Glucose and Vascular Inflammation

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2 Abstract

Background: Endothelial cells form a highly dynamic barrier between blood and tissues and can be activated in response to angiogenic and inflammatory stimuli. Endothelial cells rely on glycolysis for ATP production, and it was recently demonstrated that inhibition of endothelial glycolysis attenuates angiogenic activation. We wanted to examine the metabolic profile of inflammatory activated endothelial cells and explore the role of glycolysis in endothelial inflammatory activation.

Methods: Human umbilical vein endothelial cells were stimulated with IL-1β and the rates of glucose uptake, glycolysis and fatty acid oxidation were determined using radioactively labelled substrates. The expression of the metabolic enzymes PFKFB3 and CPT1A were determined by qPCR and confirmed on the protein level by immunoblotting. Glycolysis was partially inhibited using 3PO, a small molecule inhibitor of PFKFB3, and IL-1β induced adhesion molecule expression was assessed using cell ELISA and qPCR. Activation of NFκB was assessed using a NFκB luciferase assay kit and immunoblotting against phosphorylated IκBα, IKKα/β and JNK following stimulation with IL-1β and TNF. The levels of pan-O-GlcNAcylation in whole cell lysates were determined by immunoblotting.

Results: The endothelial glucose uptake was increased by approximately 60% after stimulation with IL-1β ($P<0.001$), correlating with a steady increase in the rate of glycolysis and a decrease in fatty acid oxidation. The increase of glycolysis was associated with increased expression of the glycolytic enzyme PFKFB3, while CPT1A, the rate limiting enzyme of fatty acid oxidation was not significantly affected by inflammatory activation. Inhibition of glycolysis attenuated endothelial upregulation of the adhesion molecules E-selectin and VCAM-1, correlating with reduced phosphorylation of key signalling molecules in both the NFκB and the JNK pathways.

Conclusion: We have shown that endothelial cells upregulate their rate of glycolysis in response to IL-1β stimulation and that this correlates with an increased expression of the rate limiting glycolytic enzyme PFKFB3. Pharmacological inhibition of glycolysis reduced the upregulation of adhesion molecules involved in in leukocyte recruitment by attenuating activation of inflammatory signalling cascades, raising the question whether PFKFB3 could be a potential therapeutic target in inflammatory disease. The exact molecular mechanisms by which 3PO inhibits phosphorylation of signalling molecules has not yet been determined and will be targeted in future experiments.

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3 Abbreviations

3PO – 3-(3-pyridinyl)-1-(4-pyridinyl)-2-preopen-1-one

(R)AGE – (Receptor for) Advanced glycation end-products

ATP – Adenosine triphosphate

CPT1A – Carnitine palmitoyl transferase 1A

ELISA – Enzyme-linked immunosorbent assay

FAO – Fatty Acid oxidation

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

HBP – Hexosamine biosynthesis pathway

HUVEC – Human umbilical vein endothelial cells

IL-1β – Interleukin 1β

NFκB – Nuclear factor κB

O-GlcNAc – O-linked β-N-acetyl glucosamine

PFKFB3 – 6-phopsphofructo-2-kinase/fructose-2,6-bisphosphatase 3

PPP – pentose phosphate pathway

qPCR – Quantitative polymerase chain reaction

ROS – Reactive oxygen species

RT-PCR – Reverse transcriptase polymerase chain reaction

SD – standard deviation

TCA – Tricarboxylic acid

TNF – Tumour necrosis factor

UDP – Uridine diphosphate

VCAM-1 – Vascular cell adhesion molecule 1

VEGF – vascular endothelial growth factor

4 Introduction

Vascular endothelial cells form the inner layer of all blood vessels. Once believed to be a mostly quiescent passive barrier between the blood and tissue, the endothelium is now known to be a highly dynamic system that orchestrates important biological functions, including regulation of vascular tone, maintenance of blood fluidity, transport of molecules across the blood vessel wall and recruitment of immune cells to tissues. In homeostasis endothelial cells remain quiescent through cell-cell mediated Notch signalling and the transcription factor Krüppel-Like factor 4 (KLF4) (Doddaballapur et al., 2014; Noseda, Chang, & McLean, 2004). While endothelial cells may be quiescent for years they are highly dynamic and react rapidly to stimuli such as hypoxia, vascular endothelial growth factor (VEGF) and inflammatory stimulation such as IL-1β and TNF (Eppihimer, Wolitzky, Anderson, Labow, & Granger, 1996; Haraldsen, Kvale, Lien, Farstad, & Brandtzaeg, 1996; Michiels, Arnould, & Remacle, 2000; Nash, Baca, Wright, & Scotney, 2006). In addition to their role in blood vessel formation endothelial cells are also important in the immune response by recruiting leukocytes in response to proinflammatory cytokines or bacterial endotoxin (Collins et al., 1995). It has been shown that both hypoxia and angiogenesis induces a glycolytic shift that is at least in part dependent on the enzyme 6-phosphofructokinase-2/fructose-2,6-bisphosphatase (PFKFB3)(De Bock et al., 2013; Obach et al., 2004; Xu et al., 2014). Metabolic shifts following activation is not confined to endothelial cells. Immune cells, including dendritic cells, macrophages and T cells have been shown to rely on a glycolytic shift to activate their effector functions (C.-H. Chang et al., 2013; Krawczyk et al., 2010; Rodríguez-Prados et al., 2010; Tannahill et al., 2013). Considering the importance of glycolysis for angiogenic endothelial activation and for inflammatory effector functions in immune cells we asked if glycolysis could also play a role in inflammatory activation of endothelial cells.

4.1 Metabolism of endothelial cells

Endothelial cells show a characteristic metabolic profile as there is a reliance on cytoplasmic glycolysis even when oxygen is abundant; as much as 85% of the energy requirement can be covered by glycolysis alone (De Bock et al., 2013). Glycolysis produces 2 molecules of ATP and 2 molecules of pyruvate per glucose molecule while complete oxidation of glucose through the tricarboxylic acid (TCA) cycle followed by oxidative phosphorylation (oxPhos) (also referred to as cellular respiration) results in up to 36 molecules of ATP per glucose molecule (Zheng, 2012). However, because cytoplasmic glycolysis occurs very rapidly, the cell can still

produce more ATP over a given time as long as long as the availability of glucose is not limited (Vander Heiden, Cantley, & Thompson, 2009).

While it might seem paradoxical that endothelial cells, the cells with the closest contact to oxygen through the blood, should rely on glycolysis rather than cellular respiration there are several possible explanations as to why this may be advantageous. By relying on glycolysis endothelial cells can reserve oxygen to the underlying tissue (De Bock et al., 2013), convey tolerance to hypoxia (Mertens, Noll, Spahr, Krutzfeldt, & Piper, 1990), glucose has a higher diffusion limit than oxygen (Gatenby & Gillies, 2004), the mitochondria may be used for other purposes such as cell signalling (Quintero, Colombo, Godfrey, & Moncada, 2006) and glycolysis can produce ATP more rapidly (Vander Heiden et al., 2009). Glucose intermediates can diverge through several branches in addition to the breakdown to pyruvate, including the catabolic pathways the pentose phosphate pathway (PPP), the hexosamine biosynthetic pathway (HBP) (Figure 4.1) (Goveia, Stapor, & Carmeliet, 2014).

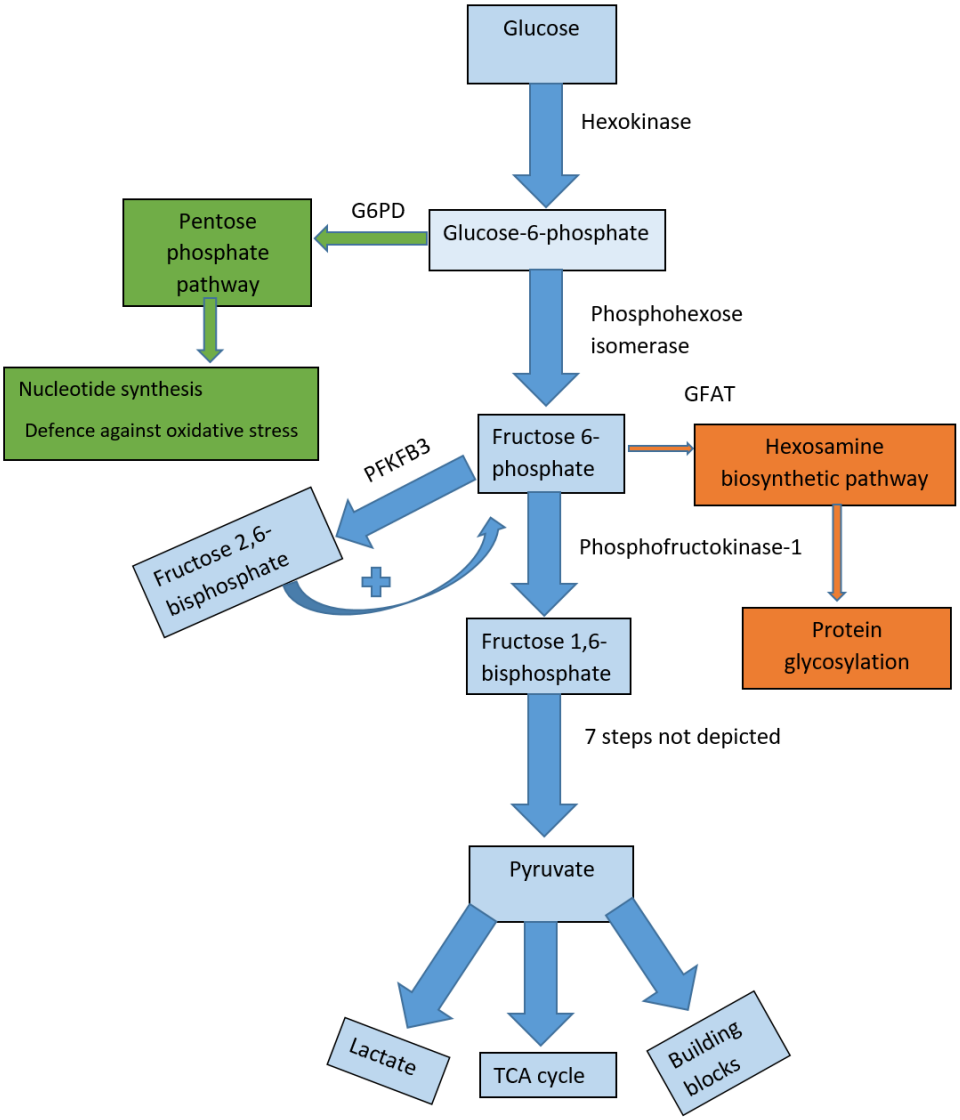

Figure 4.1 **Schematic overview of glycolysis in healthy endothelial cells**. Glycolysis is a 10-step pathway leading to the production of 2 molecules of ATP and 2 molecules of pyruvate which can either be metabolized further to lactate, oxidized in the TCA cycle followed by oxPhos or be used as building blocks. Glycolysis is positively regulated by the production of fructose 2,6-bisphosphate through the activity of the enzyme PFKFB3. The formation of fructose 2,6-bisphosphate is the commitment step of glycolysis, prior to this glucose may be metabolized in the PPP which is important for maintenance of redox balance and the HBP important for protein glycosylation.

4.1.1 Glycolysis

Glycolysis is a 10-step catabolic pathway that results in the net production of 2 molecules of ATP. The major rate-limiting step of glycolysis is the third step involving the activity of phosphofructokinase-1 (PFK-1) that is also the step where glucose is first committed to glycolysis (Nelson & Cox, 2013). The activity of PFK-1 is subject of regulation through allosteric inhibition and activation. Adenosine triphosphate (ATP) reduces the binding affinity to its substrate, fructose-6-phophate, while AMP and ADP relieves this tonic inhibition

(Chesney et al., 1999; Clem et al., 2008; Nelson & Cox, 2013). The activity is further modulated through the actions of the enzyme PFKFB3, which produces fructose 2,-6-bisphosphate, a potent allosteric activator of PFK1 (Clem et al., 2008; De Bock et al., 2013). PFKFB3 inhibition has been explored as a potential strategy for treatment of cancer and pathological angiogenesis because of its importance in regulating glycolysis due to low cytotoxicity compared to a complete blockade of glycolysis (Clem et al., 2008, 2013; Schoors et al., 2014; Xu et al., 2014).

4.1.2 Hexosamine biosynthesis pathway and O-GlcNAc

The hexosamine biosynthesis pathway (HBP) is a branch of glucose metabolism. Fructose-6-phopsphate, the substrate of PFK-1 is also the substrate of the rate-limiting enzyme in HBP, glutamine:fructose-6-phosphate amidotransferase (GFAT). The HBP produces uridine diphosphate (UDP)-glucose, a substrate used for protein glycosylation. The activity of GFAT is negatively regulated by protein kinase A which phosphorylates GFAT on Ser205 and by feedback inhibition from UDP-glucose (Broschat et al., 2002; Q. Chang et al., 2000).

The HBP can affect the activity of thousands of proteins through a post-translational modification involving the attachment of O-linked N-acetylglucosamine to serine and threonine residues on proteins. This protein modification, called O-GlcNAcylation, can affect the activity of proteins in several ways by preventing protein-protein interactions leading amongst other things to reduced availability of these sites for activating and inhibitory phosphorylation (Bond & Hanover, 2015).

4.1.3 Pentose phosphate pathway

The pentose phosphate pathway is another branch of glucose metabolism which is important for the production of nucleotides and maintenance of redox balance in endothelial cells. One of the main products of PPP is NADPH. NADP can be used to reduce glutathione, which acts as a buffer against endogenous H_2O_2 (Eelen, de Zeeuw, Simons, & Carmeliet, 2015), thereby controlling oxidative stress.

4.1.4 Fatty acid oxidation

While glycolysis is the main source of energy production and fatty acid oxidation, and (FAO) only contributes with 5-10% of the total ATP, FAO is by no means unimportant (Patella et al., 2015; Schoors et al., 2015). In proliferating endothelial cells FAO is important for the production of deoxyribonucleotides (dNTPs) (Schoors et al., 2015). The rate limiting enzyme of FAO, carnitine palmitoyl transferase 1 A (CPT1A) has been shown to be important for the formation of vascular tubes involved in the vascular permeability (Patella et al., 2015). CPT1A

facilitates the transport of long chain fatty acids into the mitochondria by coupling fatty acid CoA to carnitine to facilitate transport into the mitochondria. Fatty acids are metabolized in the mitochondria by β-oxidation to generate acetyl CoA which can subsequently enter the TCA cycle or be used as a building block for many cellular pathways (Nelson & Cox, 2013). The activity of CPT1A is negatively regulated by malonyl CoA, a substrate for fatty acid synthesis (McGarry, Mannaerts, & Foster, 1977).

4.2 Inflammation

Inflammation is the body's first response to cellular damage or infection. An early step in inflammation is the recruitment of leukocytes from the bloodstream to the tissue. Microbial products and/or substances released from damaged cells activate tissue-resident cells like macrophages to produce inflammatory cytokines. Upon exposure to such cytokines endothelial cells will start expressing the selectins E-selectin and P-selectin on their luminal surface. These selectins associate with L-selectin on the surface of leukocytes to mediate rolling on post-capillary venules (Arfors et al., 1987; Lawrence & Springer, 1993; Vestweber, 2015). P-selectin and chemokines are stored in Weibel-Palade bodies in endothelial cells and can be secreted shortly after activation making P-selectin appear on the vascular walls shortly after inflammatory activation (Eppihimer et al., 1996). Binding of selectins will slow down the leukocytes and chemokines will activate integrins that associate with the cell adhesion molecules such as intercellular cell adhesion molecule (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) resulting in slow rolling. (Vestweber, 2015). This tighter adhesion results in leukocytes crawling along the walls of the vessel until they can migrate from the bloodstream to the tissue through cell-cell junctions or, in some cases, through the body of endothelial cells (Vestweber, 2015).

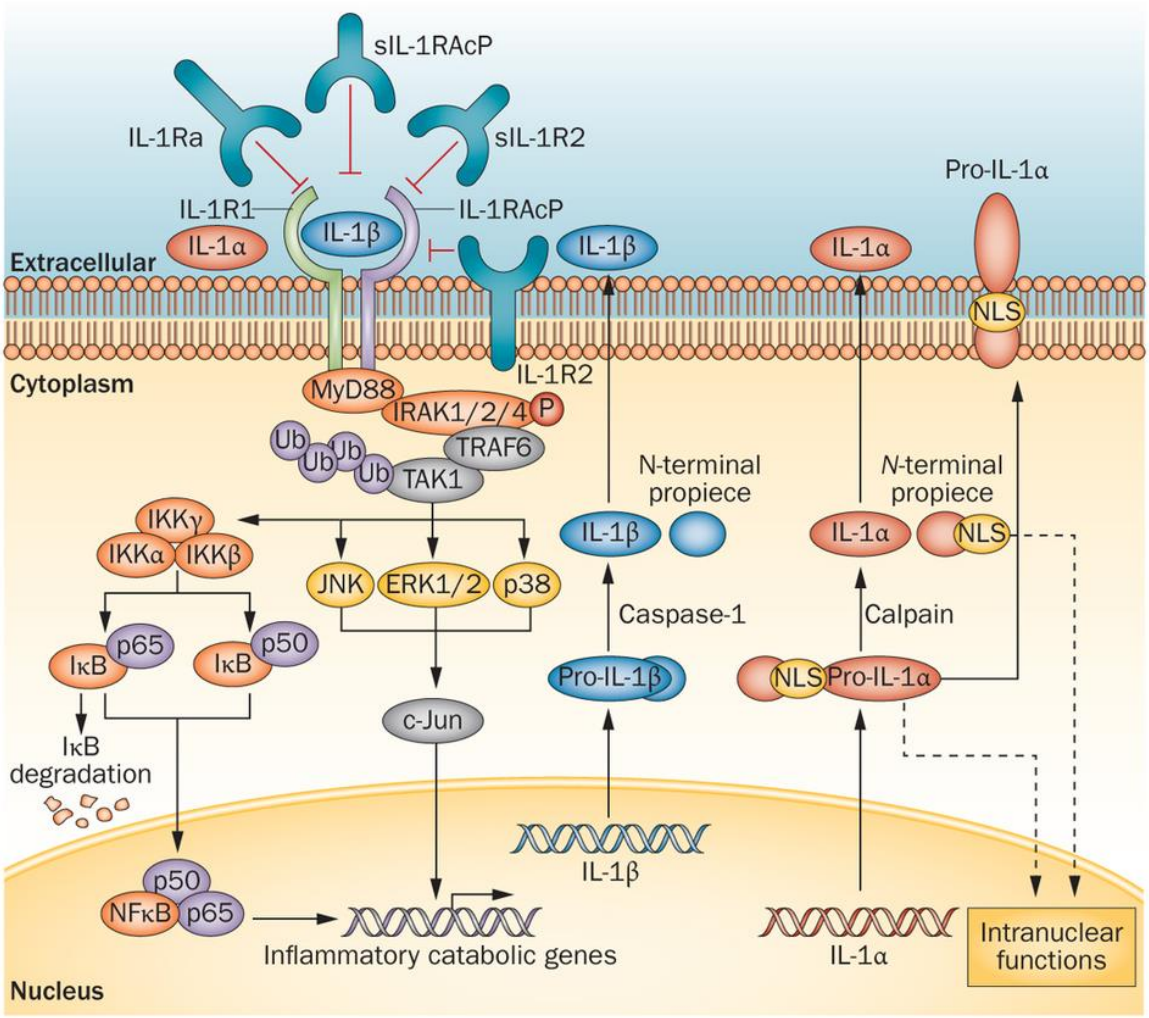

Figure 4.2 **IL-1 induced activation of key inflammatory pathways**. IL-1β induces activation of inflammatory pathways through the NFκB pathway and the MAP kinase pathways. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Rheumatology] (doi:10.1038/nrrheum.2013.160), copyright (2014).

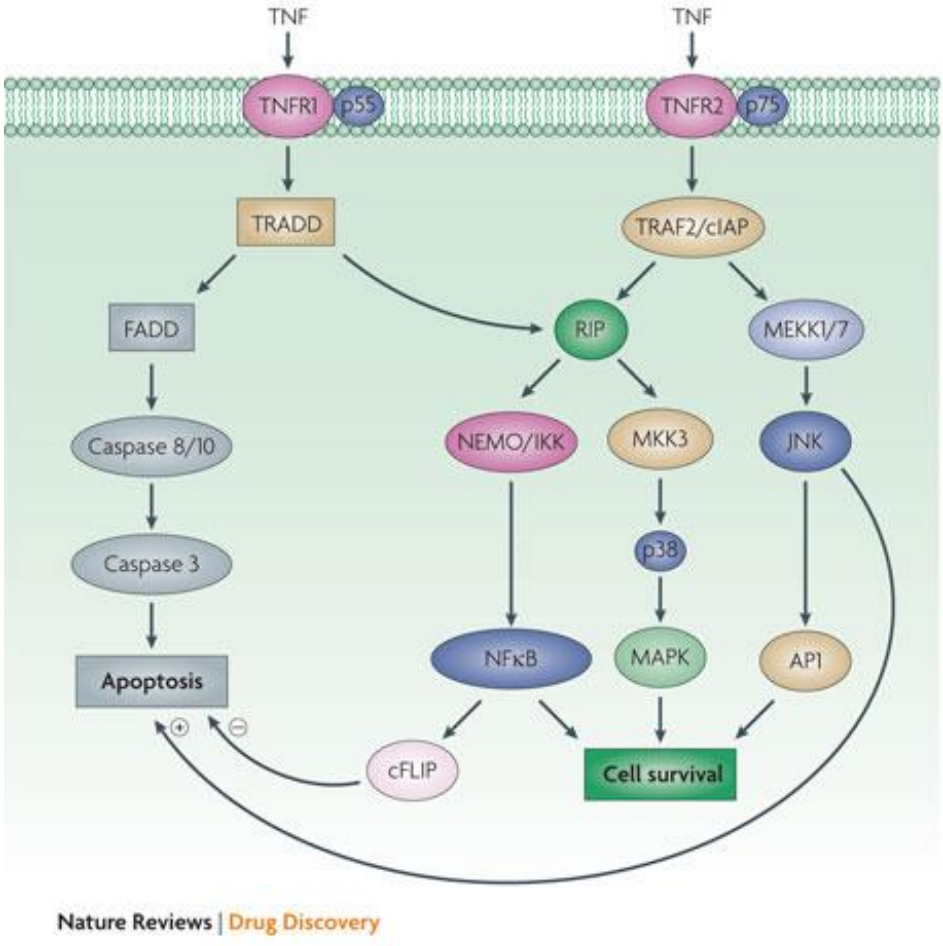

Figure 4.3 **General TNF signalling cascade**. TNF may act on the two TNF receptors, TNFR1 or TNRF2. In endothelial cells TNRF2 is the major isotype promoting cell survival and transcription of genes involved in inflammation. Inflammatory activation is mediated through the NFκB pathway, the MAPK pathway and the JNK pathway. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery (doi:10.1038/nrd3030), copyright 2010.

While inflammation is a crucial part of the innate immune response, chronic inflammation is a global health problem. Repression of inflammation by targeting the NFκB pathway has been shown to reduce insulin resistance and prolong lifespan in mice (Hasegawa et al., 2012), and vascular inflammation has also been shown to promote cardiovascular disease through the development of atherosclerosis (Libby, Ridker, & Maseri, 2002).

4.2.1 NFκB pathway

The transcription Nuclear Factor κB (NFκB) is the major mediator of inflammation in endothelial cells (Kempe, Kestler, Lasar, & Wirth, 2005). Activation of NFκB can happen through the canonical and non-canonical pathway (Sun, 2010). The canonical NFκB complex consists of P50, P65 and Inhibitor of NFκB α (IκBα). Activation of the NFκB complex requires the phosphorylation and subsequent ubiquitylation and proteosomal degradation of IκBα

(Alkalay et al., 1995; Delhase, Hayakawa, Chen, & Karin, 1999; Viatour, Merville, Bours, & Chariot, 2005). The phosphorylation of IκBα is mediated by the IκB kinase (IKK) complex consisting of IKKα, IKKβ and IKKγ, also called NFκB essential modulator (NEMO), IKKβ being the major player in canonical NFκB activation (Israel, 2010).

4.2.2 JNK pathway

The stress-activated protein kinase/c-Jun N-terminal kinase (JNK) is a protein in the mitogen activated protein kinase (MAPK) family that can be activated by stressful stimuli such as cytokines or UV-radiation (Hibi, Lin, Smeal, Minden, & Karin, 1993; Risbud & Shapiro, 2014). JNK phosphorylates c-Jun, a subunit of the Activating protein 1 (AP-1) complex, a transcription factor that induces transcription of several inflammatory genes (Wang et al., 2013).

4.2.3 Metabolism and vascular inflammation

While the relationship between acute inflammation and endothelial metabolism is not yet known, the relationship between chronic vascular inflammation and dysfunctional metabolism has been subject to much attention (Eelen et al., 2015). Conditions such as diabetes mellitus and atherosclerosis are both associated with vascular inflammation and dysfunctional metabolism (Bakker, Eringa, Sipkema, & Van Hinsbergh, 2009; Eelen et al., 2015; Ramasamy et al., 2005).

In diabetic endothelial cells hyperglycaemia mediates an accumulation of glucose and glycolysis intermediates that leads to the formation of advanced glycation end-products (AGE) resulting in inflammatory activation by acting on receptors for AGE (RAGE) (Eelen et al., 2015; Pober & Sessa, 2007; Ramasamy et al., 2005) Glucose is also shunted into the polyol pathway leading to depletion of NADPH which in combination with reduced oxidative PPP (oxPPP) activity eventually leads to a reduction in glycolysis by preventing the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Eelen et al., 2015). Protein glycosylation furthermore reduces the production of NO, an important vasodilator, by repressing endothelial nitric oxide synthetase (eNOS) (Du et al., 2001). O-GlcNAcylation has been associated with the pathology of diabetes and inflammation as it may enhance NFκB signalling by preventing inactivation of IKKβ by preventing phosphorylation of inhibitory serine residues (Kawauchi, Araki, Tobiume, & Tanaka, 2009) and O-GlcNAcylation of P65 may promote dissociation from IκBα even in the absence of phosphorylation (W. H. Yang et al., 2008).

5 Project description

Based on the strong reliance of endothelial cells on glycolysis for angiogenesis activation(De Bock et al., 2013; Xu et al., 2014), and the glycolytic shifts observed in immune cells following inflammatory activation (C.-H. Chang et al., 2013; Kelly & O'Neill, 2015; Tannahill et al., 2013), we designed our project to answer the following questions:

1. Is endothelial metabolism modulated by inflammatory activation?
2. Can we target glycolysis to regulate inflammatory activation of endothelial cells?
   2.1. Does inhibition of glycolysis modulate endothelial cell activation?
   2.2. (If an effect is observed in 2.1): By which molecular mechanism does inhibition of glycolysis modulate endothelial cell activation.

6 Material and methods

6.1 Materials

6.1.1 Cell cultures

Umbilical cords were collected after informed consent at the birth clinic at Rikshospitalet, Oslo University Hospital. Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion as described (Jaffe, Nachman, Becker, & Minick, 1973).

Recombinant human epidermal growth factor (EGF), basic fibroblast growth factor (FGF), recombinant long R3 insulin growth factor (long R3 IGF), recombinant human vascular endothelium growth factor (VEGF), recombinant human interleukin-1β (IL-1β) and recombinant human tumour necrosis factor (TNF) were obtained from R&D Systems (Abingdon, UK). MCDB 131 without L-glutamine. fetal calf serum, gentamicin, fungizone, and l-glutamine were purchased from Invitrogen, and trypsin-EDTA was from Bio-Whittaker (Walkersville, MD), hydrocortisone and heparin was purchased from Sigma.

6.1.2 RNA extraction

Trizol® was obtained from Sigma-Aldrich (Sigma), 1-Bromo-3-chloropropane and ethanol was obtained from Sigma, Phase-lock gel 2ml tubes were obtained from 5-Prime (discontinued).

6.1.3 RT-PCR

SuperScript® III Reverse Transcriptase system (Sigma), Oligo dT primer (GE Health Care), dNTP (GE health Care), RNasin® Plus RNASE inhibitor (Promega) and DEPC treated H_2O.

6.1.4 qPCR

HotStart Taq polymerase (Qiagen), EvaGreen®, 20x (Biotium), Stratagene Mx3005p (Agilent Technologies) and analysed by Stratagene MxPro software (Agilent Technologies).

6.1.5 Western blot

Lysis buffer: 2% SDS, 5mM EDTA 5mM Tris pH 6.8, 5 mM NaF, 5 mM tetrasodium pyrophosphate phosphatase inhibitor (Sigma: P5726) protease inhibitor (Sigma: P8340).

Sample buffer: β-mercaptoethanol, glycerol and bromophenol blue.

4-20% Mini Protean TGX gels, Trans-Blot® Turbo™ Transfer System Chemi-Doc MP, Trans-Blot Turbo Mini Nitrocellulose Transfer Packs Blotting-Grade Blocker, Image lab software

were obtained from Bio-Rad, Pierce ™, Restore Plus Western Blot Stripping Buffer BCA Protein Assay Kit, SuperSignal™ West Dura Extended Duration Substrate were obtained from Thermofisher Scientific, Full Rainbow Marker was obtained from GE Health Care, BSA was bought from Sigma.

6.1.6 Cell ELISA

Paraformaldehyde/Lysine/Periodate (PLP) 0,5% (made as described (McLean & Nakane, 1974)), BSA (Sigma), crystal violet (Norges Apotekerforening), TMB Microwell Peroxidase Substrate System (KPL), plate reader (Tecan Sunrise) and Magellan6 software were used for measuring OD.

6.1.7 Radiolabelled tracers

All radiolabelled reagents were purchased from Perkin Elmer.

^{3}H-thymidine was used to measure the rate of thymidine incorporation Glucose, ^{14}C-2-deoxy-D-Glucose was used to measure glucose uptake; D-[5-3H(N)]-, 1mCi (37MBq) was used to determine the rate of glycolysis and ^{3}H 9-10-palmitic acid was used to measure the rate of fatty acid oxidation. Samples were read on a TriCarb 2810 Liquid Scintillation analyser (Perkin Elmer).

6.1.8 Luciferase assay

NFκB firefly luciferase reporter lentivirus was obtained from Kerafast, Luciferase Assay Systems protocol from Promega, fluorescence was detected using a Luminoskan Ascent from Labsystems.

6.1.9 Antibodies

Table 5.1: Antibodies

Specificity	Dilution	Company	Product number	Use
α-selectin	1:200	R&D Systems	BBA16	Cell ELISA
α-VCAM-1	1:1000	Gift from J. Harland (WA)	N/A	Cell ELISA
α-Mouse IgG (λ-chain) HRP	1:3000	Sigma	A 3673	Cell ELISA
α-VCAM	1:1000	R&D Systems	BBA19	WB
α-RL2	1:1000	Thermofisher scientific	MA1-072	WB

α-IKKβ	1:1000	Cell Signalling	#8943	WB
α-pIKKα/β	1:1000	Cell Signalling	#2697	WB
α-JNK	1:1000	Cell Signalling	#9252	WB
α-pJNK	1:1000	Cell Signalling	#9251	WB
α-CPT1A	1:1000	AbCam	ab128568	WB
α-PFKFB3	1:2000	AbCam	ab181861	WB
α-β-tubulin	1:20000	AbCam	ab6046	WB
α-Mouse IgG (H+L) HRP	1:20000	Jackson Immunoresearch	715-035-151	WB
α-Goat IgG (H+L) HRP	1:10000	Santa Cruz Biotechnology inc.	sc-2020	WB
α-Rabbit IgG(H+L) HRP	1:20000	Jackson Immunoresearch	711-035-152	WB
α-IκBα	1:1000	Cell Signalling	#4814	WB
α-pIκBα	1:1000	Cell Signalling	#2859	WB

6.1.10 Primers

Table 5.2: Primers		
Target	Sequence	[$MgCl_2$] (mM)
PFKFB3 forward	GTC CCT TCT TTG CAT CCT CTG	1.5
PFKFB3 reverse	CCT ACC TGA AAT GCC CTC TTC	
CPT1A Forward	CAG CAC TTT CAG GGA GTA GC	1.5
CPT1A reverse	ATC AAT CGG ACT CTG GAA ACG	
VCAM-1 forward	AGT TGA AGG ATG CGG GAG TAT	2.0
VCAM-1 Reverse	GGA TGC AAA ATA GAG CAC GAG	

HPRT1 forward	AAT ACA AAG CCT AAG ATG AGA GTT CAA GTT GAG TT	2.0
HPRT1 Reverse	CTA TAG GCT CAT AGT GCA AAT AAA CAG TTT AGG AAT	
E-selectin forward	GCT CTG CAG CTC GGA CAT GTG G	2.0
E-selectin reverse	GGC AGC TGC TGG CAG GAA CA	

6.2 Methods

6.2.1 Cell cultures

HUVECs were cultured in MCDB 131 containing 7.5% fetal calf serum, 10 ng/ml EGF, 1 ng/ml basic fibroblast growth factor, 2 mM l-glutamine, 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, and 250 ng/ml fungizone (complete HUVEC medium) for culture expansion. When seeded for experiments the medium was changed to MCDB131 containing 2% FCS, 5ng/ml EGF, 10, ng/ml FGF, 2mM L-glutamine, 0.2 µg/ml hydrocortisone, 50 µg/ml gentamicin, 250, µg/ml fungizone, 0,5 ng/ml VEGF, 20 ng/ml long R3 IGF, 5 mg/ml ascorbic acid, 22.5 µg/ml heparin. This medium was chosen because VEGF promotes a glycolytic phenotype (De Bock et al., 2013) which was important for our purposes, and because cells remain healthier in confluent cultures than when cultured over time in the growth medium. Cells were frozen with DMSO/FCS and stored in liquid nitrogen.

All cell cultures were incubated at 37 ^{O}C in a humidified 5% CO_2, 95% air incubator and all cell culturing work was done in sterile LAF benches and sterile equipment. Cells were trypsinized using Trypsin + EDTA 0.05%, seeded onto 0.1% gelatine coated plastic growth surfaces and used at passage levels from 2-5. For experiments cells were seeded at a density of $3.8x10^4$ cells per cm^2.

All the experiments are performed using primary cells (HUVEC) isolated from umbilical cords donated from women giving birth at Oslo University Hospital Rikshospitalet. The approval from the regional ethics committee does not give the opportunity to obtain information of the health records of the donors. Because many complications during pregnancy, including gestational diabetes and pre-eclampsia, can be expected to affect endothelial functions, significant variability between cells from different donors can be expected. To reduce the likelihood of errors related to the health of the donor all experiments are repeated with different

donors and sometimes using pooled samples. While cell lines could reduce the variation between experiments and increase replicability this doesn't always yield representative results as some cell lines differ from primary cells in many respects (Galley, Blaylock, Dubbels, & Webster, 2000; Lidington, Moyes, McCormack, & Rose, 1999).

6.2.2 Microscopy

To assess the health and growth of the cell cultures we used an inverted phase contrast microscope (Leica DMIL 4X, 10X and 40X objectives).

6.2.3 RT-PCR and qPCR

Total RNA was extracted from cell lysates homogenized in TRIzol and precipitated using the company protocol (Sigma) using 1-bromo-3-chloropropane (1/10 of the TriZol volume) rather than chloroform to reduce DNA contamination when precipitating, RNA was resuspended in DEPC H_2O and stored at -70 ^{O}C. cDNA was synthesized using Superscript Transcriptase III and an oligo dT primer to amplify mRNA, the reaction was terminated at 85 ^{O}C.

Gene specific primers annealing to exon-exon junctions were designed using primer BLAST and used to amplify short fragments of cDNA. The fluorescent dye EVA Green was used for detection of PCR product and sequences were amplified using HotStart *Taq* polymerase. The results were analysed using ΔΔCT to get a measure of relative expression. qPCR setup was performed with technical triplicates using the median value for each triplicate for analysis. Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) was used for normalization.

qPCR cycles: HotStart: 95 ^{O}C 15 minutes.

Denaturing step: 95 ^{O}C 30 seconds

Annealing step: 60 ^{O}C 30 seconds

Elongation step: 72 ^{O}C 30 seconds (record at the end of step)

6.2.4 Western blot

Cell lysates are collected in lysis buffer containing 2% SDS for proteins supplemented with phosphatase inhibitor (Sigma P5726) and protease inhibitor (Sigma P8340). For some samples protein concentration was measured using Pierce BCA Protein Assay Kit to ensure that the same amount of protein was loaded in each well. In preparation for Western blot 15 µl sample buffer, containing bromophenol blue, glycerol and β-mercaptoethanol, per 100 µl lysis buffer was added to the samples and then heated on heating block at 95 ^{O}C for 5 minutes. Proteins

were separated using SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane using the Bio-Rad Trans-Blot Turbo Transfer system. Membranes were blocked with 5% milk, then incubated overnight with appropriate antibodies diluted in 1% milk, 3% BSA or 5% BSA, followed by incubation with Horseradish peroxidase (HRP) conjugated secondary antibodies. Signal was detected by adding SuperSignal™ West Dura extended Duration Substrate) and chemo luminescence was measured using Bio-Rad ChemiDoc MP system. The relative density was calculated using ImageLab software (Bio-Rad) using volume tools and adjusted intensity. When analysing phospho-proteins special measures were taken to prevent loss of phosphorylation, samples were frozen at -70 ^{O}C rather than at -20 and samples were heated to 65 ^{O}C for 10 minutes. Membranes were blocked in 5% BSA as milk contains casein, a phospho-protein, which results in a higher background (Abcam troubleshooting). β-tubulin was used as a loading control and for normalization for all samples ([protein of interest]/[β-tubulin]). When measuring protein phosphorylation normalization was performed against the level of total protein ([phosphorylated protein of interest]/[protein of interest]/[β-tubulin]). This is a semi-quantitative method as the detection is dependent on the exposure time and the affinity of the antibody.

6.2.5 Cell based ELISA

Cells were seeded in 96 well plates at a density of approximately $3.8x10^4$ cells per cm^2 were fixed using 0.5% PLP for 10 minutes in preparation for cell ELISA (Enzyme-linked immunosorbent assay) then stored in 4 ^{O}C. Cells were incubated with 0.1% saponin for 5 minutes then incubated with primary antibodies for 45 minutes on shaking table. The wells were washed and then incubated with HRP conjugated secondary antibodies for 45 minutes. 100 µl TMB solution was added to each well and incubated under constant shaking for 5-10 minutes. The reaction was then stopped using 1M H_2SO_4. The optical density (OD) was measured using a microplate plate reader and Magellan6 software at wavelength 450 nm. To correct for cell density cells were incubated in crystal violet (0.1% in PBS) for 4 minutes, then thoroughly washed. Crystal violet was solubilized in 33% acetic acid and OD was measured at 550 nm.

6.2.6 Statistical analyses

Statistical analyses were performed using GraphPad Prism 7.01 (2013) using two-tailed t-test for normally distributed data. For Non-parametric data or data where the likelihood of making type I error was judged too high Mann Whitney U test was used. Results are considered significant when $p<0.05$.

6.3 Methods used in experiments performed by other research group members

6.3.1 Glucose uptake

Cells were seeded at a density of $3.8x10^4$ cells/cm^2 and cultured for 96 hours before stimulation with IL-1β for 16 hours. Glucose uptake was measured by culturing cells in medium containing 0.5 µCi/ml ^{14}C-2-deoxy-D-glucose was added for 10 minutes at 37 ^{O}C. Cells were washed 3 times in cold PBS and lysed in 0.2M NaOH and the level of ^{14}C was measure using a TriCarb 2810 Liquid Scintillation analyser.

6.3.2 Rate of Glycolysis and fatty acid oxidation

Metabolic fluxes were measured using radioactive tracers. HUVECs were seeded at a density of $3.8x10^4$ cells/cm^2 and cultured for 96 hours before being stimulated with IL-1β for the given time. Radiolabelled trackers were added to the medium for the last 2 hours of incubation. The rate of glycolysis following IL-1β stimulation was determined by using 5^{3}H-D-glucose (0.4 µCi/ml), the rate of FAO was determined using ^{3}H 9-10 palmitic acid (0.4 µCi/ml) in medium supplemented with 100 µM cold carnitine and 50 µM carnitine. The supernatants were transferred to glass bottles with rubber stoppers and incubated for 48 hours to capture the ^{3}H converted to ^3H_2O by glycolysis or FAO. Evaporated H_2O was captured in hanging wells containing a Whatman paper soaked with H_2O. Radioactivity was measured using a TriCarb 2810 Liquid Scintillation analyser. After harvesting the supernatants, the cells were lysed in RIPA buffer and protein concentration was measured using Pierce BCA Protein Assay kit for normalization.

6.3.3 Thymidine incorporation

Rate of proliferation was determined using the incorporation of the radio labelled thymidine (^{3}H-thymidine). HUVECs were seeded at a density of $3.8x10^4$ cells/cm^2 then stimulated with IL-1β for the given time. ^{3}H thymidine was added for the last 2 hours of incubation. Cells were lysed in 0.2 M NaOH and thymidine incorporation was measured using TriCarb 2810 Liquid Scintillation analyser.

6.3.4 Luciferase assay

HUVECs were seeded at a density of $1.5*10^4$ cells/cm^2, cultured overnight and transduced with NFκB Firefly luciferase reporter lentivirus. 72 hours after transduction cells were reseeded at $6.2x10^4$ cells/cm^2 in a black 96 well plate (clear bottom) and cultured for 24 hours before IL-1β stimulation (1ng/ml) in the presence of 3PO (20µM) for 4 hours. Cells were lysed in 20 µl cell culture lysis buffer using standard Luciferase Assay Systems protocol from Promega

(catalogue #E1500). Quantification of luciferase was performed by adding 50 µl luciferin and measuring bioluminescence using Luminoskan Ascent Instrument (Labsystems).

7 Results

7.1 Endothelial cells undergo a metabolic shift following IL-1β stimulation

To determine if the metabolism of endothelial cells changes in response to inflammatory stimulation we first tested the cellular glucose uptake by measuring internalization of ^{14}C-2-deoxy-D-glucose following stimulation with IL-1β. We observed a significant increase in glucose uptake ($p<0.001$) in cells that had first been stimulated with IL-1β (1ng/ml) overnight (Figure 7.1 a). The rate of glycolysis was further investigated by measuring the flux of 5-^{3}H-glucose. The rate of glycolysis increased steadily following IL-1β treatment (Figure 7.1 b)), and by 4 hours the rate of glycolysis was significantly higher than control ($p<0.05$). The rate of fatty acid oxidation was determined by measuring ^{3}H 9-10-palmitic acid. In contrast to glycolysis FAO was decreased significantly ($p<0.05$) 24 hours after IL-1β stimulation (Figure 7.1 c). To assess whether the increased rate of glycolysis was associated with an increase in proliferation cells were incubated with ^{3}H-thymidine to measure thymidine incorporation. Proliferation in the IL-1β treated cells was reduced by approximately 50% ($p<0.0001$) following IL-1β stimulation (Figure 7.1 d).

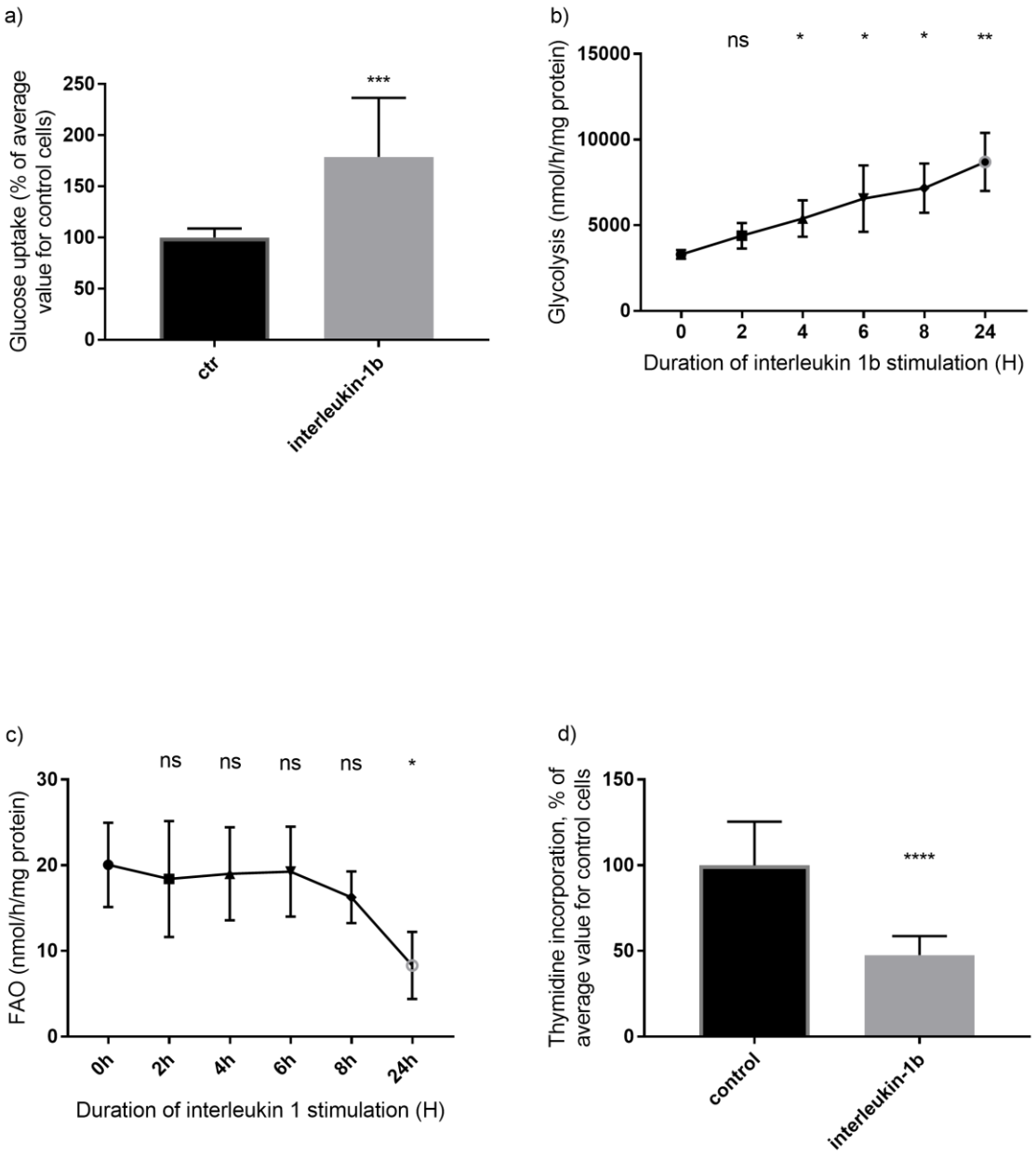

Figure 7.1 **Endothelial Cells undergo a metabolic shift following IL-1β stimulation (1ng/ml).** a) Glucose uptake was increased following overnight stimulation with IL-1β (n=9) b) The rate of glycolysis increased steadily in the 24 hours following IL-1β stimulation (n=3) c) The rate of FAO decreased gradually following IL-1β stimulation (n=3) d) The proliferation of endothelial cells decreased after overnight stimulation with IL-1β. Graphs display mean +/-standard deviation (SD) of three independent experiments, each using HUVECs from different donors *p<0.05 **p>0.01 *** P>0.001 ****p<0.0001 (two-tailed t-test). Experiments performed by Johanna Hol.

7.2 IL-1β stimulation induces PFKFB3 expression

As the initial experiments showed significant metabolic changes in response to inflammatory activation we wanted to investigate whether this correlated with a change in gene expression of

the enzymes involved in the rate limiting steps of glycolysis and FAO. HUVECs were seeded at a density of $3.8x10^4$ cells/cm^2 then stimulated with IL-1β (1 ng/ml) for 2, 4, 6, 8 and 24 hours and harvested in TRIzol, RNA was extracted for RT-PCR followed by qPCR to determine the relative expression levels of the genes encoding the rate limiting enzyme 6-phosphopfructo kinase 2/fructose-2,6-bisphosphatase (PFKFB3) in glycolysis and carnitine palmitoyl transferase 1A (CPT1A) in FAO.

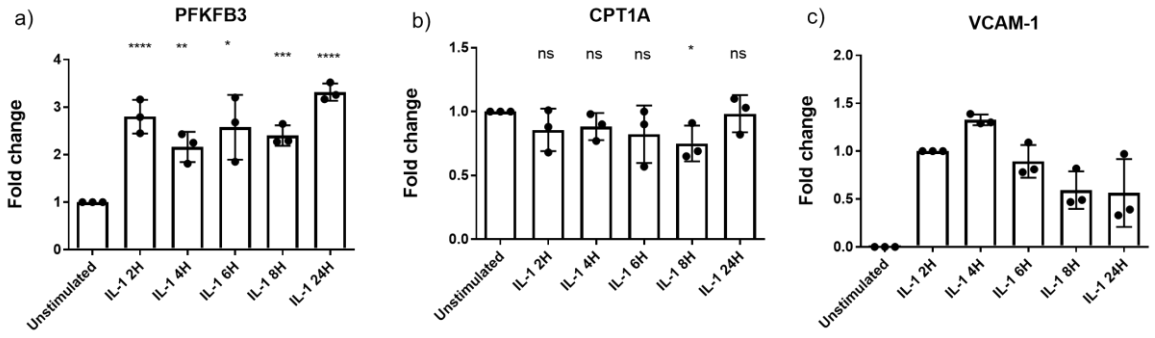

Figure 7.2 **Transcriptional regulation of metabolic enzymes in inflammatory activated endothelial cells.** Panels show transcript levels of (**a**) *PFKFB3*, (**b**) *CPT1A* and (**c**) *VCAM1* in HUVECs stimulated with IL-1β for the indicated time. Bars show mean values +/- SD from three independent experiments, each using HUVECs from a different donor and stimulated in duplicate wells and with technical triplicates for qPCR. qPCR was performed as described in material and methods sections and relative gene expression was determined by the ΔΔCT method using *HPRT* as housekeeping gene. The levels of *VCAM1* were calculated relative to the 2-hour time point as the amplitude of the response differed between donors from (10-fold to 1000-fold maximal induction compared to unstimulated cells). *P<0.05 **P<0.01 ***P<0.001 ****P<0.0001 (two-tailed t-test)

An increase in expression of *PFKFB3* was observed 2 hours after IL-1β stimulation ($p<0.001$) (Figure 7.2 a) and the levels remained elevated throughout the time course of the experiment. The expression levels of *CPT1A* remained relatively stable throughout the experiment (Figure 7.2 b)), but a significant decrease was observed at 8 hours ($p<0.05$). The *VCAM1* levels were set relative to 2-hour stimulation as the amplitude of the response varied between different donors (Figure 7.2 c). The kinetics of the *VCAM1* response were consistent with published literature (Haraldsen et al., 1996).

Protein levels can be influenced by other factors in addition to transcription. To evaluate if protein levels correlated with transcript levels, samples were harvested for immunoblotting against PFKFB3 and CPT1A. Relative quantities were estimated by densitometry using volume tools in ImageLab 4.1 build 16 (Bio-Rad laboratories 2012). The protein levels were consistent with the transcript levels (Figure 7.3 a): Levels of PFKFB3 (Figure 7.3 b) were significantly

increased after 2 hours ($p<0.01$), at which point they levelled out for the rest of the time point measured, CPT1A (Figure 7.3 c) did not change significantly from the levels in unstimulated cells at any time point. Levels of VCAM-1 (Figure 7.3 d) followed the expected pattern, being strongly induced by IL-1β, peaking at 4-8 hours followed by a decrease at 24 hours.

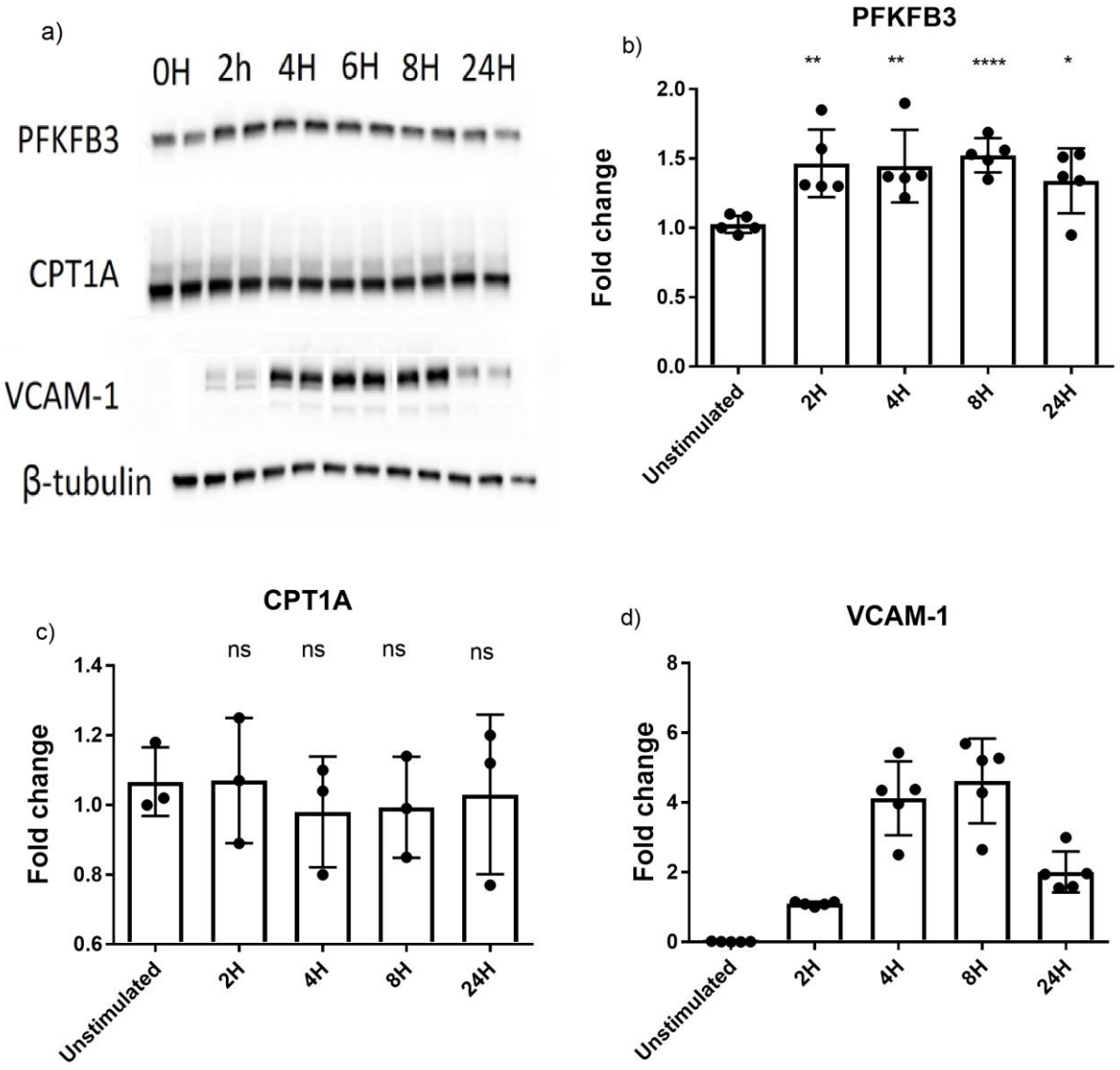

Figure 7.3 **Regulation of metabolic enzymes at the protein levels in inflammatory activated endothelial cells.** a) Representative Western blot showing the cytokine induced changes in the protein levels of PFKFB3, CPT1A, VCAM-1 and β-tubulin. Densitometric analyses of protein levels normalized to β-tubulin relative to control for (b) PFKFB3 (n=5), (c) CPT1A (n=3) and (d) VCAM-1 (n=5). Bars represent mean values +/- SD from five (or three) independent experiments, each using HUVECs from a different donor *$p<0.05$ **$p<0.01$ ****$p<0.0001$ (two-tailed t-test).

7.3 Pharmacological inhibition of PFKFB3 reduces the endothelial upregulation of adhesion molecules

The small molecule inhibitor 3PO has been shown to transiently reduce the rate of glycolysis and attenuate angiogenic endothelial activation and *in vitro* and *in vivo* and leukocyte migration in mouse models of psoriasis and colitis (Clem et al., 2008; Schoors et al., 2014). We sought to investigate whether treatment with 3PO would affect endothelial induction of adhesion molecules in response to inflammatory stimulus. Such upregulation of endothelial surface molecules is an important step in inflammatory processes, as it regulates the recruitment of leukocytes to the site of inflammation. To evaluate the effect of 3PO on the induction of E-selectin and VCAM-1 we analysed HUVECs stimulated with IL-1β (0.5 ng/ml) for 3 hours in the presence or absence of 3PO (20μM). Cell were fixed in PLP and analysed using a cell-based ELISA (enzyme linked immunosorbent assay) previously established in the Haraldsen group (Haraldsen et al., 1996). We observed that treatment with 3PO significantly inhibited the upregulation of both E-selectin (Figure 7.4 a) (P<0.01)) and VCAM-1 (Figure 7.4) (P<0.0001). The inhibitory effect was even more pronounced when cells were pre-treated with 3PO 30 minutes prior to IL-1β stimulation.

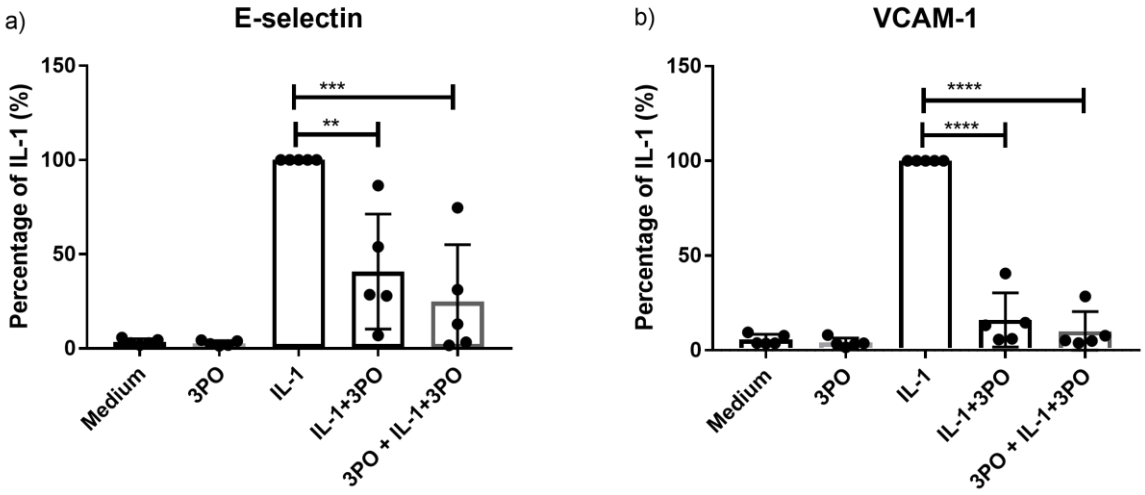

Figure 7.4 **Pharmacological inhibition of PFKFB3 reduces the endothelial upregulation of adhesion molecules.** Cells were stimulated with IL-1β for 3 hours in the presence or absence of 3PO, and level of E-selectin, (**a**) and VCAM-1 (**b**) were measured using cell ELISA. Graphs show data from five independent experiments, each using HUVECs from a different donor. Data are normalized to the level of expression in IL-1β stimulated cells in each experiment. Bars show mean +/- SD. **p<0.01 ***p<0.001 **** p<0.0001. (two-tailed t-test).

So far, our results showed that 3PO treatment reduced the upregulation of both VCAM-1 and E-selectin protein in response to IL-1β, however, it remained to be confirmed that this was accompanied by inhibition on the transcription level. To evaluate the effect of 3PO on transcription of genes encoding adhesion molecules, HUVECs were stimulated by IL-1β (1ng/ml) for 2 hours in the presence or absence of 3PO (20μM), and RNA was harvested for qPCR. We found that treatment with 3PO reduced the expression of *SELE* (encoding E-selectin) by approximately 40% ($p<0.001$) (Figure 7.5 a)) and *VCAM1* by approximately 70% ($p<0.0001$) (Figure 7.5 b).

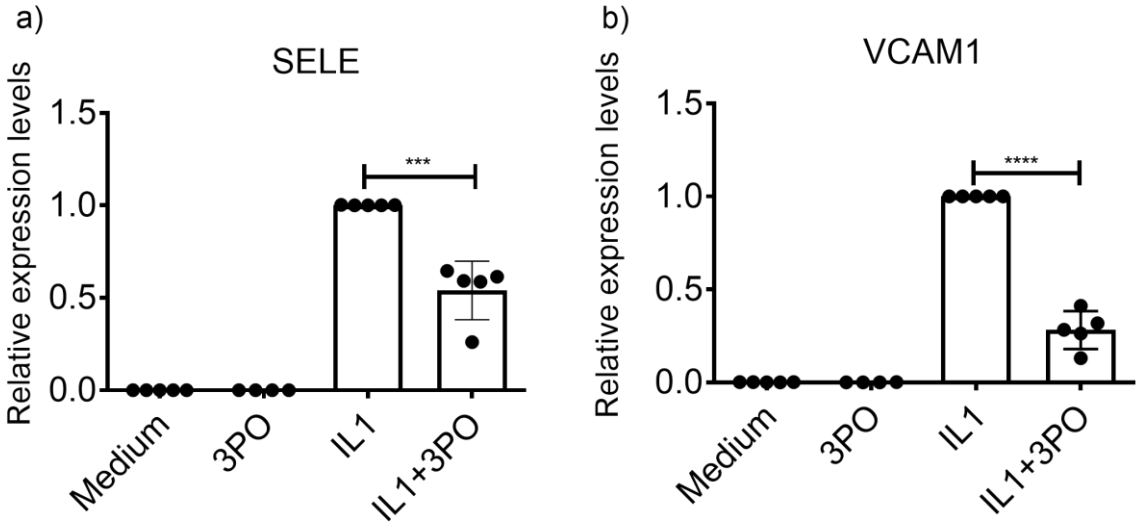

Figure 7.**5 Pharmacological inhibition of PFKFB3 reduces the gene expression of adhesion molecules** Relative transcription levels of (**a**) *SELE* and (**b**) *VCAM1* following stimulation with IL-1β (1 ng/ml) for 2 hours in the absence or presence of 3PO (n=5) Bars show mean +/- SD from five independent experiments, each using HUVECs from a different donor. *** $P<0.001$ **** $p<0.0001$ (two-tailed t-test).

7.4 3PO inhibits cytokine induced activation of the NFκB pathway

IL-1β stimulation of endothelial cells activates the NFκB, p38 and JNK pathways (Pollheimer et al., 2012). Moreover, NFκB is considered a major regulation of inflammatory activation in endothelial cells (Kempe et al., 2005). To investigate the mechanisms behind the reduction in induction of adhesion molecules we first considered the effects on NFκB signalling. The activation of NFκB was assessed by using a luciferase assay. Cells were transduced with NFκB firefly luciferase reporter lentivirus, seeded into 96 well plates and stimulated with IL-1β either in the presence or absence of 3PO (20 μM) in triplicates. We observed that 3PO treatment significantly reduced the NFκB activation in response to IL-1β ($p<0.0001$) (Figure 7.6).

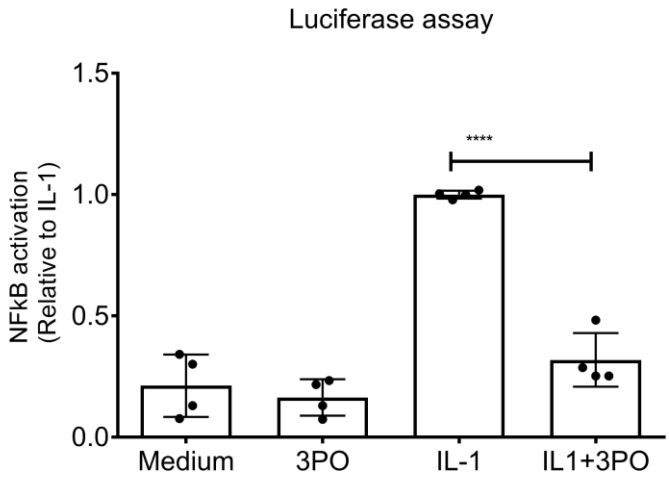

Figure 7.6 **3PO inhibits IL-1β induced NFκB activation in endothelial cells.** HUVECS expressing NFκB Firefly Luciferase reporter were stimulated with IL-1β for 4 hours in the absence or presence of 3PO. Bars show mean +/-SD from four independent experiments, each using HUVECs from a different donor. ****p<0.001 (two-tailed t-test).

IκBα impose tonic inhibition of NFκB in the absence of stimulation. An early step in the NFκB signalling pathway is the phosphorylation, ubiquitylation and degradation of IκBα that allows NFκB to be activated and migrate to the nucleus (Alkalay et al., 1995; Naumann & Scheidereit, 1994). To assess at which step of the pathway 3PO inhibited NFκB signalling, we stimulated cells with IL-1β (1ng/ml) for 5 minutes or 60 minutes with or without pre-treatment with 3PO (20μM) and harvested samples for immunoblotting. We found that 3PO treatment significantly reduced the IL-1β induced phosphorylation of IκBα after 5 minutes (P<0.001), but the effect after 60 minutes varied and did not reach significance (*Figure 7.7*).

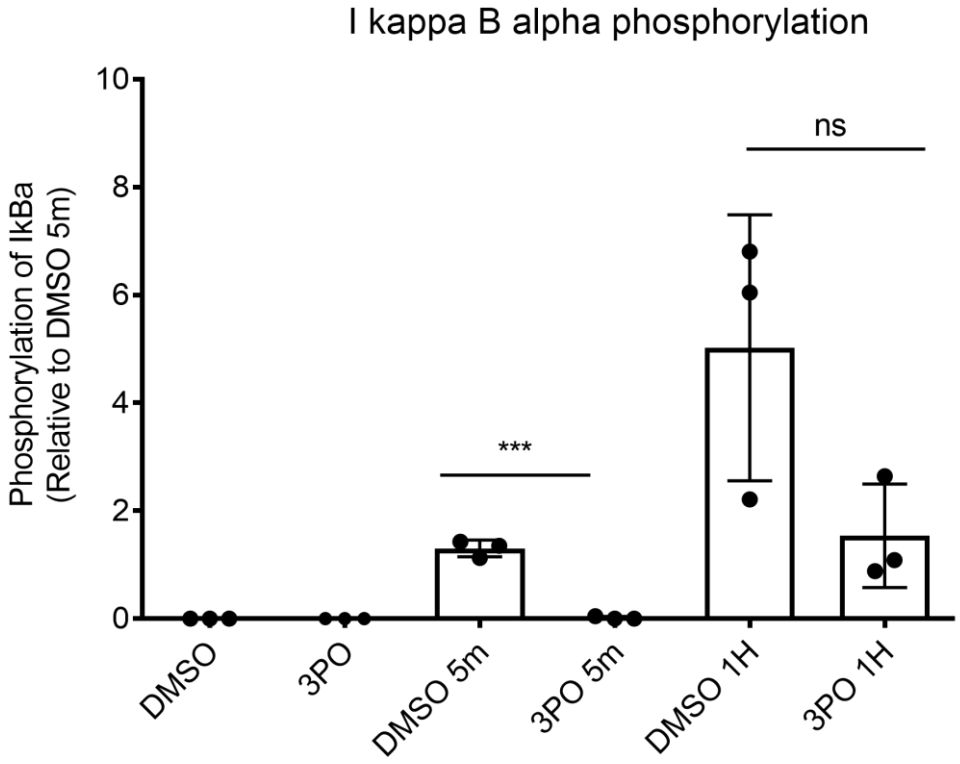

Figure 7.7 **3PO inhibits IL-1β induced phosphorylation of IκBα.** Relative quantities of phosphorylation of IκBα estimated by densitometry. Phosphorylation of IκBα after 5 minutes of IL-1β stimulation is significantly reduced in the 3PO treated cells compared to vehicle. Bars show mean +/- SD from three independent experiments, each using HUVECs from a different donor *** $p<0.001$ (two tailed t-test).

We next asked whether 3PO selectively inhibited phosphorylation of IκBα or if upstream NFκB signalling was also attenuated. Phosphorylation of IκBα is regulated by the IκB kinase (IKK) complex, mainly by the subunit IKKβ. We therefore assessed the effect of 3PO on phosphorylation and activation of the IκB kinases (IKKα and IKKβ). HUVECs were again treated with 3PO 30 minutes prior to being stimulated with IL-1β (1 ng/ml) and harvested after 5 and 60 minutes of stimulation and protein phosphorylation was investigated by immunoblotting with phospho-specific antibodies.

We found that 3PO treatment significantly reduced the phosphorylation of IKKα/β after stimulation with IL-1β after 5 minutes of IL-1β stimulation, but that the effect on phosphorylation 60 minutes after stimulation varied between HUVEC donors and did not reach significance when results were pooled (Figure 7.8 b)).

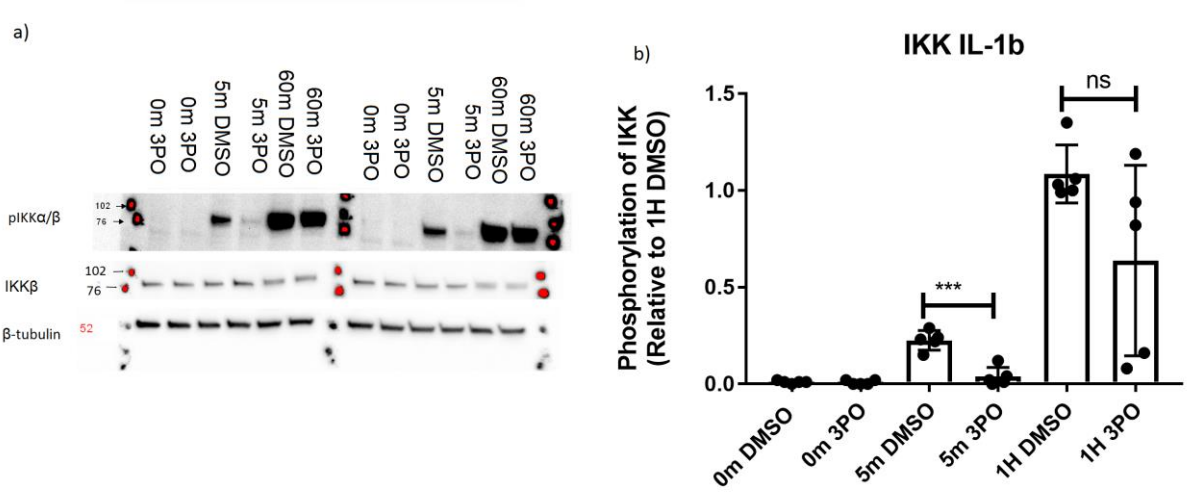

Figure 7.8 **3PO inhibits phosphorylation of IKKα/β following IL-1 stimulation** a) Representative blot showing the differences in IKKα/β phosphorylation in IL-1β stimulated cells treated with 3PO cells compared to vehicle. b) Densitometric quantification of IKKα/β phosphorylation normalized to total IKKβ and β-tubulin. Bars show mean +/- SD from five independent experiments, each using HUVECs from different donors. ns $p>0.05$ ***$p<0.001$ (two-tailed t-test).

The NFκB signalling pathway is activated by several mediators induce that inflammation, including IL-1β and another pro-inflammatory cytokine, tumour necrosis factor (TNF). Because the signalling cascades between the receptors for IL-1β and TNF and activation of IKKα/β include some shared and some separate mediators we next asked if 3PO also inhibited IKK-activation in response to TNF. Cells were pre-treated for 30 minutes with 3PO (20 µM) or DMSO and stimulated with TNF (1 ng/ml) for 5 minutes or 60 minutes and phosphorylation was analysed using immunoblotting (Figure 7.9 a)). Densitometric quantification revealed that 3PO caused a significant decrease in IKKα/β phosphorylation ($p<0.01$) after 5 minutes of stimulation, but not after 60 minutes of stimulation, also when signalling was activated by TNF (Figure 7.9 b).

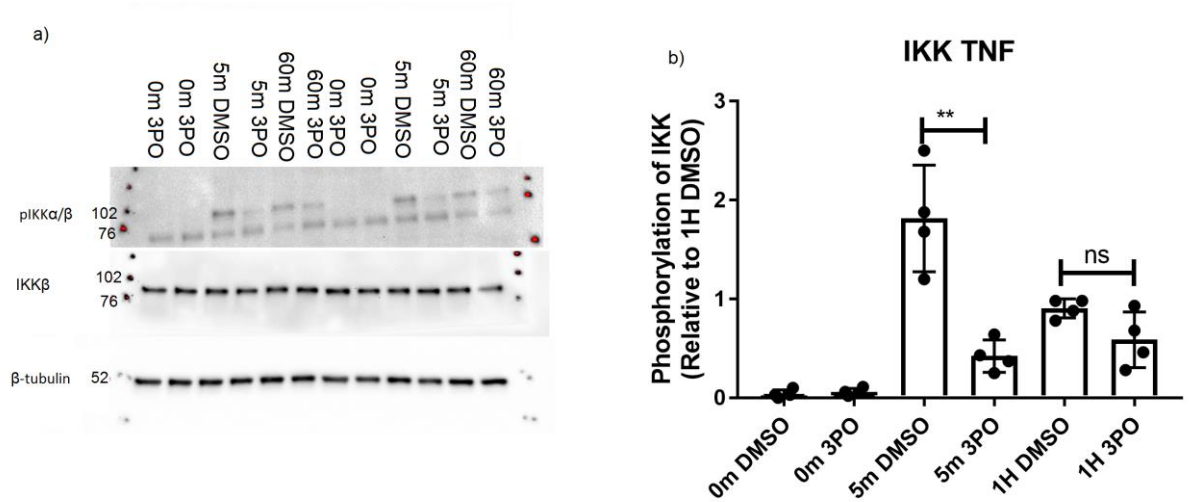

Figure 7.9 **3PO inhibits phosphorylation of IKKα/β following TNF stimulation:** a) Representative Western blot of IKKα and IKKβ phosphorylation following TNF stimulation (1ng/ml) pre-treated with vehicle or 3PO (20 µM). b) Densitometric quantification of IKK phosphorylation relative to cells stimulated with TNF for 1 hour. Bars show mean +/- SD of four independent experiments, each using HUVECs from a different donor. ns $p>0.05$ ** $p<0.01$ (two-tailed t-test).

Phosphorylation of IKK follows activation of the TAK1/TAB1/TAB2/TRAF6 complex (Pober, 2002). Another downstream target of this complex is the stress activated kinase JNK (Zhou, Connell, & MacEwan, 2007). We next investigated if 3PO would also affect phosphorylation of this kinase and found that the phosphorylation of JNK was significantly reduced after both 15 and 30 minutes of IL-1β stimulation (Figure 7.10 a)-b)) and after 15 minutes, but not after 30 minutes of TNF stimulation (Figure 7.10 c)-d).

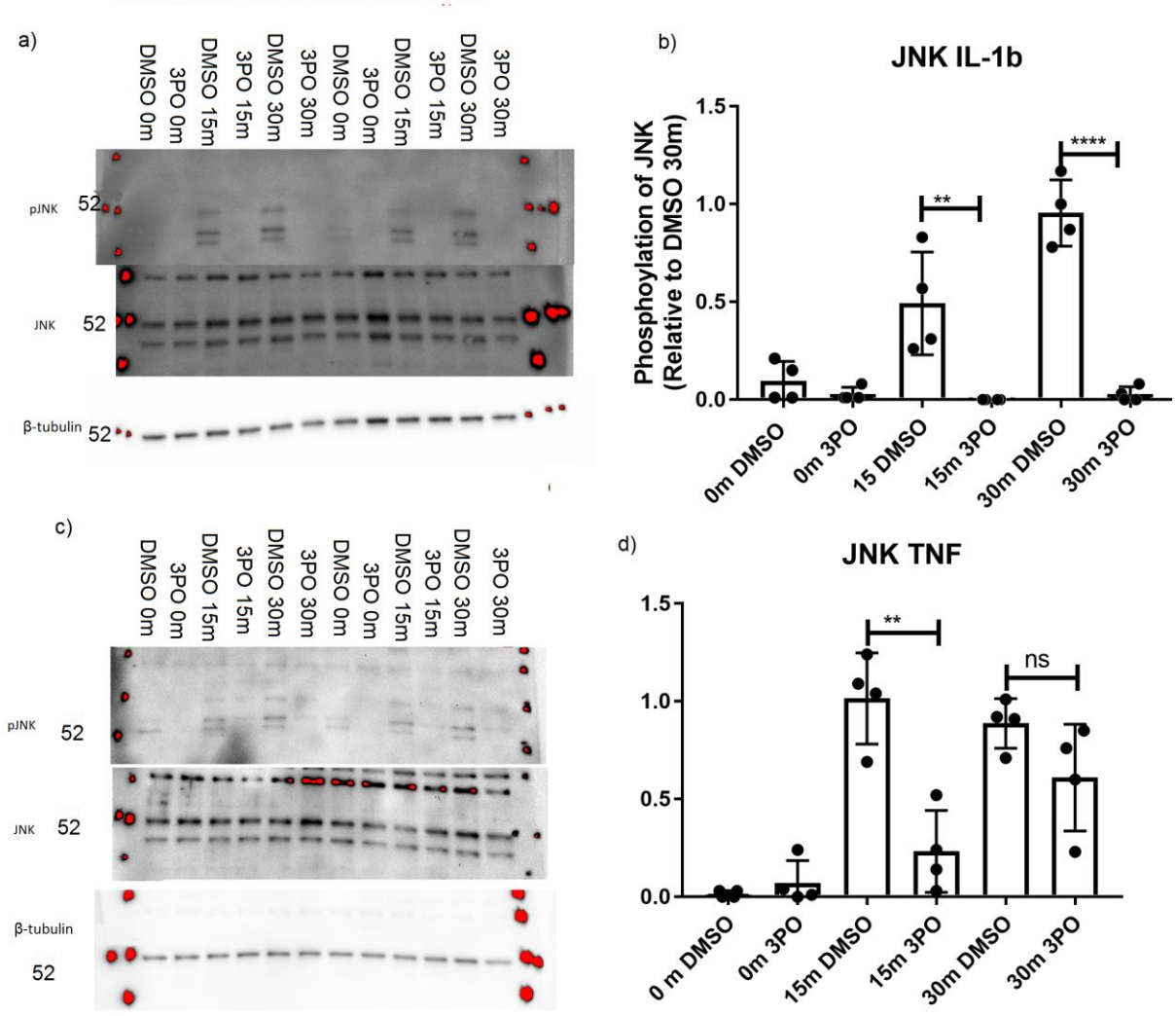

Figure 7.10 **3PO inhibits phosphorylation of JNK.** a) Representative Western blot of JNK phosphorylation following IL-1β stimulation with vehicle or 3PO pre-treatment b) Densitometric quantifications of JNK phosphorylation in IL-1β stimulated cells pre-treated with either vehicle or 3PO. c) Representative Western blot of JNK phosphorylation following TNF stimulation with vehicle or 3PO d) Densitometric quantification of JNK phosphorylation following TNF stimulation in cells with pre-

treatment of vehicle or 3PO. Bars represent mean value +/- SD from four independent experiments, each using HUVECs from different donors. ns $p>0.05$ ** $p<0.01$ **** $p<0.0001$

In conclusion, we observed that 3PO inhibits the activation of several major signalling pathways in response to the proinflammatory cytokines IL-1β and TNF, explaining the observed inhibition of endothelial adhesion molecule upregulation. Our results indicate that 3PO either exerts a broad inhibitory effect on phosphorylation, or that it affects activation of signalling molecules that are common for IL-1β and TNF signalling, and at the level upstream of the TAK1/TAB1/TAB2/TRAF6 complex.

7.5 3PO stimulation does not significantly alter pan-O-GlcNAcylation

O-GlcNAcylation on serine and threonine residues can be impaired when glycolysis is impaired by inhibition with 2-deoxy-D-glucose as this leads to a depletion of fructose 6-phosphate, a substrate of the HBP (Lund, Elias, & Davis, 2016); inhibition of subsequent steps of glycolysis leading to an accumulation of fructose 6-phosphate can result in an increased flux into the HBP (Du et al., 2000; Schoors et al., 2015). Increased O-GlcNAcylation has been associated with both increased and reduced vascular inflammatory activation (Xing et al., 2008; W. H. Yang et al., 2008). Furthermore, O-GlcNAcylation can affect the phosphorylation of several components of the NFκB cascade, including IKKβ (Kawauchi et al., 2009; Xing et al., 2011; W. H. Yang et al., 2008). Because 3PO has the potential to alter O-GlcNAcylation, either by increasing the flow through the HBP (Du et al., 2000) or by unspecific action on the glutamine fructose-6-phosphate amidotransferase (GFAT), which is the rate limiting step for β-linked O-GlcNAcylation (possibility suggested by Clem, 2008). To check whether the cells from the previous experiment exhibited any change in pan-O-GlcNAcylation. Membranes were incubated with RL2 antibody against O-GlcNAcylated proteins (MA1-072) (ThermoFisher) and lanes were compared using densitometry between whole lanes then normalized to β-tubulin. No consistent change was observed for any of the stimulated cells (Figure 7.11 a)-b)

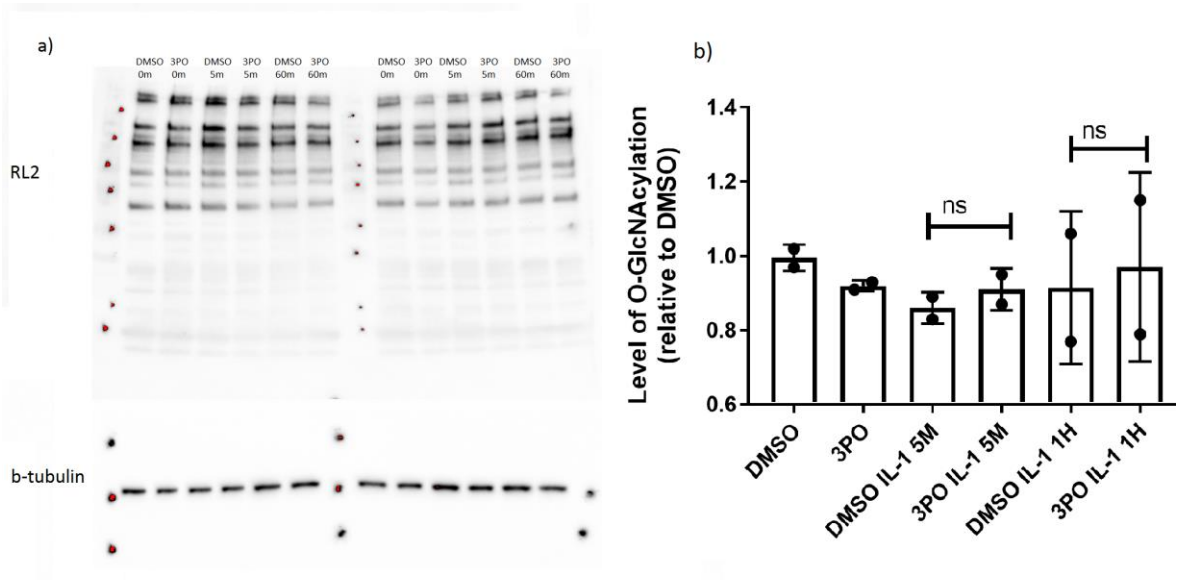

Figure 7.11 **Pan-O-GlcNAcylation of proteins is not significantly altered by 3PO treatment** a) Representative Western blot for cells stimulated with IL-1β (1ng/ml) in the presence of vehicle or 3PO (20μM). b) Densitometric quantification of stimulated cells relative to unstimulated cells from 1 experiment with duplicates. ns P>0.05 (two-tailed t-test)

We hypothesized that the change in O-GlcNAcylation may not be prominent enough to be detected in whole cell lysates at this timepoint and that it might be more pronounced at a later timepoint or at a different dose of 3PO. We Therefore incubated cells in 3PO at concentrations ranging from 5-20 μM of 3PO (increments of 5 μM) with IL-1β (1ng/ml) for 2 hours before harvesting proteins extracts. We observed a trend towards an increased level of O-GlcNAcylation (Figure 7.12 a)), but this failed to reach significance (Mann Whitney U) (Figure 7.12 b)). To reduce the probability of failing to detect a significant effect the experiment should be repeated with cells from more donors.

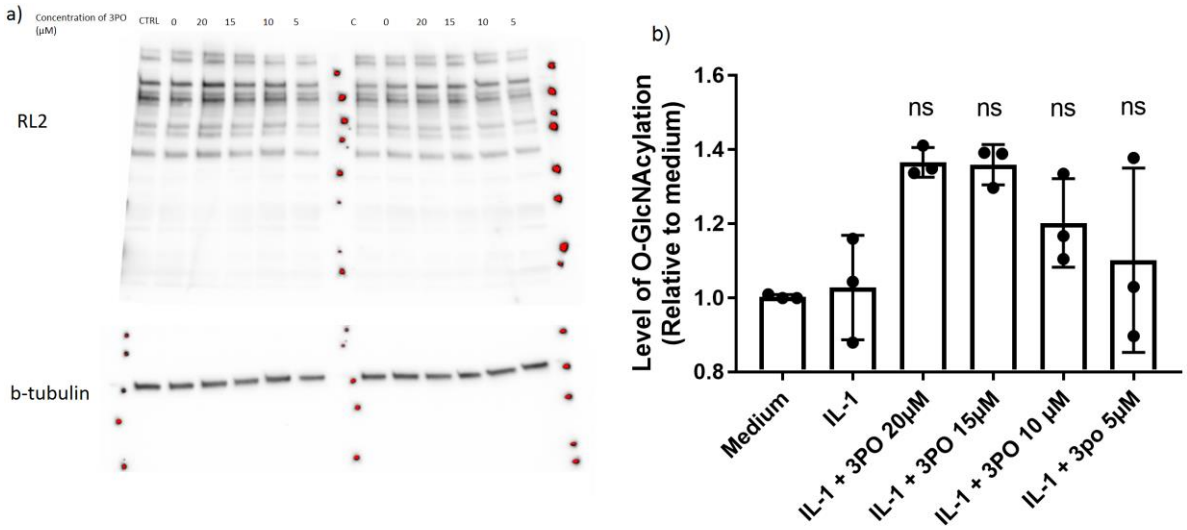

Figure 7.12 **Pan-O-GlcNAcylation is not significantly altered in 3PO treated cells** a) representative blot of cells after stimulation with IL-1β for 2 hours in the presence of varying concentration of 3PO. b) Densitometric quantifications of whole lanes for pan-O-GlcNAcylation. Bars show mean +/- SD representative of three independent experiments, each using HUVECs from different donors. ns $P>0.05$ $p<0.05$ (Mann Whitney U)

8 Discussion

The data presented in this thesis show that the rate of glycolysis dramatically increases when endothelial cells are activated by the inflammatory cytokine IL-1β, and that pharmacological inhibition of glycolysis attenuates endothelial upregulation of adhesion molecules by impeding early phosphorylation events in canonical inflammatory signalling cascades.

8.1 Endothelial metabolism shifts following inflammatory activation

Our results show a significant increase in both glucose uptake and glycolysis following IL-1β stimulation for the first 24 hours. This is similar to the angiogenic situation reported by De Bock et al (2013), where the glycolytic machinery relocalised to sites of rapid actin polymerisation and promoted cytoskeletal rearrangements associated with angiogenic sprouting. A high rate of glycolysis is often associated with a proliferative cellular state. This was also true in the in the angiogenic context, where inhibition of glycolysis induced reversible cell cycle arrest (De Bock et al., 2013; Schoors et al., 2014) In contrast we observed that proliferation was reduced by approximately 50%, as shown by a reduction in thymidine uptake. This is in agreement with previous reports showing that inflammatory activation inhibits endothelial cell division (Pollheimer et al., 2012). This implies that energy and building blocks provided by the inflammatory-induced glycolytic phenotype are used to fuel other processes than proliferation. A metabolic shift towards increased glycolysis upon inflammatory activation has been reported for several immune cells including monocytes, macrophages, dendritic cells and T cells (C.-H. Chang et al., 2013; Cheng et al., 2014; Ghesquière, Wong, Kuchnio, & Carmeliet, 2014; Kelly & O'Neill, 2015; Krawczyk et al., 2010; Tannahill et al., 2013). In these cells a transition from cellular respiration to cytoplasmic glycolysis is required to deliver optimal effector functions in response to inflammatory stimuli (C.-H. Chang et al., 2013; Tannahill et al., 2013). On the other hand glycolysis has also been linked to cellular survival and an improved ability to resist oxidative stress, in that respect acting as an indirect break on inflammation (Huo et al., 2010, 2012; Z. Yang, Fujii, Mohan, Goronzy, & Weyand, 2013).

8.2 Cytokine stimulation induced PFKFB3 expression

We have demonstrated that IL-1β stimulation of endothelial cells induce a higher rate of glycolysis and a reduction in fatty acid oxidation. We hypothesized that this shift in metabolism might be accompanied by altered expression of key metabolic enzymes and decided to test the expression of *PFKFB3* and *CPT1A*. PFKFB3 has previously been shown to be increased in endothelial cells in response to angiogenic stimulation and regulates endothelial cell glycolysis

during new vessel formation (De Bock et al., 2013). CPT1A is the rate limiting enzyme for FAO in most cells, including endothelial cells (Schoors et al., 2015). Inhibition of CPT1A in endothelial cells also inhibits cellular calcium homeostasis and disrupts endothelial barrier function, a finding that may couple FAO to inflammation induced vascular permeability (Patella et al., 2015).

IL-1β stimulation resulted in an increase of both PFKFB3 transcription level and protein level, which is likely one of the driving forces behind the increased rate of glycolysis observed.

There was a significant change in *CPT1A* on transcriptional level after 8 hours, but this was not seen at any of the other time points tested, nor on Western blot. The reason for this could be due to regulation of protein activity rather than regulation of transcription and translation. This could be due to an increase in malonyl CoA which reduces the activity of CPT1A (McGarry, Leatherman, & Foster, 1978) or by other mechanisms which could attenuate FAO.

8.3 Pharmacological inhibition of PFKFB3 inhibits the cytokine induced activation of Endothelial cells

The early results indicated that cytokine stimulation of endothelial cells increased the rate of glycolysis through an increased expression of PFKFB3. To determine if this was a requirement in inflammation we investigated the effects of inhibiting glycolysis with the small, molecule inhibitor 3PO, which is proven to be an effective inhibitor both *in vitro and* in vivo (Clem et al., 2008; Schoors et al., 2014). Notably, this molecule exerts a partial and transient inhibition of glycolysis, in contrast to the stronger irreversible effect of 2DG that we have observed to be highly cytotoxic in endothelial cells. Our experiments revealed that there is a marked decrease in the protein levels of E-selectin and VCAM-1, which are involved in leukocyte migration (Lawrence & Springer, 1993; Vestweber, 2015). As it has been shown that glycolytic enzymes may also modulate mRNA translation (C.-H. Chang et al., 2013) we further quantified the transcript levels of adhesion molecules. There was a clear correlation between the decrease on protein and mRNA level implicating that the observed effect was upstream of gene transcription. While there are several pathways involved in inflammation the NFκB pathway is the most important in endothelial cells (Kempe et al., 2005; Zhou et al., 2007).

Further investigation of NFκB using a NFκB luciferase reporter based system confirmed that NFκB activation was indeed decreased following 3PO treatment. Activation of NFκB requires the dissociation from the inhibitory cytoplasmic chaperone IκBα (Alkalay et al., 1995; DiDonato, Hayakawa, Rothwarf, Zandi, & Karin, 1997; W. H. Yang et al., 2008). We found

that the phosphorylation state of IκBα was also strongly reduced by inhibition of glycolysis, explaining the observed inhibition of NFκB activation in 3PO treated cells. The phosphorylation state of IKKβ is another marker of NFκB activation (Israel, 2010; Karin, 1999). We observed a clear reduction in early phosphorylation of IKKβ in 3PO treated cells. This effect was also observed when stimulating with TNF indicating that 3PO either directly reduced phosphorylation of IKKβ or activation of an upstream mediator common for both TNF and IL-1β signalling or that 3PO elicited broad effects on phosphorylation. We further examined the JNK pathway and observed similar inhibition of activating phosphorylation, therefore showing that the effects were not limited to IKKβ phosphorylation. Our results therefore show that pharmacological blockade of glycolysis can dampen the activation of numerous signalling mediators involved in inflammatory activation of the endothelium, but the mechanism has yet to be pinned down. It has been proven that PFKFB3 inhibition does not affect ATP levels or energy charge in the basal state (De Bock et al., 2013; Schoors et al., 2014), but this experiment should be repeated in inflammatory activated endothelial cells to exclude that this increase in glycolysis is a requirement to provide ATP to the signalling cascade following cytokine stimulation. In the same context, it would be useful to evaluate the effects on inhibiting phosphorylation of proteins not affected by inflammatory activation.

8.4 3PO does not significantly alter the level of pan-O-GlcNAcylation in whole cell lysates

Because O-GlcNAcylation occurs on serine and threonine residues and are known to inhibit both activating and inhibitory phosphorylation at these sites, we next asked if inhibition of glycolysis affected the level of pan-O-GlcNAcylation in endothelial cells. From the literature, we found that 3PO could have the potential to either inhibit or promote O-GlcNAcylation: First Clem et al. (2008) proposed that 3PO could also inhibit fructose 6-phosphate binding to GFAT, the rate limiting enzyme of the HBP, thus reduce protein O-GlcNAcylation. In hyperglycaemic conditions there is an increased flux into the HBP caused by inhibition of GAPDH and accumulation fructose 6-phosphate (Du et al., 2000; Eelen et al., 2015), which is linked to the pathology of diabetes by promoting activation of NFκB by preventing the inactivating autophosphorylation of IKKβ (Kawauchi et al., 2009) and by preventing NFκB from associating with IκBα (W. H. Yang et al., 2008).

Western blot analysis of whole cell lysates revealed no differences in pan-O-GlcNAcylation, but we did observe a trend towards an increase at later time points after stimulation. Repeating the number of experiments with an increased number of donors would be necessary to show

whether this could reach the chosen level of significance ($p<0.05$). However, it would be easier to understand biological significance by evaluating the effect on the identified signalling molecules by immunoprecipitation.

If we confirm that am increased flux into the HBP, most likely caused by an increased availability of fructose 6-phosphate, this is highly interesting: In fact, the effect is opposite of O-GlcNAcylation in hyperglycaemic conditions, like diabetic patients, O-GlcNAcylation promotes NFκB activation (Kawauchi et al., 2009; W. H. Yang et al., 2008). The increase in O-GlcNAcylation in diabetes is related to dysfunctional glycolysis in endothelial cells leading to decrease in PPP and a decreased activity of the enzyme GAPDH. Inhibition of glycolysis leads to an accumulation of the fructose 6-phosphate, the substrate for GFAT, the rate limiting enzyme of HBP (Eelen et al., 2015). O-GlcNAcylation has also been shown to be related to decreased NFκB activation and a decrease in inflammation in wound healing (Xing et al., 2008, 2011). Our results indicated that 3PO might have a dose-dependent increase in O-GlcNAcylation after 2 hours. This would need to be further confirmed by checking if any of the proteins involved in the inflammatory activation have the modification using immunoprecipitation

Considering methodological limitations of our experiments, we have observed that some post-translational modifications may be lost due to sample treatment. For future experiments samples for evaluation of O-GlcNAcylation will therefore be treated the same way as samples intended for evaluation of phosphorylation levels by freezing samples at -70 ^{O}C and heated at 65 ^{O}C rather than at 95 ^{O}C.

9 Concluding remarks and future perspectives

We have shown that endothelial cells undergo a metabolic shift following cytokine stimulation. This metabolic shift correlates with an increase in the glycolytic enzyme PFKFB3, which could be a likely mediator of the increase. The reason for decreased fatty acid oxidation remains unknown, but it could be due to regulation of enzymatic activity. By targeting glycolysis using the small molecule inhibitor 3PO the induced expression of adhesion molecules and activation of NFκB was decreased, but the mechanisms for this reduction in inflammatory activation has yet to be determined. One reason could be a global decrease in phosphorylation caused by either lack of ATP or by increased O-GlcNAcylation, thus preventing phosphorylation of serine/threonine residues. In addition, other early signalling events other than phosphorylation

could be inhibited by 3PO and cause downstream effects on targets described here. Based on *in vitro* results on inflammatory activation blockade of glycolysis may have therapeutic potential in inflammatory disorders.

Future experiments should consider the effects on phosphorylation in signalling cascades and determine if the effects could be explained by energy depletion or if there is another mechanism linking increased glycolysis and inflammation. A good way to study this could be by enrichment of phospho-proteins followed by high resolution mass spectrometry. Another venue to explore is the use of alternative inhibitors that also reduce the rate of glycolysis without disrupting it completely. This could aid us in determining if the inhibitor 3PO has off-target effects that inhibits inflammation or if the effect is mediated through reducing the rate of glycolysis. One could also consider using RNA interference against PFKFB3, but should keep in mind that a reduction in enzyme activity over time might result in compensatory mechanisms that could complicate interpretation of results. Future experiments should also include an *in vivo* model to assess if 3PO inhibits endothelial inflammatory activation in experimental inflammation.

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