Published in final edited form as:

ACS Infect Dis. 2017 March 10; 3(3): 216–224. doi:10.1021/acsinfecdis.6b00180.

HK2 Recruitment to Phospho-BAD Prevents Its Degradation, Promoting Warburg Glycolysis by *Theileria*-Transformed Leukocytes

Malak Haidar^{†,‡}, Anne Lombès^{†,‡}, Frédéric Bouillaud^{†,‡}, Eileen J. Kennedy[§], and Gordon Langsley^{†,‡,*}

[†]Inserm U1016, CNRS UMR8104, Cochin Institute, Paris 75014 France

[‡]Laboratoire de Biologie Cellulaire Comparative des Apicomplexes, Faculté de Médecine, Université Paris Descartes – Sorbonne Paris Cité, Paris 75014, France

[‡]Laboratoire de Mitochondries, Bioénergétique, Métabolisme et Signalisation, Faculté de Médecine, Université Paris Descartes — Sorbonne Paris Cité, Paris 75014, France

[§]Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, Georgia 30602, United States

Abstract

Theileria annulata infects bovine leukocytes, transforming them into invasive, cancer-like cells that cause the widespread disease called tropical theileriosis. We report that in *Theileria*-transformed leukocytes hexokinase-2 (HK2) binds to B cell lymphoma-2-associated death promoter (BAD) only when serine (S) 155 in BAD is phosphorylated. We show that HK2 recruitment to BAD is abolished by a cell-penetrating peptide that acts as a non-phosphorylatable BAD substrate that inhibits endogenous S155 phosphorylation, leading to complex dissociation and ubiquitination and degradation of HK2 by the proteasome. As HK2 is a critical enzyme involved in Warburg glycolysis, its loss forces *Theileria*-transformed macrophages to switch back to HK1-dependent oxidative glycolysis that down-regulates macrophage proliferation only when they are growing on glucose. When growing on galactose, degradation of HK2 has no effect on *Theileria*-infected leukocyte proliferation, because metabolism of this sugar is independent of hexokinases. Thus, targeted disruption of the phosphorylation-dependent HK2/BAD complex may represent a novel approach to control *Theileria*-transformed leukocyte proliferation.

*Corresponding Author: (G.L.) Phone: +33 140516592. Fax: +33 140516570. gordon.langsley@inserm.fr.

Author Contributions

M.H. performed the biochemical experiments. G.L. conceived the study, and results were interpreted by M.H., A.L., F.B., E.J.K., and G.L. E.J.K. provided peptide expertise, M.H. prepared figures, and M.H. and G.L. wrote the manuscript with inputs and comments from A.L., F.B., and E.J.K.

The authors declare no competing financial interest.

ORCID

Gordon Langsley: 0000-0001-6600-6286

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfec-dis.6b00180.

Figure S1: global PKA activity is not changed by peptide treatment. Figure S2: microscopic imaging of cell-penetrating nonphosphorylatable (S155A) and phospho-mimic (S155D) BAD peptides. Figure S3: HK2 is readily detected in *Theileria*-infected B cells (TBL20) (PDF)

Graphical abstract

Keywords

Theileria; hexokinase; BAD; glycolysis; Warburg; oxidative phosphorylation

INTRODUCTION

Tropical theileriosis is an economically important bovine disease, widespread in North Africa, the Middle East, India, and China.¹ It is caused by the protozoan parasite *Theileria annulata*, which affects domestic cattle (*Bos taurus* and *Bos indicus*) and Asian buffalo (*Bubalus bubalis*).^{2,3} *T. annulata* infects B cells and monocytes/macrophages¹ that display many characteristics typical of tumors such as resistance to apoptosis, uncontrolled proliferation independent of exogenous growth factors, and increased ability to disseminate in vivo.^{4–6} However, the transformed state of infected leukocytes is completely reversed upon drug-induced parasite death⁷ and infected macrophage tumorigenic virulence attenuated by multiple in vitro passages.⁸ Attenuated infected macrophages are used as live vaccines against tropical theileriosis.

In mammalian cells, there are two different bioenergetic pathways that depend on alterations in oxygen status. Under aerobic conditions, cells metabolize one molecule of glucose into approximately 34 molecules of ATP via oxidative phosphorylation that is dependent on the mitochondrial respiratory chain. In contrast, cells metabolize one molecule of glucose into two molecules of lactate and produce two molecules of ATP under hypoxic conditions.⁹ In 1956, Otto Warburg discovered that cancer cells tend to convert glucose into lactate to produce energy rather than utilizing oxidative glycolysis, a phenomenon that became known as the Warburg effect.^{10,11} Originally it was believed that solid tumors used Warburg glycolysis because they lacked oxygen and experienced hypoxia. However, it is currently thought that the switch away from oxidative glycolysis by proliferating cells is an attempt to reduce reactive oxygen species (ROS) generated by the respiratory chain. This thinking is particularly applicable to *Theileria*-transformed leukocytes that do not form solid tumors in vivo and are, thus, unlikely to experience hypoxia. *Theileria*-transformed macrophages express HK2/HK1 and pyruvate kinase isozyme M1 and M2 (PKM1/PKM2) ratios typical of Warburg glycolysis.¹² Hexokinase 2 (HK2) is a key glycolytic enzyme that phosphorylates

glucose to yield glucose-6-phosphate, and augmented HK2 expression typifies Warburg glycolysis.¹³

The Bcl-2 family protein BAD has a well-established role in the regulation of glucose metabolism separate from its role in apoptosis.^{14–16} BAD belongs to a subset of Bcl-2 family members, known as BH3-only pro-apoptotic proteins, which share sequence homology only within the α -helical BH3 motif, also known as the minimal death domain.¹⁴ In hepatocytes under the influence of insulin, phosphorylation of the BH3 suppresses its apoptotic activity and triggers the metabolic function of BAD. The impact of BAD on cellular metabolism depends on its ability to bind glucokinase (GK),^{14,16} where GK binding to BAD requires phosphorylation of S155 (mouse BAD numbering), which is the target of multiple kinases such as RSK1,^{17,18} MAPKs, ERK1/2, PI3K/AKT, and PKA.¹⁹ Phosphorylation of BAD is required and sufficient for its metabolic function.¹⁶ Furthermore, BAD and GK reside in a mitochondrial complex together with PKA, PP1 catalytic units, and WAVE-1 to integrate glycolysis and apoptosis.¹⁵ An association between HK2 and BAD has not been reported for transformed leukocytes.

PKA activity is high in *Theileria*-infected leukocytes, which results in constitutive phosphorylation of S155 on BAD.^{20,21} Therefore, we asked if PKA-mediated phosphorylation of S155 in BAD resulted in the recruitment of HK2 to mitochondria in *Theileria*-transformed macrophages. We also asked if ablation of the HK2/BAD association would affect the type of glycolysis used by *Theileria*-transformed leukocytes and hence their proliferation status. Here, we report that inhibited phosphorylation of S155 in *Theileria*-transformed macrophages provoked dissociation of HK2 from BAD, whereupon HK2 becomes ubiquitinated and degraded by the proteasome. Loss of HK2 obliged *Theileria*-transformed macrophages to switch from high lactate producing Warburg to HK1-dependent oxidative glycolysis. The switch was associated with a drop in transformed leukocyte proliferation. Therefore, PKA-mediated recruitment of HK2 to BAD protects HK2 from proteasome degradation and promotes both Warburg glycolysis and transformed macrophage proliferation.

RESULTS

HK2 and BAD Form a Complex in *Theileria*-Transformed Macrophages

T. annulata infection of leukocytes induces PKA-mediated phosphorylation of S155 of BAD.²¹ However, classical chemical inhibition (ATP analogues such as H89 or KT5720) of kinase activity ablates PKA-mediated phosphorylation toward its myriad substrates, thereby clouding assignment of the physiological consequences of phosphorylation of a specific site in a given substrate. For this reason, we chose to use cell-penetrating, competitive BAD substrate peptides to specifically knockdown S155 phosphorylation without altering overall PKA activity. The S155A peptide functions as a nonphosphorylatable BAD substrate and not as a catalytic inhibitor of PKA, because overall constitutive PKA activity^{20,21} was unchanged in treated macrophages (Figure S1). Because the S155A peptide actually inhibits phosphorylation of BAD on S155, this peptide likely serves as a nonphosphorylatable pseudosubstrate occupying substrate-binding sites for kinases (PKA, RSK, etc.) targeting S155 in BAD. By contrast, the S155D peptide does not act as a competitive inhibitor for

BAD S155 phosphorylation, because it mimics the phosphorylation product. Using these two peptides we examined the phosphorylation status of S155 using a phosphospecific antibody and observed decreased phosphorylation when infected macrophages were treated with a nonphosphorylatable version of the BAD peptide, S155A (Figure 1A). As expected, no dampening of S155 phosphorylation was observed with the phosphomimic S155D peptide (Figure 1A). Both synthetic peptides were FITC labeled, enabling visual confirmation that they were successfully taken up by macrophages (Figure S2).

HK2 is a glycolytic enzyme implicated in the first step of aerobic glycolysis that is located on the outer membrane of mitochondria, where it phosphorylates glucose to glucose-6-phosphate.^{22,23} To assess whether HK2 and BAD resided together in a complex on mitochondria in *Theileria*-transformed macrophages, both HK2 and BAD were independently immunoprecipitated, and precipitates were probed with anti-BAD and anti-HK2 antibodies, respectively. This revealed that BAD associates with HK2 in *Theileria*-transformed macrophages. However, when they are treated with the non-phosphorylatable S155A BAD peptide, but not when treated with the phosphomimic S155D peptide (Figure 1B), the amount of HK2 that is immunoprecipitated together with BAD decreases. Thus, treating *Theileria*-transformed macrophages with the nonphosphorylatable BAD peptide appears to reduce HK2 binding to BAD and hence HK2 recruitment to the mitochondria. The 50% reduction in phosphorylation of BAD implies that the stability of the whole complex¹⁵ is phosphorylation-dependent and that the complex dissociates when S155 phosphorylation is down-regulated.

As levels of HK2 diminished in transformed macrophages upon ablation of S155 BAD phosphorylation, we investigated whether HK2 became ubiquitinated and degraded by the proteasome. To determine whether the phosphorylation status of BAD triggers ubiquitination-dependent proteasomal degradation of HK2, macrophages were treated with the proteasome inhibitor MG132. HK2 was first immunoprecipitated with an anti-HK2 antibody, and the precipitate was probed with an anti-mono- and -polyubiquitin antibody (Figure 1C). Down-regulation of BAD phosphorylation by the S155A peptide and dissociation of the BAD/HK2 complex clearly induced ubiquitination of HK2, whereas the phosphomimic peptide (S155D) did not. Thus, in *Theileria*-transformed macrophages, phosphorylation of S155 recruits HK2 to BAD and protects HK2 from ubiquitin-mediated proteasomal degradation.

Subcellular Location of BAD and HK2

To determine the effects of the nonphosphorylatable and phosphomimic peptides on the subcellular localization of HK2 and BAD, cell imaging was performed (Figure 2). When macrophages (M) are treated with the nonphosphorylatable S155A BAD peptide, the HK2 signal is lost (Figure 2), whereas no effect was observed upon treatment with the phosphomimic peptide (S155D). This strongly argues that levels of HK2 depend on the phosphorylation status of S155 BAD. Furthermore, we confirmed the mitochondrial localization of HK2 by examining its association with the α -1 subunit of succinate dehydrogenase (SDHA), the flavoprotein subunit of mitochondrial complex II.²⁴ Figure 2B shows HK2 in green and SDHA-1 in red. Merging the two images shows areas of overlap in

yellow that are consistent with HK2 being localized on mitochondria. When *Theileria*-transformed macrophages are treated with the nonphosphorylatable S155A peptide, phosphorylation of S155 BAD is ablated and the HK2 signal is lost due to HK2 degradation (Figure 1C).

HK2 Degradation Induces Transformed Macrophages To Switch from High Lactate Producing Warburg to Oxidative Glycolysis

Theileria-provoked leukocyte transformation induces infected host cells to perform Warburg glycolysis that produces high amounts of lactate.^{12,25,26} Therefore, production of lactate was measured and found diminished when *Theileria*-transformed macrophages and B cells were treated with cell-penetrating S155A peptide (Figure 3A). A significant decrease in transformed macrophage lactate output (ECAR) and an increase in oxygen consumption (OCR) was also obvious by metabolic analysis of live cells (Figure 3B, left). Seahorse measurements allow an estimation of the mitochondrial reserve respiratory capacity (RRC), and this increased 2.2-fold over basal OCR, consistent with *T. annulata*-infected macrophages having an important reserve capacity under normal conditions. Upon treatment with the S155A peptide, the reserve capacity increased 2.9-fold over basal OCR, showing that in the absence of HK2 there is a more profound adaptation toward mitochondrial respiration (Figure 3B, right). HK1 protein levels were analyzed by western blot and found to be unchanged whether macrophages were treated or not with the two peptides (Figure 3C). Thus, loss of HK2 renders the balance of HK1/HK2 in favor of HK1. Furthermore, the predominance of HK1 is associated with a change to oxidative glycolysis in cells treated with the nonphosphorylatable BAD substrate peptide, which correlates with decreased BAD phosphorylation on S155.

BAD Phosphorylation Promotes Proliferation of *T. annulata*-Transformed Leukocytes

T. annulata infection of BL20 reprograms these cells that are now expressing HIF1- α .²⁷ Consequently, when growing on glucose, TBL20 proliferates more quickly (compare BL20 to TBL20), likely due to its expressing more HK2 (Figure S3). Only TBL20 and macrophage (M) proliferation decreases when phosphorylation of BAD is inhibited following treatment with the non-phosphorylatable S155A BAD peptide (Figure 4). No adverse effect on proliferation was observed upon treatment with the phosphomimic (S155D) peptide (Figure 4). Moreover, neither peptide altered proliferation of immortalized, but noninfected, B cells (BL20). Cell viability was >85% in all of the experiments, showing that dissociation of HK2 from BAD did not provoke loss of viability.

Effect of Carbon Source on Proliferation of *Theileria*-Infected Macrophages

Tumors are exposed to dramatically different nutrient conditions, including variation in oxygen and carbohydrate supplies. Metabolism of cancer cells and indeed that of all proliferating cells is adapted to switch between carbohydrate metabolism via glycolysis or mitochondrial TCA cycle and oxidative reactions. We monitored transformed macrophage proliferation for 24–48 h in the presence of either the nonphosphorylatable (S155A) or phosphomimic (S155D) peptides (Figure 5). Changing the medium from 10 mM glucose to 10 mM galactose (glucose deprivation) induced a reduction in proliferation of *Theileria*-transformed macrophages, suggesting that they have adapted to growing on glucose.

However, the metabolism of galactose does not require hexokinase activity, so, expectedly, treatment with the non-phosphorylatable S155A peptide that induced HK2 degradation (Figure 1C) had no effect on cells metabolizing galactose. By contrast, treatment notably affected proliferation of macrophages metabolizing glucose (Figure 5, left), demonstrating that when metabolizing galactose, the dependence on hexokinase activity is bypassed and loss of HK2 is not detrimental (Figure 5, right).

DISCUSSION

The majority of transformed cells have undergone a “metabolic switch” in their glycolysis termed the Warburg effect.¹⁰ *Theileria*-transformed leukocytes acquire many of the characteristics of cancer cells such as producing the energy required for their uncontrolled proliferation via the Warburg effect.^{12,25,28} Moreover, they have up-regulated the HIF-1 α transcription factor and consequently preferentially express genes characteristic of Warburg glycolysis such as *hk2*, *pkm2*, and *ldha*. The Bcl-2 family member BAD plays an important regulatory role in glycolysis, where its activity is regulated by phosphorylation in response to growth factors and survival.^{29,30} We have previously described that in *Theileria*-transformed B cells, PKA is constitutively active and BAD is permanently phosphorylated at S155.²¹

Transformed leukocytes express HK2, and we reasoned that S155 phosphorylation of BAD might recruit HK2 to the mitochondria and in such a way contribute to their preferential use of Warburg glycolysis.^{12,28} Therefore, we used a cell-penetrating nonphosphorylatable S155A BAD substrate peptide to disrupt HK2/BAD complexes by inhibiting BAD S155 phosphorylation and asked whether loss of the association affected Warburg glycolysis performed by *Theileria*-transformed leukocytes. Importantly, treating *Theileria*-transformed macrophages with the nonphosphorylatable S155A BAD peptide ablated the interaction of HK2 with BAD, whereupon HK2 became ubiquitinated and degraded, whereas the amount of HK1 remained unchanged. Upon degradation of HK2, *Theileria*-transformed macrophages and B cells consumed more oxygen and produced less lactate. These findings are consistent with a switch from Warburg to oxidative glycolysis provoked by loss of HK2.

The oxidative stress of continuously proliferating *Theileria*-transformed leukocytes induces a HIF-1 α -driven Warburg effect^{12,28} (for a review see ref 25). We demonstrate that upon degradation of HK2, *Theileria*-transformed leukocytes consumed more oxygen and produced less lactate, consistent with loss of HK2, thereby forcing a switch from Warburg to oxidative glycolysis. Thus, in addition to HIF-1 α -driven *hk2* transcription, phosphorylation-dependent binding of HK2 to BAD represents an alternative post-translational strategy to regulate the ability of HK2 to phosphorylate glucose while simultaneously protecting complex-bound HK2 from proteasomal degradation so that it can participate in Warburg glycolysis. Interestingly, *N*-acetylglucosamine stemming from infection by Gram-positive bacteria leads to dissociation of HK2 from mitochondria and activation of the inflammasome.²³ This directly contrasts to *Theileria* infection of leukocytes that promotes HK2 recruitment to mitochondria and favors Warburg glycolysis. However, in our study, we did not address whether recruitment of HK2 to mitochondria might also dampen

inflammasome activation, a process that is potentially beneficial to intracellular parasite survival.

Proliferation was not as robust when *Theileria*-transformed macrophages grew on galactose as compared to glucose. However, it did not affect viability, and this may be due to constitutive PKA²⁰ activity protecting them from glucose deprivation.³¹ Galactose is converted into galactose-1 phosphate by galactokinase, and the Leloir metabolic pathway that catabolizes D-galactose is independent of hexokinase activity. Consequently, growth of *Theileria*-transformed macrophages in the presence of galactose became insensitive to decreased BAD S155 phosphorylation via treatment with the S155A peptide, as loss of HK2 was not detrimental on this sugar source. This demonstrates the target specificity of the S155A peptide, as its effects are no longer observed when HK2 activity is bypassed.

In conclusion, in *Theileria*-transformed leukocytes the switch from oxidative to Warburg glycolysis that accompanies their uncontrolled proliferation involves constitutive activation of HIF-1 α , PKA, and S155 phosphorylation of BAD, and phosphorylation of S155 promotes recruitment of HK2 to mitochondria-localized BAD. Sequestered in a BAD complex, HK2 is protected from ubiquitination and degradation by the proteasome such that HK2 levels predominate those of HK1, and this favors Warburg over oxidative glycolysis. Protection from ubiquitin-mediated proteasomal degradation is, therefore, a novel, alternative way of regulating HK2 levels over and above HIF-1 α -driven *hk2* transcription. In nontransformed hepatocytes, cell-penetrating phosphomimic peptides that disrupt the BAD/GK complex counteract gluconeogenesis and improve glycemia in models of diabetes and insulin resistance, and it has been proposed that phospho-BAD mimetics may restore functional β cell mass in diabetes.³⁰ We propose that ablating HK2 recruitment to BAD could negatively affect the proliferation of any cell that preferentially uses Warburg glycolysis, as proteasomal degradation of HK2 would render them reliant of HK1-mediated oxidative glycolysis for growth. On the basis of these findings, disruption of BAD S155 phosphorylation may serve as a useful strategy for the treatment not only of tropical theileriosis but also of cancer due to tumor dependence on Warburg glycolysis.

MATERIALS AND METHODS

Cell Culture and Reagents

Cells used in this study are *T. annulata*-transformed macrophages,³² where transformed macrophages used correspond to passage 62. Noninfected BL20 cells³³ and *T. annulata*-infected BL20 (TBL20) cells used have been previously characterized in our laboratory.³⁴ All cells were incubated at 37 °C with 5% CO₂ in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U of penicillin, 0.1 mg/mL streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and in addition 5% 2-mercaptoethanol for BL20 and TBL20, respectively.

Media for Metabolic Manipulation

For cell culture under glucose free conditions, glucose-free RPMI (Gibco, Life Technologies, Paisley, UK) was supplemented with 10 mM galactose. As control, medium

supplemented with 10 mM glucose was used. Media were further supplemented with 10% heat-inactivated dialyzed FBS, 2 mM L-glutamine, 100 U of penicillin, 0.1 mg/mL streptomycin, and HEPES.

Peptide Synthesis

The amino acid sequence of BAD is based on the bovine sequence (VKKKKIKREIKIAAQRYGRE-LRRMSDEFHV), where S155 (S119 in bovine BAD) in wild type BAD was replaced by a non-phosphorylatable alanine (**A** in bold, VKKKKIKREIKIAAQRYGRELRRMADEFHV). The penetrating peptide to facilitate cell entry is underlined. As a negative control, S155 (S119 in bovine BAD) was replaced by a phosphorylation-mimic aspartate (**D** in bold, VKKKKIKREIKIAAQRYGRELRRMDDEFHV). All synthetic peptides were labeled on the C-terminus with FITC and HPLC purified at 95%, as described.³⁵

BAD Phosphorylation Experiments

The phosphorylation of BAD on S155 was ablated using the nonphosphorylatable S155A peptide described above. A concentration of 10 μ M was used for both peptides incubated for 2 h at 37 °C in 5% CO₂ for all experiments performed in this study except for proliferation assays (10 μ M for 24 and 48 h). No effect on S155 phosphorylation was observed when transformed macrophages were treated with peptides at 5 μ M for 2 and 24 h (data not shown). All experiments were done independently three times in duplicate.

Proliferation Assay

M and BL20/TBL20 cells were treated each day with 10 μ g/mL S155A and S155D peptides, respectively. Infected leukocyte viability was checked throughout. All experiments were done independently three times in duplicate. The error bars represent SEM values between three biological replicates.

Proteasome Inhibition

To block the catalytic activity of the proteasome, cells were treated for 2 h with the proteasome inhibitor MG132 (Santa Cruz Biotechnology sc-351846) at a final concentration of 10 μ M.

western Blot Analysis

Cells were washed with cold PBS and lysed on ice for 30 min in the lysis buffer and then centrifuged at 13000 rpm for 15 min at 4 °C to eliminate cellular debris. Protein concentration was determined by using the Bradford method (by reading the optical density at 595 nm). Cell extracts were mixed with 5 $\times$ Laemmli and denatured at 95 °C for 5 min. Proteins were separated by migration through a denaturing SDS-PAGE gel and electrotransferred onto a nitrocellulose membrane (Protran). The membrane was blocked by 5% nonfat milk–TBST (for antibody of interest) or 3% nonfat milk–PBST (for anti-actin antibody) for 1 h at room temperature. Antibodies used in immune-blotting were as follows: rabbit polyclonal antibody anti-phospho-BAD (S155) (Santa Cruz Biotechnology sc-101641) diluted 1/100 in 5% BSA–TBST, rabbit anti-BAD (Cell Signaling 9292S)

diluted 1/200 in 5% BSA–TBST, rabbit polyclonal antibody anti-HK2 (Cell Signaling 2867S) diluted 1/1000 in 5% BSA–TBST, and rabbit monoclonal antibody anti-HK1 (Cell Signaling 2804) diluted 1/1000 in 5% BSA–TBST, all incubated overnight at 4 °C. Goat polyclonal antibody anti-actin (Santa Cruz Bio-technology I-19) diluted 1/2000 in 3% BSA–PBST was incubated for 1 h at room temperature. After washing, membranes were incubated with peroxidase-conjugated secondary antibody (mouse anti-IgG, rabbit anti-IgG, and goat anti-IgG (Santa Cruz Biotechnology) diluted 1/5000 for 1 h at room temperature. After washing, membranes were developed using Super Signal detection kit (Thermo Fisher).

Co-immunoprecipitations (Co-IPs)

Co-IPs were conducted with protein extracts of *Theileria*-transformed macrophages together with a goat anti-HK2 antibody (Santa Cruz Biotechnology sc-6521) or an anti-BAD antibody (Santa Cruz Biotechnology sc-8044). HK2 precipitates were transferred to western blots and probed with the anti-BAD antibody, a rabbit anti-HK2 (Cell Signaling 2867S), and a mouse monoclonal mono- and polyubiquitinated conjugate antibody (FK2; Enzo BML-PW8810-0100). BAD precipitates were transferred to western blots that were probed with the goat anti-HK2 antibody. Normal IgG was used as a negative control, and the whole cell lysate without IP was included as positive control.

Immunofluorescence Microscopy

Cells (5×10^4) were plated on glass coverslips coated with poly-L-lysine. Cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized using 0.5% Triton X-100. After a 30 min treatment with blocking solution (3% BSA and PBST buffer, 0.1% Tween20), cells were stained for 2 h with the primary antibodies (anti-HK2, Santa Cruz Biotechnology sc-6521; anti-BAD, Santa Cruz Biotechnology sc-8044), and a mouse anti-SDHA-1 antibody.²⁴ After three washings with PBS, cells were incubated with the secondary antibody (Alexa fluor 594 goat anti-rabbit IgG A11037, Alexa fluor 488 rabbit anti-goat IgG A11078, and Alexa fluo594 goat anti-mouse IgG A11005) for 45 min in the dark. After three additional washings, slides were stained with 4,6-diamidino-2-phenylindole (DAPI; diluted 1/ 1000; Sigma-Aldrich) for 5 min to visualize the nuclei. Labeled preparations were mounted in Dako and analyzed by inverted microscopy (Leica DMI6000s). Acquisitions were made with metamorphous software.

Oxygen Consumption and Lactate Output Measurements

Oxygen consumption and lactate output rates were measured in accordance with the manufacturer's instructions (Seahorse Bioscience). Experiments were replicated in six wells and averaged for each experimental condition.

Lactate ELISA

Lactate production was also measured using Sigma lactate colorimetric assay kit (MAK064). Cells (5×10^5) were collected in the lactate assay buffer provided with the kit and homogenized, and then changes in lactate output were measured using a microplate reader, with OD 570 nm.

Statistical Analysis

Data were analyzed with the Student's *t* test. All values are expressed as the mean $\pm$ SEM. Values were considered to be significantly different when *p* values were <0.05 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the help of Thomas Guilbert of Cochin Institute's Imagerie Cellulaire Plateform. M.H. was supported by a Ph.D. fellowship from the National Council for Scientific Research, CNRS, Beirut, Lebanon. G.L. acknowledges support from an ANR grant (11 BSV3 01602) and Labex ParaFrap (ANR-11-LABX-0024), and all authors acknowledge support from INSERM and the CNRS. E.J.K. acknowledges support from NIH (CA188439).

References

1. Dobbelaere D, Heussler V. Transformation of leukocytes by *Theileria parva* and *T. annulata*. *Annu Rev Microbiol.* 1999; 53:1–42. [PubMed: 10547684]
2. Robinson PM. *Theileriosis annulata* and its transmission—a review. *Trop Anim Health Prod.* 1982; 14(1):3–12. [PubMed: 6805112]
3. Dumanli N, Aktas M, Cetinkaya B, Cakmak A, Koroglu E, Saki CE, Erdogmus Z, Nalbantoglu S, Ongor H, Simsek S, et al. Prevalence and distribution of tropical theileriosis in eastern Turkey. *Vet Parasitol.* 2005; 127(1):9–15. [PubMed: 15619369]
4. Fell AH, Preston PM. Proliferation of *Theileria annulata* and *Theileria parva* macroschizont-infected bovine cells in scid mice. *Int J Parasitol.* 1993; 23(1):77–87. [PubMed: 8468139]
5. Irvan A, Morrison W. Immunopathology, immunology and immunoprophylaxis of *Theileria* infections in immune responses in parasitic infections: immunology, immunopathology, and immunoprophylaxis. *Protozoa.* 1987; 3:223–274.
6. Tretina K, Gotia HT, Mann DJ, Silva JC. *Theileria*-transformed bovine leukocytes have cancer hallmarks. *Trends Parasitol.* 2015; 31(7):306–314. [PubMed: 25951781]
7. Dobbelaere D, Coquerelle T, Roditi I, Eichhorn M, Williams R. *Theileria parva* infection induces autocrine growth of bovine lymphocytes. *Proc Natl Acad Sci U S A.* 1988; 85(13):4730–4734. [PubMed: 3133661]
8. Somerville RP, Adamson RE, Brown CG, Hall FR. Metastasis of *Theileria annulata* macroschizont-infected cells in scid mice is mediated by matrix metalloproteinases. *Parasitology.* 1998; 116(Part 3):223–228. [PubMed: 9550215]
9. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science.* 2009; 324(5930):1029–1033. [PubMed: 19460998]
10. Warburg O. On the origin of cancer cells. *Science.* 1956; 123(3191):309–314. [PubMed: 13298683]
11. Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer.* 2011; 11(5):325–337. [PubMed: 21508971]
12. Metheni M, Echebli N, Chaussepied M, Ransy C, Chereau C, Jensen K, Glass E, Batteux F, Bouillaud F, Langsley G. The level of H₂O₂ type oxidative stress regulates virulence of *Theileria*-transformed leukocytes. *Cell Microbiol.* 2014; 16(2):269–279. [PubMed: 24112286]
13. Jin Z, Gu J, Xin X, Li Y, Wang H. Expression of hexokinase 2 in epithelial ovarian tumors and its clinical significance in serous ovarian cancer. *Eur J Gynaecol Oncol.* 2014; 35(5):519–524.
14. Danial NN. BAD: undertaker by night, candyman by day. *Oncogene.* 2008; 27(Suppl 1):S53–S70. [PubMed: 19641507]
15. Danial NN, Gramm CF, Scorrano L, Zhang CY, Krauss S, Ranger AM, Datta SR, Greenberg ME, Licklider LJ, Lowell BB, et al. BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. *Nature.* 2003; 424(6951):952–956. [PubMed: 12931191]

16. Danial NN, Walensky LD, Zhang CY, Choi CS, Fisher JK, Molina AJ, Datta SR, Pitter KL, Bird GH, Wikstrom JD, et al. Dual role of proapoptotic BAD in insulin secretion and beta cell survival. *Nat Med*. 2008; 14(2):144–153. [PubMed: 18223655]
17. Fang X, Yu S, Eder A, Mao M, Bast RC Jr, Boyd D, Mills GB. Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. *Oncogene*. 1999; 18(48):6635–6640. [PubMed: 10597268]
18. Shimamura A, Ballif BA, Richards SA, Blenis J. Rsk1 mediates a MEK-MAP kinase cell survival signal. *Curr Biol*. 2000; 10(3):127–135. [PubMed: 10679322]
19. Russo P, Catassi A, Cesario A, Servent D. Development of novel therapeutic strategies for lung cancer: targeting the cholinergic system. *Curr Med Chem*. 2006; 13(29):3493–3512. [PubMed: 17168719]
20. Haidar M, Echebli N, Ding Y, Kamau E, Langsley G. Transforming growth factor beta2 promotes transcription of COX2 and EP4, leading to a prostaglandin E2-driven autostimulatory loop that enhances virulence of *Theileria annulata*-transformed Macrophages. *Infect Immun*. 2015; 83(5):1869–1880. [PubMed: 25690101]
21. Guernon J, Dessauge F, Traincard F, Cayla X, Rebollo A, Bost PE, Langsley G, Garcia A. A PKA survival pathway inhibited by DPT-PKI, a new specific cell permeable PKA inhibitor, is induced by *T. annulata* in parasitized B-lymphocytes. *Apoptosis*. 2006; 11(8):1263–1273. [PubMed: 16761111]
22. Wilson JE. Hexokinases. *Rev Physiol, Biochem Pharmacol*. 1995; 126:65–198. [PubMed: 7886381]
23. Wolf AJ, Reyes CN, Liang W, Becker C, Shimada K, Wheeler ML, Cho HC, Popescu NI, Coggeshall KM, Arditi M, et al. Hexokinase is an innate immune receptor for the detection of bacterial peptidoglycan. *Cell*. 2016; 166:624–636. [PubMed: 27374331]
24. Sverdllov AL, Elezaby A, Behring JB, Bachschmid MM, Luptak I, Tu VH, Siwik DA, Miller EJ, Liesa M, Shirihai OS, et al. High fat, high sucrose diet causes cardiac mitochondrial dysfunction due in part to oxidative post-translational modification of mitochondrial complex II. *J Mol Cell Cardiol*. 2015; 78:165–173. [PubMed: 25109264]
25. Metheni M, Lombes A, Bouillaud F, Batteux F, Langsley G. HIF-1alpha induction, proliferation and glycolysis of *Theileria*-infected leukocytes. *Cell Microbiol*. 2015; 17(4):467–472. [PubMed: 25620534]
26. Medjkane S, Weitzman JB. A reversible Warburg effect is induced by *Theileria* parasites to transform host leukocytes. *Cell Cycle*. 2013; 12(14):2167–2168. [PubMed: 23803730]
27. Durrani Z, Weir W, Pillai S, Kinnaird J, Shiels B. Modulation of activation-associated host cell gene expression by the apicomplexan parasite. *Theileria annulata* *Cell Microbiol*. 2012; 14(9):1434–1454. [PubMed: 22533473]
28. Medjkane S, Perichon M, Marsolier J, Dairou J, Weitzman JB. *Theileria* induces oxidative stress and HIF1alpha activation that are essential for host leukocyte transformation. *Oncogene*. 2014; 33(14):1809–1817. [PubMed: 23665677]
29. Gimenez-Cassina A, Garcia-Haro L, Choi CS, Osundiji MA, Lane EA, Huang H, Yildirim MA, Szlyk B, Fisher JK, Polak K, et al. Regulation of hepatic energy metabolism and gluconeogenesis by BAD. *Cell Metab*. 2014; 19(2):272–284. [PubMed: 24506868]
30. Ljubicic S, Polak K, Fu A, Wiwczar J, Szlyk B, Chang Y, Alvarez-Perez JC, Bird GH, Walensky LD, Garcia-Ocana A, et al. Phospho-BAD BH3 mimicry protects beta cells and restores functional beta cell mass in diabetes. *Cell Rep*. 2015; 10(4):497–504. [PubMed: 25640178]
31. Wilderman A, Guo Y, Divakaruni AS, Perkins G, Zhang L, Murphy AN, Taylor SS, Insel PA. Proteomic and metabolic analyses of S49 lymphoma cells reveal novel regulation of mitochondria by cAMP and protein kinase A. *J Biol Chem*. 2015; 290(36):22274–22286. [PubMed: 26203188]
32. Singh S, Khatri N, Manuja A, Sharma RD, Malhotra DV, Nichani AK. Impact of field vaccination with a *Theileria annulata* schizont cell culture vaccine on the epidemiology of tropical theileriosis. *Vet Parasitol*. 2001; 101(2):91–100. [PubMed: 11587838]
33. Theilen GH, Rush JD, Nelson-Rees WA, Dungworth DL, Munn RJ, Switzer JW. Bovine leukemia: establishment and morphologic characterization of continuous cell suspension culture, BL-1. *J Natl Cancer Inst*. 1968; 40(4):737–749. [PubMed: 4171552]

34. Moreau MF, Thibaud JL, Miled LB, Chaussepied M, Baumgartner M, Davis WC, Minoprio P, Langsley G. *Theileria annulata* in CD5(+) macrophages and B1 B cells. *Infect Immunity*. 1999; 67(12):6678–6682. [PubMed: 10569790]
35. Kennedy EJ, Scott JD. Selective disruption of the AKAP signaling complexes. *Methods Mol Biol*. 2015; 1294:137–150. [PubMed: 25783883]

Figure 1.

HK2 and BAD reside in a complex. (A) western blot analysis using specific anti-phospho-BAD S155, anti-BAD, and anti-actin antibodies and transformed macrophages treated or not with S155A and S155D peptides. Treatment with the S155A peptide induced a 50% reduction in the phospho-BAD S155 signal. (B) Immunoprecipitation analyses with anti-HK2 and anti-BAD antibodies using whole cell lysates derived from *Theileria*-transformed macrophages (M) treated or not with the penetrating synthetic peptides (S155A and S155D). The left upper panel (IP-HK2) shows western blot of the HK2 precipitate probed with an anti-BAD antibody, and the upper right panel (IP-BAD) shows western blot of the BAD precipitate probed with an anti-HK2 antibody. Ablation of BAD S155 phosphorylation by treatment with the S155A peptide diminished the formation of BAD/HK2 complexes. When BAD is precipitated, the amount of associated HK2 is nearly undetectable. The lower panel shows western blot analysis of total cell extracts used for both immunoprecipitations probed

with anti-HK2, anti-BAD, and anti-actin antibodies, the latter used as a loading control. (C) Cells were treated with 10 μ M MG132 and with either S155A or S155D peptides. Whole cell lysates were subjected to western blot analysis. Treatment with MG132 protected HK2 from degradation and markedly promoted the accumulation of ubiquitinated HK2 only in transformed macrophages treated with the S155A peptide. The decreased amounts of HK2 detected by western blot are therefore due to ubiquitin-mediated proteasome degradation of HK2.

Figure 2.

Subcellular localization of BAD and HK2. Immunofluorescence images were obtained with anti-HK2 and anti-BAD antibodies, and transformed macrophages were left untreated or treated with phosphoresistant (S155A) or phosphomimic (S155D) peptides. (A) Treatment with peptide S155A ablated HK2 staining (green). (Right) HK2/BAD co-localization was analyzed by Manders method of pixel intensity correlation measurements using ImageJ/Fiji-Coloc2 plugin. (B) Mitochondrial localization as shown by SHDA-1 red staining and that of HK2 (green) is lost when BAD phosphorylation is ablated following treatment with the S155A peptide. (Right) HK2/SHDA-1 co-localization was analyzed by using the Manders method of pixel intensity correlation measurements using ImageJ/Fiji-Coloc2 plugin, and an average for three independent cells is given. DNA was stained with DAPI (blue). Bars represent 10 μ M. The white arrow in panel A (top left) indicates parasite nuclei.

Figure 3.

HK2 degradation provokes a switch from Warburg to oxidative glycolysis (A) *Theileria*-transformed leukocytes were treated with the S155A and S155D peptides, and lactate output was measured by ELISA. Only S155A treatment diminished lactate output. (Left) *, $p < 0.05$ compared to untreated macrophages (M). (Right) #, $p < 0.05$ compared to BL20; §, $p < 0.05$ compared to *T. annulata*-infected BL20 (TBL20). (B) (Left) Oxidative measurements of lactate output (ECAR) and oxygen consumption (OCR) by *Theileria*-transformed macrophages. *, $p < 0.05$ compared to untreated macrophages (M). (Right) S155A peptide treatment caused an increased mitochondrial reserve respiratory capacity (RRC). (C) HK1 levels compared to actin following peptide treatments of transformed macrophages showing no obvious change in HK1 levels.

Figure 4.

Phosphorylation of BAD S155 affects proliferation of *T. annulata*-transformed leukocytes. *T. annulata* infection of immortalized B cells (BL20) increases their proliferation (compare TBL20 to BL20) when growing on glucose (10 mM). Inhibition of BAD S155 phosphorylation following treatment with the phosphoresistant peptide S155A diminishes the number of cells of only TBL20 cells and transformed macrophages (M), whereas no effect was observed with the BAD phosphomimic peptide S155D. *, $p < 0.05$ compared to TBL20. #, $p < 0.05$ compared to M. Cell viability was $>85\%$ (not shown). The error bars show SEM values from three biological replicates.

Figure 5.

Type of glycolysis affects proliferation of *Theileria*-infected macrophages. The proliferation of *Theileria*-transformed macrophages represented by the number of cells appears slower in media supplemented with galactose, as opposed to glucose (compare left-and right-hand panels). In addition, when galactose (10 mM) is the provided sugar, the dependency on hexokinase activity is bypassed and no effect on proliferation was observed following treatment with BAD peptides compared to cells cultivated in glucose (10 mM). *, $p < 0.05$ compared to untreated macrophages (M). Cell viability was >90% (not shown). Error bars show SEM values from three biological replicates.