and posteriorly the potassium channels. This electric impulse travels through the axon of the neuron, promoting the release of other neurotransmitters stored in vesicles in the terminations of the axon[3]. Neurons which axons are wrapped in myelin conduct the nerve impulse faster[2].

In the CNS, proliferation of neuronal precursors and their differentiation occur mostly before birth, when the CNS is being developed. However, there are some neural stem cells that can differentiate into functional neurons during adult life, although being a very slow process[6]. Because most neurons cannot be renewed, it is important to protect neurons, otherwise severe damage could occur. Glial cells are the ones with the role to support, connect, and shield the central and peripheral nervous systems' neurons[2].

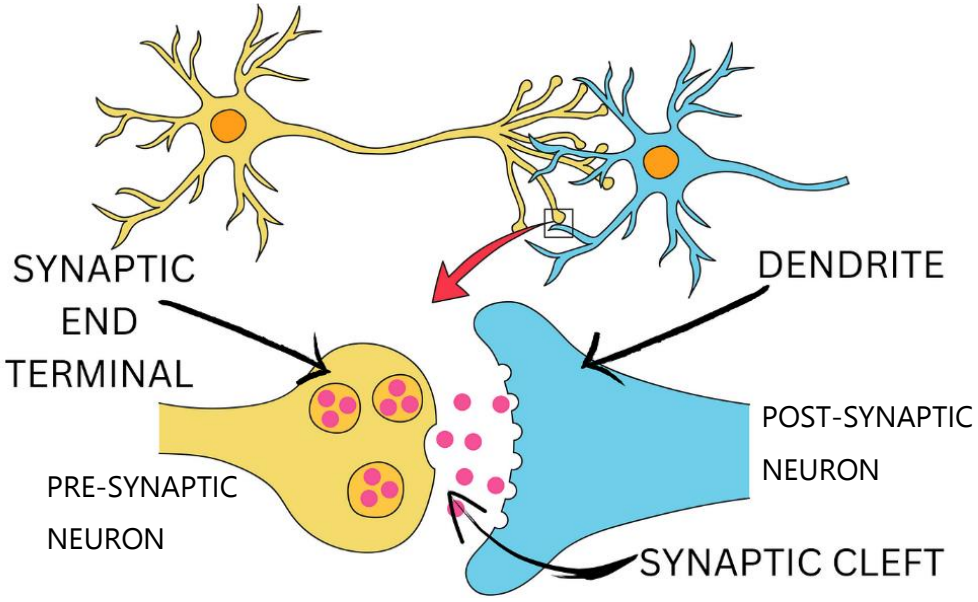


Figure 1.1 — Representation of the synaptic cleft. The end terminals of the pre-synaptic neuron contain small vesicles filled with neurotransmitters. When an action potential hits the neuron, the vesicles fuse with the membrane, releasing neurotransmitters into the synaptic cleft. They are going to be recognized by receptors on the post-synaptic neuron's dendrites, potentially generating an action potential, depending on the type of neurotransmitter and the receptors involved. Adapted from Psychology Records[7].

1.1.2 Glial cells

Frequently, neurons are considered as the protagonists of the CNS. However, glial cells are as important as neurons, since they play vital roles in homeostasis, synaptic modulation,

and response to injury and disease[2]. Glial cells comprise various types of cells present in the CNS with distinct functions. The three principal glial cells in the CNS are oligodendrocytes, astrocytes and microglia (**Figure 1.2**).

Oligodendrocytes are the cells involved in the myelination process of the neurons' axons. Myelin is an extension of oligodendrocytes plasmatic membrane covering neuronal axons, which is essential for: (i) fast conduction of the action potential and (ii) the maintenance of axonal integrity[8].

Astrocytes are the most abundant glial cells in the CNS and perform a variety of supportive functions, including regulation of the extracellular environment, maintenance of the blood-brain barrier (BBB), metabolic support for neurons, and modulation of synaptic transmission[2].

Microglia are the resident immunocompetent cells in the CNS[9]. Since they are very relevant in the development of this project, microglia are described in more detail in the next section.

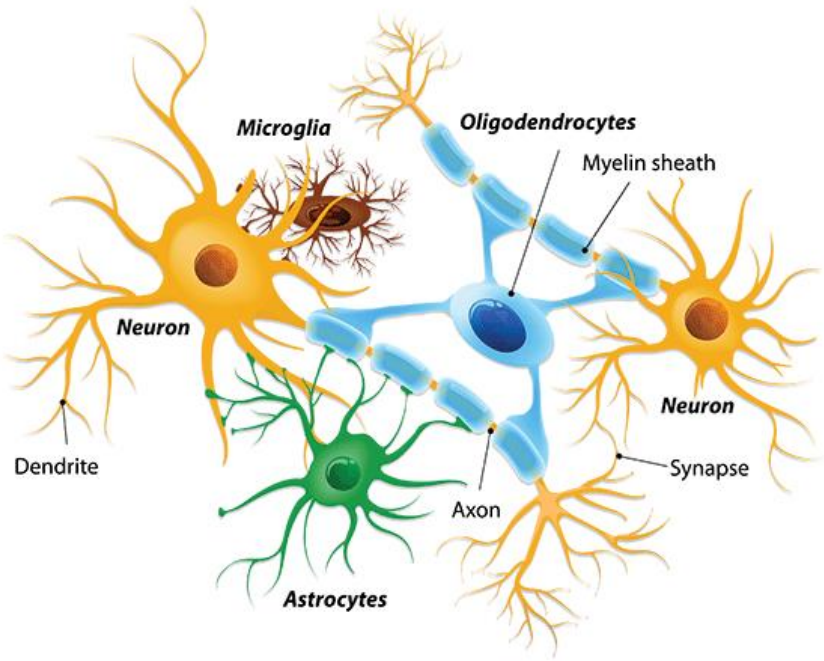


Figure 1.2 — Representation of glial cells in the CNS: astrocytes, oligodendrocytes and microglia. They are involved in roles of support of the neurons and protection of the CNS from invasive pathogens and molecules. From Li[10].

1.1.2.1 Microglia

In mammals, almost every part of the organism is supervised by the immune system to ensure security and homeostasis, and CNS is no exception. The peripheral immune system

cannot reach CNS, because of BBB, a specialized layer of cells (mainly endothelial cells, pericytes and astrocytes) that offer protection against harmful molecules, virus, bacteria, and other substances that could be present in the blood, including immune cells[11]. Therefore, CNS has particular and specialized immune cells called microglia, that are glial myeloid cells, being essential to the healthy environment of the organism[9,12].

This phagocytic and immunocompetent cell population in the CNS is in charge of modulating inflammation in response to infection and damage[9,13]. When faced with a pathogen or damage molecular pattern, recognized by Toll-like Receptors (TLRs), microglia initiate pro-inflammatory gene expression processes, leading to the secretion of numerous pro-inflammatory cytokines and reactive oxygen species (ROS), and to the adaptation of their morphology to rounder shape, turning into an active state, that also may involve a metabolic shift from an oxidative to a glycolytic metabolism[14] (**Figure 1.3**).

All these inflammatory processes aim to eliminate the pathogen or harmful molecules by disrupting the conditions that favor their spread and promoting their removal. Beside controlling inflammation, microglia can also release neurotrophic molecules[15], and these cells also participate in the modulation of synaptic homeostasis[16] being essential for the healthy development and activity of neurons[17,18]. Previously, it was believed that microglia exhibited two distinct activation states: one purely neurotoxic and the other purely neuroprotective (M1/M2 profiles)[19]. However, it is now understood that microglia display a spectrum of activation, releasing different neurotoxic or neuroprotective molecules depending on the context[20]. The precise conditions that drive microglia to produce these factors remain unclear. Thus, neuroinflammation is important to maintain the clearance of pathogens, damaged cells, and other harmful substances, as well as the homeostasis of the CNS. However, it is important to notice that an exacerbated or chronic inflammatory immune response leads to neurologic pathologies, for example potentiating and accelerating neurodegenerative diseases, as well as worsening consequences[21,22].

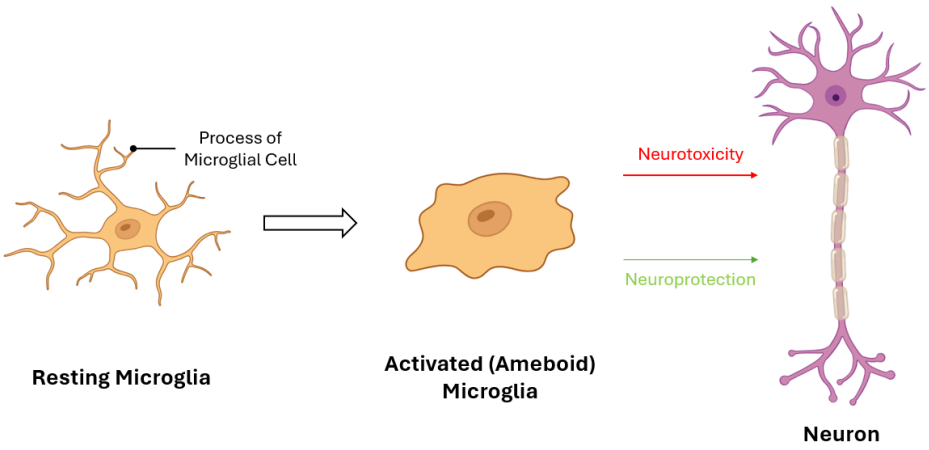


Figure 1.3 — Microglial cell response. Microglial cells are found resting in the CNS. When sensing pathogens or damage molecular patterns, microglia turn into an activated state, promoting the inflammation process. Microglia can also get into a state that promotes neuroprotection or a state promoting the degeneration of neurons. It is important to note that between these states there are many intermediate states. Chronically overactivated microglia can be dangerous leading into a chronic inflammation, in cases of stroke or Alzheimer's Disease, degenerating neurons uncontrollably, worsening the stage of the disease.

1.2 Carbon Monoxide (CO)

Carbon monoxide (CO) is a gas that can be generated by hydrocarbon combustion. It is broadly known to be toxic when inhaled in high quantities, poisoning, approximately, 137 *per* 1 million people, every year worldwide[23,24]. In a molecular level, CO binds to hemoglobin with a higher affinity than molecular oxygen generating a different hemoglobin complex called carboxyhemoglobin, that may compromise the delivery of molecular oxygen to tissues[25]. In 1857, it was published the first precise description of CO toxicity in the organism[23]. Likewise, it was also shown that CO is cytotoxic because of its interaction with cytochrome c oxidase, restricting the electron flow in the electron transport chain (ETC)[26,27].

However, CO is also a gasotransmitter endogenously produced in mammals. In 1949, CO was found as an endogenous product of metabolism, found in the human exhaled air[28]. In the last two decades, CO has been studied as a molecule that can bring benefits when applied in low concentrations[29]. Actually, CO has been described as a neurotransmitter and a vasomodulator, with anti-inflammatory and anti-apoptotic properties[30–33].

1.2.1 Heme Oxygenase (HO)

CO is generated by a reaction performed by Heme Oxygenase (HO) (**Figure 1.4**). A heme group mainly originating from lysed erythrocytes is converted into biliverdin, free iron and

CO[34]. HO activity contributes (i) to the removal of toxic free heme groups (probably originated by dying cells) and (ii) to the production of cytoprotective and homeostatic molecules: bilirubin, free iron and CO. Biliverdin is catalyzed into bilirubin by biliverdin reductase being antioxidant[35]. Free iron is prooxidant but rapidly interacts with iron proteins to express more ferritin, which is a cytoprotective molecule[36]. Finally, CO has anti-inflammatory and cytoprotective roles that have been being studied in the last decades[37]. Thus, HO has an important role in the oxidative homeostasis of the cell, metabolizing heme groups, which are very oxidizing in the cell environment[38].

HO-1 and HO-2 are the two main isoforms of the enzyme found in human cells, being the first one inducible and the second constitutive[35]. HO responses to numerous types of stress, namely oxidative stress, ischemia, hypoxia, abnormal body temperature and inflammation[38]. Therefore, HO will be activated or upregulated when cells face these conditions, promoting the surveillance and maintenance of cellular homeostasis [35]. Moreover, it has been shown the crucial role of HO in certain organs, for example, brain, heart, intestine, liver, and lung[39,40].

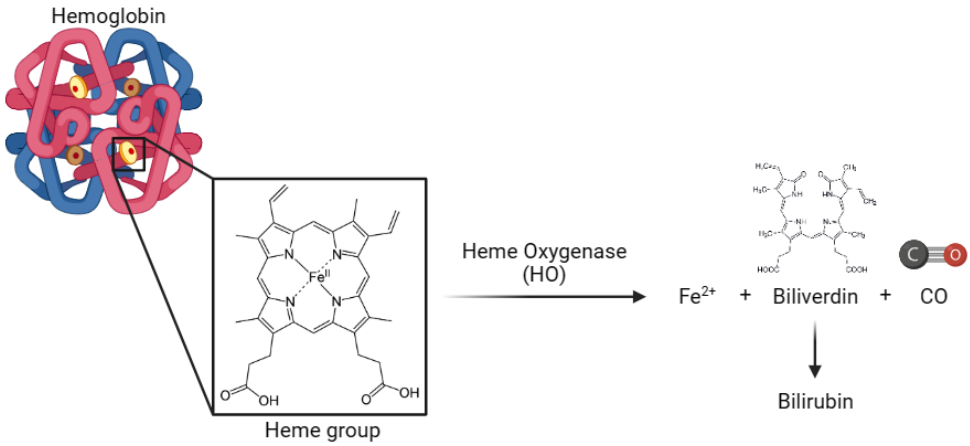


Figure 1.4 — Heme oxygenase (HO) converts heme groups, derived from dead cells, into biliverdin (which will turn into bilirubin by the action of biliverdin reductase), free iron and carbon monoxide (CO).

1.2.2 CO-Releasing Molecules (CORMs)

Since CO has been discovered as a potential therapeutic molecule, it has been important to develop a manner to deliver it to the cells, exogenously, and both *in vitro* and *in vivo*. Researchers started to approach clinical applications by inhalation of CO gas. However, there were many limitations associated, for example, inhaled CO is not tissue specific and could lead to a systemic hypoxia[41]. Thus, it was important to create a safer way to deliver CO into the cells. So, CO-Releasing Molecules (CORMs) emerged as a solution to overcome this issue.

CORMs are chemical complexes designed to release controlled amounts of CO in biological systems and under specific conditions[42]. They bring the great advantage of minimizing the toxicity risk of high levels of CO. CORMs must be designed to encompass parameters, such as water solubility, biocompatibility, chemical stability, toxicity profile, absorption, metabolism, and excretion mechanisms to ensure their correct functionality[42].

Through the years, many CORMs have been designed, but only a few of them were able to act properly *in vivo* and *in vitro*. An example of that is ALF-826 (**Figure 1.5**), which is a novel and non-commercial molybdenum-based CORM. Since molybdenum is present in some human enzymes, the organism metabolism presents the ability to excrete the molybdenum containing compound after releasing CO moieties, thus it does not show any toxicity issues[43,44]. There are studies showing its anti-neuroinflammatory capacity through the deliverance of CO[43,44].

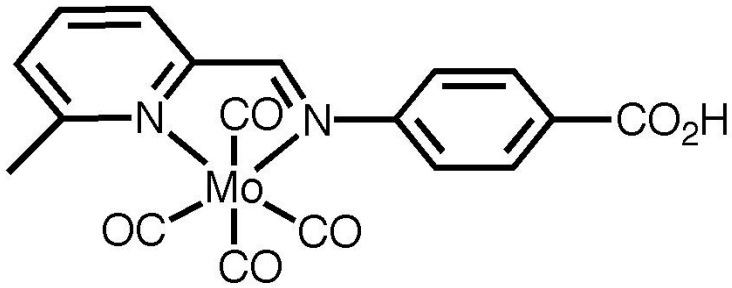


Figure 1.5 — Molecular structure of CORM ALF-826.

1.2.3 CO's Biological Effects

During the studies of the biological processes of CO, it has been relevant to identify in which pathways CO interferes or is included in. CO is a chemically inert gasotransmitter and interacts essentially with the metal catalytic centers of certain proteins, containing reduced iron[45]. In fact, contrary to nitric oxide (NO), CO only binds to Fe^{2+}, that limits the possibility of interactions between CO and Fe^{3+}- heme center proteins in the cell[45].

Great efforts have been made through the years to uncover the pathways CO participates and still more research is needed to better understand the CO impact, especially in the CNS. However, it is already broadly known that CO has biological roles related to inflammation, cell death, mitochondrial metabolism and biogenesis[23]; and in many cases it depends on ROS signaling (**Figure 1.6**). The next sections describe the most impactful pathways CO interacts with, with a special focus on the CNS.

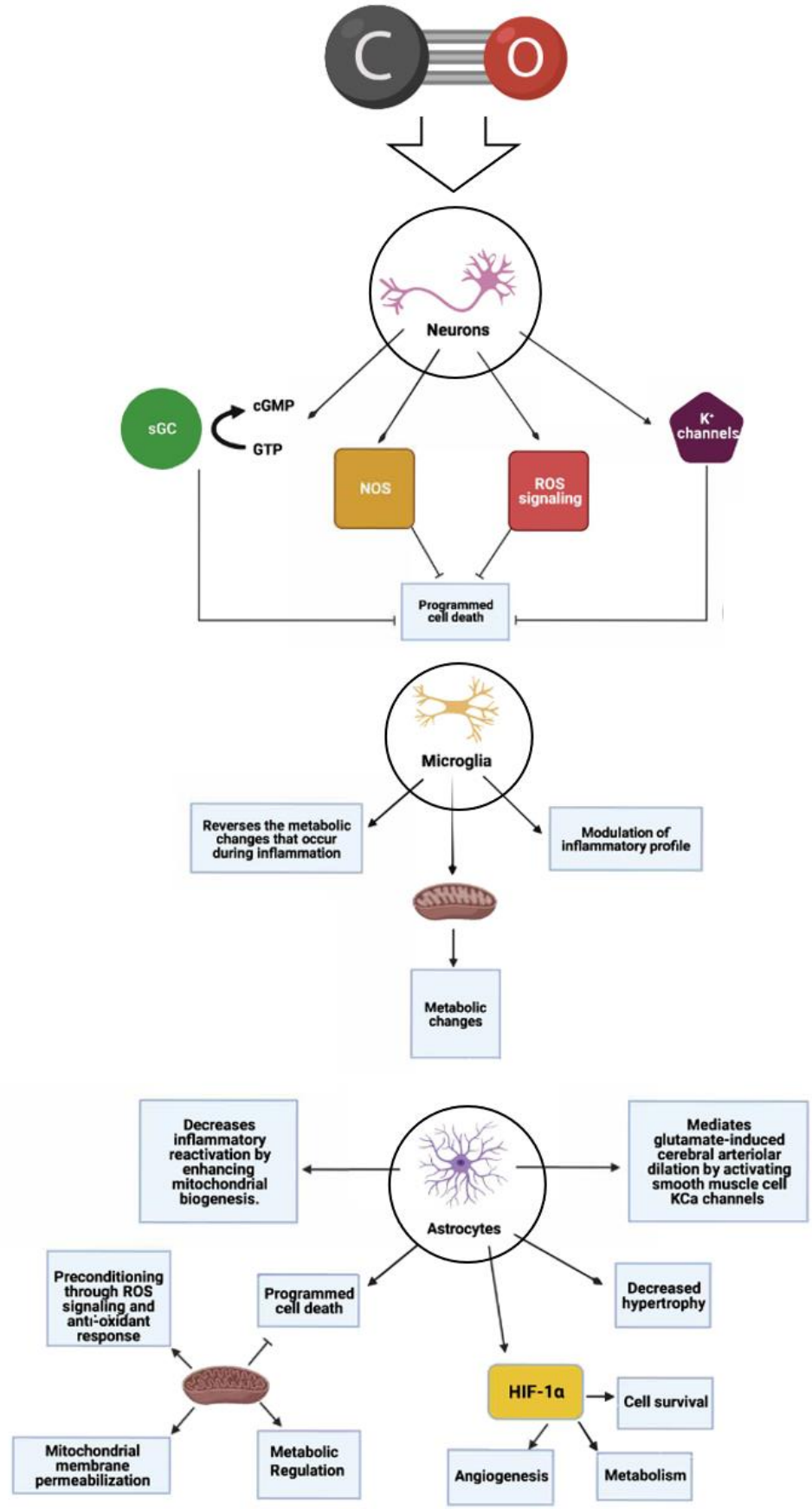

C
O
Neurons
sGC
cGMP
GTP
NOS
ROS signaling
K+ channels
Programmed cell death
Microglia
Reverses the metabolic changes that occur during inflammation
Modulation of inflammatory profile
Metabolic changes
Decreases inflammatory reactivation by enhancing mitochondrial biogenesis.
Mediates glutamate-induced cerebral arteriolar dilation by activating smooth muscle cell KCa channels
Astrocytes
Preconditioning through ROS signaling and anti-oxidant response
Programmed cell death
Decreased hypertrophy
HIF-1α
Cell survival
Mitochondrial membrane permeabilization
Metabolic Regulation
Angiogenesis
Metabolism

Figure 1.6 — CO's Biological Effects in the CNS. CO interacts essentially with Fe^{2+}- catalytic center proteins. CO can regulate the cells death of cells, is involved in the ROS signaling and preconditioning related with mitochondria, interacts with pro-survival factors, metabolic changes and modulation of inflammation. Adapted from Siracusa *et al*[46].

1.3 CO and CNS

1.3.1 CO and Neuroinflammation

CO has been studied for limiting severe and chronic neuroinflammation, once it has anti-inflammatory features. In fact, several studies showed that CO has an anti-neuroinflammatory role in stroke and multiple sclerosis[47,48], promoting a lower secretion of pro-inflammatory cytokines and NO[48,49].

CO and NO are small signaling molecules that play significant roles in inflammation, modulating each other's function. They can either enhance or inhibit inflammatory responses depending on the context. An example of that is that CO can protect against shock and reverse pulmonary hypertension by modulating NO production[50]. NO can also act as an inducer of the HO-1, leading to an increase of CO production[51]. This dynamic interplay helps regulate inflammatory processes, modulating tissue protection and inflammation.

CO showed to be neuroprotective when cells face ischemia and reperfusion in *in vivo* models, such as perinatal Wistar rats, as also in preventing cell death. CO treatment, in *in vivo* models, present significant uplifts in terms of cell loss and tissue inflammation, verifying a lower neurodegenerative progression[52–55].

CO will interact with many cell pathways, regulating the generation of cytoprotective and inflammatory molecules, for example, ROS signaling and mitochondrial metabolism, that are going to be explored in detail in the next sections.

1.3.2 ROS Signaling modulated by CO

ROS are reactive molecules that are produced in virtually all cells. Hydrogen Peroxide (H_2O_2), Superoxide ion ($O2^-$), Singlet Oxygen ($^1O_2$) and Hydroxyl Radical ($^{\bullet}OH$) are some examples of ROS[56].

Mitochondria is one of the organelles where ROS is most produced. An abnormal electron flow on the ETC leads to bigger leakage of electrons out of the chain, reacting with oxygen and producing ROS[57]. Peroxisomes are also key producers of ROS by their normal metabolism[58]. It is important to note that high levels of ROS can lead to oxidative damage in proteins,

DNA, and membranes, potentially leading to cell death. For that, both mitochondria and peroxisome have enzymes capable of converting ROS into less reactive molecules, such as Superoxide Dismutase (SOD), Catalase and Glutathione (GSH) Peroxidase[59]. It is broadly known that, in aging process, there is a higher production of cellular ROS and a weaker capacity of response by the enzymes, worsening cellular status[60]. However, it is proven that low levels of ROS also act as signaling molecules, with important homeostatic and beneficial functions.

ROS generation is one of the most studied effects of CO in the cell. As a preconditioning-like action, CO-induced low levels of mitochondrial ROS act as signaling molecules, which in turn stimulate several biological responses: cellular differentiation, cytoprotection, anti-inflammatory or modification of cell metabolism[37]. Cells and tissues exposed to low doses of CO rapidly produce ROS, being crucial regulators of cellular signaling, influencing processes such as immune cell activation and vascular remodeling[61].

As CO binds to transition metals, proteins containing a heme group are its main target. One of the most studied examples is cytochrome *c* oxidase. When CO binds to cytochrome *c* oxidase, it slows down the flow of electrons, which accumulates electrons, especially at complex III (**Figure 1.7** and **Figure 1.8**). As a result, coenzyme Q's ubisemiquinone state has a longer lifetime, which increases its tendency to degrade O_2 into superoxide (O_2^-). These ROS are then subsequently function as signaling molecules[45]. This inhibition can be partial and transitory, only producing low levels of ROS, leading to beneficial effects[62].

Also, CO has shown powerful neuroprotective roles, controlling the production of low concentrations of ROS not only by the interference of cytochrome *c* oxidase but via NADPH oxidase, present in the cell membrane (**Figure 1.7**). Basuroy's group verified that both endogenous and exogenous CO inhibit NADPH oxidase's ability to produce superoxide anion, hence averting oxidative stress-induced cell death in cerebral endothelial cells. Additionally, ROS originating from NADPH triggered a pathway for cell survival by boosting CO synthesis through constitutive HO-2[63,64].

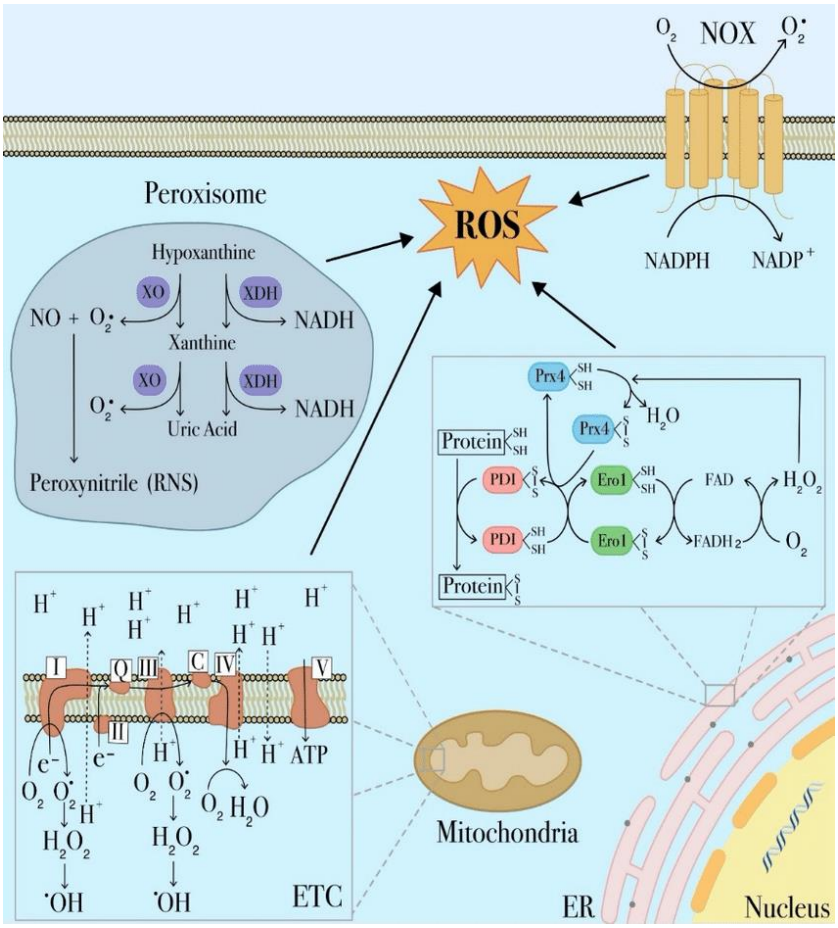


Figure 1.7 — Principal sites of ROS generation in cells. ROS can derive mainly from the peroxisomal metabolism, NADPH oxidase (present in the cell membrane) and mitochondria (from the ETC). As it has been studied for the last years, CO interacts essentially with mitochondria and NADPH oxidase. From Trombetti *et al.*[65].

Additionally, regarding neuroinflammation, the literature shows uncertainty about the effect of low doses of CO in the induction of antioxidant enzymes such as (i) Manganese Superoxide Dismutase (MnSOD), mostly present in mitochondria, (ii) Catalase, mostly present in peroxisomes and (iii) GSH, mostly present in cytosol and mitochondria, potentially mitigating oxidative damage. Studies show that CORM-3 might increase SOD-3 activity and decrease SOD-1 and SOD-2 activity[66]. Some have also speculated that CO might interact with catalase through its heme moiety[67]. However, an interaction like this is unlikely since CO binds strongly to Fe^{2+}, and the heme moiety present in catalase presents Fe^{3+} [68].

It is not known yet the action mechanisms of CO in the peroxisomes. CO could interfere in a way that peroxisomal ROS bring beneficial functions to the cell as it happens with mitochondrial ROS. Thus, this brings an interesting point to uncover in this project.

As it was mentioned before, high concentrations of CO induce rapid and high quantities of ROS production, becoming toxic to the cells. However, when it is present in low

concentrations, CO also induces a rapid production of ROS, this time turning into a crucial role in signal transduction[67] (**Figure 1.8**). These ROS have the ability to function as signaling molecules that control a variety of biological processes, including the activation of immune cells and vascular remodeling[61]. This ROS production activates several key pathways and transcription factors, including p38 mitogen-activated protein kinases (p38 MAPK), Peroxisome proliferator-activated receptor-γ (PPARγ), and Hypoxia-inducible factor 1- α (HIF1α). Each of these pathways contributes to the anti-inflammatory and protective effects of CO, that will be more explored below.

CO exerts anti-inflammatory effects in response to lipopolysaccharide (LPS) through the hyperphosphorylation of the p38 MAPK pathway, via ROS generation[69]. The ROS produced lead to the activation of p38 MAPK, which is necessary for CO's inhibition of pro-inflammatory cytokines like tumor necrosis factor α (TNF-α)[61]. Inhibition of ROS production using antioxidants or mitochondrial inhibitors like antimycin A blocks the CO-induced activation of p38 MAPK, highlighting the importance of ROS in this signaling pathway[61].

CO pretreatment conditions immune cells by upregulating PPARγ, a nuclear receptor involved in regulating inflammation and metabolism[70]. CO-induced ROS are crucial for the activation of PPARγ, leading to anti-inflammatory effects[32]. PPARγ activation inhibits the expression of pro-inflammatory genes and transcription factors such as NF-κB and STAT1[71]. The protective effects of CO are mediated, at least in part, through PPARγ, as evidenced by experiments showing that blocking ROS production inhibits CO-induced PPARγ expression[67].

About HIF1α, CO rapidly induces HIF1α stabilization in macrophages in a ROS dependent manner, leading to increased expression of Transforming growth factor β (TGFβ), an anti-inflammatory cytokine involved in cell survival[72]. CO-induced HIF1α and TGFβ protect cells from cell death provoked by a total deprivation of oxygen, an interesting link to the study of stroke[72].

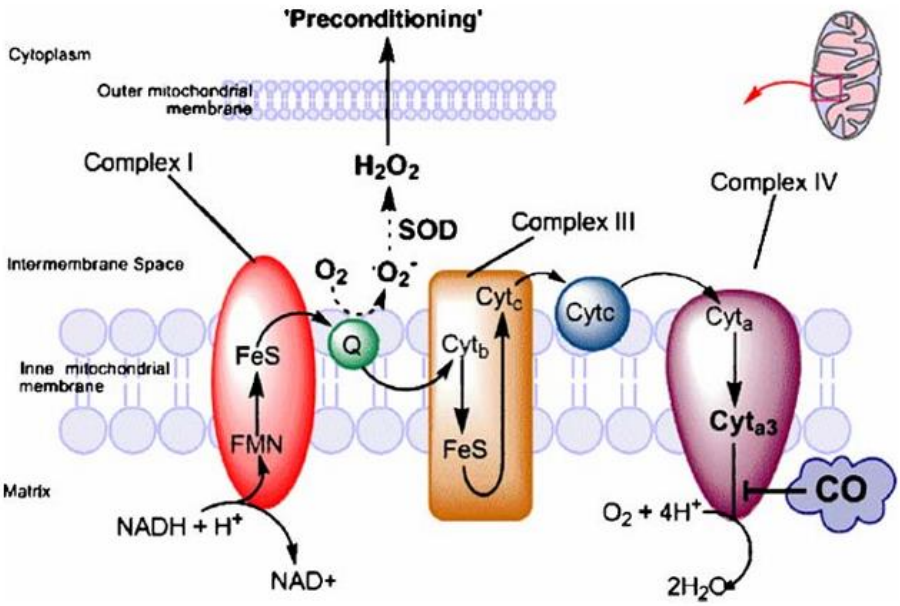


Figure 1.8 — Schematic demonstrating how CO inhibits the mitochondrial chain of electron transport. CO causes the rate of electron transport to slow down, which allows electrons to build up, especially at complex III. Consequently, Coenzyme Q (Q) has a longer lifetime in the ubisemiquinone state, which increases the likelihood of reducing O_2 into superoxide. Then, superoxide could be enzymatically converted to other ROS, which together change signaling pathways and cause a conditioning response. From Bilban *et al.*[67].

1.3.3 CO impact on Mitochondria

Not only CO has a great impact on ROS production on the mitochondria, but also on its metabolism and biogenesis. Several experiments showed that low intracellular levels of CO stimulate mitochondrial biogenesis and upregulates the expression of biogenesis promoting factors, like nuclear-respiratory factor-1 and -2 (Nrf-1/-2), peroxisome proliferator-activated receptor gamma co-activator-1α (PGC-1α) and mitochondrial transcription factor (TFAM)[37], namely in macrophages, cardiomyocytes and primary culture of mouse astrocytes.

Cells are pre-treated with CO, upregulating these factors, promoting mitochondrial biogenesis, leading to a better cellular maintenance[73]. It is important to notice that there is a lot of data about the impact of CO in mitochondria in different cell types. However, in all of them CO had a big influence on the mitochondrial biogenesis, having a cytoprotective and anti-inflammatory role in the cells[37].

The balance between elimination and biogenesis of mitochondria is essential to have a homeostatic environment in the cell[74,75]. Mitochondria can be generated by *de novo* biogenesis or by fission. They get eliminated by a process of selective autophagy called mitophagy[76]. Nuclear translocation of transcription factor EB (TFEB) is a molecule that regulates these processes

and is modulated by CO, being cytoprotective[77]. Although knowing that CO has an impact in these processes, it is not already well known the exact pathways it is included.

Other pathways of the mitochondrial metabolism are also modulated by CO. Mitochondrial Membrane Permeabilization (MMP) is involved in apoptosis control and is inhibited by low concentrations of CO, protecting the cell against death in a ROS dependent manner[78]. Also, CO seems to have a mild-uncoupling effect, interacting with proteins present in the mitochondrial membrane, generating a leakage in the proton flow, promoting ROS signaling in enough quantities to improve metabolism and potentiating its anti-inflammatory role[79]. There are also evidences that CO promotes a decrease in glycolysis, an increase in oxygen consumption, ATP production and in pentose phosphate pathway[37]. However, these pathways still need to be better investigated.

1.4 CO and Neurological Diseases

As CO has neuroprotective and anti-inflammatory functions, it holds great potential as a molecule to prevent or treat diseases. In fact, there are already *in vitro* and *in vivo* studies showing that CO brings benefits to cases of neurological diseases, for example, Alzheimer's Disease, Parkinson's Disease and stroke. Due to the research background of our lab, there will be a greater focus in stroke in this project.

1.4.1 CO and Stroke

Stroke can be divided into two subgroups: ischemic stroke and hemorrhagic stroke. In ischemic stroke, there is a blockage of a brain vessel that obstructs blood supply to a certain part of the brain, while, in hemorrhagic stroke, a rupture happened in a brain vessel[80]. In both cases, there is oxidative and cell damage, cell death and exacerbated neuroinflammation[81].

Both ischemic and hemorrhagic strokes involve significant inflammation and oxidative stress, which can lead to brain damage. In cases of ischemic stroke, CO has shown neurological benefits by the induction of HO-1 expression[82], inhibition of NO synthesis[83] and enhancing of mitochondrial function[84] (**Figure 1.9**). In hemorrhagic strokes, HO-1 activity participates in heme clearance and bilirubin production, resulting in a reduction of vasospasm and lipid peroxidation[85]. Also, low-dose CO treatment post-hemorrhage improves neurological outcomes and reduces inflammation[86], showing a big potential for therapeutic outcomes.

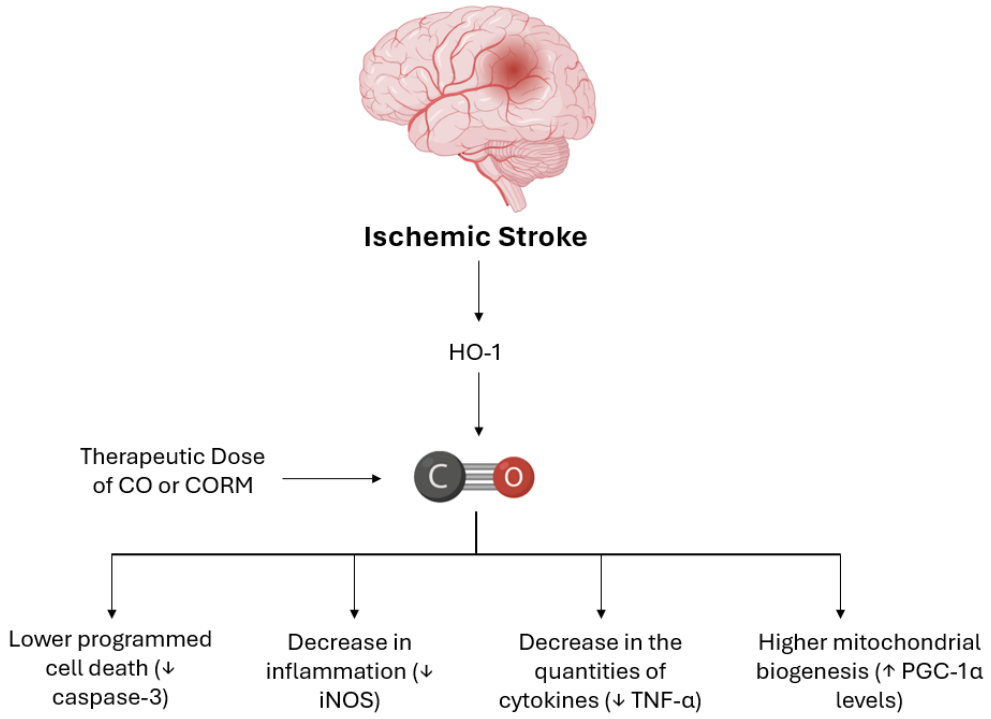


Figure 1.9 — Neuroprotection of CO in ischemic stroke. After a therapeutic dose of CO, it shows to promote a lower programmed cell death (less caspase-3), a decrease in inflammation (lower iNOS), a decrease in the quantities of cytokines (less TNF-α) and a higher mitochondrial biogenesis (higher PGC-1α levels)[87].

1.5 Peroxisomes

Peroxisomes are small single-membrane organelles found in virtually all eukaryotic cells. Their main role is to control the complex lipids metabolism. Some of the peroxisome metabolism, like detoxification of reactive anionic species and amino acids metabolism, is shared with other organelles[88]. In contrast, β-oxidation of very-long chain fatty acids (VLCFAs), synthesis of ether lipids and α-oxidation of branch-chained fatty acids are processes that occur exclusively in peroxisome[89].

Peroxisomal homeostasis depends on the balance between their synthesis and elimination[90]. Formation of new peroxisomes can happen by fission of pre-existing peroxisomes[91] or by *de novo* synthesis[92]. Pexophagy is the process called to the selective autophagy of peroxisomes, the most commonly via to their elimination[93].

Peroxisomes are dynamic organelles that interact with other cell compartments, such as mitochondria, endoplasmic reticulum, lysosomes, and lipid droplets[94–97]. Mitochondria and peroxisome share many features in their metabolism. Both are involved in the β-oxidation of fatty acids and the ROS oxidative balance[97,98]. Mitochondria are the most studied organelle in

inflammation[99]. Mitochondria participate in the modulation of the inflammatory stage, where cells need to switch their metabolism from a fatty acid oxidation to a fatty acid synthesis, as long with the activation of inflammasome and other molecules, for example mitochondrial antiviral-signaling proteins[100].

Only in the last few years, peroxisome emerged as an organelle that also modulates immune response, since it shares characteristics with mitochondria that could be related with the inflammatory process. The information about peroxisome role in the immune response is still not totally clear and needs to be more in-depth studied.

1.5.1 Peroxisomes, Metabolism and Cell Stress

Peroxisomes are essential organelles in the maintenance of the cellular environment. They respond to numerous types of stress, like oxidative stress, hypoxia, starvation, cold exposure, and noise, that can derive from the environment or from their own metabolism[101] (**Figure 1.10**). For that, they have the ability to adapt themselves in terms of shape, abundance, size, and localization[102]. Peroxisomes recently emerged as organelles that actively participate in signaling beside their functions in the passive metabolization of molecules.

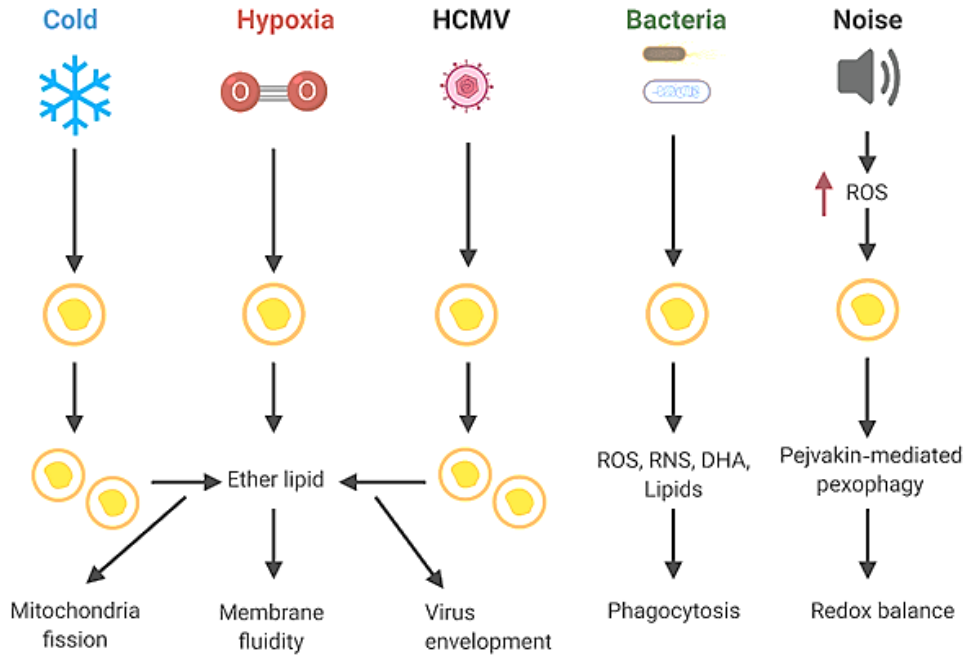


Figure 1.10 — Peroxisomes are involved in many processes of the cell, including cold, hypoxia, Human Cytomegalovirus (HCMV), phagocytosis when faced with pathogens, and noise. From He *et al.*[101].

1.5.1.1 β-oxidation of VLCFAs

Peroxisome metabolism can encompass many pathways. Herein, we will focus on β-oxidation of VLCFAs. These metabolic processes are reviewed in He *et al.* (2021)[101].

For β-oxidation of VLCFAs to occur, these molecules need to be linked to an acyl-CoA by acyl-CoA synthetase (ACSL) consuming a molecule of ATP[103]. They need to pass through the peroxisomal membrane by transmembrane channels of ATP-binding cassette transporter D subfamily (ABCD1-3)[104]. Then, the first step of the reaction is catalyzed by Acyl-CoA oxidase 1 (Acox1), generating a molecule of H_2O_2[88] (**Figure 1.11**). After this first step, the molecule will go through processes catalyzed by other enzymes called multifunctional enzyme 1 or 2 (MFE1/2) and 3-ketoacyl-CoA thiolase, that generates a new acyl-molecule that can react again with Acox1, repeating the cycle until the fatty acid is short enough to be transferred to mitochondria and be totally metabolized[101].

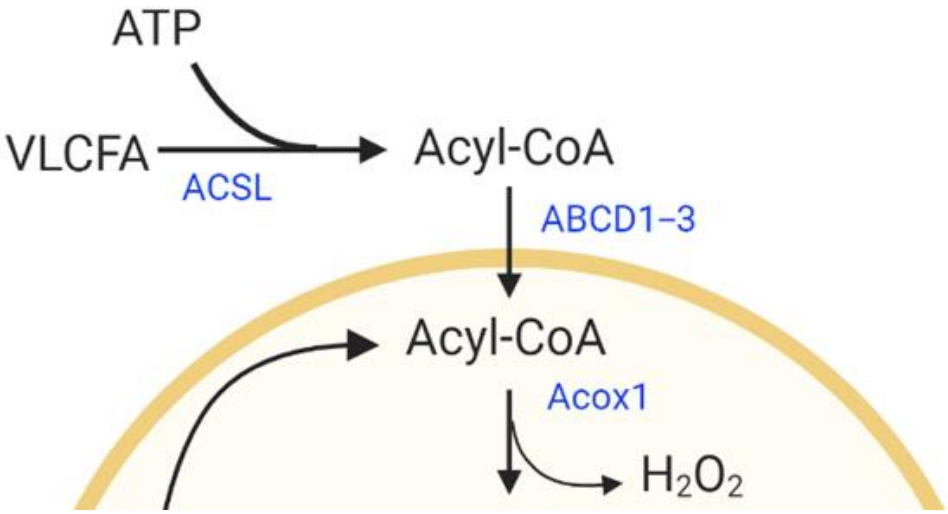


Figure 1.11 — First steps of the β-oxidation of VLCFAs. VLCFAs are converted into Acyl-CoA and enter in the peroxisome through molecular proteins named ABCD1-3. Then, Acox1 is the first enzyme to catabolize Acyl-CoA, releasing a molecule of H_2O_2. From He *et al.*[101].

1.5.1.2 Oxidative Stress

Peroxisomes are one of the major sites of H_2O_2 production in eukaryotic cells, being able to produce up to 35% of all H_2O_2 in some animal tissues[105]. Actually, their name derived from this molecule, as they are great producers of peroxides, along with mitochondria[105]. Since high quantities of ROS can cause damage to the cell, peroxisomes present mechanisms to defend the cell against oxidative stress[106]. Catalase is the main antioxidant enzyme in peroxisome, reducing H_2O_2 into H_2O[106].

Studies show that when catalase is not present in the cell or is not correctly acting, cells present a higher number of peroxisomes (to compensate the loss of catalase activity), and

higher levels of ROS production that consequently affect mitochondria as a downstream effect, demonstrating a correlation between the oxidative metabolism in these two organelles[107].

Humans with a gain-of-function mutation in Acox1 present higher levels of H_2O_2, presenting faster neurodegeneration, loss of glial cells and a higher risk of cancer since ROS will cause damage to DNA. In contrast, Acox1 loss-of-function leads to an accumulation of VLCFAs, potentiating glial degeneration[108]. Also, cells lacking Acox1 and ABCD1 are not oxidatively homeostatic, increasing the production of ROS and RNS[109].

Pexophagy can also be triggered by ROS and hypoxia. Peroxisomal ROS activate ataxia-telangiectasia mutated (ATM) kinase, that is a molecule sensitive to DNA damage[110]. ATM gets into peroxisomal matrix through Peroxisomal Biogenesis Factor 5 (PEX5), an import receptor of peroxisomal matrix proteins, and phosphorylates Ser141 of PEX5[111]. This will promote an ubiquitylation that will promote the pexophagy machinery[111]. The peroxisomal ROS generation will not only interfere with pexophagy but also with biogenesis. PEX5 has a Cys residue near its C-terminal domain that is sensitive to oxidative environments. This residue is important to the regulation of peroxisomal biogenesis, getting inhibited when cell faces a higher ROS production[112]. However, this information is not clear yet, since other studies show that there is a compensatory effect when ROS levels are higher[107].

Other types of stresses can also stimulate peroxisome to intervene. For example, cold promotes peroxisomal biogenesis producing more ether lipids, helping mitochondrial fission and, consequently, thermogenesis[113]. Also, noise promotes ROS production, generating an oxidative imbalance. For that, Pejvakin-mediated pexophagy helps to eliminate peroxisomes restoring oxidative homeostasis[114] (**Figure 1.10**).

1.5.1.3 Peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are fatty acid-activated transcription factors of nuclear hormone receptor superfamily that regulate energy metabolism[115].

As of right now, PPARα, PPARβ/δ, and PPARγ are the three subtypes of PPAR that are known. PPARα mediates its functions by controlling fatty acid transport, esterification and oxidation[115,116]. PPARβ/δ participates in fatty acid oxidation and also in the regulation of blood glucose levels[115,116]. In addition, PPARγ participates mainly in energy storage, promoting adipogenesis and lipid synthesis, being highly expressed in white adipose tissue, however, also expressed in the liver, skeletal muscles, intestine and immune cells[115,117].

More specifically, PPARγ significantly influences inflammation and oxidative stress, particularly in the context of brain injuries and neurodegenerative diseases[116]. Upon activation, it

translocates into the nucleus, where it modulates gene transcription, promoting anti-inflammatory and antioxidant responses[118]. These include the inhibition of pro-inflammatory transcription factors like NF-κB, decreasing the expression of cytokines and iNOS[115,119]. PPARγ also upregulates anti-inflammatory genes in order to mitigate immune cells activation[120].

As it is known, oxidative stress is a major contributor to brain damage, mainly by the action of ROS. PPARγ activation enhances the expression of antioxidant enzymes like catalase and SOD, which help neutralize ROS[121]. In models of ischemia, PPARγ agonists decrease lesion volume, reduce oxidative stress markers, and improve neurological outcomes[122], being a very interesting target to the development of novel therapeutics in this study area.

1.5.2 Peroxisomes and Immune Response

For several years, peroxisomes were thought to be organelles only involved in the lipid metabolism. Mitochondria have been always very studied as a metabolically active organelle controlling the immune system and the immune response, involved in the protection against pathogens. However, over the time, it was speculated that peroxisomes could also be involved in the regulation of the immune response and inflammation, since they share many features with mitochondria[98]. Actually, peroxisomes are involved in the regulation of the oxidative environment in the cell, they degrade molecules like prostaglandins[123] and leukotrienes[124], that can modulate inflammation, and they can synthesize and degrade polyunsaturated fatty acids that are associated with inflammation mediators like protectins, resolvins and maresins[125,126]. Additionally, peroxisomal metabolism is very important to the correct levels of eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA), that are important anti-inflammatory lipids[127]. All these processes showed to be important when the cell faces a pathogenic attack, promoting processes like phagocytosis, inflammation and immune cell activation and proliferation[128].

Peroxisomes and mitochondria seem to cooperate regarding the activation of immune system. DiCara's group reviewed the immune and inflammatory functions of the main peroxisomal proteins, evidencing that peroxisome is an important organelle in these processes[128]. An example of that is that an *ABCD1* deletion in macrophages and monocytes, which are cells that phagocyte, led to a malfunctioning transport of VLCFAs into the peroxisome, making them less capable to phagocyte[129].

Besides the peroxisomal proteins, many peroxisomal metabolites have important roles in the process of inflammation and immunity. Many lipids are involved in the process of phagocytosis and maturation of immune cells such as EPA, DPA and plasmalogens, as it was said

before. Likewise, fatty acids are involved in the activation of macrophages and T cells[130]. The ROS homeostasis also have roles of immune cell activation, as long with host defense and phagosome formation[131,132].

Focusing more on inflammation, the anti-inflammatory function of peroxisomes relies on peroxisomal β-oxidation activity, as the overexpression of TNF-α proteins results from the deletion of important peroxisomal β-oxidation enzymes[133]. In cases when peroxisomes are malfunctioning, the peroxisomal metabolism affects in a manner that unbalanced levels of ROS will be generated mainly through mitochondria, since it is a downstream organelle to the peroxisome[107,134]. Mitochondrial ROS will promote the inflammation process through oxidative stress.

In X-linked Adrenoleukodystrophy (X-ALD), which is the most common peroxisomal disease, patients experience cases of inflammation, namely neuroinflammation, having immune cells passing through the BBB, and infiltrating in the brain[135]. In models that mimic this disease, it was observed higher levels of secreted pro-inflammatory cytokines, like Interleukin 6 (IL-6) and Interleukin 8 (IL-8) via mitogen-activated protein kinases (MAPK) pathway, promoted by many transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[136,137]. There was also an accumulation of VLCFAs and a downregulation of the catalase activity, potentiating the process of neuroinflammation[138]. These studies clearly evidenced the link that had been already thought to occur between the peroxisome and the inflammatory process (**Figure 1.12**).

Peroxisomes also participate in the antiviral processes, signaling type III interferon response from stimuli received through viral-recognition receptors, such as TLRs[131].

In summary, peroxisomes play a crucial role in regulating various immune processes by influencing membrane composition, impacting receptors, and signaling pathways. The altered lipid profile due to peroxisomal defects affects vesicular trafficking, mitochondrial function, and immune pathways such as NF-κB. Peroxisomes contribute to the secretion of immune mediators, influence ROS signaling, regulate phagocytosis, and participate in antiviral responses, closely collaborating with mitochondria in energy metabolism and lipid catabolism.

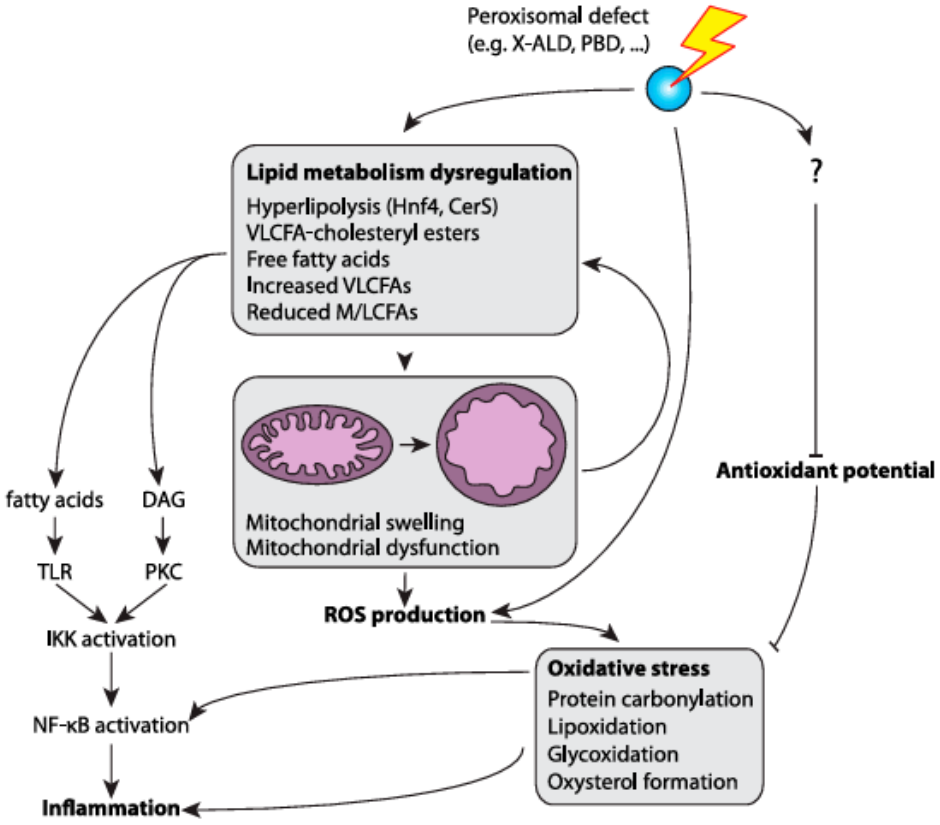


Figure 1.12 — Peroxisomal malfunction and Inflammation. A peroxisomal defect will affect the normal lipid metabolism, promoting disfunctions on mitochondria. Higher levels of ROS will be produced, generating oxidative stress, potentiating the process of inflammation and activation of the immune system. From Di Cara *et al.*[128].

1.5.3 Peroxisomes and Neurological Diseases

Peroxisomes have shown to be essential for the function of numerous cells in the organism, namely in CNS cells.

Alzheimer's disease is a neurodegenerative disease that causes dementia mainly due to a loss of neurons in hippocampus. There is a continuous accumulation of tau and β-amyloid proteins[139]. However, a study showed that when peroxisomes are stimulated to proliferate in this type of cells, there is a reduction of the neurodegenerative effects of the accumulation of β-amyloid proteins, due to a higher catalase activity[140]. Actually, it has already been observed a diminished quantity of peroxisomes in Alzheimer's patient cells[141].

In Parkinson's disease, there is a degeneration of neurons in substantia nigra, leading to motor deficiency including rigidity to patients' movements through the time[141]. It is common to observe an accumulation of damaged mitochondria and α-synuclein proteins in these patients' cells, demonstrating a problem with the recycling pathways of the cells[142]. In models

where PEX5 is absent, there is a faster accumulation of α-synuclein, demonstrating some type of correlation between the peroxisome activity and the development of Parkinson's[143].

As it was addressed before, stroke can be divided into two subgroups: ischemic stroke and hemorrhagic stroke. In a study using *in vitro* mouse primary cells and *in vivo* mouse brain, it was observed an increase in peroxisomal volume, and consequently in catalase activity and in peroxisomal numbers, after ischemic stroke[144]. Chen's group were motivated by these results and developed a study to correlate post-stroke dementia and D-amino acid oxidase (DAO), a peroxisomal enzyme that oxidizes D-serine, and its potential as a biomarker. They verified that these patients presented higher DAO levels in their plasma, suggesting that peroxisomal biogenesis is promoted in the chronic stage of stroke[145].

In conclusion, many neurological disorders share peroxisomal dysfunction in the CNS, which results in disturbed cellular homeostasis and adds to the pathophysiology of those conditions.

2

OBJECTIVE

Therefore, our hypotheses and scientific questions were based on literature data:

(i) CO is anti-inflammatory, and anti-inflammatory lipids are metabolized in peroxisomes. Thus, does CO control lipid metabolization in peroxisomes leading to production of anti-inflammatory lipids?

(ii) CO regulates mitochondrial metabolism, and peroxisome oxidates very long and long-chain fatty acid into short fatty acids (such as acetate) that fuel mitochondrial metabolism. Then, the next question is whether CO improves mitochondrial metabolism via acetate production at peroxisomes. In particular when glucose availability is limited, like in ischemic stroke.

(iii) CO acts via ROS generation and signaling in a preconditioning like manner and ROS are also produced in peroxisomes. The final question is: can CO induce ROS production at peroxisomal level?

Therefore, the main objective of this thesis is to understand whether the anti-inflammatory role of CO is dependent on the peroxisomal activity. Assays were performed to analyze neuroinflammation and peroxisome activity in microglial BV2 murine cells. To compare if the peroxisome is necessary in this correlation, there were used wild-type (WT) microglial cell line and two other cell lines knockout (KO) for essential genes to the normal function of the peroxisome, Acox1 and ABCD1/ABCD2.

3

METHODS

3.1 Main Buffers Composition

The composition of the main buffers used in the Methods are described in **Table 1**.

Table 1 — Composition of the Main Buffers used in the Methods.

BUFFER	COMPOSITION
PBS 10X	1.54 M NaCl, 15.4 mM KH_2PO_4, 34mM Na_2HPO_4; pH=7.2
RIPA 10X	50 mM Tris-HCl, 150 mM NaCl, 3.4 mM SDS, 24 mM sodium deoxycholate, 40 mM Triton X-100, 1% protease inhibitors (Invitrogen)
LB 5X	1.5 mM Tris-HCl, 10% (w/v) SDS, 25 mL glycerol (Sigma), 2.5 mL β-mercaptoethanol (Sigma), 2.5 mL bromophenol blue (Sigma); pH=6.8
T-TBS 10X	3 M NaCl, 1 M Tris-HCl
1% (W/V) BSA	1g BSA (EMD Milipore), 100 mL T-TBS 1x

3.2 BV2 Cell Line Cultures

Wild-Type (WT) BV2 murine microglia cells were supplied by ICLC, Genova, Italy. Acox1 KO and ABCD1/ABCD2 DKO BV2 murine microglia cells were supplied by Stephan Savary's Laboratoire Bio-PeroxIL EA7270, University of Bourgogne Franche-Comté, Dijon, France. All BV2 cells' tests were carried out up to passage numbers 11–35. The culture media used for the cells was RPMI-1640 (Sigma-Aldrich), to which 10% fetal bovine serum (FBS), 4 mM L-glutamine (Thermo-Fisher Scientific), 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Thermo-Fisher Scientific) were also added. Cell lines were maintained at 37 °C and 5% CO_2 in a humified incubator.

3.3 BV2 Cell Freezing and Thawing Procedures

For the freezing process, the cell suspension that remained on the T-flask, after being scrapped, was centrifugated for 5 minutes at 500 g. Then, the pellet was resuspended in 1 mL of a 90% FBS + 10% Dimethyl sulfoxide (DMSO) solution. The cells were kept in cryotubes at -80 ºC.

For the thawing process, the cells were firstly washed in Phosphate-buffered saline (PBS 1×) and centrifuged for 5 minutes at 500 g. Then, the pellet was resuspended in RPMI-1640 supplemented culture media.

3.4 BV2 Cell Seeding

In terms of cell seeding, WT, Acox1 KO, and ABCD1/ABCD2 DKO BV2 cells were plated for 48 hours, in 24-well and 96-well plates. BV2 cells were first counted using a hemocytometer, after being scrapped from the T-flask, using a cell scrapper. The hemocytometer is a specialized microscope slide with defined dimensions of 0.1 mm of depth. The grid formed using a certain volume of cell suspension gives us an accurate concentration of cells. As we can see in **Figure 3.1**, the number of cells present in three different green squares were counted, calculated the mean, and multiplied by 10^4, obtaining the number of cells *per* milliliter.

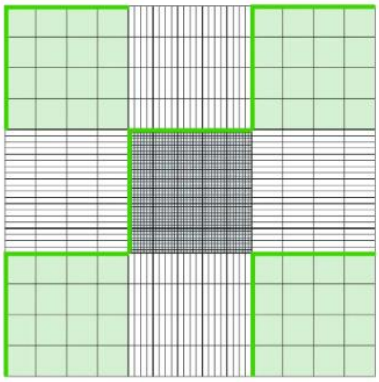


Figure 3.1 — Hemocytometer. The cells present in three different green squares are counted, the media is calculated and, when multiplied by 10^4, the number of cells *per* milliliter is obtained.

Since all three BV2 cell lines have different growth rates, there are different seeding densities to consider (**Table 2**). Experiments were essentially performed in 24-well and 96-well plates, which have 0.32 and 1.9 cm^2, respectively.

Table 2 — WT, Acox KO and ABCD1/ABCD2 DKO BV2 seeding densities *per* cm^2 *per* 48 hours (24 hours of growth + 24 hours of treatments).

CELL LINES	CELLS/CM^2
WT BV2	1.5×10^4
ACOX1 KO BV2	2.0×10^4
ABCD1/ABCD2 DKO BV2	3.0×10^4

WT and KO BV2 cells were usually plated in 12 wells each. The scheme bellow (**Figure 3.2**) demonstrates a global line of thinking, since the cell counting to the volumes needed to cell seeding, for a 24-well plate or a 96-well plate, knowing that each well shelters 0.5 ml and 0.15 ml, respectively.

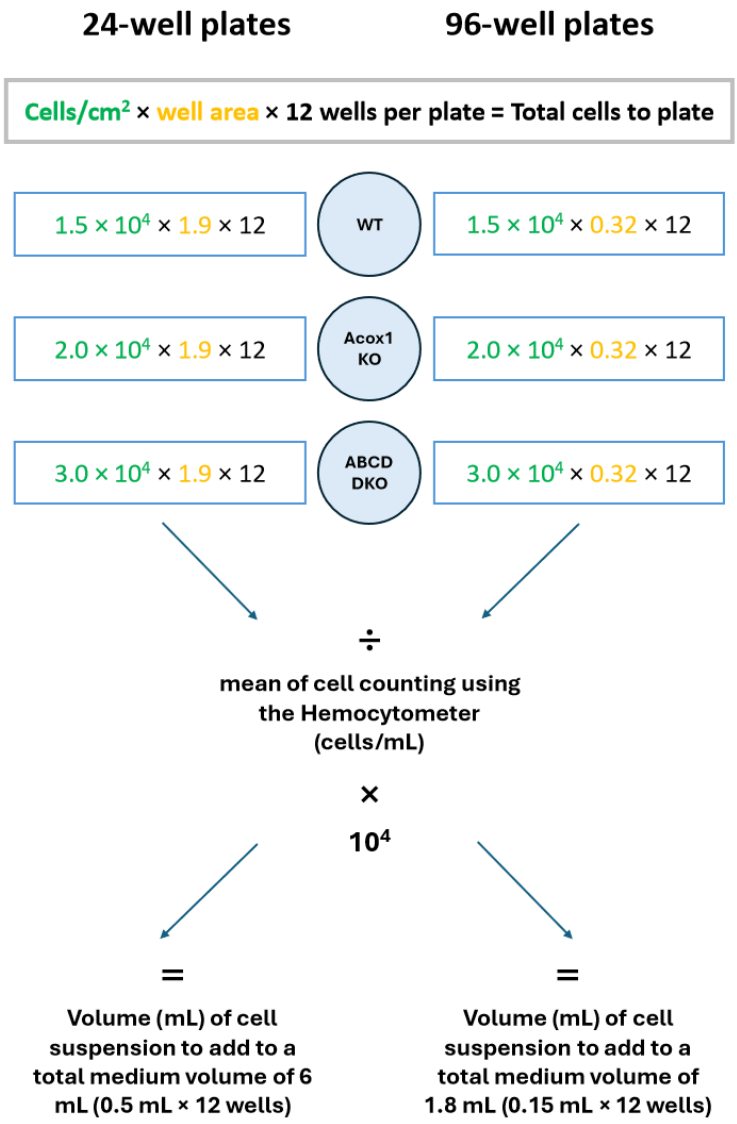


Figure 3.2 — Global Scheme of Seeding BV2 cells.

3.5 Cell Culture Treatments

For every assay, after 24 hours of seeding, plated cells were treated with a CO-Releasing Molecule (50 µM of ALF-826) for 1 hour before LPS (1 µg/mL, O111:B4 LPS, Sigma-Aldrich) was joined for another 23 hours (**Figure 3.3A**). Due to a recurrent problem in the induction of inflammation by LPS in WT BV2 cells, several strategies were taken to try to understand the reason for such an event. These topics will be covered in greater detail in the Results section.

There were always made three replicates of the same condition to guarantee more reliable results. The designs of the plates are shown in the **Figure 3.3B**. After the 23 hours of LPS finished, cells were either kept alive (in case of ROS Quantification) or scrapped from the well.

Both the supernatant and the pellet were collected for future experiments. Pellets were stored at -20 ºC in different volumes of Lysis tampon, Radio-Immunoprecipitation Assay (RIPA 1×) Buffer, depending on the experiment.

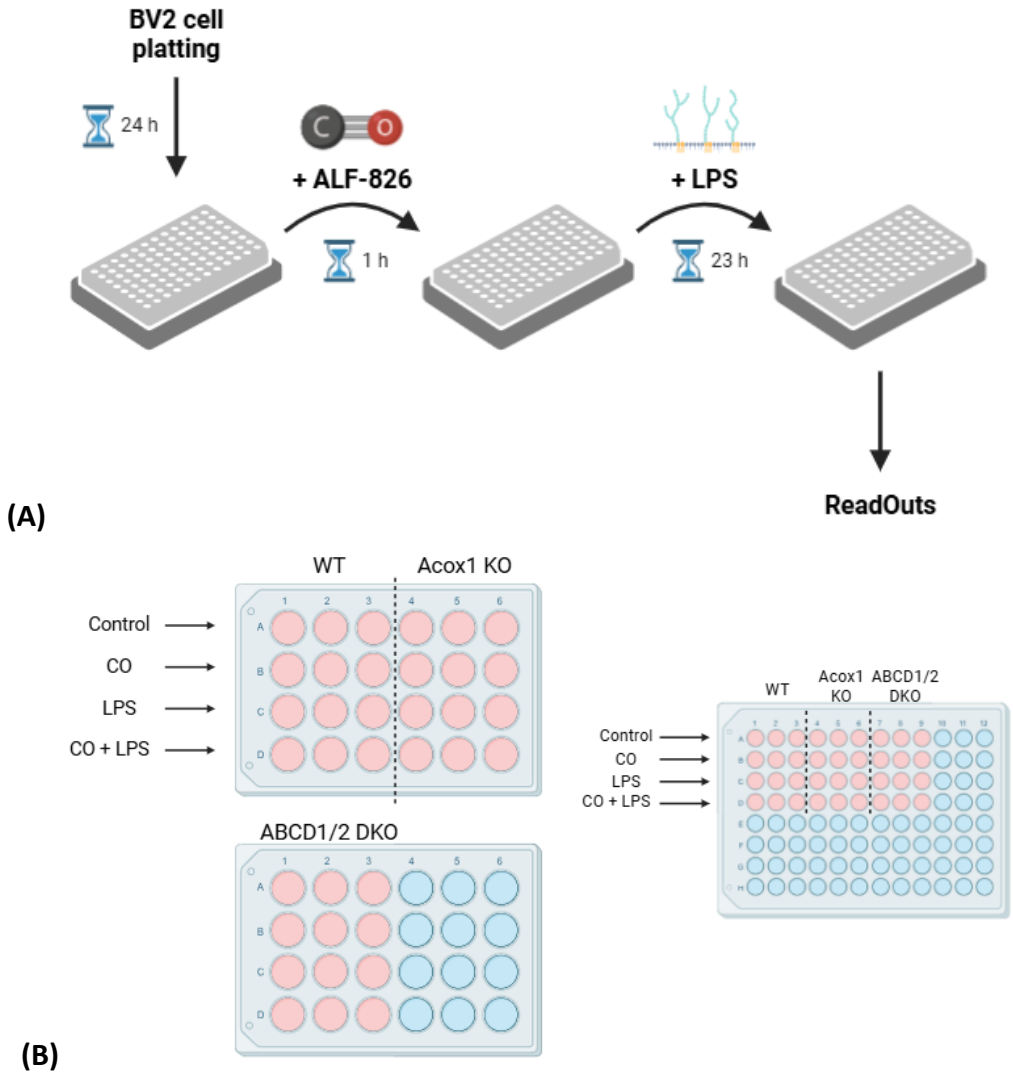


Figure 3.3 — (A) Treatment of BV2 cells with ALF-826 and LPS, and respective incubation times. (B) Schematic representation of Cell Treatment design in 24-well (left) and 96-well (right) plates.

In some experiments, 2 mM of 3-Amino-1,2,4-triazole (3-AT), a catalase inhibitor, was applied to the cell culture for 23 hours after 1 hour of treatment with CO. The following steps were performed similarly as described above.

3.6 CO Preparation, Storage and Exposure

ALF-826 was made up to a final concentration of 2.5 mM in 0.1 M $NaHCO_3$ at pH 8.4. The CORM was stored at -80 ºC and shielded from light after being filtered using a 0.2 µm filter. We used the thawed aliquots right away. Cells were subjected to 50 µM of ALF-826 throughout the indicated intervals.

3.7 Griess Reaction Assay

The colorimetric Griess reaction test was used to measure the nitrite (NO_2^-) in the microglia supernatant, an indirect way to measure inflammation. When microglia get inflamed, it releases NO to the medium surrounding, being oxidized into NO_2^-, a more stable molecule. Griess reagent will react with NO_2^- and form a colorimetric molecule, with its absorbance peak at 540 nm (**Figure 3.4**).

BV2 cells were treated with ALF-826 (1 hour) and LPS (23 hours), as mentioned in Section 3.5. After gathering the microglia culture medium from the 24-well plates, it was centrifuged for a second time for 5 minutes at 10 000 g, to get rid of any cellular debris. Then, 100 µL of supernatant were added to a 96-well transparent plate (in triplicates). After that, there were added 100 µL of Griess Reagent to the wells, incubated at 37 ºC for 10 minutes and absorbance (at 540 nm) was determined using a Tecan Infinite F200 PRO microplate reader.

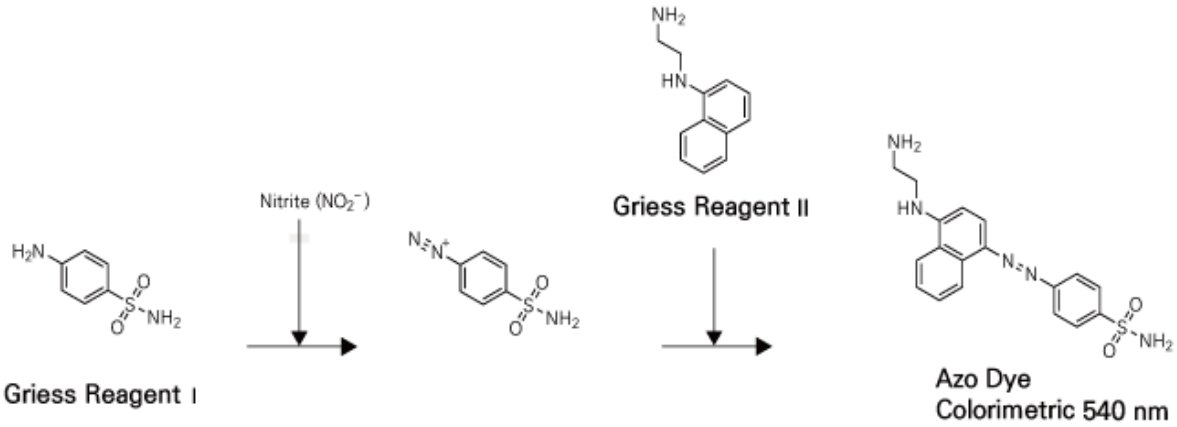


Figure 3.4 — Griess Reagent reacts with nitrite (NO_2^-) forming a colorimetric molecule detectable at 540 nm of absorbance. From Biomax[146].

3.8 BCA Protein Quantification

To quantify the total proteins in the sample, it was used a Pierce BCA Protein Assay Kit (Thermo Scientific). The quantity of protein is proportionally direct to the absorbance at 560 nm, result of the reaction of the proteins present in the sample with the kit's working reagent. With the kit's BSA (albumin), it is constructed a calibration line through consecutive dilutions.

In each well of a 96-well transparent flat bottom plate, it is placed 10 µL of sample or BSA, forming three reading replicates. All the samples were diluted 10 times in MilliQ water. Subsequently, it is added 100 µL of Working Reagent (in a ratio of 50:1 of Reagent A to Reagent B, respectively). Then, the plate is incubated at 37 ºC for 30 minutes and absorbance is read at 560 nm using a Tecan Infinite F200 PRO microplate reader. Afterwards, the absorbance is converted to concentration using the calibration line.

3.9 ROS Generation Assay

ROS were measured in BV2 cell cultures using 2',7'-dichlorofluorescein diacetate (H_2DCFDA) (Invitrogen), which is converted to 2',7'-dichlorofluorescein (DCF) when entering the cell (**Figure 3.5**). DCF emits fluorescence at 530 nm, which can be detected using a microplate reader.

BV2 cells were treated with ALF-826 (1 hour) and LPS (23 hours), as mentioned in Section 3.5. For this experiment, BV2 cells needed to be seeded in a 96-well black flat bottom plates. After the plates were centrifuged in a microplate rotator, the supernatant was discarded and added a solution of 5 µM of H_2DCFDA in PBS 1×. The plates were incubated at 37 ºC for 20

minutes and fluorescence intensity was measured using a Tecan Infinite F200 PRO microplate reader (λ_{ex} 485 nm/λ_{em} 530 nm).

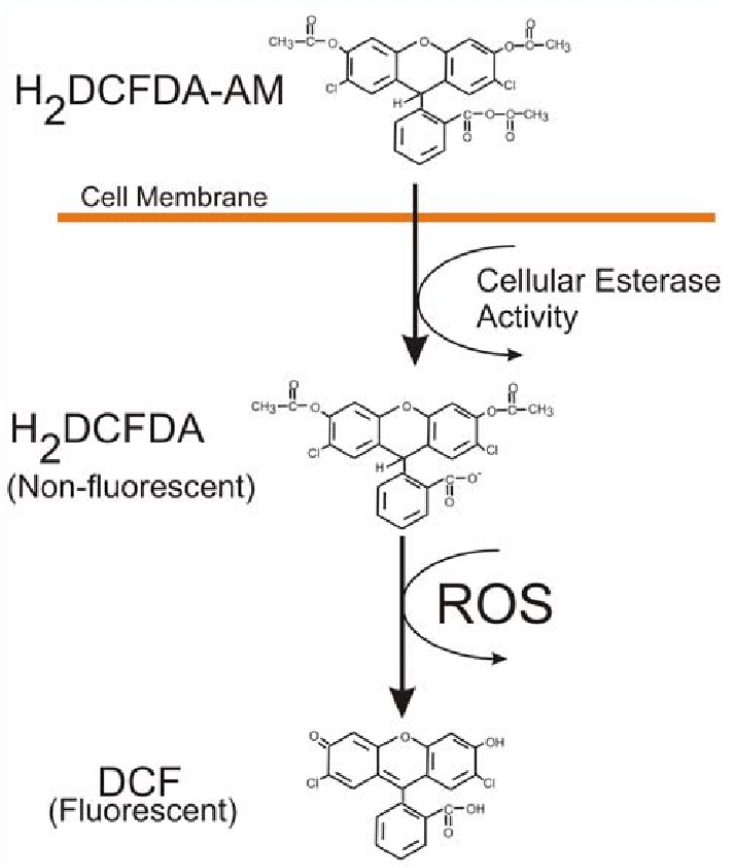


Figure 3.5 — H_2DCFDA-AM passes through the cell membrane, reacts with ROS and produces DCF, a fluorescent compound, detectable by microplate readers. From Held *et al*[147].

3.10 Chemiluminescence Immunoblotting for Catalase and PEX19 Quantification

Cells were collected from 24-well plates, washed using PBS 1×, and lysed with RIPA 1×. Then, the protein was quantified using the BCA Protein Quantification Method (Section 3.8), to guarantee a similar quantity of samples of 20 µg *per* lane. The samples were mixed with Loading Buffer (LB) 5×. Posteriorly, the samples were incubated for 10 min at 95 ºC.

The samples were separated using polyacrylamide gel electrophoresis–sodium dodecyl sulphate (SDS PAGE) on a 10% gel. The band size reference was a NZYColour Protein Marker II (NZYtech). After that, proteins were electrically transferred to a nitrocellulose membrane (Amersham Protran 0.45 NC; GE LifeSciences) and blocked overnight using 1% (m/v) Bovine Serum Albumin (BSA) in PBS 1×.

Membranes were labelled with primary and secondary antibodies (**Table 3**), being washed three times with Tween-Tris-buffered saline (T-TBS) Buffer in the end of each incubation. After, the membranes were subjected to Enhanced chemiluminescence (ECL) Clarity Western Detection Reagent (Bio Rad) for 1 minute, immunoblots were exposed to identify the reactive bands to an X-ray film in a Dark Chamber (using a Developer and a Fixer). The intensity of the bands was quantified using ImageJ.

Table 3 — Primary and Secondary antibodies used in Immunoblotting.

ANTIBODY		DILUTION USED	REFERENCE
ANTI-CATALASE (GOAT)	Primary	1:2000	R&D System REF AF3398
ANTI-PEX19 (RABBIT)	Primary	1:2000	ThermoFisher REF PA5-22129
ANTI-GOAT	Secondary	1:5000	Abcam REF ab6741
ANTI-RABBIT	Secondary	1:5000	Amersham REF NA934V

3.11 Catalase Activity Assay

This method of determining the specific catalase activity is based on the consumption of H_2O_2 *per* minute of each sample. There is added a known concentration of H_2O_2 (20 mM), which is being consumed and can be measured at an absorbance of 240 nm.

BV2 cells were treated with ALF-826 (1 hour) and LPS (23 hours), as mentioned in Section 3.5. For this experiment, BV2 cells were scrapped and collected from 24-well plates in 50 µL of RIPA 1×.

Firstly, since H_2O_2 is not stable, we needed to check the stock concentration, diluting it 400 times, and measuring its absorbance at 240 nm. Then, dividing the absorbance by the Molar Extinction Coefficient (22 $M^{-1}.cm^{-1}$ for microplates) and multiplying by the dilution factor 400, we get the stock concentration of H_2O_2. With that concentration, we prepared a 400 mM H_2O_2 working solution.

For this assay, we use a specialized 96-well transparent flat bottom plate able to measure absorbance at 240 nm. *Per* well, we added 10 µL of sample lysate, 10 µL of Tris-HCl (1 M, pH 7.4) to keep the optimal conditions for the enzymatic activity, 10 µL of 400 mM H_2O_2 working solution and 170 µL of MilliQ water. For each sample, there were three reading replicates. Rapidly, we measured the absorbance at 240 nm for 3 minutes.

Finally, the samples protein was quantified by BCA Protein Quantification protocol, mentioned above, with the aim of normalization of the results obtained. Then, using **Equation 1**, we calculated the specific catalase activity.

$$Specific\ catalase\ activity = \frac{\Delta Abs_{240nm}.min^{-1}}{22} \times 10^6 \times \frac{200}{10} \times \frac{1}{[protein]mg.l^{-1}}$$

Equation 1 — Specific catalase activity (in µmol of H_2O_2 consumed/min/mg of protein). $\Delta Abs_{240nm}.min^{-1}$ refers to the slope of consumed H_2O_2; 22 $M^{-1}.cm^{-1}$ is the Molar Extinction Coefficient; 10^6 converts mol to µmol; 20 (200÷10) is the dilution factor; [protein] $mg.L^{-1}$ represents the normalization with total protein of the sample.

3.12 Quantification of TNF-α by ELISA

Enzyme-linked immunosorbent assay (ELISA) was per formed to measure TNF-α microglia cell extract levels, using the respective Standard ABTS ELISA Development Kits (PeproTech). BV2 cells were treated with ALF-826 (1 hour) and LPS (23 hours), as mentioned in Section 3.5. For this experiment, BV2 cells were scrapped and collected from 24-well plates in 50 µL of RIPA 1×. All experiments were performed in accordance with the respective manufacturer's instructions: Absorbance values were measured at 415 nm, with wavelength correction set at 560 nm, using an Infinite F200 PRO microplate reader (Tecan).

3.13 Statistical Analysis

Results are presented as mean ± standard error of the mean (SEM), with at least three biological replicates being per formed for all experiments. All statistical analyses were performed using 2 tails unpaired t-student test, in order to calculate p-values to determine the randomness chance. $p < 0.05$ were considered significant.

4

RESULTS

4.1 Optimization of Inflammatory Stimulus in WT BV2 cell line

As it will be addressed in the Section 4.3 of the Results, NO Quantification Assays were performed in order to evaluate the level of inflammation in WT, Acox1 KO and ABCD1/ABCD2 DKO BV2 cell lines. NO is indirectly quantified by nitrite levels using Griess reagent (Section 3.7). It is known that microglial cells release high amounts of NO following stimulation of inflammation induced by LPS. For many years in our lab, 0.5 µg/mL of LPS has been applied to BV2 cell line for promoting neuroinflammation and release of NO[43,44]. Nevertheless, LPS did not induce NO production in WT cells, indicating no inflammatory response. Thus, this first section addresses many approaches and optimizations that were made in order to understand what was causing WT BV2 cell line not to respond to the inflammatory stimuli of LPS.

First, we tested a LPS dose-response experiment using LPS from three different origins. Five different concentrations (0.5, 1, 5, 10 and 20 µg/mL) of three different LPS originating from different serotypes of *Escherichia coli* (O26:B6; O111:B4; O55:B5) were applied. The three different LPS were used to discard the possibility of LPS O26:B6, the one used previously in our lab, was not inducing inflammation.

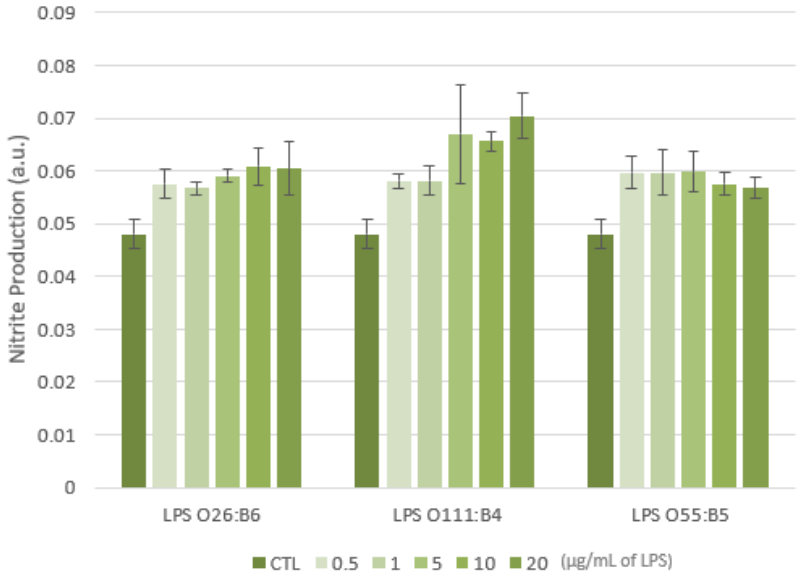


Figure 4.1 — LPS Dose Response through NO Quantification Assay in WT BV2 cells. Supernatant was collected from WT BV2 cells pre-treated with LPS for 24 hours. There were used three different LPS from different *Escherichia coli* serotypes (O26:B6, O111:B4 and O55:B5) and five different concentrations of each (0.5, 1, 5, 10 and 20 µg/mL). *n*=1.

As it can be observed in **Figure 4.1**, the two optimal concentrations were 5 and 20 µg/mL of LPS O111:B4. However, even these two concentrations did not reach the normal levels of NO production (about 0.1 a.u. for the optimal setting conditions), which is the expected value of BV2 cells when inflamed.

There was a possibility that the problem was the Griess Reagent, which is used in the experiment for reacting with nitrite, generating a pink color that is measured by absorbance. For that reason, a nitrite calibration curve, using different concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/mL) was constructed to verify if the Griess Reagent was actually working. As we can observe in **Figure 4.2**, the calibration curve is correct, meaning that the Griess Reagent was working.

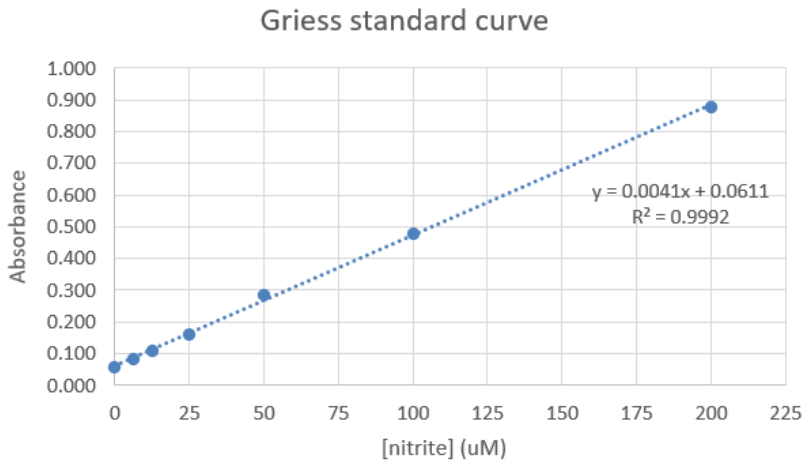


Figure 4.2 — Calibration line using different concentrations of Nitrite with the aim to verify Griess Reagent was working correctly. *n*=1.

There was also the possibility of the 96-well plates used were damaged. Thus, the experiment was repeated in the same conditions with new plates. However, the results did not alter after this change (data not shown).

Then, literature and previous experience in our lab demonstrated passage number affects a cell line's characteristics over time. Actually, the WT BV2 cell line used was over passage number 40. Therefore, a passage number 7 cell line was thawed in order to repeat the same experiment performed with the older cell line. The results, showed in **Figure 4.3**, were very favorable at the time, once the majority of the LPS stimuli induced inflammation in the WT cells, surpassing the expected value of 0.1 a.u.. However, in the following experiments, the values of NO production did not reach this peak no more, bringing this issue back again, indicating that the potential problem could have its origin in cell culture medium.

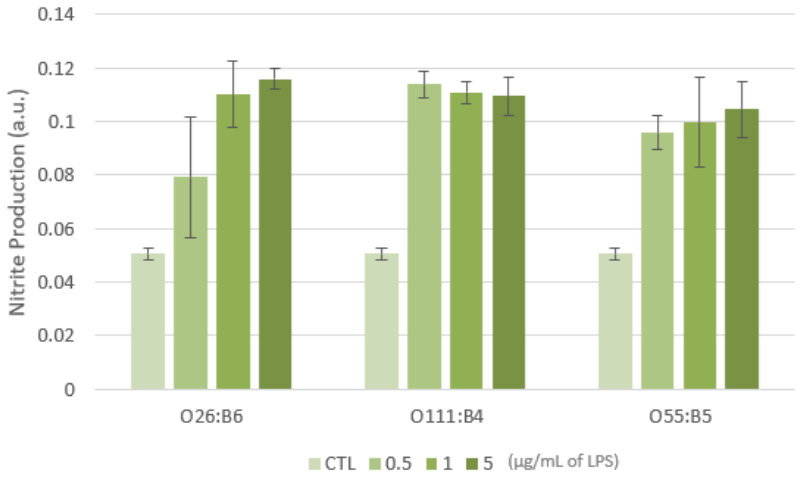


Figure 4.3 — LPS Dose Response using passage number 7 WT BV2 cells by NO Quantification Assay. Supernatant was collected from WT BV2 cells pre-treated with LPS for 24 hours. There were used three different LPS (O26:B6, O111:B4 and O55:B5) and three different concentrations of each (0.5, 1 and 5 µg/mL). *n*=1.

Because shortly after cell thawing in the first experiment, BV2 cells respond to LPS, it may indicate that cells have adapted to the culture medium a while after thawing. In fact, it is broadly known that FBS is a key supplement in culture media providing growth factors to cells, protecting them from harmful molecules and toxic agents. Moreover, FBS composition is dependent on its origin and lot., thus several hypotheses were taken into account. First, it was hypothesized that the WT cells could get overprotected with 10% of FBS present in the medium. Therefore, it was performed the same experiment, using WT BV2 cell lines with low passage number and three different concentrations of FBS (10, 5 and 2.5%), in order to disclose if the concentration of FBS was causing cells not to respond to LPS.

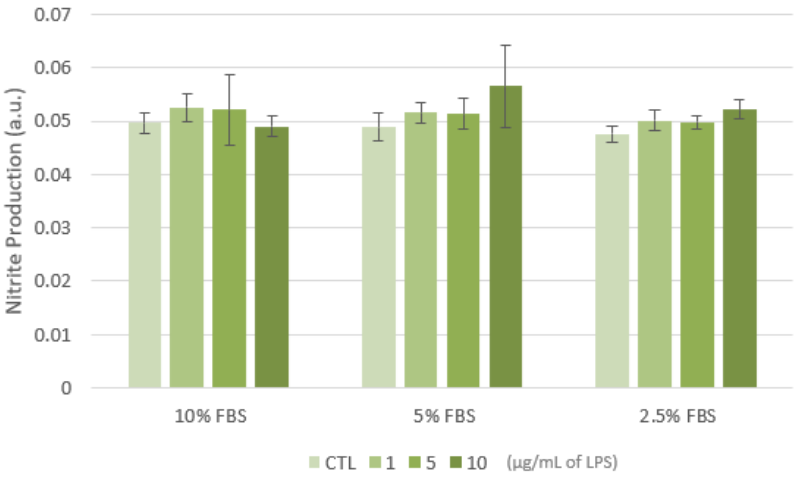


Figure 4.4 — LPS Dose Response in WT BV2 cells, using three different concentrations of LPS O111:B4 (1, 5 and 10 µg/mL) and three different concentrations of FBS (10, 5 and 2.5%) with the aim to understand if the FBS was over-protecting cells. Supernatant was collected from WT BV2 cells pre-treated with LPS for 24 hours. *n*=1.

As it can be seen in **Figure 4.4**, the results were not positive, not reaching the peak of 0.1 a.u., as it was expected.

Then, two different sources of sera were used (a different lot of FBS and a horse's serum) in order to assess if that could be the problem. At the same time, the experiment was performed in the usual condition of 24 hours of LPS stimuli, but also for 6 hours of LPS stimuli, in order to understand if a shorter time of exposition to LPS could prevent a more aggressive feature of LPS. In fact, it was also hypothesized that 24 hours would be too long leading to cell death.

Nevertheless, no inflammatory response was found (**Figure 4.5**), thus the issue may not be due to the FBS, once neither the concentrations nor the different lots solved the issue of the induction of inflammation by LPS.

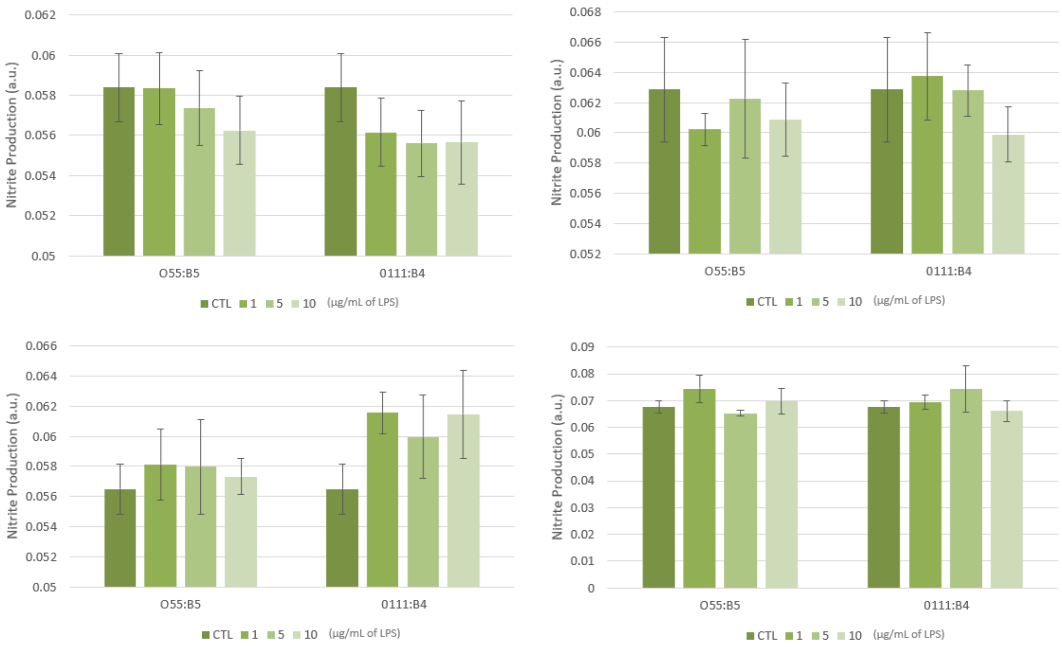


Figure 4.5 — LPS Dose Response in WT BV2 cells using different lots of sera (horse's serum (left) and a different lot of FBS (right)). Supernatant was collected from WT BV2 cells pre-treated with LPS. Top panel shows the NO Quantification Assay performed after 6 hours of LPS (1, 5 and 10 µg/mL of LPS O55:B5 and O111:B4) and bottom panel shows the same experiment performed after 24 hours of LPS.

Lastly, and still concerning medium composition, there is literature regarding BV2 cell line and neuroinflammation that use DMEM-F12 as a culture medium. Thus, RPMI-1640 was substituted by DMEM-F12 to verify if this could overcome the issue. One key difference between these media is that DMEM-F12 contains glutamine (2.5 mM), whereas RPMI-1640 does not. In order not to change the original protocol, glutamine was also supplemented to DMEM-F12 leading to a final concertation of 6 mM of glutamine instead of the used 2 mM glutamine in RPMI-1640. As it can be seen in **Figure 4.6**, using DMEM-F12 instead of RPMI-1640 solved the issue, since all the values of the three different LPS reached the expected value of 0.1 a.u.. This can be happening due to an involvement of glutamine in the inflammatory response.

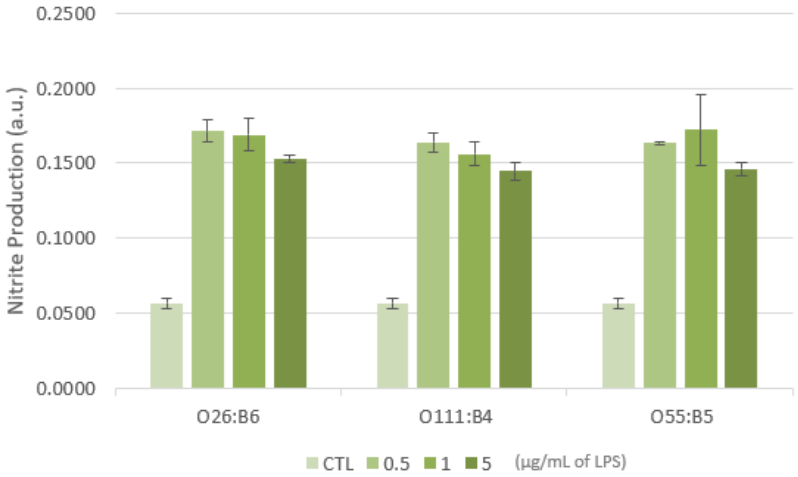


Figure 4.6 — LPS Dose Response in WT BV2 cells using DMEM-F12 instead of RPMI-1640. Supernatant was collected from WT BV2 cells pre-treated with LPS. There were used three different LPS (O26:B6, O111:B4 and O55:B5) and three different concentrations of each (0.5, 1 and 5 µg/mL). *n*=1.

Thus, the main reason for BV2 not to respond to LPS may be identified. However, more assays are needed to guarantee stability of results and to optimize glutamine concentration. It is important to highlight that these optimization steps have been performed during the development of this Master thesis. Moreover, the optimization was done only for WT BV2 cell line since KO cell lines always responded to LPS.

4.2 How does CO alter peroxisomal activity?

In order to understand whether and how CO affects the peroxisomal activity, a Catalase Activity Assay was performed in the WT cell line. Catalase is an antioxidant enzyme mainly present inside the peroxisome thus it is a good indicator of peroxisomal activity[148]. It degrades peroxides, essentially derived from the peroxisomal metabolism, namely lipid peroxidation. The literature is controversial regarding catalase activity in response to LPS-induced inflammation[149–151]. It is known that when catalase is inhibited in macrophages, the inflammation gets more exacerbated[152]. However, there is no data about changes in its activity in microglia. Also, the literature does not refer to anything about the CO's impact on catalase activity, becoming a novel approach in this thesis project.

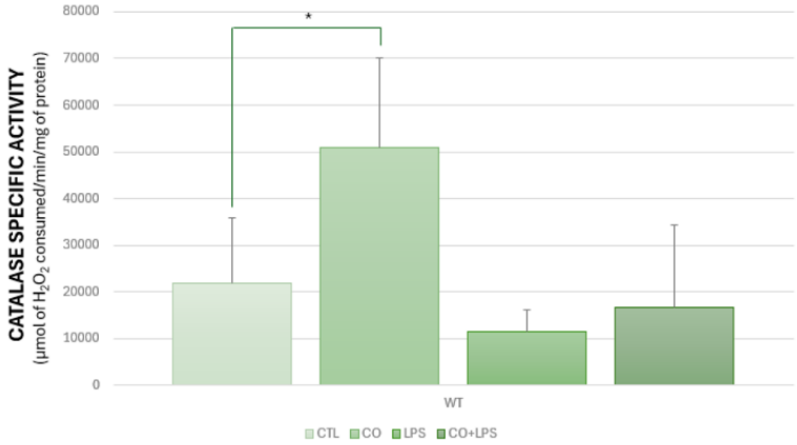


Figure 4.7 — Catalase activity increases when WT BV2 cells are treated with CO. Cells were treated with 50 µM of ALF-826 for 24 hours and 1 µg/mL of LPS for 23 hours. For the group treated with both CO and LPS, ALF-826 was added 1 hour before LPS. n=4; data analyzed with 2 tails unpaired t test; *p<0.05.

Figure 4.7 shows the enzymatic activity of catalase in the four groups: Control (CTL), CO, LPS and CO + LPS. As it can be observed, low doses of CO significantly increase catalase activity. One can speculate it can be due to CO's anti-neuroinflammatory features. LPS seems to decrease catalase activity, and in CO pre-treated cells, LPS increases catalase activity when compared to the LPS group. Nevertheless, no statistical difference was found and regarding LPS effect on catalase activity we also obtained many other incongruent results, apparently in accordance with the literature. In conclusion, CO increases catalase activity, which may be explained by three options: (i) catalase activity is increased because of a conformational change, (ii) CO stimulates peroxisomal biogenesis, increasing its population or (iii) catalase is activated by the antioxidant response triggered by low levels of ROS produced by CO.

For that reason, Western Blot against Catalase were performed in order to understand if the peroxisomal population changed (i) when peroxisome is not functional and (ii) when cells are treated with CO and LPS. Since catalase is primarily found in the peroxisome, it serves as an indirect way to measure the peroxisomal population.

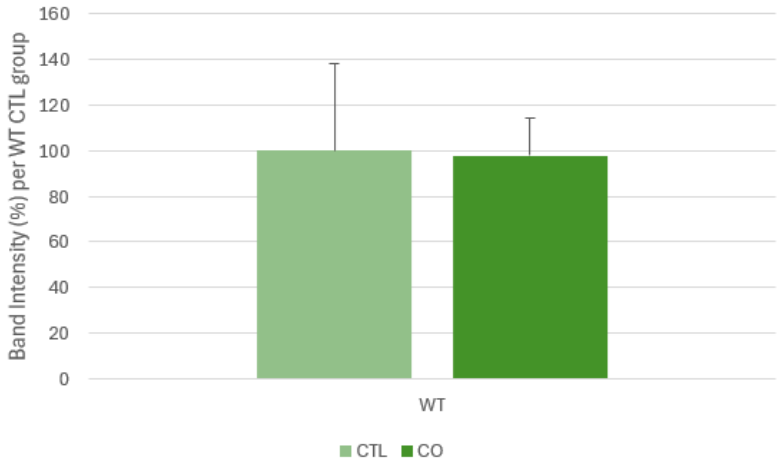


Figure 4.8 — Treatment with CO does not alter the peroxisomal population in WT BV2 cells. Cells were treated with 50 μM of ALF-826 for 24 hours. Catalase expression was assessed by Western Blot. *n*=4; data analyzed with 2 tails unpaired *t* test.

First, catalase expression was compared in WT BV2 cells treated with CO to determine if the previously observed increase in catalase activity was due to increased expression. As we can see in **Figure 4.8**, there is no significant differences between CTL and CO groups. Thus, the previously observed increase in catalase activity is likely due to enhanced enzymatic activity rather than increased enzyme expression.

Then, we compared the effect of LPS in the expression of catalase in WT BV2 cells. However, the results obtained were not consistent (data not shown), needing further experiments to draw conclusions.

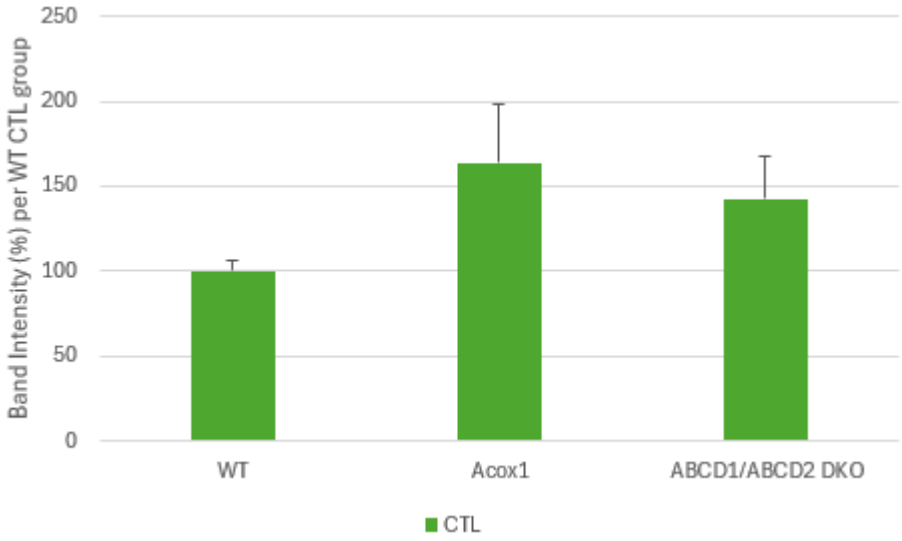


Figure 4.9 — Catalase Expression increases when peroxisome is malfunctioning. Samples were collected from untreated WT, Acox1 KO and ABCD1/ABCD2 DKO BV2 cells. Catalase expression was assessed by Western Blot. *n*=3; data analyzed with 2 tails unpaired *t* test.

Finally, we wanted to understand if a peroxisomal malfunction can interfere with the expression of catalase. As we can see in **Figure 4.9**, both KO cell lines show increased catalase expression, it may indicate a compensatory response to impaired peroxisomal function. This may help offset the loss of peroxisomal activity or counteract the excess ROS generated by peroxisome deficiency.

It was also applied a catalase inhibitor, in order to understand if a catalase inhibition increased or not peroxisomal population. **Figure 4.10** shows that no conclusion can be taken since high variable results were obtained in the presence of 3-AT concerning catalase expression.

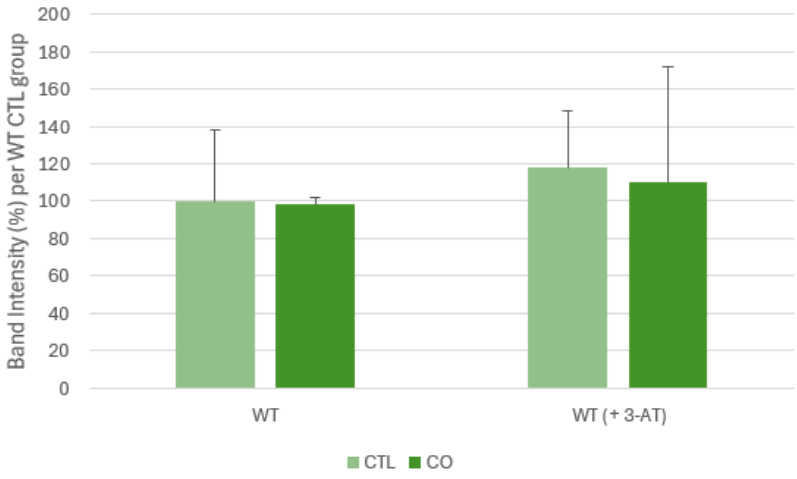


Figure 4.10 — CO impact on peroxisomal population when catalase is inhibited in WT BV2 cells. Cells were treated with 50 µM of ALF-826 for 24 hours and with 2 mM of 3-AT for 23 hours. For the group treated with both CO and

3-AT, ALF-826 was added 1 hour before 3-AT. Catalase expression was assessed by Western Blot. *n*=2; data analyzed with 2 tails unpaired *t* test.

4.3 Is peroxisome involved in the anti-inflammatory CO response?

With the aim of understanding if the peroxisome is necessary for CO to be anti-neuroinflammatory, WT and KO BV2 cells for peroxisomal enzymes were tested.

First, WT BV2 cells were treated with a CORM ALF-826 at 50 µM for 1 hour followed by LPS treatment at 0.5 µg/mL (LPS) for 23 hours (**Figure 4.11**). According to what was previously published[43,44], CO protects against neuroinflammation, by partially preventing cellular NO production assessed by quantification in the culture supernatant. There is only one biological replica because of all technical problems described in Section 4.1.

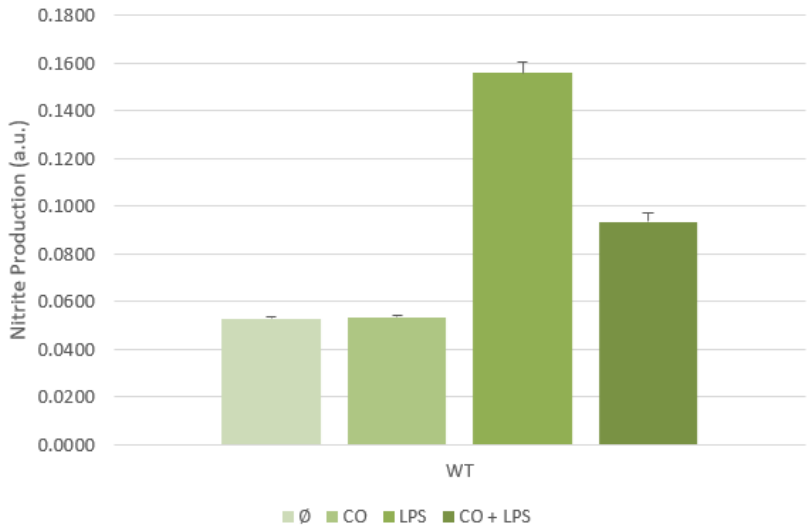


Figure 4.11 — CO reduces the inflammatory characteristics of WT BV2 cell's secretions. Cells were treated with 50 µM of ALF-826 for 24 hours and 1 µg/mL of LPS for 23 hours. For the group treated with both CO and LPS, ALF-826 was added 1 hour before LPS. The supernatant from WT BV2 cells was analyzed to measure nitrite concentrations using Griess colorimetric assay. *n*=1.

Then Acox1 KO and ABCD1/ABCD2 DKO BV2 cell lines were challenged with LPS, in the presence or absence of CO. In Acox KO and ABCD1/ABCD2 DKO cells, peroxisomes are dysfunctional because they are unable to efficiently metabolize VLCFAs and other lipid substrates, leading to the accumulation of toxic metabolites and impaired peroxisomal functions. Acox1 KO cells lack Acox1, the first enzyme involved in β-oxidation of VLCFAs and ABCD1/ABCD2 DKO cells lack ABCD1 and ABCD2 transporters, two of the main transporters of VLCFAs into the peroxisome.

As we can see in **Figure 4.12**, Acox1 KO and ABCD1/ABCD2 DKO cells show a similar profile as the WT cells, meaning CO is still able to prevent neuroinflammation. ABCD1/ABCD2 DKO cells seem to have a lesser reversion of nitrite production compared to Acox1 KO, potentially meaning that these cells could depend more on the peroxisome for CO to promote anti-neuroinflammation. However, both these cell lines profiles suggest that peroxisome might not be necessary in the process of anti-neuroinflammation promoted by CO.

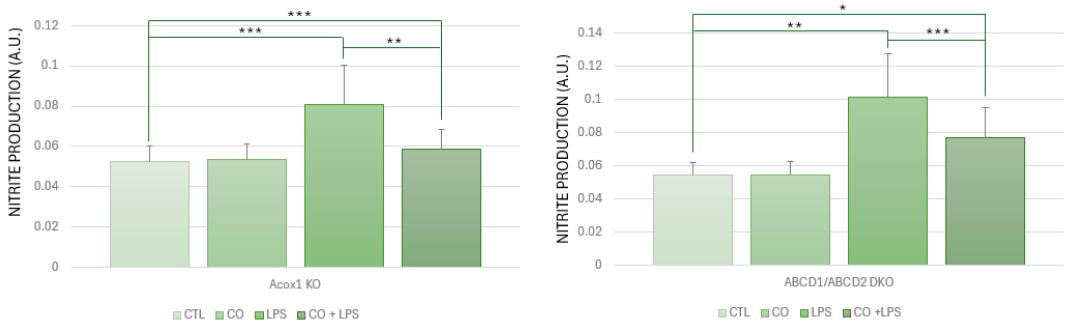
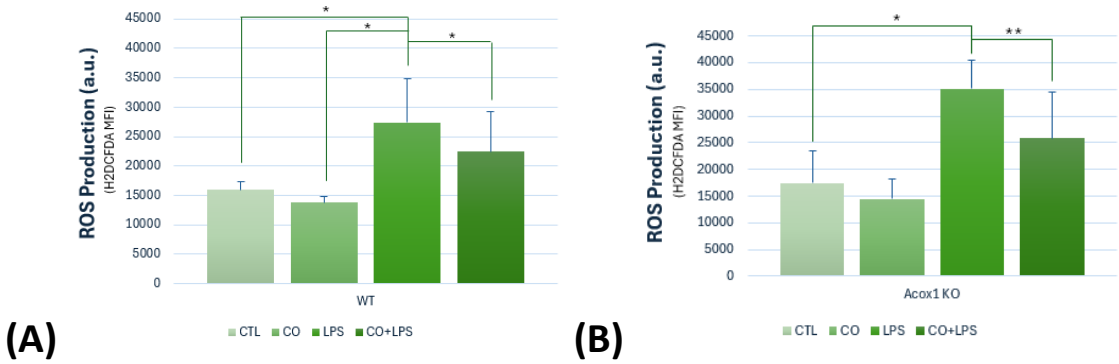


Figure 4.12 — CO partially attenuates the inflammatory profile of Acox1 KO (left) and ABCD1/ABCD DKO (right) BV2 cells. Cells were treated with 50 µM of ALF-826 for 24 hours and 1 µg/mL of LPS for 23 hours. For the group treated with both CO and LPS, ALF-826 was added 1 hour before LPS. The supernatant of the two cell lines was analyzed to measure nitrite concentrations using Griess colorimetric assay. Acox1 KO: n=9; ABCD1/ABCD2 DKO: n=7; data analyzed with 2 tails unpaired t test; *p<0.05, **p<0.01; ***p<0.001.

4.4 Is the peroxisome involved in ROS production when cells face inflammation?

In order to complement the NO quantification assays, ROS quantification assays were performed to assess neuroinflammation. It is described in the literature that LPS provokes stress to cells, generating high quantities of ROS, turning into a good inflammation biomarker. Then, it is expected to see a reversion in the ROS production of CO + LPS group of WT cells. If Acox1 KO and ABCD1/ABCD2 DKO cells do not present this profile, it could mean that peroxisome could be involved in the anti-neuroinflammatory process of CO.



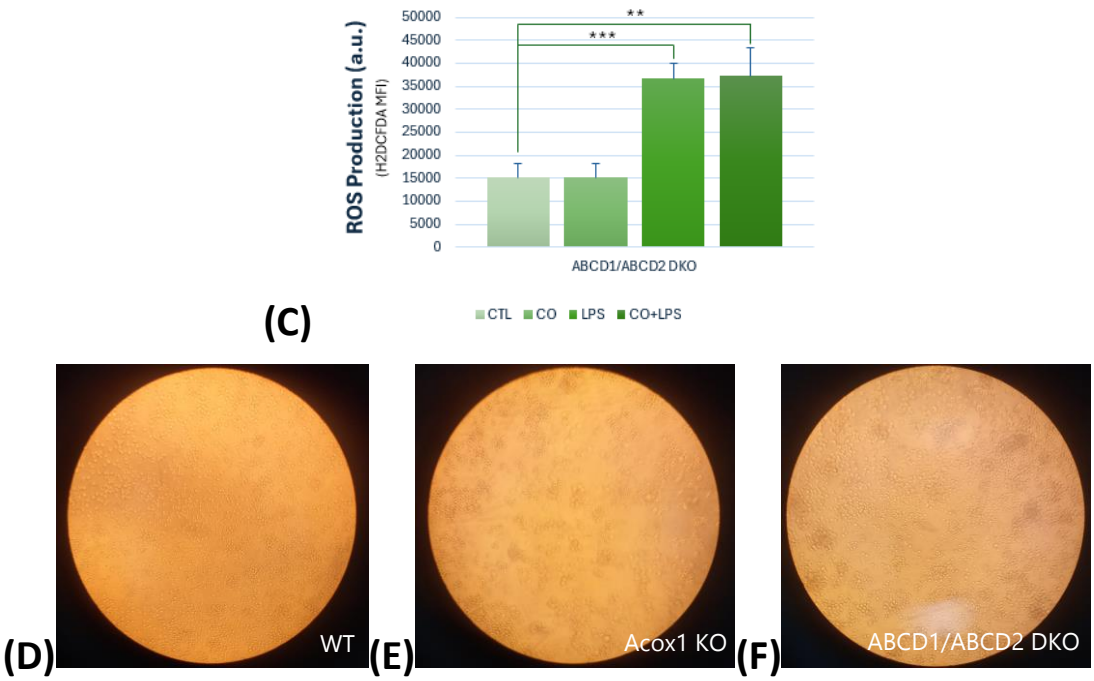


Figure 4.13 — CO decreases ROS production in WT (A) and Acox1 KO (B) BV2 cells but does not exhibit the same effect in ABCD1/ABCD2 DKO (C) BV2 cells. Cells were treated with 50 μM of ALF-826 for 24 hours and 1 μg/mL of LPS for 23 hours. For the group treated with both CO and LPS, ALF-826 was added 1 hour before LPS. Intracellular H_2O_2 was measured by fluorescence using H_2DCFDA. Similar concentrations of cells were seeded in each well for WT (D), Acox1 KO (E) and ABCD1/ABCD2 DKO (F) BV2 cells. WT; n=5; Acox1 KO: n=5; ABCD1/ABCD2 DKO: n=5; data analyzed with 2 tails unpaired t test; *p<0.05, **p<0.01; ***p<0.001.

Figure 4.13A shows ROS production by WT BV2 cell line under different conditions. We can see an increase when cells are treated with LPS, which was expected. In CO + LPS group, there is a reduction on the ROS production, showing the anti-neuroinflammatory role of CO. It is important to notice that the ROS quantification is not as specific as NO quantification in characterizing inflammation, since ROS can be generated by numerous reasons and pathways.

In **Figure 4.13B**, we can observe a similar profile from Acox1 KO compared to WT cells. The results obtained are coherent with the NO quantification assay, since the patterns are similar. CO keeps inhibiting inflammation, even though Acox1 is not present.

In **Figure 4.13C**, we can see that, when ABCD1 and ABCD2 are absent, CO seems to depend on the peroxisome to inhibit ROS generated by LPS. This can be correlated with the partial reversion these cells presented at NO quantification assay. The results seem to indicate that CO may depend on the peroxisomal activity for reducing ROS production in response to inflammation, when ABCD1 and ABCD2 transporters are not present.

In both KO cell lines, there is a significantly greater increase in ROS production compared to the WT, when treated with LPS. This highlights the role of peroxisomes in cellular redox

response and in inflammation, as studies indicate that dysfunctional peroxisomes lead to heightened inflammatory responses[128].

4.5 Is peroxisome involved in the expression of TNF-α regarding inflammation?

Another strategy to measure neuroinflammation was the evaluation of TNF-α in the secretome of the three cell lines under different conditions. ELISA Assay was performed to compare the expression of TNF-α, a pro-inflammatory cytokine in the presence and absence of CO and/or LPS. These conditions were imposed on WT, Acox1 KO and ABCD1/ABCD2 DKO cell lines. It is expected to observe, in the WT cells, an increase in TNF-α concentration in response to LPS and a reversion of TNF-α in CO pre-treated cells.

Figure 4.14 shows the ELISA assay for the three cell lines. As it is observable, the results obtained from the WT cell line were not expectable since LPS did not promote TNF-α production and inflammatory response, thus, the experiment needs to be repeated. Moreover, when this assay was performed the concentration of glutamine in the medium was not yet optimized. Thus, we cannot take any conclusion from the KO cell lines without a right profile from the WT cells.

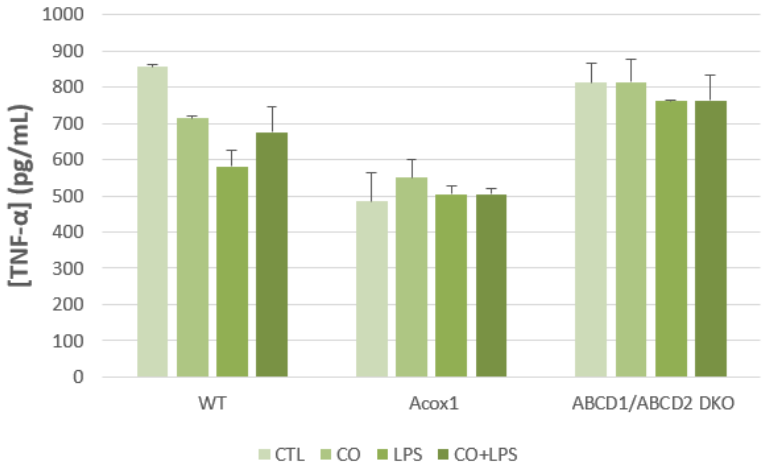


Figure 4.14 — TNF-α Quantification for WT, Acox1 KO and ABCD1/ABCD2 DKO BV2 cells. Cells were treated with 50 μM of ALF-826 for 24 hours and 1 μg/mL of LPS for 23 hours. For the group treated with both CO and LPS, ALF-826 was added 1 hour before LPS. The supernatant of the two cell lines was analyzed to measure TNF-α concentrations via ELISA. *n*=1.

5

Discussion and Conclusion

5.1 Optimization of LPS-induced neuroinflammation in WT BV2 cell line

First it was necessary to disclose why LPS was not able to promote an inflammatory response, namely NO production in WT BV2 cells. Many efforts were made to understand what factor was restraining the induction of inflammation to WT BV2 cells. In fact, using DMEM-F12 instead of RPMI-1640 led LPS group reach the expected value of NO production. A likely explanation is that, because DMEM-F12 is a more nutrient-rich medium than RPMI-1640, it stimulates BV2 cells to have a stronger metabolic response to inflammation triggered by LPS.

Also, using DMEM-F12, the concentration of glutamine was higher (6 mM) when compared with RPMI-1640 (2 mM). With a higher concentration of glutamine, the results show that WT BV2 cells respond to LPS stimulation. Actually, glutamine has been described as an anti-inflammatory molecule, that can reduce high levels of NO production[153] and inhibit the initiation of this process by downregulating TLR4 in intestinal cells[154]. Then, the results of a higher concentration of glutamine lead to more inflammation seem inconsistent, as glutamine is generally known for its anti-inflammatory properties, making it unlikely to promote inflammation.

Regarding TLR4, this receptor is the one responsible for activating a signal transduction when cells are in contact with LPS. LPS cannot interact directly with TLR4, binding to it *via* the adapter protein myeloid differentiation factor-2 (MD-2)[155]. Subsequently, this complex forms a dimer, initiating intracellular signaling. If glutamine was downregulating the expression of TLR4, a reduced production of NO would be expected due to decreased TLR4 signaling. Before the change of medium, it was hypothesized that the previous cells could have lost the expression of one of these proteins and could not respond to the stimulation of LPS. However, even

the passage number 7 cells must have lost the expression of these proteins, which is very unlikely to occur. Also, a spontaneous overexpression of Interleukin 1 Receptor Associated Kinase M (IRAK-M) and Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1 (SHIP1) could have inhibited the TLR4 response to LPS[156,157]. Using antagonists of this pathway is a potential strategy to understand this problem. Still, negative and positive control cells could have helped to understand if the problem is coming from the cells or other factors. For example, WT BV2 cells from a different source could serve as a positive control to clarify this.

Another possibility is that glutamine added to RPMI-1640 was degraded. Unlike DMEM-F12, RPMI-1640 does not contain glutamine by default, which might affect the response to LPS, not because of its anti-inflammatory role, but due to a lack of glutamine needed for metabolism. Theoretically, a degraded glutamine should have also caused a higher NO production, due to the anti-inflammatory feature of glutamine. Again, this hypothesis is unlikely since inflammation was induced to other cells using the same glutamine. Besides glutamine, there are still other factors present in RPMI-1640 that are not found in DMEM-F12, such as biotin, vitamin B_{12} and Para-Aminobenzoic Acid (PABA). However, none of these compounds were found to be anti-inflammatory. Also, RPMI-1640 contains high levels of inositol and choline. Choline is crucial for producing acetylcholine, a neurotransmitter that is part of the cholinergic anti-inflammatory pathway, which regulates the immune response and reduces inflammation[158]. GSH is also present in RPMI-1640, which is a powerful reducing agent that helps to neutralize free radicals, detoxification and regulate immune system, reducing inflammation[159]. These two last compounds could explain the reduced ability to trigger inflammation, still being unlikely since RPMI-1640 was always used in inflammation experiments in BV2 cells in our lab. To investigate whether these differences affect inflammation induction, it would be useful to test WT BV2 cells with an anticholinergic, a GSH inhibitor and/or RPMI-1640 medium supplemented with 6 mM of glutamine. No compounds present in DMEM-F12 were found to be pro-inflammatory to help stimulate inflammation in WT BV2 cells.

Despite these considerations, there were still other factors that could have been tested. It was considered that the issue might be related to LPS. The KO cell lines responded to LPS stimulation. However, since these cells do not present their peroxisome fully functional, they do not represent good controls to inflammation assays. When FBS is not well heat inactivated, LPS can be sequestered by FBS proteins. Lipopolysaccharide binding protein (LBP)[160,161], High-density lipoprotein (HDL), Low-density lipoprotein (LDL), albumin and hemoglobin[162] are examples of proteins present in FBS that can sequester LPS and restrain its action. If FBS was not

heat inactivated, or inactivated for a shorter period, this hypothesis could justify what happened. However, since it was tested many lots of FBS, it is unlikely that all of them were not well heat inactivated. LPS can also self-aggregate, generating a weaker induction of inflammation[163]. However, some of the LPS solutions used in the cells were also used before, when LPS induced inflammation in WT BV2 cells, making this explanation unlikely to be happening.

A high confluence of cells in the wells could also hinder LPS stimulation. However, many cell concentrations were tried along the seeding procedure and no change was found.

Another possibility was the presence of mycoplasma. As the literature affirms that mycoplasma reduces the sensitivity of BV2 cells to the inflammation induction of LPS[164]. Actually, cells can survive long periods of time contaminated with mycoplasma, altering cell metabolism, growth rate and changes in DNA[165], potentially causing the decrease of sensitivity of BV2 cells to LPS induction. However, this just could have happened if all the frozen cells were contaminated with mycoplasma since passage number 7, which is not true since LPS stopped inducing inflammation in the last year.

A possible solution to this problem is the use of another inflammation stimuli such as TNF-α and Interferon γ (IFN-γ), once they are recognized by different receptors in the cell and can induce inflammation in a more robust manner. Even though NO quantification assays did not show inflammation in WT BV2 cells, LPS-treated cells had a more rounded shape, suggesting that LPS may have had an effect.

5.2 CO's impact in catalase activity and expression

Regarding catalase activity as a measure of peroxisomal function, CO increases significantly catalase activity. As it is known that low concentrations of ROS induced by CO activates antioxidant cell mechanisms, such as GSH and HO-1[166]. This might be accompanied by an increase in other antioxidant enzymes like catalase, as part of a coordinated response to enhance the cell's ability to handle oxidative stress. Therefore, there are three possible explanations for CO to stimulate catalase activity: (i) CO activates catalase through an antioxidant response to low ROS levels produced by CO, (ii) CO stimulates the growth of peroxisomes, increasing their number or (iii) CO causes a conformational change in catalase.

First, CO promotes activation of p38 MAPK[167], NF-κB[168] and PPARγ[169], factors that upregulate catalase activity[170–172]. To confirm if this possibility is true, expression levels of these factors must be measured. Nevertheless, this might be accompanied by an increase in expression of catalase. Cells could also be upregulating catalase as a protective mechanism in response to

CO exposure and its excessive ROS generation, which could lead to oxidative damage. However, this is not the case since these cells are only exposed to low concentrations of CO.

To understand if there is actually an increase in catalase expression, Western Blot assays were performed. Western blot results show no evidence that CO increases catalase activity, suggesting that CO does not promote catalase expression. Another possibility to occur is a conformational change in the enzyme. There was hypothesis in past papers that CO could interact with catalase's heme moiety, increasing its activity[67]. However, it is less likely, since catalase contains Fe(III)[173,174] and CO interacts only with Fe(II)[175]. It is still possible that CO induces a conformational change in the enzyme to enhance its activity, but studies regarding enzyme conformational changes are needed to confirm the potential interaction between CO and catalase.

There was no significant difference of catalase activity induced by LPS. Actually, during the experiments, the results of this condition were not consistent, impeding conclusions. This might have happened because of the malfunction found in LPS inflammation induction.

Additionally, we wanted to test how a peroxisomal malfunction would affect the expression of catalase. Actually, the results obtained showed an increase in the expression of catalase when peroxisome is not functioning well, namely when Acox-1 or ABCD1/ABCD2 are knocked out. This may occur because the cell is either compensating for an imbalance or trying to protect itself from increased ROS production[128]. Raas's group observed that cells lacking Acox1 present an increase in catalase activity[176]. Then, we speculate that it must happen due to an increase in its expression. To determine more precisely if the peroxisomes are suffering more biogenesis or it is just catalase that is being more expressed, it would be useful to examine the expression of other peroxisomal proteins that are not involved in antioxidant defense, such as PEX19. Several Western Blot assays targeting PEX19 were conducted; however, no conclusive data was obtained, and further optimization of the experimental conditions is required. This would help assessing whether the overall production of peroxisomal proteins is increasing (suggesting enhanced peroxisomal biogenesis) or if only antioxidant-related proteins are being upregulated in response to the oxidative stress.

Regarding pharmacological inhibition of catalase using 3-AT, it is described in the literature that a catalase inhibition increases peroxisomal biogenesis[107]. However, our results show no increase in its expression, making it impossible to find a conclusion. Walton's group only found an increase in peroxisomal biogenesis after 48 hours of 3-AT treatment and not 24 hours, needing a repetition of the experiments[107].

5.3 Peroxisomal role in CO's antioxidant and anti-inflammatory features

Since CO shows to have an impact on catalase activity, an abundant antioxidant enzyme in peroxisome, could peroxisomes be involved in CO's anti-inflammatory effects? Although WT BV2 cells responded to LPS producing the expected levels of NO only once, CO reverted inflammation in activated microglial cells, which is in accordance with the literature [43,44].

Therefore, if the peroxisome was necessary for the anti-inflammatory action of CO in Acox1 KO and ABCD1/ABCD2 DKO cell lines, CO should not limit neuroinflammation stimulated by LPS. The results obtained present a partial reversion of NO production promoted by CO in LPS-inflamed cells, in both cell lines, suggesting that the CO's anti-neuroinflammatory effect might not be dependent on peroxisome activity.

It is already described in literature that peroxisomal deficiencies lead to inflammation[128]. This can justify the fact that LPS stimulates KO cells easier and not WT cells. Acox1 deficiency triggers Interleukin 1 (IL-1) inflammation pathway[177] and ABCD1/ABCD2 deficiency accumulate elevated levels of VLCFAs[178], generating stress to the cells. This can exacerbate inflammatory response to these cells, limiting the anti-inflammatory role of CO. However, this indicates that CO's action depends on low levels of cellular stress, a claim that is not supported by the existing literature.

Even though being less specific, ROS increased levels can also indicate inflammatory response. ROS are produced when cells face oxidative stress, promoted by inflammation or other sources of biological stress, being less specific for that reason.

One of the first results we observe is that both KO cell lines present a higher levels of ROS production, probably due to an accumulation of VLCFAs inside or outside the peroxisomes, generating oxidative stress. Also, the results obtained showed that CO reverts ROS production when cells are stimulated with LPS in Acox1 KO cell line, a similar profile as the WT cell line. In contrast, in ABCD1/ABCD2 DKO cells, CO did not revert ROS production in LPS stimulated cells, indicating that ABCD1 and ABCD2 may participate in the anti-inflammatory role of CO. But why does this happen only in ABCD1/ABCD2 DKO cell line?

Actually, accumulation of fatty acids leads to oxidative stress and excess of ROS damages, eventually masking the anti-inflammatory effect of CO. In ABCD1/ABCD2 DKO cells, impaired VLCFA import might lead to the accumulation of these fatty acids or their toxic metabolites in cytosol[179]. This could enhance oxidative stress, overwhelming the antioxidant response

that CO typically induces, thereby preventing the reversion of ROS production. The disruption of VLCFA metabolism in ABCD1/ABCD2 DKO cells might cause metabolic imbalances, leading to secondary effects such as mitochondrial alterations[180]. This could result in higher baseline ROS levels or an inability to control ROS levels when exposed to LPS, even in the presence of CO. However, this is less likely since the values of the control groups in all the cell lines were similar. These compensatory mechanisms might be less effective in ABCD1/ABCD2 DKO cells, leading to the observed failure to revert ROS production. Also, ABCD1/ABCD2 DKO cells might exhibit broader disruptions in peroxisomal function, affecting not only β-oxidation but also the detoxification processes linked to peroxisomes, thus exacerbating ROS production even when CO is applied. However, ABCD1/ABCD2 DKO cells may present and express ABCD3, another Long Chain Fatty Acids (LCFAs) transporter, allowing these molecules to enter in peroxisome by other pathway[181]. There are no studies about the expression of ABCD3 when ABCD1/ABCD2 are not expressed. It would be expected that this protein might be more highly expressed to compensate for the loss of the other two transporters. However, this hypothesis had to be more explored.

But why does this not happen in Acox1 KO cells? A possibility is an adaptation of the cell line to overcome the dysfunction present in peroxisome, enhancing other pathways. Acox1 is the first enzyme to act in β-oxidation of VLCFAs. So, contrary to ABCD1/ABCD2 cell line, VLCFAs might enter peroxisome, being accumulated inside of it. The use of alternative Acyl-CoA Oxidases (Acox2 and Acox3) and an increase of β-oxidation in mitochondria are unlikely, since there is no evidence of crosstalks between these enzymes and mitochondria do not have the machinery to catabolize VLCFAs. The most probable possibilities are: (i) an activation of PPAR-α, activating a higher expression of fatty acid metabolism genes[182] or (ii) cells could have rewired their metabolism to rely more on glycolysis or amino acid catabolism for energy, reducing the need for fatty acid oxidation as a primary energy source[183] (generating, in the same way, oxidative stress).

TNF-α can also be a good molecule to measure inflammation in BV2 cells. Regarding TNF-α quantification by ELISA, the results were inconclusive since LPS did not induce TNF-α increase, which is largely described in the literature[44]. Therefore, more ELISA assays are needed to describe the alterations in cytotoxic cytokines in response to LPS-induced inflammation and to assess the peroxisome involvement in the CO-induced anti-inflammatory response.

Overall, glutamine may play a key role in LPS-induced inflammation, though further experiments are needed as results conflict with existing literature. GSH and choline, present in RPMI-1640, may also influence inflammation in WT BV2 cells due to their anti-inflammatory

properties. CO increases catalase activity but does not seem to affect catalase expression, though results were not statistically significant. Then, CO might increase catalase activity due to a conformational change in the enzyme. Catalase is highly expressed when peroxisomes present defects. In peroxisome-deficient cells, NO production profile was similar to WT cells, but CO did not revert LPS-induced ROS production in ABCD1/ABCD2 DKO cells, suggesting a link between peroxisomes and CO's anti-inflammatory effects. Future studies should explore glutamine, GSH and choline's role in neuroinflammation and CO's connection to peroxisomal dysfunction and oxidative stress.

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