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Review

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**The emerging role of metabolic regulation in the functioning of Toll-like receptors
and the NOD-like receptor Nlrp3.**

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Abstract

While it has long been suspected that inflammation participates in the pathogenesis of metabolic disorders such as the insulin resistance that occurs in type 2 diabetes, recent work suggests that this is not the only important interaction between metabolism and inflammation. Inroads into the understanding of the relationship between metabolic pathways and inflammation are indicating that signaling by innate immune receptors such as TLR4 and Nlrp3 regulate metabolism. TLRs have been shown to promote glycolysis, whilst Nlrp3-mediated production of IL-1 β causes insulin resistance. A key role for the hypoxia-sensing transcription factor HIF1 α in the functioning of macrophages activated by TLRs has also recently emerged. This review will assess recent evidence for these complex interactions and speculate on their importance for innate immunity and inflammation.

Keywords: Metabolism, glycolysis, HIF1 α , TLRs, insulin resistance.

Innate immune system acts as the first line of defense against infection partly by recognising highly conserved sets of molecular targets called pathogen-associated molecular patterns (PAMPs) via a limited number of germline-encoded receptors known as pattern-recognition receptors (PRRs). These PRRs include Toll-like receptors (TLRs) and NOD-like receptors (NLRs), both of which initiate downstream signaling pathways that culminate in the activation of antimicrobial pro-inflammatory immune responses.

TLRs recognise specific components of viruses, bacteria, fungi and parasitic protozoa. In mammals 12 members of the TLR family have been identified. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surface of various immune cells, whereas TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular compartments such as the endoplasmic reticulum and endosomes. TLR4, the best characterised TLR, is highly expressed on dendritic cells (DCs) and macrophages and binds to lipopolysaccharides (LPS) from Gram-negative bacterial cell walls. All TLRs are type I transmembrane receptors, characterized by an extracellular leucine rich repeat (LRR) domains and an intracellular Toll-IL1 receptor-Resistance (TIR) domain. The TIR domain is necessary for the recruitment of various adaptor molecules to activate the downstream signaling pathways, such as those that activate NF-kappaB (NF- κ B), leading to the transcription of typical pro-inflammatory cytokines such as interleukin-1 beta (IL-1 β), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF α) [1].

NLRs are involved in the recognition of host-derived and microbial ‘danger’ associated molecules which are produced under conditions of cellular stress or injury. Activation of a subset of NLRs leads to the assembly of high-molecular-mass complexes called inflammasomes. There are currently four inflammasomes identified, comprising NLRP3, NLRP1, NLRC4 and AIM2. Inflammasome activation has been shown to lead to the generation of active caspase 1, a requisite for the production of the mature form of the archetypal inflammatory cytokine, IL-1 β [2] .

Recently, evidence has emerged that both TLRs and inflammasomes impact on metabolism. TLR activation leads to the so-called ‘Warburg effect’, involving a switch

to glycolysis, similar to that occurring in tumours [3]. The NLRP3 inflammasome on the other hand has been shown to cause insulin insensitivity and may be important for the pathogenesis of type 2 diabetes (T2D) [4]. In this review we discuss these recent findings and present a model whereby metabolic regulation in the TLR and NLRP3 systems might be a key controlling process in innate immunity. This area is a new and exciting frontier in the role of innate immunity in the sensing of metabolic imbalance, as would occur in infection and tissue injury. This process could be critical for the restoration of homeostasis following such insults.

Glycolysis is necessary for macrophage and dendritic cell function

Generally it is understood that mammalian cells acquire energy in the form of ATP by anaerobic glycolysis when oxygen is limiting or oxidative phosphorylation when oxygen is available. Whilst the aerobic pathway may provide more energy per glucose molecule (32 ATP) it is relatively slow. Glycolysis on the other hand only produces 2 ATP per glucose molecule, but it can do so very rapidly. It is this response that may be vital for immune cells such as macrophages and DCs in hypoxic environments such as those encountered at sites of inflammation. In support of this there is evidence to suggest that both macrophages and dendritic cells (DCs) require the glycolytic pathway not only for rapid energy metabolism but importantly for immune function.

Mounting an immune response to infection is energy intensive. In the presence of pathogens or pathogen products, immune cells can shift from quiescence to a highly active state within hours of stimulation. Recent data has shown that DCs can do this by switching their core metabolism from oxidative phosphorylation to glycolysis [3]. Whilst resting DCs predominantly use the more conservative catabolic program of oxidative phosphorylation, TLR sensing activates the switch to glycolysis. DCs stimulated with multiple TLR agonists up-regulate Glucose transporter 1 (GLUT1) expression, have increased lactate production and a decrease in mitochondrial O₂ consumption, all hallmarks of glycolysis [3, 5]. Importantly it was shown that this is mediated by the PI3K/Akt pathway, as DCs lacking Akt1 or treated with the PI3K inhibitor LY294002 exhibited significantly reduced LPS-induced glycolysis. In contrast, DC retrovirally

transduced to express an active form of Akt had increased background levels of glycolysis and an increased glycolytic response to LPS. Interestingly, the suppression of mitochondrial fatty acid β -oxidation triggered by LPS was unaffected by the inhibition of PI3K by LY294002, the absence of Akt1, or the expression of the active form of Akt. Instead AMP kinase (AMPk), a central regulator of catabolic metabolism and oxidative phosphorylation in eukaryotic cells appears to be responsible for the energy production by oxidative phosphorylation in resting DCs, as reduction in AMPk levels boosted LPS-induced glycolysis [3]. This shift in metabolism is not only necessary for the viability and maturation of DCs but supports the development of demanding cellular functions including the transcription, translation and secretion of an array of inflammatory mediators.

DCs are however not the only immune cells reliant on glycolysis. Macrophages are also highly dependent on glycolysis for the production of ATP. Addition of glycolytic inhibitors reduces activity of these myeloid cells whereas mitochondrial inhibitors have not been shown to affect their ability to mount an inflammatory response [6]. In addition to generating ATP quickly to meet cellular demands, increasing glycolysis has also been shown to protect activated macrophages from apoptosis [7]. Activation of macrophages by interferon gamma ($\text{IFN}\gamma$) and LPS inhibits mitochondrial respiration. This is caused by release of large quantities of nitric oxide (NO) produced by the inducible NO synthase, consequently arresting oxidative phosphorylation and preventing mitochondrial ATP production [8]. Ordinarily this would cause mitochondrial collapse and apoptotic cell death but macrophages are able to remain viable and functional due to the switch to glycolysis. ATP produced by increased glycolysis is consumed by the mitochondria to maintain the mitochondrial membrane potential [7]. Glycolysis is therefore necessary for both activation of cells and protection from cell death and helps explain how macrophages can survive and function in oxygen deprived environments.

The molecular basis of increased glycolysis in response to TLR activation is poorly understood but a recent study highlights the importance of the bifunctional isoenzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) [9]. PFK2 catalyses the

synthesis and degradation of fructose 2,6-bisphosphate (Fru-2,6-P₂), which is the most potent activator of 6-phosphofructo-1-kinase, a key regulatory enzyme in glycolysis. Two isoforms of PFK2 exist, L-PFK2 which is expressed in the liver, is less active and causes degradation of Fru-2,6-P₂, and the more active ubiquitous form, u-PFK2 which increases synthesis of Fru-2,6-P₂. Activation through multiple TLR pathways causes a preferential shift to the u-PFK2 isoform, with L-PFK2 protein levels almost undetectable. Expression of u-PFK2 caused an increase in the conversion of glucose to lactate, whilst attenuation of u-PFK2 expression impaired macrophage viability upon activation [9]. Together these data suggest a direct link between TLR activation and glycolysis.

HIF is induced by LPS

Another mechanism that could link LPS and TLR4 to enhanced glycolysis is the transcription factor HIF1 α (HIF1 α) [10]. Hypoxia-inducible factor (HIF) is the principle transcriptional regulator of hypoxic adaption and directs cells to produce ATP via glycolysis [11].

HIF consists of a constitutively active β -subunit and an oxygen labile α -subunit, which exists in three forms termed HIF1 α , -2 α and -3 α . In the presence of oxygen HIF1 α protein is rapidly turned over. Conserved proline residues of HIF1 α are hydroxylated by a family of three prolyl hydroxylases (PHD1-3) [12]. This allows recognition by an E3 ubiquitin ligase containing the von Hippel-Lindau (VHL) protein which leads to ubiquitin-mediated targeting of HIF1 α for proteasomal destruction [13]. In addition, a conserved asparagine residue of HIF1 α undergoes hydroxylation by Factor Inhibiting HIF (FIH) in the presence of oxygen. This asparaginyl hydroxylation prevents recruitment of co-activators to the HIF complex, thereby preventing downstream target gene transcriptional activation [14,15]. In hypoxic conditions reactive oxygen species (ROS) produced by the mitochondria induce H₂O₂-mediated HIF1 α expression by targeting PHD. Hydrogen peroxide (H₂O₂) oxidises Fe²⁺ to Fe³⁺, reducing Fe availability which is required by PHD to hydroxylate HIF1 α . These data show that lack of mitochondrial respiration increases expression of HIF1 α . When degradation of HIF1 α is prevented it translocates to the nucleus, binds HIF1 β and other co-activators and initiates

transcription of target genes which contain conserved Hypoxia Response Elements (HRE), [16] including those involved in glycolysis, erythropoiesis, angiogenesis and proliferation.

HIF1 α is transcriptionally induced by LPS and is required to control multiple myeloid cell functions [5, 17-19]. Several signaling intermediates have been reported to be involved, including NF- κ B [20, 21], ROS [22-24], PHD [18] and p42/p44 mitogen-activated protein kinases (MAPKs) [20].

LPS induces HIF1 α mRNA expression in human monocytes through NF- κ B binding to the promoter of the HIF1 α gene [20, 21]. Inhibitor of NF- κ B (I κ B) kinases, responsible for activation of NF- κ B, are also critical in the upregulation of HIF1 α expression as macrophages deficient in IKK- β (β subunit of I κ B) have decreased expression of the HIF1 α target, GLUT1 [21]. In addition, LPS-induced HIF1 α was accompanied by a significant increase in phosphorylation of the MAP kinase intermediate p44/42. Activation of p44/42 appears to be critical for LPS-induced HIF1 α activation, since inhibition of the upstream kinase MEK1/2 by PD 98059 and RNAi directed against p44/42 significantly reduced HIF1 α mRNA and protein accumulation [20].

Other studies indicate that HIF1 α induction by LPS is dependent on ROS generation [23, 24]. Wang and colleagues demonstrated that young Mcl1^{+/-} mice (Mcl1 encodes a mitochondrial protein necessary for ubiquinone biosynthesis) have reduced oxygen consumption and mitochondrial function but sustained mitochondrial oxidative stress. LPS treatment of these mice led to an increase in HIF1 α expression and elevation of levels of pro-inflammatory cytokines [23]. Sphingosine kinase 1 (SphK1) has also recently been shown to mediate LPS-induced ROS activation of HIF1 α . Extracellular signal-regulating kinase, PLC-1 γ and PI3 Kinase were found to be critical for the LPS-induced SphK1 activation [22].

Additionally, it has been shown that LPS increases HIF1 α protein accumulation through decreasing the transcription of PHD2 and PHD3 in macrophages in a TLR4-dependent

manner [18]. HIF1 α directly binds to the promoter of the TLR4 locus and up-regulates TLR4 expression during O₂ deprivation [10].

HIF1 α up-regulates the expression of several glycolytic enzymes but there is also evidence to suggest glycolysis controls HIF1 α expression. Culturing various normal and tumour cell lines in glucose, lactate or pyruvate (end products of glycolysis) increases HIF1 α levels. Pyruvate, which was shown to be the most potent inducer, also promotes HIF1 α DNA- binding and increases transcription of vascular endothelial growth factor (VEGF), erythropoietin (EPO), GLUT3, aldolase A [25] and plasminogen activator inhibitor-1 (PAI-1) [26] indicating that glycolysis-induced HIF1 α is functional. However a mechanism of pyruvate-regulated HIF1 α expression has yet to be determined.

The mitochondrial tricarboxylic acid cycle (TCA) links glycolysis and oxidative phosphorylation. By manipulating the rate limiting enzyme, succinate dehydrogenase (SDH) which converts succinate to fumarate in the TCA cycle, HIF1 α expression can be enhanced [27]. Succinate directly inhibits PHD activity by product inhibition, since PHD also converts α -ketoglutarate to succinate. Also, blocking glycolysis with 2-Deoxyglucose (2DG) causes an increase in succinate dehydrogenase activity, decreasing succinate, which in turn enhances PHD activity leading to degradation of HIF1 α . Addition of succinate also increases ROS production and rescues HIF1 α protein expression and transcriptional activity. 2DG thereby causes increased turnover of HIF1 α . Succinate also rescued the effect of 2DG on HIF1 α expression [27]. These data highlight the balance required between glycolysis and oxidative phosphorylation to regulate HIF1 α expression.

The stabilisation of HIF1 α by glucose may also involve the pentose phosphate pathway (PPP) rather than glycolysis [28]. The PPP pathway generates reducing equivalents, in the form of NADPH, for reductive biosynthetic reactions. Treatment of a mast cell line HMC with the PPP inhibitor 6-aminonicotinamide (6-AN) prevented the glucose induced stabilisation of HIF1 α . Addition of NADPH enhanced HIF1 α protein expression whereas silencing glucose-6-phosphate dehydrogenase, the rate-limiting enzyme responsible for

NAPDH, prevented HIF1 α stabilisation [28]. Glucose metabolism is clearly necessary for the stabilization of HIF1 α , but it is yet to be determined which metabolite(s) is responsible. Since TLRs promote glycolysis, it is likely that the PPP is also enhanced, which could impact on HIF1 α stability.

These data support an inter-dependent relationship between HIF1 α and LPS resulting in positive feedback which may amplify macrophage activity at sites of hypoxia and infection (Figure 1).

HIF is essential for myeloid function

HIF seems to be critical in innate cell development and function as it is required for DC and macrophage maturation ([3, 5, 29, 30]. Knocking down HIF decreases the ability of the DC to up-regulate co-stimulatory factors and reduces the potential of DCs to stimulate the proliferation of allogenic T cells [5]. In mice with myeloid-specific ablation of the HIF1 α subunit, HIF1 α deficiency results in an 80% reduction of ATP [31]. The metabolic defect in HIF1 α deletion in macrophages results in impairment of energy-demanding processes such as aggregation, migration and invasion [31]. In addition to its key role in regulating metabolism and energy generation, Cramer *et al* showed that HIF1 α mediates macrophage inflammatory responses. Compared to control mice, myeloid HIF1 α ^{-/-} mice displayed reduced acute skin inflammation triggered by 12-O-tetradecanoylphorbol- 13-acetate (TPA), as indicated by decreased edema and leukocyte infiltration [31]. When induced to develop arthritis, these mice also showed compromised synovial infiltration, pannus formation and cartilage destruction, suggesting ameliorated chronic inflammatory responses mediated by HIF1 α deficient macrophages. In addition, in patients, HIF1 α was shown to be abundantly expressed by macrophages in inflamed rheumatoid synovia, while being absent in healthy synovia [32]. In infection models, loss of myeloid HIF1 α resulted in decreased bacterial killing of group A *Streptococcus* and *P. aeruginosa* by macrophages *in vitro* and *in vivo* [33]. Furthermore, exposure to these pathogens and LPS induced HIF1 α activity in macrophages in a TLR4-dependent fashion [18].

Loss of HIF1 α in macrophages leads to significant decreases in the production of TNF α , IL-1 β and IL-1 α , but not IFN γ or the anti-inflammatory cytokines IL-4 and IL-10. Therefore HIF1 α appears to be necessary for the transcription of proinflammatory cytokines [18]. This identifies proinflammatory cytokines as potential HIF target genes.

Inhibition of glycolysis by caspase 1

In a manner opposite to TLRs, the NLRC4 inflammasome might actually inhibit glycolysis. As glycolysis is essential for macrophage survival and activation, the cleavage of glycolytic enzymes is therefore predicted to be an essential step toward cell death. Indeed it's been shown that the glycolytic enzymes, fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, α -enolase and pyruvate kinase can be specifically targeted by active caspase 1. These enzymes were processed in peritoneal macrophages from wild type mice infected with *S. Typhimurium*, but not in those lacking caspase 1 [34]. Since NLRC4 senses *S. Typhimurium* to activate caspase-1, this implicates the NLRC4 inflammasome in this process [35]. In addition, the rate of glycolysis in the caspase 1 deficient cells was higher, further supporting the inhibitory role for caspase 1-mediated inactivation of glycolytic enzymes [34]. These data provide evidence that inflammasomes inhibit glycolysis. This may therefore restrict intracellular pathogen replication by depleting energy stores quickly and allowing the infected host to undergo caspase 1-mediated cell death.

Inflammation and metabolic disorders

Glycolysis is clearly important for DC and macrophage function. High glucose levels however can have adverse effects. Chronic hyperglycemia associated with over nutrition leads to insulin resistance. Insulin is secreted by pancreatic beta cells (β cells) and promotes glucose uptake in the muscle and reduces gluconeogenesis in the liver and lipolysis in adipose tissue. Insulin resistance results in defective nutrient metabolism and glucose intolerance. Eventually pancreatic beta cells can no longer compensate for

insulin resistance and type 2 diabetes with elevated circulating glucose develops. Obesity is the fundamental cause of insulin resistance and type 2 diabetes.

Obesity is characterized by increased storage of Fatty Acids (FA) in an expanded adipose tissue mass and a state of chronic inflammation. Recent evidence suggests that both TLR4 and Nlrp3 are critical in this process with IL-1 β as the key inflammatory cytokine. TLR4 induces pro-IL-1 β whilst Nlrp3 activates caspase 1 leading to production of the mature active cytokine. Adipose tissue has been viewed upon as the instigator of insulin resistance [36,37]. There is accumulation of macrophages in adipose tissue in obese patients [38]. This is associated with increased levels of TNF α . TNF α induces insulin resistance by disrupting insulin signal transduction through the phosphorylation of inhibitory serine of insulin receptor substrate 1 (IRS1) [39]. Mice lacking functional TNF α are protected against obesity-induced insulin resistance. In addition, TNF α is also over-expressed in the adipose and muscle tissues of obese humans, and when administered exogenously leads to insulin resistance [40]. However, attempts to block TNF signaling in obese individuals with T2DM have so far yielded disappointing results [41]. In addition, IL-1 β accumulates in adipose tissue and mediates insulin resistance. Wild type mice injected with IL-1 β have decreased insulin sensitivity compared to IL-1 β null mice, which do not show insulin resistance when fed a high fat diet [42]. Recent data suggest that Nlrp3 is responsible for sensing obesity-associated inducers of caspase 1, such as lipotoxic ceramides and palmitate, a saturated free fatty acid, to initiate macrophage activation and IL-1 β secretion [4, 42]

Insulin resistance leads to the pancreas over-working to produce more and more insulin to 'mop up' glucose in the blood metabolic stress signals recruit monocytes to clear dying β cells. Increased numbers of macrophages are observed in pancreatic islets from patients with type 2 diabetes [43]. Hyperglycemia induces IL-1 β secretion by both macrophages and pancreatic cells β -cells [44-46] creating an inflammatory environment which has cytotoxic effects on β -cells [45] [47] inducing them to undergo apoptosis [48]. In β cells, glucose induces ROS generation which subsequently activates Nlrp3-dependent IL-1 β secretion. This is mediated by thioredoxin-interacting protein (TXNIP)

which directly binds Nlrp3. This suggests oxidative stress is responsible for IL-1 β secretion in these cells [49]. In macrophages however in addition to glucose, the amyloid polypeptide (IAPP) which is secreted along with insulin in the pancreas, is required for Nlrp3-dependent IL-1 β processing [50]. Glucose also activates caspase 1 in human and murine adipose tissue. Glucose-induced activation of TXNIP mediates IL-1 β mRNA expression levels and intracellular pro-IL-1 β accumulation in adipose tissue [51]. In addition, mice deficient in either Nlrp3, caspase 1, or IL-1 β fed a normal-chow diet have improved insulin sensitivity, supporting the role of Nlrp3 in regulating glucose homeostasis [52].

The mechanisms by which IL-1 β attenuates insulin signaling include interactions of IL-1 β -dependent signalling proteins with insulin receptor and/or IRS proteins, such as members the SOCS family. SOCS1, SOCS3 and SOCS6 have been identified as the principle inhibitors of the insulin pathway by interfering with IRS-1 and IRS-2 phosphorylation or by targeting IRS for proteasomal degradation [53].

Why would the NLRP3/IL-1 system cause insulin resistance? There are two possibilities. Insulin resistance in the periphery might spare glucose for leukocyte function in immune cells. Or perhaps under conditions of hyperglycemia, IL-1 limits glucose uptake and metabolism to prevent generation of ROS or other metabolites, made as a result of enhanced glucose metabolism (Figure 2) which would be damaging.

Perspective

The interface between metabolism and innate immunity is of great interest. TLRs and Nlrp3 may sense metabolic disturbance caused by tissue injury, obesity and infection, and trigger processes to restore homeostasis. These innate receptors also impact directly on metabolic events during this process. HIF1 α may be especially important in these pathways and insulin resistance an important output from the Nlrp3 inflammasome. Further molecular details are needed and where specificity lies in these events is an important question. Future work will reveal the importance of metabolic processes for inflammation in both health and disease.

Figure legends

Figure 1: Interplay between TLR4, glycolysis and HIF1 α

Activated DCs and macrophages shift their metabolism to glycolysis for energy and biosynthesis to support demanding cellular function. HIF1 α is a key regulator of the expression of glycolytic enzymes. Its regulation by hypoxia is well known but how it is regulated under normoxic conditions is less well understood, however TLR4 signalling and glycolysis may play an important role.

Figure 2: TLR4, Nlrp3, hyperglycemia and hyperlipidemia in IL-1 β production

TLR4 and Nlrp3 act in concert to produce the pro-inflammatory cytokine IL-1 β . TLR4 will promote glucose uptake which is metabolised by glycolysis. An increase in succinate may occur (although precisely how is unknown), inhibiting PHD, stabilising HIF1 α which could have a role in the induction of pro-IL-1 β . In addition, succinate can induce ROS, which also decreases PHD activity, but in addition are required for Nlrp3 inflammasome activation. The source of the ROS appears to be mitochondria. Hyperlipidemia will generate lipids such as ceramides and palmitate, which also activate Nlrp3, as does IAPP deposited in the pancreas in type 2 diabetes. Nlrp3 activates caspase-1 leading to production of IL-1 β , which acts in a negative feedback manner to block insulin signaling and glucose uptake. These events may be critical in the pathogenesis of type 2 diabetes.

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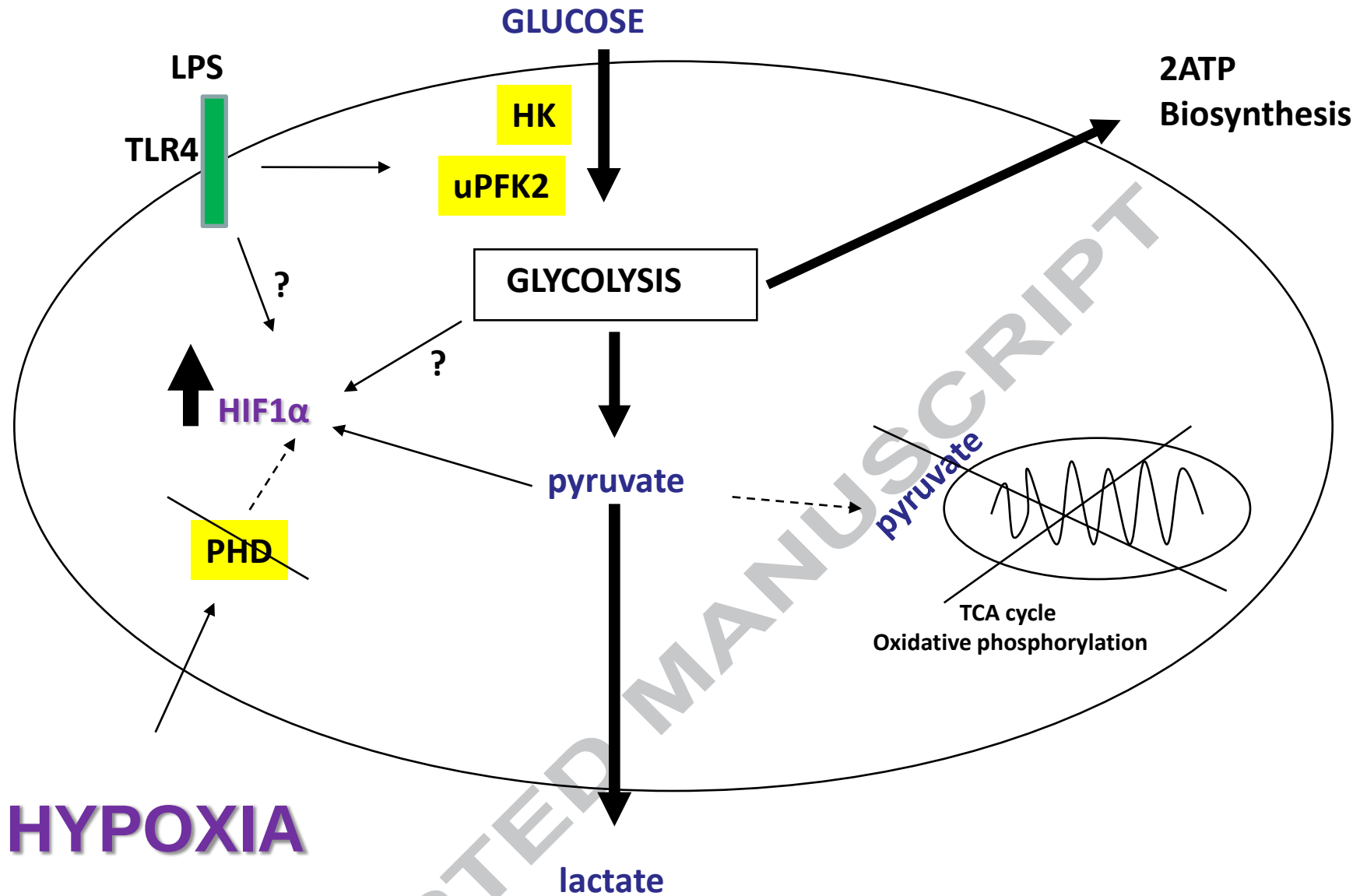


Figure 1

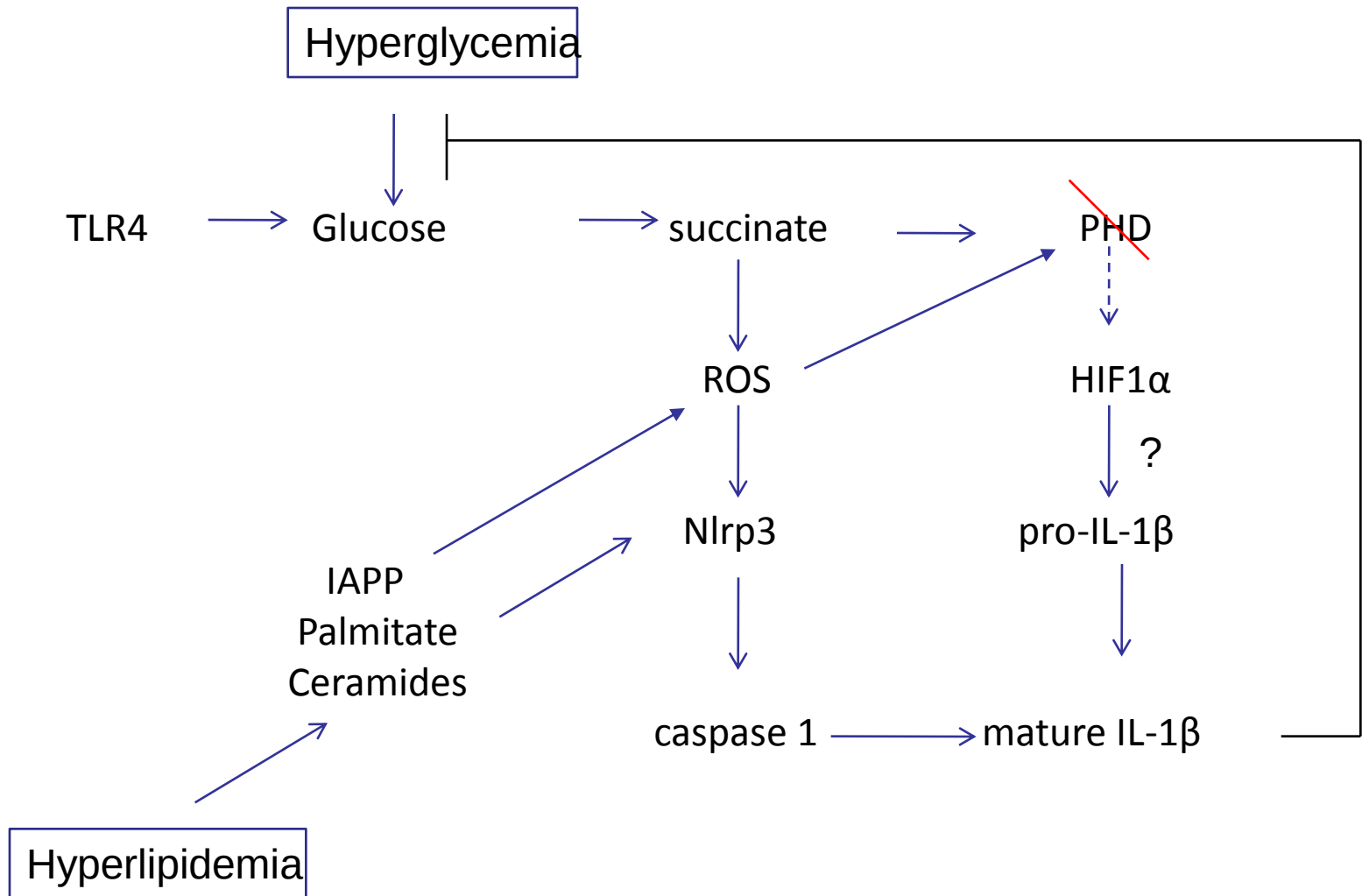


Figure2