Role of AMPK in Herpes Simplex Virus Type 1 Replication in Endothelial Cells

Dissertation

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SUMMARY

After successful invasion of susceptible hosts, systemic distribution of viral pathogens often requires the interaction of viruses with cells of the vascular system, of which endothelial cells are an indispensable part. Among the viruses that could interact with endothelial cells in order to reach target organs or establish infection, is the herpes simplex virus type 1 (HSV-1). HSV-1 is a highly contagious ubiquitous pathogen belonging to the *Herpesviridae* family. In addition to 'cold sores', this pathogen may also play an important role in the development of Alzheimer's disease, severe septic processes as well as atherosclerosis. The latter represents a major global health problem leading to life-threatening diseases like stroke and myocardial infarction. Currently, no virus-specific vaccines are available to prevent HSV-1 infections. Hence, the identification of host cell proteins, which are involved in HSV-1 replication and the modulation of their activities, might be a promising strategy for future antiviral therapies. Among others, targets in host cell metabolism, which is essentially required for virus replication are of major interest. The current thesis addresses the question of whether and how one such candidate, the AMP-activated protein kinase (AMPK) known to be a master regulator of various metabolic pathways, affects HSV-1 replication in endothelial cells. To this end, human umbilical vein endothelial cells were infected with HSV-1 and alterations caused by this infection including various signaling pathways, autophagy and inflammatory and antiviral responses were examined as compared to control cells. Furthermore, we studied the role of AMPK and downstream targets using pharmacological tools and the RNAi technology.

Our data confirms that HSV-1 replicates in endothelial cells. HSV-1 infection led to alteration of several major signaling pathways, i.e., activation of the Protein Kinase B (PKB/Akt) Akt/mechanistic target of rapamycin complex 1 (mTORC1) pathway and stress kinases; c-Jun N-terminal kinases (JNK) and inhibition of extracellular signal-regulated kinase (ERK). We show that the Akt/mTORC1 pathway is essentially involved in HSV-1 replication and that HSV-1 replication is controlled by AMPK even though AMPK signaling was not changed by infection. An increase in viral concentration was observed in AMPK knockdown and knockout cells and, on the other hand, a decrease in viral concentrations was seen in endothelial cells treated with AMPK activators. AMPK could protect endothelial cells against HSV-1 replication via mTORC1 inhibition, since AMPK activators led to decreased phosphorylation of p70 ribosomal S6 kinase (p70S6K), a downstream target of mTORC1. In addition, AMPK

activators inhibited acetate incorporation into endothelial cells suggesting that inhibition of fatty acid or cholesterol synthesis, which are known to be important for viral replication, could be a mechanism for the antiviral role of AMPK. In line with this, downregulation of acetyl CoA carboxylase 1 (ACC1), a key enzyme in fatty acid biosynthesis, prevented HSV-1 replication in endothelial cells. We could further show that proteins involved in early autophagy play a protective role against HSV-1 replication. However, this effect was not a consequence of AMPK and was not mediated by autophagy. In contrast, autophagy may even exhibit a pro-viral role.

Collectively, our data indicates a protective role of AMPK against HSV-1 replication in endothelial cells via inhibition of mTORC1 and lipid synthesis. We provide a better insight into HSV-1 infection of the endothelium along with demonstrating a complex interaction of HSV-1 with the autophagy pathway and revealing heightened defense mechanisms of endothelial cells to HSV-1 infection. This data could pave way for developing antiviral strategies using AMPK, a host cell protein that, according to the current global situation is a need of the hour.

ZUSAMMENFASSUNG

Nach Virusinfektionen setzt die lokale oder systemische Verteilung von Viren häufig eine Interaktion mit vaskulären Endothelzellen voraus. Zu den Viren, die mit Endothelzellen interagieren, gehört auch das herpes simplex virus Typ 1 (HSV-1). HSV-1 ist ein hoch ansteckendes ubiquitär vorkommendes Virus aus der Familie der Herpesviridae. Es verursacht primär sogenannte Herpesbläschen, spielt aber auch eine Rolle bei Alzheimer-Erkrankung, schwerer Sepsis oder Atherosklerose. Diese Erkrankungen stellen globale Gesundheitsprobleme dar, beispielsweise ist Atherosklerose die Ursache für lebensbedrohliche Erkrankungen wie Schlaganfall oder Myokardinfarkt. Gegenwärtig stehen keine Impfstoffe gegen HSV-1-Infektionen zur Verfügung. Um die HSV-1-Therapien zu verbessern, werden auch Proteine der Wirtszellen als vielversprechende pharmakologische Targets untersucht und Strategien zu deren Modulierung erprobt. Dabei sind unter anderem auch Proteine, die den Metabolismus der Wirtszellen regulieren, von Bedeutung, da diese für die Vermehrung der Viren essentiell sind. Die vorliegende Arbeit untersucht, ob und wie die AMP-aktivierte Proteinkinase (AMPK), ein Masterregulator des zellulären Metabolismus, die Replikation des HSV-1 in vaskulären Endothelzellen beeinflussen kann. Dazu wurden Endothelzellen, die aus humanen Nabelschnurvenen oder aus der Lunge von Mäusen gewonnen wurden, mit HSV-1 infiziert. Die dadurch ausgelösten Veränderungen von intrazellulären Signalwegen, Autophagie sowie inflammatorischen und antiviralen Antworten wurden untersucht und mit denen in nicht-infizierten Kontrollzellen verglichen. Um die Rolle der AMPK zu klären, wurden pharmakologische AMPK-Aktivatoren und AMPK-spezifische siRNA zur Downregulation der Kinase eingesetzt.

Unsere Daten bestätigen, dass sich HSV-1 in Endothelzellen replizieren kann. Nach Infektion mit HSV-1 sind Veränderungen verschiedener wichtiger Signalwege der Zelle nachweisbar, zum Beispiel die Aktivierung des Proteinkinase Akt/mechanistic target of rapamycin complex 1 (mTORC1) -Signalweges, die Stimulierung von Stresskinasen und die Hemmung der extracellular signal-regulated kinase 1 (ERK1). Wir zeigen, dass der Akt/mTORC1-Weg eine essentielle Rolle in der HSV-1-Replikation spielt. Die Virusreplikation wird auch durch AMPK kontrolliert, obwohl die HSV-1-infektion der Zellen den AMPK-Signalweg selbst nicht verändert. Die Downregulation der katalytischen Untereinheiten der AMPK führt zur einer erhöhten Virusreplikation, während pharmakologischen Aktivatoren deren Hemmung bewirken. Wir zeigen, dass

die protektive Wirkung der AMPK auf verschiedenen Mechanismen beruht. Dazu gehören die Hemmung des mTORC1-Weges, was anhand einer verminderten Phosphorylierung der ribosomalen S6-Kinase, eines mTORC1-Targets, nachgewiesen wurde. Andere Wirkmechanismen betreffen metabolische Prozesse. AMPK-Aktivatoren vermindern den Einbau von radioaktiv markiertem Acetat in die neutrale Lipidfraktion der Zellen, was auf eine Hemmung von Fettsäure- und/oder Cholesterolsynthese schließen lässt. Beide stellen für die Virusreplikation essentielle Prozesse dar. Das zeigt sich in einer Verminderung der HSV-1-Replikation, wenn Acetyl-CoA-Carboxylase 1 (ACC1), das Schlüsselenzyme der Fettsäuresynthese, durch siRNA herunterreguliert oder die Hydroxymethylglutaryl-CoA-Reduktase (HMGCR), das Schlüsselenzyme der Cholesterolsynthese, durch Simvastatin gehemmt wird. Zusätzlich zur protektiven Rolle der AMPK zeigen wir, dass die Proteine ULK1 und Beclin1, die in die Initiierung der Autophagie einbezogen sind und durch AMPK aktiviert werden können, ebenfalls vor HSV-1 schützen. Die protektive Wirkung dieser Proteine ist jedoch unabhängig von AMPK und wird nicht über Autophagie vermittelt. Autophagie scheint im Gegenteil die Virusreplikation zu unterstützen.

Zusammenfassend ist festzustellen, dass AMPK vor HSV-1-Infektion von Endothelzellen schützt und dies auf eine Hemmung von mTORC1 und eine Verminderung der Lipidsynthese zurückzuführen ist. Die hier vorgestellten Daten erweitern zudem die Kenntnisse über die Replikation von HSV-1 in Endothelzellen und die Bedeutung der Autophagie in diesem Prozess. Sie öffnen den Weg für die Entwicklung neuer antiviraler Strategien durch Aktivierung der AMPK in Wirtszellen, der möglicherweise nicht nur für hSV-1 sondern auch für die Behandlung weiterer Viruserkrankungen genutzt werden kann.

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1. INTRODUCTION

1.1 Introduction to viruses

The origin and the development of the concept of viruses dates back to the 19th century. Foot-and-mouth disease virus was the first ever animal virus to be discovered causing diseases in farm animals [1]. From the 19th century when scientists were only beginning to understand the new entity, to the current novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causing the global pandemic in 2020 the study and knowledge of viruses has exploded in an incredible manner.

Essentially, viruses are submicroscopic infectious pathogens, which can replicate only inside living cells. They are capable of infecting a broad range of hosts from eukaryotes like humans, animals and plants to even prokaryotes like bacteria and archaea. The size of viruses in general ranges from as small as 20 nm up to 200-300 nm [2]. Every virus particle (virion) consists of a core containing genetic material and a protein coat, the capsid, which depending on the type of virus can be either enveloped or not. The genetic material codes for essential proteins important for virus replication in the host cell. Viruses therefore completely rely on the host's metabolic and genetic functions [3].

The interaction between virus and host cell, the infection of cells and the replication cycle of viruses are unique to the type of virus. Not every virus is capable of causing diseases in the host and some viruses can also live in symbiosis with the host. The ability of a virus to infect and cause a disease in the host cell is referred to as viral pathogenesis [4].

1.1.1 Viral taxonomy

Given the abundance of viruses in nature and the huge amount of information available, a virus classification system is of utmost importance. Biologists have used several classification systems in the past, based on the morphology (icosahedral, helical), the type of host they infect (plant, animal, bacteria), the presence or absence of an envelope or the disease they cause in the host [5]. Two important classification systems are widely used, the International Committee on Taxonomy of Viruses [ICTV] classification, which has been adapted from the Lwoff, Horne, and Tournier (the LHT system) and sorts viruses based on the family, genus and species method [6] and the most commonly used Baltimore system, proposed by David Baltimore, categorizes

virions in 7 classes based on the type of genetic material present (e.g., single stranded RNA, double stranded DNA, etc.) [7].

1.1.2 Medical relevance

The ever developing field of virology has led to a better understanding of numerous viruses and its effects on the living world, but, due to the constant evolving nature of viruses and their ability to adapt to a variety of hosts, our grasp on this subject is still limited. There are thousands of viruses known to researchers and new viruses are being discovered frequently. Many viruses are environmentally and genetically important and play a role in evolution. Viruses are also being used for vaccine developments. Of note, some viruses are responsible for deadly human diseases like acquired immune deficiency syndrome (AIDS), Influenza, Ebola, etc. and with the latest Covid-19 pandemic it is becoming more and more clear, how important it is to understand viral diseases, to widen our knowledge and come up with effective antiviral strategies [8].

1.2 Herpes simplex virus type 1 (HSV-1)

HSV-1 is an extremely contagious and ubiquitous pathogen, which belongs to the *Herpesviridae* family (the Greek word *herpein* means “to creep”), and the human alpha (α)-herpesviruses subfamily. Besides HSV-1, this subfamily includes herpes simplex virus type 2 (HSV-2) and the varicella zoster virus (VZV). HSV-1 is a double-stranded DNA virus [9]. According to WHO, an estimated 67% of the world population or around 3.7 billion people below the age of 50 were infected with HSV-1 in 2016, making it a very common and endemic pathogen. The main route of HSV-1 transmission is direct contact with an affected individual. The virus is also known to be transmitted to newborn infants by infected mothers. Most HSV-1 infections are asymptomatic or show mild symptoms like blisters and open sores, more commonly known as “cold sores” but sometimes infections can also lead to severe complications or even life-threatening consequences like encephalitis, meningitis, and blindness in newborns or immunocompromised individuals [10,11]. The HSV subtypes type 1 and 2 share numerous epitopes but mostly differ in their transmission route. HSV-1 is mainly transmitted by oral contact to cause infection in or around the mouth and HSV-2 is transmitted through genital contact during sex, causing infection in the genital or anal

area. However, in some cases HSV-1 can also cause genital herpes and HSV-2 oral herpes [12].

One of the most striking characteristics of HSV-1 and (α)-herpesviruses is that after the primary infection usually occurring in childhood most commonly in the oral mucosa, the virus is capable of travelling to the neurons and establishing lifelong latency in the ganglia of sensory neurons. This latent virus can reactivate multiple times in an infected individual and cause recurrent disease, which varies in severity and its transmissibility between individuals [13].

1.2.1 HSV-1 structure

The HSV-1 virion is enveloped, spherical and its size ranges from 150-200 nm in diameter. As shown in figure 1, it comprises of the core containing the genetic material, the double-stranded linear DNA.

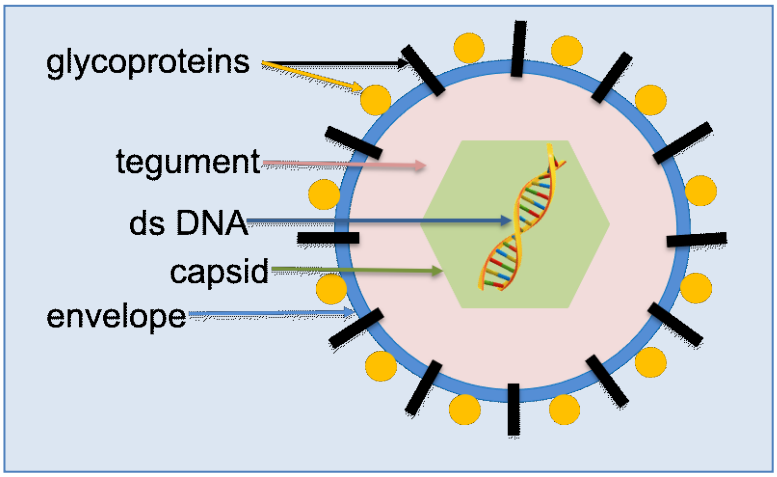


Figure 1: The HSV-1 virion
The HSV-1 virion consists of double-stranded DNA (ds DNA) enclosed in an icosahedral capsid surrounded by a proteinaceous layer called tegument. The virion is enveloped by means of a lipid bilayer with various membrane proteins (glycoproteins) embedded in it (adapted from viralzone.expasy.org [7]).

This viral DNA is surrounded by a complex icosahedral capsid or nucleocapsid consisting of 162 capsomeres and six viral proteins (VPs). The capsid is embedded in a proteinaceous layer called the tegument, which consists of about 22 VPs important for regulating the viral replication cycle. A lipid bilayer called the envelope surrounds the tegument and anchors about 16 membrane proteins of which 12 were identified to be glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and gN). These proteins are important in mediating the entry of the HSV-1 virion in the host cells [9,10,14,15].

1.2.2 HSV-1 genome

The double-stranded DNA constituting the HSV-1 genome can range from 150 to 250 kbp in size and could have a guanine-cytosine (GC) content of 60-75%. The genome as shown in figure 2, encodes for at least 84 protein-coding open reading frames (ORF), the long non-coding RNA latency-associated transcript (LAT) and numerous miRNAs. Each viral transcript encodes a single protein and does not contain introns [16]. HSV-1 contains three origins of replication (Ori) within the genome: two copies of Ori_S located in the short arm and one copy of Ori_L in the long arm of the genome [17].

During the course of HSV-1 replication in host cells, the viral genes are expressed in a cascade manner, one gene turning on and regulating the next. Accordingly, they are classified into three classes: α or immediate-early (IE) genes, β or early (E) and γ or late (L) genes [18].

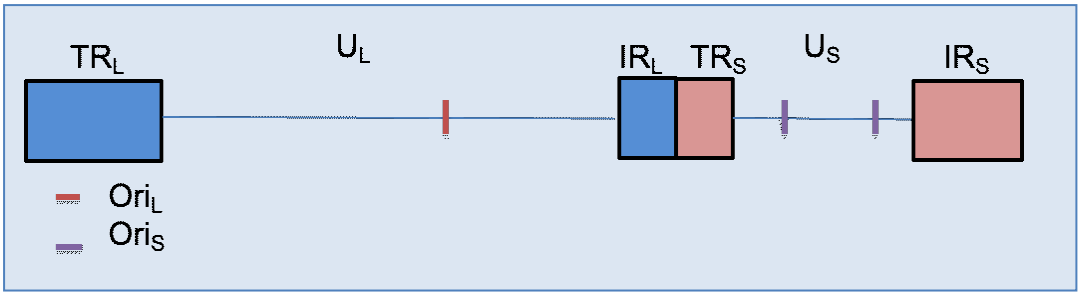


Figure 2: The HSV-1 genome
The HSV-1 genome is about 150-250 kbp long. It consists of a long (L) and short (S) component with unique sequences (U_L and U_S). Each unique sequence comprises of inverted repeats (TR_L and IR_L, TR_S and IR_S). Three origins of replication exist, one in the long arm called Ori_L and two in the short arm called Ori_S. The genome encodes for various protein-coding open reading frames (ORF), the long non-coding RNA latency-associated transcript (LAT) and numerous miRNAs (adapted from viralzone.expasy.org [7]).

1.2.3 HSV-1 replication and infection cycle

The HSV-1 lytic or productive life cycle as shown in figure 3 involves several key steps: entry of the virion into the host cell, viral gene expression, viral DNA replication, virion assembly and egress of progeny virions.

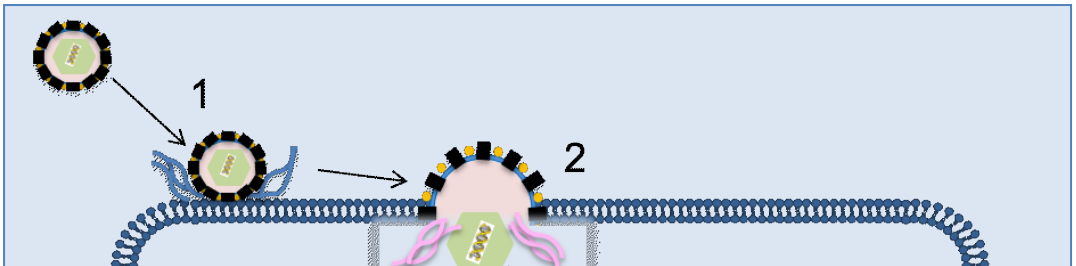


Figure 3: The HSV-1 replication cycle
The HSV-1 replication cycle comprises of the following steps: 1. attachment and adsorption on the cell surface 2. Fusion with the cell membrane 3. Entry into the cell 4. The capsid and genome enter the nucleus for transcription of genes and replication 5,8. Translation of proteins necessary for replication and envelope production takes place in the cytosol and is shuttled to the nucleus. 6,7. Viral genome is made in several copies and packaged into capsids. 9. Capsid containing genome is packaged into the envelope and 10. egressed out of the cell (adapted from viralzone.expasy.org [7]).

1.2.3.1 Virion attachment and entry into the cell

The entry of HSV-1 into the host cell can take place by either fusion of the viral envelope to the host cell membrane or via endocytosis of the virion and fusion with the intracellular vesicles. The process of entry of the virion is dependent on the cell type; however, fusion of the viral envelope mediated by the interaction of the viral surface glycoproteins with host cell surface receptors is a vital step in the infection cycle [19]. Glycoproteins gB and gC interact with the heparan sulfate moieties, which are proteoglycans receptors on host cell surfaces, and initiate virus attachment. Glycoprotein gB is imperatively required for penetration while gC, although not essential, contributes to adsorption and penetration of the virus into cells [20]. Glycoprotein gB can also bind to immunoglobulin-like type 2 receptor on the cell surface and glycoprotein gD interacts with various cellular receptors, which are called herpes virus entry (Hve) proteins e.g., Nectin 1 and 2, herpes virus entry mediator (HVEM) and 3-*O*-sulfated heparan sulfate (3-*O*-S-HS). After binding to any of the

above receptors, gD activates gB and gH/gL heterodimer, which allows fusion of the viral envelope to the cell membrane and, finally, virus entry [21].

1.2.3.2 Viral gene expression

Upon virus entry, viral capsid and tegument proteins are released into the host cell cytoplasm. The exposed tegument proteins (e.g., US3, UL37, etc.) interact with host transport machinery (dynein, dynactin, kinesin 1) and pave the way for the viral genome to reach the cell nucleus [22]. In addition, various other tegument proteins such as the virion host shutoff (VHS) RNase, VP16 and UL47 are shuttled to the nucleus [23,24]. Once the capsid reaches the cell nucleus membrane, tegument proteins, VP1/2 and UL37 are thought to interact with the host nuclear pore complex (NPC) which mediate transport in and from the nucleus. The viral genome is then released into the nucleus, and this is called uncoating [25].

The entry of the viral genome causes a series of changes in the host cell. Immune responses are activated from the host cell (see 1.6.4) whereas the virus tries to block the host cellular pathways to increase its replication efficiency. If the virus overcomes the host response, the next step of viral gene expression begins. Viral mRNA is synthesized via the host RNA polymerase II. Viral proteins lead to the sequential expression of IE, E and L genes [24]. VP16 is the most crucial protein required for the expression of IE genes consisting of Infected Cell Protein (ICP) genes, mostly important in blocking host antiviral response. IE genes in turn activate expression of E genes, which are involved in viral DNA replication. Thereafter, the L genes are expressed and encode structural viral proteins important in assembly of new virions [18].

1.2.3.3 Viral DNA replication

As mentioned above, once the E genes are expressed, viral DNA replication begins in the nucleus and can start at any one of the three origins of replications. In the first step, UL9 and ICP8, which are DNA binding proteins, bind to the AT-rich regions and distort the origin of the double-stranded DNA causing a conformational change which prevents re-annealing of the DNA strands. Then, the helicase-primase complex consisting of UL5, UL8, and UL52 unwinds the double-stranded DNA and synthesizes short RNA primers. This initiates replication. Next the polymerase complex consisting

of viral DNA polymerase (Pol) and UL42 (processivity subunit) are recruited to the replication fork and synthesize the leading and lagging DNA strand [26].

1.2.3.4 Virion assembly and release

Once the capsid proteins (VP5, VP19, etc.) are translated in the cytosol, they are transported back to the nucleus for packaging. The empty capsids are assembled in the nucleus and then the viral proteins (e.g., UL6, UL15, etc.) help in packing the viral DNA in the empty capsids [27]. After capsid assembly and DNA packaging is complete, the virus particles move out of the nucleus generally by fusion of the capsid to the nuclear membrane. It matures in the cytoplasm forming the tegument and envelope with the help of cytoplasmic organelles of the cell [28]. The complete virus particles or virions then 'egress' or bud out of the cell generally via exocytosis also known as viral shedding, where the UL20 protein seems to be of importance. These virions will in turn to infect other host cells [29].

1.2.3.5 Viral Latency

As stated earlier, HSV-1 possesses a remarkable feature of producing lifelong latent infections in individuals and can lead to productive replication by reactivation. The sensory neurons of the trigeminal ganglia (TG) are the main site of latent infection [13], although certain other sites are documented. Although not clearly understood, inhibition of VP16, important for IE genes expression, may lead to the latent infection cycle and a virion devoid of the tegument and envelope may be transported to the neurons. During latency, the HSV-1 genome is maintained in a circularized form and viral transcription is limited to expressing LAT, which is processed into a stable intron and miRNAs. The function of LAT is not well known, it is thought to help maintain latency and to prevent the infected neurons from cell death. The miRNAs are shown to maintain no or little expression of the viral lytic genes [30].

HSV-1 reactivation can be caused by various triggers such as stress, low immunity, UV exposure, trauma and hormonal changes. To progress into an active lytic cycle the virus needs to overcome the expression of LAT and miRNAs and increase the expression of proteins needed for producing infectious virions, of which ICP0 seems to be important [31].

1.2.4 Current anti-HSV-1 treatments

Existing treatments against herpes infection include several antiviral drugs. The widely used drugs are acyclovir, penciclovir and famciclovir, which represent first-line treatments. These compounds are nucleic acid analogs and interfere with the viral DNA polymerase, which in turn interrupts viral DNA replication. However, to be recognized by the viral DNA polymerase, the drugs need to be activated intracellularly via phosphorylation by viral thymidine kinases (TK, UL23 gene) or sometimes also by cellular kinases. Since these drugs target the viral genome replication, they clearly are only active against only productive infections and reduce the number of new virions in the infected cell. A major drawback of these compounds is the generation of mutations in the viral TK thus rendering the drugs ineffective. This is most commonly seen in immunocompromised individuals. The use of acyclovir, penciclovir and famciclovir is also limited in children and pregnant women [32].

The second-line treatment against HSV-1 infections includes drugs like foscarnet and ganciclovir, which do not need to be activated by viral genes. However, numerous side effects have been reported by their usage thus making them unfavorable for antiviral therapy [33]. Current drugs being investigated in clinical trials include brincidofovir, amenamevir, pritelivir, nelfinavir mesylate and other botanical compounds. Brincidofovir is also a nucleic acid analog but conjugated to a lipid having higher retention in the cell. Amenamevir and pritelivir act as inhibitors of the viral helicase-primase and nelfinavir mesylate, a protease inhibitor, inhibits the maturation and export of viral particles [34].

Even though considerable research has been carried out and various antiviral drugs are currently being studied in clinical trials, there is still no licensed virus-specific vaccine available to prevent and treat herpes infections (active and latent) making it more important to develop new antiviral strategies causing lesser side effects in the infected individuals. One important field of study could be to target host cell proteins as an effective means of antiviral strategy.

1.2.5 HSV-1 and cellular signaling interactions

The interaction between the host cell and the infecting virion particle is extremely tight knit and multilayered, seen clearly based on its replication cycle. For efficient infection

and replication, the virus modulates the host cell signaling pathways in its favor. The host cell in return generates an obvious antiviral response by increasing production of cytokines and chemokines. There is constant pressure on both the pathogen and the host to combat and evolve against one another.

HSV-1 is known to interact with various cellular signaling pathways. One, is the phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) pathway, which is involved in regulation of various cellular processes by activating downstream targets and plays an important role in cell cycle, growth and proliferation [35,36]. Others are the mechanistic target of rapamycin (mTOR) pathway, important in protein translation [37], etc. as well as diverse host cell proteins for effective replication in host cells. In baby hamster kidney and HeLa cells, HSV-1 activates the p38 mitogen-activated protein kinase (p38MAPK)-dependent pathway, important in stress-related response, and prevents destabilization of B-cell lymphoma 2 (Bcl-2) thereby leading to inhibition of apoptosis [38]. HSV-1 also seems to interact with the autophagy pathway and maneuvers it in ways, which vary from cell to cell type [39,40]. The interaction of HSV-1 with cellular signaling pathways affects the host cell and its responses. Some interactions are crucial for efficient viral replication. Targeting these interactions could help in inhibiting HSV-1 replication in the cells and may represent effective antiviral therapies.

For instance, heat shock protein 90 inhibitor AT-533 has been shown to block HSV-1 egress form the nucleus and assembly of the virion in Vero cells [41]. BX-795, an inhibitor of phosphoinositide-dependent kinase-1 (PDK1) by blocking PDK1/Akt/mTOR signaling, inhibits HSV-1 replication by blocking JNK/p38 pathway in HEC-293T, HeLa, Vero and HEC-1-A cells [42]. Therefore, identification and understanding such host cell factors and their interaction with HSV-1 are of importance. Most of these interactions seem dependent on cell type, thus, recognizing proteins which play a role across various cell types would be of advantage.

1.3 Endothelium

The endothelium comprises a single layer of squamous cells called endothelial cells (ECs). This monolayer lines the inner wall of the entire vascular system and forms a barrier between the vessel wall and circulating blood. The endothelium barrier is

semipermeable and regulates transfer of small and large molecules to the underlying tissues.

ECs maintain vascular homeostasis through a range of metabolic and synthetic functions like control of vascular tone, blood flow and blood pressure, synthesis or release of vasoactive substances, angiogenesis, inflammation and immune responses. ECs have autocrine, paracrine and endocrine actions and are extremely dynamic and active in nature. Due to their numerous functions, the structural and functional integrity of the endothelium is of utmost importance. Endothelial dysfunction or injury can lead to a variety of pathological problems like atherosclerosis, allograft vasculopathy, hypertension, congestive heart failure, primary pulmonary hypertension, sepsis and inflammatory syndromes (figure 4) [43–45].

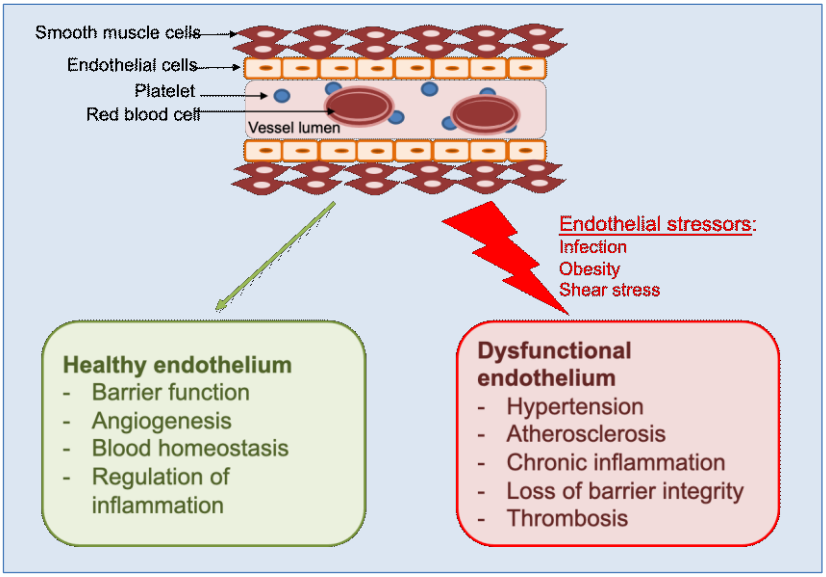


Figure 4: The endothelium and its functions
The endothelium consists of cells that line the inner wall of the entire vasculature. They serve a variety of functions. Upon stress, by various agents such as infections, the integrity of the endothelium is lost giving rise to several complications (adapted from R. Triggle et al; 2012 [46]).

During atherogenesis, for instance, functional alterations of the endothelium such as increased adhesivity and permeability allow the incorporation of lipids and inflammatory cells into the vessel wall resulting in the formation of atherosclerotic plaques or atheroma's. This restricts blood flow to vital organs in the body and causes life-threatening diseases such as coronary artery disease, myocardial infarction or stroke [47]. In addition to various risk factors like hypertension, diabetes mellitus, smoking, and a positive family history, studies have also shown that various infectious

pathogens play a role in the pathogenesis of endothelial dysfunction and atherosclerosis [48].

1.4 HSV-1 and the endothelium

After successful invasion of susceptible hosts, systemic distribution of viral pathogens often requires the interaction of viruses with cells of the vascular system, of which endothelial cells form an important part. Viruses (or virus-infected leukocytes) can migrate through the endothelial barrier or can directly infect endothelial cells via specific receptor molecules [49].

Even though the primary site of HSV-1 infection is the epithelium, the virus is known to interact with vascular endothelium, which also could be one of the latent herpetic infection sites. The reactivation of HSV-1 in the endothelium is thought to promote endothelial dysfunction and to aggravate atherosclerosis [50]. The role of HSV-1 as a risk factor for atherosclerosis was first discussed decades ago when Marek's disease herpesvirus, a chicken herpesvirus, led to atherosclerosis of large muscular arteries in an animal model, which closely resembled chronic atherosclerosis in human arteries [51]. Since then, several studies have strengthened this claim. A possible mechanism of HSV-1 causing atherosclerosis could involve adherence of monocytes to the infected endothelium with subsequent lipid accumulation in macrophages and vascular smooth muscle cells and increasing thrombin deposition in atherosclerotic plaques [11]. In addition to its possible role in the pathogenesis of atherosclerosis, HSV-1 infection may lead to other complications in the host via endothelium damage. Thus, understanding the mechanism of HSV-1 infection of endothelial cells could lead to a better control of disease in the host.

1.5 5’ Adenosine monophosphate (AMP)-activated protein kinase (AMPK)

AMPK is a heterotrimeric serine-threonine protein kinase. It acts as an energy sensor and a master regulator of cellular energy pathways. On discovery, the kinase was known only for its role in phosphorylation and inactivation of key enzymes involved in lipid biosynthesis; however, since then many targets of the protein have become evident. The kinase plays an important role in regulating metabolic enzymes, transcription and translation factors, proliferation and growth pathways, as well as

epigenetic processes. AMPK is implicated in the pathogenesis of various disorders including metabolic diseases, inflammation and infection [52,53].

1.5.1 Structure

The heterotrimeric complex of AMPK consists of three subunits. The catalytic α-subunit determines the protein kinase activity and is encoded by the protein kinase AMP-activated-α (PRKAA) gene. PRKAB and PRKAG genes encode the regulatory β- and the γ-subunits, respectively. The AMPK α- and β-subunits have two isoforms, and the γ-subunit has three isoforms. The combinations of these make up 12 different AMPK variants. As shown in figure 5, the two isoforms of the catalytic α-subunit have about 94% structural similarity and possess a serine/threonine kinase domain (KD) at the N-terminus, which determines the kinase activity. The KD consists of the “activation loop”, which is the primary site of AMPK activation and comprises a conserved threonine residue (T172), whose phosphorylation is important for AMPK activation. An autoinhibitory domain (AID), which suppresses the kinase activity of AMPK, an adenine nuclear sensor segment called α-linker and the C-terminal domain (α-CTD), which has the binding site for the β subunit, follow the KD. The β-subunit contains a glycogen-binding carbohydrate-binding module (CBM) at the N-terminus, which is myristoylated. The CBM is joined by a β-linker to the C-terminal domain (β-CTD), which interacts with α- and γ-subunits. The β-subunit represents the backbone of the AMPK structure. The three isoforms of the γ-subunit differ mostly in the lengths of the N-terminus. The N-terminal domain is followed by the β-subunit binding site and then by a conserved adenine nucleotide-binding domain interacting with ATP, ADP and AMP in a competitive manner. This binding domain is formed by two Bateman domains each containing two cystathionine β-synthase repeats (CBS), i.e., CBS 1 and 2 build Bateman 1 and CBS 3 and 4 Bateman 2, respectively. The ability of the γ-subunit to bind ATP, ADP and AMP confers AMPK with its ability to sense the energy state of the cell [52,54].

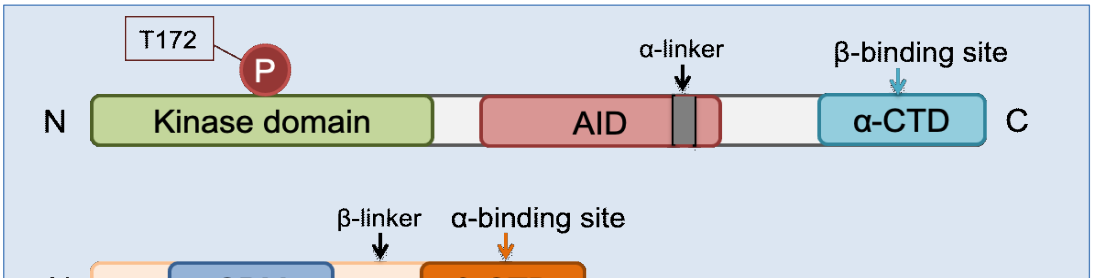


Figure 5: Structure of AMPK
The catalytic α-subunit consists of a kinase domain with the conserved threonine residue T172 (P), an AID and a binding domain for the β-subunit. The β-subunit, the backbone of AMPK, consists of the CBM and the α- and γ-subunit binding site. The γ-subunit consists of the β-subunit binding site and 2 Bateman regions, each formed by 2 CBS repeats. The Bateman domains of the γ-subunit are important for AMP/ATP binding and thus for the allosteric activation of the AMPK (adapted from Hardie, 2014 [53]).
AID: autoinhibitory domain, CTD: C-terminal domain, CBM: carbohydrate-binding module, CBS: cystathionine β synthase, NTD: N-terminal domain

1.5.2 Regulation

Being an important energy sensor, the activity of AMPK is tightly regulated by various mechanisms (figure 6). Firstly, under energy stress conditions, direct binding of AMP to the Bateman region of the γ-subunit causes allosteric activation of the kinase, which leads to about 10-fold increase of the activity [55]. In addition to AMP, ATP can increase AMPK activity by binding to the γ-subunit by a competitive mechanism; however, ATP has 5 times less binding affinity than AMP. This suggests that changes in the AMP/ATP ratio leads to an increase in the kinase activity rather than just an increase in the AMP concentration in the cell [56]. Secondly, in addition to direct allosteric activation, AMP binding promotes increased phosphorylation at the T172 residue of the catalytic subunit, which increases the kinase activity by about 100 fold [57]. The underlying mechanism is that the conformational change induced by AMP binding to the γ-subunit increases the affinity of the AMPK upstream kinase, liver kinase B1 (LKB1) and protects against Thr172 dephosphorylation by protein phosphatases (e.g., protein phosphatase 2C (PP2C)). LKB1, also known as serine/threonine kinase 11 (STK11), is a tumor suppressor and exists as a complex with STRAD (STE20-related adaptor) and CAB39/MO25 (mouse protein 25). The activity of the complex is thought to be 10-times higher than just mere LKB [58].

In addition to the AMP-dependent activation of AMPK, an AMP-independent activation can occur upon an increase of intracellular calcium ions (Ca^{2+}). Ca^{2+} stimulates the Ca^{2+}/calmodulin-activated protein kinase (CAMKK2/CAMKKβ), another upstream kinase of AMPK. Several agonists initiate an increase of intracellular Ca^{2+} via receptor coupling. In endothelial cells, these stimuli include thrombin, extracellular nucleotides, sphingosine-1-phosphate, bradykinin, ghrelin and vascular endothelial growth factor (VEGF) [59].

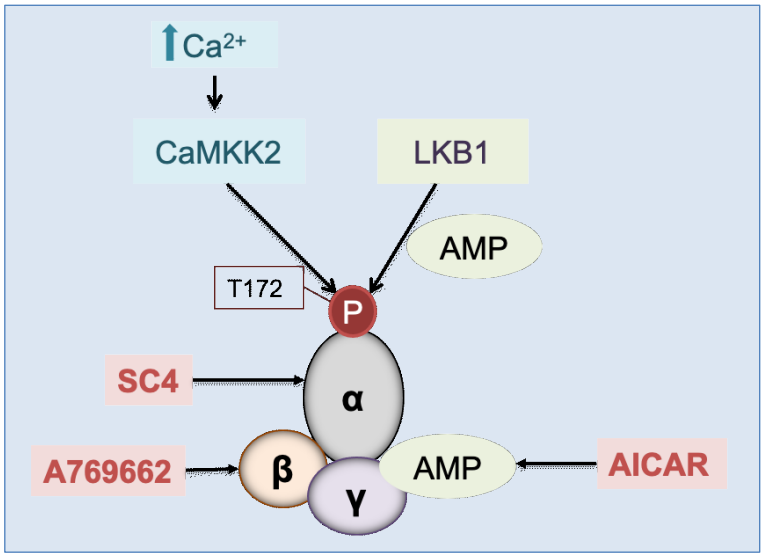


Figure 6: AMPK regulation
AMPK is allosterically activated by binding of AMP to the γ-subunit. Complete activation is achieved by phosphorylation at T172 in the α-subunit. The responsible kinases are LKB1, which acts in an AMP-dependent way, or CaMKK2 in response to an increase of intracellular calcium. The pharmacological activator AICAR is intracellularly converted into ZMP and works as an AMP mimic. A769662 and SC4 lead to allosteric activation by binding to distinct sites different from the AMP binding domain.
AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide, ZMP: AICAR 5'-monophosphate, AMPK: 5'AMP-activated protein kinase, CaMKK2: calcium/calmodulin dependent protein kinase kinase 2, LKB1: liver kinase B1

Various pharmacological AMPK activators have been developed. These are small molecule compounds, which mimic intracellular conditions or directly bind to AMPK and lead to a strong increase in the kinase activity. The compounds can be divided into three categories. One group of activators are compounds that inhibit cellular ATP synthesis (e.g., metformin, berberine, resveratrol). Pro-drugs, which act as AMP mimetics once inside the cells and lead to allosteric activation belong to the second category (e.g., AICAR which is converted to ZMP (AICAR 5'-monophosphate)). The third group involves allosteric activators, which bind to distinct sites in the AMPK complex other than the AMP binding site (e.g., A769662, which binds to the β1 subunit

and requires phosphorylation of the Ser108 residue [60], or SC4, which is thought to be a more potent activator of the α2-containing complexes [61]) [62]. In this project, A769662 (A76) and SC4 were extensively used.

1.5.3 Function

1.5.3.1 Metabolic functions

During energy stress conditions, AMPK phosphorylates key factors involved in various pathways. Broadly, activation of AMPK inhibits anabolic pathways consuming energy and stimulates catabolic pathways to increase energy production (figure 7) [63].

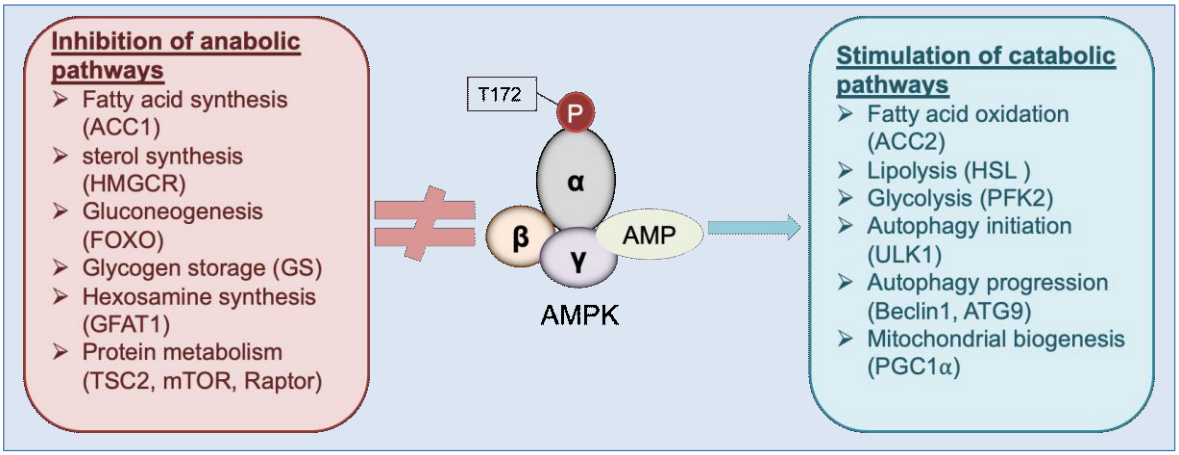


Figure 7: Metabolic regulation by AMPK
Upon activation, AMPK stimulates catabolic pathways and inhibits anabolic pathways by phosphorylating various intracellular proteins.
AMPK: 5'AMP-activated protein kinase, ACC1/2: acetyl-coenzyme A carboxylase 1/2, HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase, FOXO: fork head box protein O1, GS: glycogen synthase, GFAT1: glutamine fructose-6-phosphate amidotransferase-1, TSC2: tuberous sclerosis complex 2, mTOR: mechanistic target of rapamycin, HSL: hormone-sensitive lipase, PFK2: 6-phosphofructo-2-kinase, ULK1: Unc-51-like kinase 1, ATG9: Autophagy related gene 9, PGC1α: peroxisome proliferator activated receptor gamma coactivator 1α

AMPK was first discovered for its role in lipid metabolism. De novo fatty acid (FA), cholesterol and triglyceride (TG) synthesis are inhibited by AMPK, whereas FA uptake and β-oxidation are activated. Inhibition of FA synthesis is achieved by an inhibitory phosphorylation of ACC1, which mediates the rate-limiting step in FA synthesis, the conversion of acetyl-CoA to malonyl-CoA [64]. Cholesterol synthesis is inhibited by an inhibitory phosphorylation of the key enzyme 3-hydroxy-3-methylglutaryl-coenzyme A-reductase (HMGCR) [65]. The activation of FA catabolism is accomplished by an AMPK-mediated inhibitory phosphorylation of acetyl-CoA carboxylase 2 (ACC2), which in turn releases the malonyl-CoA-mediated inhibition of carnitine palmitoyltransferase-1 (CPT-1), the transporter of FAs to mitochondria for β-oxidation [64].

In addition, AMPK increases oxidative metabolism by increasing mitochondrial biogenesis via activation of the master regulator peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) that promotes transcription of nuclear-encoded mitochondrial genes via activation of sirtuin 1 (SIRT1) [66].
AMPK regulates glucose metabolism in certain tissues by stimulating the translocation of glucose transporters GLUT4 and GLUT1 to the plasma membrane and by stimulating glycolysis [67]. The latter is achieved by increasing hexokinase 2 expression and phosphorylating and activating 6-phosphofructo-2-kinase (PFK2) activity [68]. In addition, AMPK inhibits glycogen synthesis through inhibitory phosphorylation of glycogen synthase (GS) and activates glycogen breakdown by phosphorylating and activating glycogen phosphorylase (GP). Gluconeogenesis is inhibited by AMPK via suppressing the expression of gluconeogenic enzymes like phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) [69].
Protein synthesis, which demands a huge amount of energy in cells, is inhibited by AMPK. This occurs via inhibition of the mTORC1 via inhibitory phosphorylation of the upstream regulator tuberous sclerosis complex 2 (TSC2) and of rapamycin-sensitive adaptor protein of mTOR (Raptor), a component of the mTORC1 complex [70]. Via inhibition of mTORC1 but also via phosphorylation of Unc-51-like kinase 1 (ULK1), AMPK stimulates protein degradation through autophagy (see 1.6.2) [63].

1.5.3.2 Non-metabolic functions

In addition to metabolic functions, AMPK is able to counteract oxidative stress and inflammation. It regulates antioxidant defense by upregulating expression of genes like superoxide dismutase (SOD) and thioredoxin (TRX) via phosphorylation and activation of Fork head box protein O (FOXO), which, in turn, reduces Reactive oxygen species (ROS) levels in the cell [71]. The anti-inflammatory effects of AMPK are described below (1.6.4). Of note, AMPK is thought to play a role in antiviral defense by inhibiting different anabolic pathways required for virus replication. So far, this has been observed for human immunodeficiency virus (HIV), hepatitis C virus (HCV) and human cytomegalovirus (HCMV) [72].

1.5.3.3 Functions in endothelial cells

In endothelial cells, AMPK exhibits a metabolic function, although this is still poorly characterized, and plays an important protective role. It has been described that ACC1 and fatty acid synthesis are inhibited by AMPK in endothelial cells and fatty acid oxidation is enhanced [73,74].

AMPK has been shown to activate endothelial nitric oxide synthase (eNOS) and to increase nitric oxide (NO) production, although conflicting data exist. AMPK exhibits anti-inflammatory actions by inhibiting transactivation and cytokine-induced activation of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-kB) and by inhibiting glycerol-3-phosphate acyltransferase, necessary for de novo synthesis of diacylglycerol (DAG) and protein kinase C (PKC) activation [75]. Moreover, AMPK is essentially involved in angiogenesis induced by VEGF [59], which may be related to NO production [76], induction of autophagy [77] and inhibition of the hexosamine pathway, which normally represses angiogenesis via *O*-GlcNAcylation of angiogenic proteins [78].

1.6 Key host cell processes in virus interactions

1.6.1 Autophagy

Maintaining cellular homeostasis and survival is of utmost importance and thus, cells have developed various mechanisms against agents causing such changes in their environment. One such adaptive process is autophagy that occurs in cells in response to stress conditions such as nutrient deprivation, infections and hypoxia. Autophagy, a catabolic process, is the degradative removal of damaged or excess cellular organelles and misfolded proteins or aggregates from the cytoplasm in the lysosomes. There are three main types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy (herein further named autophagy) is the most common type of autophagy in cells (figure 8) [79,80].

During the process of autophagy, the cargo to be degraded is sequestered in double-membrane vesicles, so-called autophagosomes, and targeted to lysosomes for degradation. The initiation of autophagy begins with recruitment of the ULK1 complex to the phagophore assembly site in the isolation membrane, from which the autophagosome generation starts. The isolation membrane is thought to be derived from the endoplasmic reticulum (ER), mitochondria, trans-Golgi or endosomes. Once ULK1 is activated, it phosphorylates components of the class III PI3K (PI3KC3)

complex. This, in turn activates local phosphatidylinositol-3-phosphate (PI3P) production, PI3P then recruits the PI3P effector proteins, WD repeat domain phosphoinositide-interacting proteins (WIPIs) and zinc-finger FYVE domain-containing protein 1 (DFCP1), which trigger nucleation of the phagophore. The expansion of the phagophore leading to a functional autophagosome is then mediated by microtubule-associated protein 1 light chain 3 beta (LC3B) conjugation. This involves binding to membrane-resident phosphatidylethanolamine (PE) through which LC3B is inserted into the autophagosome membrane. The conversion of unconjugated LC3B (LC3BI) to conjugated LC3B (LC3BII) is a distinctive event in the formation of autophagosome and a vital marker for the process. Specific adaptor proteins such as the sequestosome-1 (commonly known as p62) which has cargo recognition sites and binding sites for LC3B, facilitate engulfment of various cellular components. The autophagosome then undergoes maturation via removal of autophagy proteins and recruitment of proteins such as soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNAREs), which help in the fusion with the lysosome. The double membraned mature autophagosome finally fuses with the lysosome. Acidic hydrolases in the lysosome degrade the autophagic cargo, and rescued nutrients are used up by the cell from the cytoplasm [79,81,82].

In ECs, autophagy is important against oxidative stress, excessive lipid or glucose accumulation and helps to maintain barrier function and angiogenesis [75]. It is also implicated to play a role in protecting ECs against atherosclerosis and ageing [77,83]. Autophagy seems to play a protective or supportive role in virus infection. Its antiviral response seems to be related to degradation of virions, viral components or host factors necessary for viral replication. This response is also known as virophagy. In addition, various autophagy proteins like beclin1 and other proteins encoded by autophagy genes (ATGs) are thought to play a role in innate immune responses by controlling cytokine and interferon signaling [84,85]. However, even though autophagy as a process serves to act against viral infections, viruses have evolved to use it or parts of this process for their benefit. Various viruses (HCV, Coxsackievirus B3 (CVB3), HSV-1) use membranes involved in the process as a site of replication or evasion against the hosts immune response [86–88]. Hence, it is vital to study the effect of viruses on autophagy and vice versa to develop effective means to inhibit viral infections.

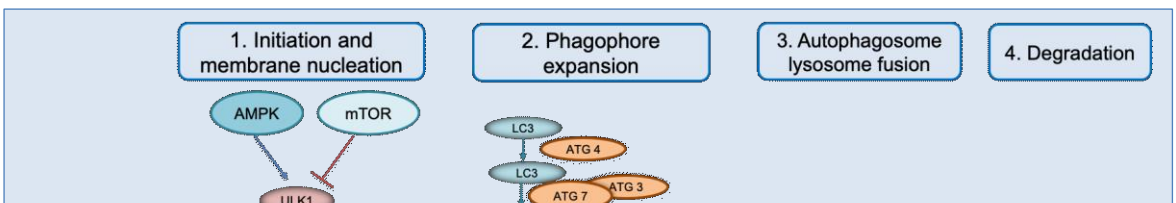


Figure 8: The autophagy process
Autophagy is initiated by activation of ULK1 complex by AMPK and inhibition of mTOR. The ULK1 complex phosphorylates the PI3K III complex and WIPI and DFCP1 addition results in membrane nucleation and phagophore formation around the cargo to be degraded. The phagophore expansion to phagosome takes place via addition of conjugated LC3B with the help of ATG 12 conjugation system. The phagosome matures to form a complete autophagosome via stripping of ATGs. The autophagosome then fuses with the lysosome to form the autolysosome which then results in degradation of the components via acid hydrolyses (Adapted from Tang et al [89]).
AMPK: 5'AMP-activated protein kinase, mTOR: mechanistic target of rapamycin, ULK1/2: Unc-51-like kinase 1/2, ATG: autophagy-related genes, FIP200: 200 kDa focal adhesion kinase family-interacting protein, P: phosphorylation, Beclin1: B-cell lymphoma-interacting protein 1, VPS34: vacuolar protein sorting 34, AMBRA1: Autophagy and Beclin1 Regulator 1, P115: general vesicular transport factor, WIPI: WD repeat domain phosphoinositide-interacting proteins, DFCP1: zinc-finger FYVE domain-containing protein 1, LC3: microtubule-associated protein light chain 3, PE: phosphatidylethanolamine

1.6.2 AMPK and mTOR crosstalk and their role in autophagy

The crosstalk between AMPK and mTOR is an important means of regulation in the cell. There are two functional complexes of mTOR: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 consists of raptor and mammalian lethal with SEC13 protein 8 (mLST8), regulates protein translation by activating ribosomal S6K and inhibiting eukaryotic initiation factor4E binding protein (4EBP1). mTORC2, consisting of rapamycin-insensitive companion of mTOR (Rictor), LST8 and mammalian stress-activated protein kinase interacting protein 1 (mSin1) plays a role in cytoskeletal organization and cell survival via Akt. mTORC1 is directly regulated by cellular energy and nutrient status, i.e., by amino acid and glucose levels, and is

activated by various upstream kinases such as class I PI3K-Akt [90]. AMPK inhibits the mTORC1 complex via various mechanisms, one being an inhibitory phosphorylation of the TSC2 complex [70] another one being the phosphorylation of raptor at conserved serine-threonine residues [91]. The interaction of AMPK and the mTORC2 complex is not completely understood, although AMPK has been shown to directly activate the mTORC2 complex to promote cell survival during energetic stress [92].

Both, AMPK and mTORC1, are known to regulate autophagy although in an opposite way. mTORC1 plays an important role in inhibiting autophagy via inhibitory phosphorylation of ULK1 at the S757 site. Contrastingly, under starvation conditions, AMPK inhibits mTORC1 and thereby frees the S757 site and, at the same time, phosphorylates ULK1 at the S555 site. Both events lead to induction of autophagy [93]. In addition, AMPK is thought to play a role in autophagosome maturation and fusion with lysosomes [94].

The activation of the mTOR pathway seems to be important in viral replication due to its role in protein translation and viral growth, as shown for the West Nile virus [95] and for HSV-1 in different cell types [37,96]. To analyze the role of AMPK in this context could thus provide more insights in HSV-1 replication.

1.6.3 Viruses and host cell metabolism

Cell metabolism comprises reactions carried out by the cell for maintaining its proper functioning. It can be divided into two categories: anabolism and catabolism. Both regulate the availability of nutrients, which then serve as building blocks or sources for energy. Carbohydrate and lipid metabolism are two such important pathways, which are also closely related to each other. Carbohydrate metabolism involves three key pathways regulating glucose homeostasis: gluconeogenesis, glucose metabolization (glycolysis and glucose oxidation) and glycogenolysis. Lipid metabolism comprises synthesis and degradation of FAs and/or complex lipids [97,98]. The choice between synthesis and degradation of metabolites is regulated tightly in the cell by various factors. AMPK as mentioned above is one such key regulator. As described in chapter 1.5.3, AMPK increases ATP levels in cells by stimulating breakdown of glucose and lipids and inhibiting their synthesis and storage.

Viruses rely completely on the host cell for their replication and need to exploit the major host metabolic pathways. Many viruses (Zika, HCMV, HSV-1) are thought to

increase glycolysis in the cells upon infection, as a means of increasing the availability of reducing equivalents and precursors for macromolecule biosynthesis. However, the use of host cell metabolism can vary from cell type to cell type and among different viruses. Some viruses also increase glucose uptake in cells. Most, but not all viruses tend to induce fatty acid synthesis and increase energy availability via fatty acid oxidation (Vaccinia, HCMV, etc.) [99,100]. In this context, AMPK may play a role. For instance, HCV enhances replication by accumulation of lipids via AMPK inhibition [101]. Thus, understanding the specific metabolic requirements for virus replication in the respective host cells could provide important approaches to hinder it.

So far, however, not much is known about the role of EC metabolism in virus replication. ECs are highly glycolytic and prefer to keep oxidative phosphorylation at a low rate, which makes them independent of oxygen, keeps levels of ROS low and allows to maintain oxygen supply to tissues. In addition, a high rate of glycolysis ensures the production of macromolecules, needed for instance for angiogenesis, and the generation of reducing factors through pathway branching off from glycolysis. Nevertheless, EC have the capability for FA oxidation (FAO). They do take up FAs but also can synthesize them and can store them in the form of lipid droplets. Lipid droplet formation in ECs avoids lipotoxicity, offers FAs for FAO and enables the release fatty acids from ECs to adjacent perivascular cell types [102,103].

1.6.4 Host cell inflammatory and antiviral responses

Inflammation is primarily a protective response against tissue injury or harmful stimuli like infectious pathogens, dead cells or irritants. Inflammatory responses rely on pattern-recognition receptors (PPRs), which recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) [104]. The PPRs consist of three group of receptors: the toll-like receptors (TLRs), the retinoic acid-inducible gene I (RIG I)-like receptors (RLRs) and the cytosolic DNA sensor cyclic, GMP-AMP (cGAMP) synthase (cGAS) – STING (stimulator of interferon genes). Activation of PRRs by PAMPs and DAMPs triggers downstream signaling and leads to the production of interferons (IFNs) and pro-inflammatory cytokines [105]. One important component of the inflammatory response is the inflammasome, which is responsible for the secretion of the proinflammatory cytokines interleukin 1 beta (IL-1β) or IL-18. The inflammasome represents a multimeric protein complex, which is assembled in the cytosol after sensing PAMPs and DAMPs and subsequently cleaving

the proinflammatory pro-cytokines to its active form [104]. Other inflammatory cytokines are translated as active forms (IL-6, IL-8) or shedded from plasma membrane-bound precursors (tumor necrosis factor alpha (TNFα)) [106]. Inflammation, although being an initially protective process, plays a role in the pathophysiology of many chronic diseases, including cardiovascular and cerebrovascular disease [107,108].

Endothelial cells activated by pro-inflammatory agonists such as TNFα, IL-1β and endotoxins play an important role in inflammatory processes. Via the activation of NF-κB signaling, cascades are activated, which lead to the expression of intracellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) on the cell surface, which, in turn, bind immune cells and promote their transmigration into the tissue. In addition, other inflammatory cytokines and chemokines are released, the inducible NO synthase is stimulated, and the endothelial cytoskeleton is reorganized thereby providing intercellular gaps for plasma protein and leukocyte extravasation [44,109,110]. The inflammatory response of endothelial cells (and other inflammatory cells) has been shown to be controlled by AMPK, which is known to inhibit NF-kB signaling via several pathways such as SIRT1, FOXO or PGC-1α. Via these actions, AMPK plays a pivotal role in regulating recruitment of inflammatory cells to the vasculature and modulating inflammatory responses thus supporting a controlled, well-regulated inflammatory response in ECs [111–113].

Viruses are one of the key stressors, against which an inflammatory reaction is produced. Upon viral infection, cells produce among other cytokines the so-called IFNs that interfere with the virus replication. IFNs induce interferon-stimulated gene (ISG) transcription via the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. There are three groups of IFNs, Type I, II and III, of which generally but not exclusively Type I-IFNs are involved in antiviral response. IFN-β is one such important IFN of this group. IFNs also play important roles in triggering adaptive immune response and antigen presentation [114]. Naturally, viruses have developed strategies to overcome and resist inflammatory responses by suppressing the PRRs (HSV-1), by shutting off host cell transcriptional and translational activities (some RNA viruses) or by taking refuge in host cell organelles to delay active infections [115,116]. Whether anti-inflammatory proteins such as AMPK support the interferon response or interfere with viral resistance mechanism is not yet known.

1.7 Aim

HSV-1, along with its primary site of infection, infects and replicates in the endothelium. This infection has been reported to be a trigger for endothelial dysfunction and to be involved in the pathogenesis of atherosclerosis. However, the infection and replication of HSV-1 in vascular endothelial cells is poorly characterized. Moreover, metabolic host cell proteins, which may be targeted to prevent or reduce virus replication in endothelial cells, are not studied although HSV-1 vaccines are unavailable and new antiviral treatment options are important. Thus, the aim of this thesis was to understand the infection of HSV-1 in the endothelium and identify the role of AMPK, the master regulator of cellular metabolic pathways, in HSV-1 replication in endothelial cells. We infected endothelial cells with HSV-1 and addressed the following questions:

- Does HSV-1 replicate in endothelial cells?
- How does HSV-1 alter endothelial signaling pathways and function?
- Does AMPK play a role in HSV-1 infection in endothelial cells?
- What mechanisms are involved in the role of AMPK against HSV-1 replication in endothelial cells?

The data obtained in this project were expected to improve our understanding of HSV-1 infection in endothelial cells and to increase the potential for future antiviral strategies against virus infections based on host cell protein modulation.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cells

All in vitro experiments were performed on human umbilical vein endothelial cells (HUVEC) isolated from fresh umbilical cords or in mouse lung endothelial cells (MLEC) isolated from mouse lungs. The preparation of HUVEC and MLEC is described in 2.2.1.1.

Viral titrations were performed on African Green Monkey Kidney cells (GMK, Vero cells) purchased from the American Type Culture Collection (ATCC®, CCL-81™).

2.1.2 Virus

All experiments were performed with the HSV-1 Kos strain, a laboratory strain, provided by the Institute for Medical Microbiology, Section for Experimental Virology, Jena University Hospital.

2.1.3 Chemicals, reagents and kits

The general chemical and reagents used throughout the project are enlisted in tables 1-3.

Table 1: Cell culture media, supplements and transfection reagents

Substance	Company / manufacturer
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich Chemie GmbH, Steinheim
Bovine serum albumin (BSA), fat-free	Sigma-Aldrich Chemie GmbH, Steinheim
Medium 199 (M199)	Lonza, Basel, Switzerland
Biotin	Sigma-Aldrich Chemie GmbH, Steinheim
Ciprofloxacin Kabi	Fresenius Kabi Deutschland GmbH, Bad Homburg
Collagenase-II	Worthington Biochemical Corporation, USA
Dulbecco's modified Eagle's medium (DMEM)	Lonza, Basel, Switzerland
Dulbecco's modified Eagle's medium (DMEM), high glucose, Glutamax	Gibco® Life Technologies Corporation, UK

Eagle's Minimum Essential Medium (EMEM)	Lonza, Basel, Switzerland
Endothelial mitogen (EM)	Hycultec GmbH, Beutelsbach, Germany
Endothelial cell growth supplement (ECGS)	Sigma-Aldrich Chemie GmbH, Steinheim
Ethanol	AppliChem GmbH, Darmstadt
Fetal calf serum (FCS)	Sigma-Aldrich Chemie GmbH, Steinheim
Gelatin	Sigma-Aldrich Chemie GmbH, Steinheim
Heparin-sodium	Sigma-Aldrich Chemie GmbH, Steinheim
Human serum	Sigma-Aldrich Chemie GmbH, Steinheim
Human Serum Albumin (HSA)	Bayer Vital GmbH, Leverkusen
L-Carnitine	Sigma-Aldrich Chemie GmbH, Steinheim
L-Glutamine	Sigma-Aldrich Chemie GmbH, Steinheim
MEM non-essential amino acid (100x)	Sigma-Aldrich Chemie GmbH, Steinheim
Penicillin/Streptomycin (100 U/ml, 100 µg/ml)	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium hydroxide	Merck KGaA, Darmstadt
Trypsin-EDTA (0.05%, 0.02 %)	Sigma-Aldrich Chemie GmbH, Steinheim
Vitamin C	Sigma-Aldrich Chemie GmbH, Steinheim
Hank’s Balanced Salt Solution (HBSS)	Sigma-Aldrich Chemie GmbH, Steinheim
SAINT-RED, SAINT sRNA	Synvolux Therapeutics B.V., Groningen, Belgium

Table 2: Chemicals and reagents

Substance/ Reagents	Company / manufacturer
2-Methyl-1-Propanol (Isobutanol)	Carl Roth GmbH Co. KG, Karlsruhe
^{14}CAcetate (Acetic acid [1-^{14}C] sodium salt) Specific activity: 56.6 µCi/µmol	Hartmann Analytic
Acrylamide 4K solution (30 % mix)	AppliChem GmbH, Darmstadt
Ammonium persulphate (APS)	AppliChem GmbH, Darmstadt
β-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Steinheim
Bovine Serum Albumin (BSA), Fraction V, pH 7,0	AppliChem GmbH, Darmstadt
Bio-Rad DC Protein Assay	Bio-Rad Laboratories Inc., Hercules, USA
Bromophenol blue	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium cacodylate trihydrate	Serva Electrophoresis GmbH, Heidelberg
Calcium chloride ($CaCl_2$)	AppliChem GmbH, Darmstadt
Chloroform	Carl Roth GmbH Co. KG, Karlsruhe
Citric acid, water free	Sigma-Aldrich Chemie GmbH, Steinheim
Complete, EDTA-free protease inhibitor cocktail tablets (PIC)	Roche Diagnostics GmbH, Mannheim
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Steinheim

Disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O)	Carl Roth GmbH Co. KG, Karlsruhe
Dithiothreitol (DTT)	Sigma-Aldrich Chemie GmbH, Steinheim
ECL Western Blotting Detection Reagent	GE Healthcare UK Limited, UK
Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetra acetic acid (EGTA)	Sigma-Aldrich Chemie GmbH, Steinheim
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich Chemie GmbH, Steinheim
Glucose	Sigma-Aldrich Chemie GmbH, Steinheim
Glutaraldehyde 25 %	Serva Electrophoresis GmbH, Heidelberg
Glycerol	Carl Roth GmbH Co. KG, Karlsruhe
Glycine	AppliChem GmbH, Darmstadt
Hydrochloric acid (37 %)	Carl Roth GmbH Co. KG, Karlsruhe
Isopropanol	Carl Roth GmbH Co. KG, Karlsruhe
Kodak GBX developer and replenisher	Sigma-Aldrich Chemie GmbH, Steinheim
Kodak GBX fixer and replenisher	Sigma-Aldrich Chemie GmbH, Steinheim
Magnesium sulphate ($MgSO_4$)	Sigma-Aldrich Chemie GmbH, Steinheim
Methanol	AppliChem GmbH, Darmstadt
N,N,N′,N′-Tetramethyl ethylenediamine (TEMED)	AppliChem GmbH, Darmstadt
Phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium dihydrogen phosphate (KH_2PO_4)	Riedel-De Haen AG, Hannover
Potassium chloride (KCl)	VEB Jenapharm-Laborchemie, Apolda
Protease inhibitor cocktail	Sigma-Aldrich Chemie GmbH, Steinheim
RNAse Away	Sigma-Aldrich Chemie GmbH, Steinheim
Scintillation Cocktail RotiSzint eco® plus LSC Universal cocktail	Carl Roth GmbH & Co. KG, Karlsruhe
Skimmed milk powder	AppliChem GmbH, Darmstadt
Sodium azide	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium chloride (NaCl)	AppliChem GmbH, Darmstadt
Sodium diphosphate tetrabasic ($Na_4P_2O_7$)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium fluoride (NaF)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium hydroxide (NaOH)	Carl Roth GmbH Co. KG, Karlsruhe
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium phosphate dibasic dihydrate (Na_2HPO_4 x 2 H_2O)	Carl Roth GmbH Co. KG, Karlsruhe
Spectra™ Multicolor Broad Range Protein Ladder (10-260kDa)	Fermentas life science, Fermentas GmbH, St. Leon-Rot
Sucrose	Sigma-Aldrich Chemie GmbH, Steinheim
Tris(hydroxymethyl)aminomethane (Tris)	AppliChem GmbH, Darmstadt
Triton X-100	Ferak Berlin GmbH, Berlin

Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate	Perkin Elmer, Waltham, USA

Table 3: Kits

Kits	Company/manufacturer
NucleoSpin® RNA isolation kit	Macherey-Nagel GmbH & Co. KG
First strand cDNA synthesis kit	Thermo Fischer Scientific, Karlsruhe
Maxima ™ SYBR ™ Green / ROX qPCR Master Mix	Thermo Fischer Scientific, Karlsruhe
LEGENDplex™ Multi-Analyte Flow Assay Kit, Human Inflammation Panel 1 (13-plex)	Biolegend, San Diego

2.1.4 Stimulators and inhibitors

All substances used as stimulators or inhibitors are enlisted in table 4 along with their final concentrations.

Table 4: Stimulators and inhibitors

Substance	Final Concentration	Company/manufacturer
AICAR	0.5, 1, 2 mM	LC-Labs, Woburn, USA
A769662	10, 50 μM	Abcam, Cambridge, USA
Akt 1/2 Kinase-Inhibitor (Akt-Inhibitor VIII)	20 μM	Sigma-Aldrich Chemie GmbH, Steinheim
Bafilomycin A1	50 nM	Enzo Life Sciences GmbH, Lorrach
3-Methyladenine	10 mM	Sigma-Aldrich Chemie GmbH, Steinheim
Torin 2	100 nM	Sigma-Aldrich Chemie GmbH, Steinheim
SC4	0.1, 1 and 10 μM	Kindly provided by Jon Oakhill, St. Vincent's Institute Medical Research, Melbourne, Australia
2-Deoxyglucose (2-DG)	20 mM	Sigma-Aldrich Chemie GmbH, Steinheim
Simvastatin	10 μM	Calbiochem R, EMD Biosciences, Inc., Darmstadt

2.1.5 Antibodies

The primary antibodies used for western blotting were dissolved in 5% BSA in Tris-NaCl (TN) -Tween buffer (10 mM Tris, 1,5 M NaCl, 0.1% Tween® 20, 0.02% sodium

azide) and stored at 4 °C. Secondary antibodies were freshly dissolved in 5% milk in TN-Tween buffer. All antibodies used for western blotting are enlisted in table 5.

Table 5: Primary and secondary antibody for western blot

Antibody against	Origin	Dilution	Product number	Company/ manufacturer
phospho-ACC (S79)	rabbit, polyclonal	1:1000	3661	CST
ACC	rabbit, monoclonal	1:1000	3676	CST
phospho-Akt (S473)	rabbit, polyclonal	1:1000	9271	CST
Akt	rabbit, polyclonal	1:1000	9272	CST
phospho-AMPKα1 (T172)	rabbit, monoclonal	1:1000	2535	CST
phospho-AMPKα1 (S485)	rabbit, monoclonal	1:1000	2537	CST
AMPKα	rabbit, polyclonal	1:1000	2532	CST
AMPKα1	rabbit, polyclonal	1:1000	2795	CST
AMPKα2	rabbit, polyclonal	1:1000	2757	CST
phospho-ATG14 (S29)	rabbit, monoclonal	1:1000	92340	CST
ATG14	rabbit, monoclonal	1:1000	96752	CST
phospho-ATG16L1 (S278)	rabbit, monoclonal	1:1000	ab195242	Abcam
ATG16L1	rabbit, monoclonal	1:1000	8089	CST
β-actin	rabbit, monoclonal	1:5000	4970	CST
Phospho-Beclin1 (S15)	rabbit, monoclonal	1:1000	84966	CST
Phospho-Beclin1 (S93)	rabbit, monoclonal	1:1000	14717	CST
Beclin1	rabbit, monoclonal	1:1000	3495	CST
phospho-ERK (T202/Y204)	rabbit, monoclonal	1:1000	9106	CST
ERK1/2	mouse, monoclonal	1:1000	9107	CST
phospho-JNK (Thr183/Tyr185)	rabbit, monoclonal	1:1000	9255	CST
JNK	rabbit, polyclonal	1:1000	9252	CST
LC3B	rabbit, monoclonal	1:1000	83506	CST
phospho-p70S6K (T389)	rabbit, polyclonal	1:1000	9205	CST
p70S6K	rabbit, polyclonal	1:1000	9202	CST
phospho-Raptor (S792)	rabbit, polyclonal	1:1000	2083	CST
Raptor	rabbit, polyclonal	1:1000	2280	CST
phospho-ULK1 (S555)	rabbit, monoclonal	1:1000	5869	CST
phospho-ULK1 (S757)	rabbit, polyclonal	1:1000	6888	CST
ULK1	rabbit, polyclonal	1:1000	4476	CST
rabbit IgG (H+L)	goat, peroxidase labeled	1:5000	074-1506	KPL
mouse IgG (H+L)	goat, peroxidase labeled	1:5000	074-18096	KPL
CD102 (MLEC isolation)	Rat, anti-Mouse	1:25	553326	BD Biosciences

Dynabeads	Sheep anti-Rat	1:25	110.35	Dynal Biotech ASA, Oslo Norway

CST: Cell Signaling Technology® Inc., Danvers, USA

KPL: Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland, USA

2.1.6 Small interfering ribonucleic acid (siRNA)

The siRNAs used are SMARTpool siRNAs, which minimize off-target effects. All siRNAs used are enlisted in table 6.

Table 6: List of siRNAs

siRNA	Target protein	Catalog number
ON-TARGET*plus* Non-Targeting Pool	Control	#D-001810-10
siGENOME Human PRKAA1 (5562) siRNA	AMPK α1	#M-005027-02
siGENOME Human PRKAA2 (5563) siRNA	AMPK α2	#M-005361-02
siGENOME Human ULK1 (8408) siRNA	ULK1	#M-005049-00
siGENOME Human BECN1 (8678) siRNA	beclin1	#M-010552-01
ON-TARGETplus Human ACACA (31) siRNA	ACC 1	L-004551-00
-	ACC 2	Sc -43597
siGENOME Human MAP1LC3B (81631) siRNA	LC3B	# M-012846-01

2.1.7 Primers

All primer pairs used for Real-time PCR are enlisted in table 7 and are obtained from Sigma-Aldrich Chemie GmbH, Steinheim.

Table 7: Primers for Real-time PCR

Primer name	Sequence
β-Actin	Forward: GGGACGACATGGAGAAAATCTG Reverse: GAAGGTCTCAAACATGATCTGGG
IFNA2	Forward: CTCATGTTTCTGCTATGACC Reverse: GTGCCTTAAGAGCTGAATAC
IFNB1	Forward: ATTCTAACTGCAACCTTTCG Reverse: GTTGTAGCTCATGGAAAGAG
IFNG	Forward: GGTAACTGACTTGAATGTCC Reverse: TTTTCGCTTCCCTGTTTTAG
IL-6	Forward: GCAGAAAAAGGCAAAGAATC Reverse: CTACATTTGCCGAAGAGC
IL-8	Forward: TGCCAAGGAGTGCTAAAG Reverse: CTCCACAACCCTCTGCAC
CCL2	Forward: AGACTAACCCAGAAACATCC

	Reverse: ATTGATTGCATCTGGCTG
TNFa	Forward: GGCTCCAGGCGGTGCTTGTTC Reverse: AGACGGCGATGCGGCTGATG
IL-1beta	Forward: CTCTCTCACCTCTCCTACTCAC Reverse: ACACTGCTACTTCTTGCCCC

2.1.8 General materials and instruments

All materials required for regular laboratory use such as pipettes, pipette tips and gloves were purchased from Eppendorf, Brand®, Meditrade® and Greiner Bio-One. Other general materials and instruments are enlisted in table 8.

Table 8: Materials and instruments

Material/Instrument	Company/manufacturer
Allegra 64R (centrifuge)	Beckman Coulter, Inc. USA
FACS Canto	Becton, Dickinson and Company, Franklin Lakes, New Jersey
Biofuge primo R (centrifuge)	Heraeus Holding GmbH, Hanau
BP310P (balance)	Sartorius AG, Göttingen
BP61 (balance)	Sartorius AG, Göttingen
Cell culture vessels	Greiner Bio-One GmbH, Frickenhausen
Cell scraper blue 25,0 cm	Greiner Bio-One GmbH, Frickenhausen
Centrifuge 5415 C	Eppendorf AG, Hamburg
Clamps for umbilical cord	Plazotta Onlineshop. München
Cuvettes	Sarstedt AG & Co., Nümbrecht
Filterpaper Chrom-Paper Sheets	Sartorius Stedim Biotech S.A., France
Fridge +4 ° C / Freezer -20 ° C	Liebherr-International Deutschland GmbH, Germany
Hamilton syringe	Hamilton Bonaduz AG, Switzerland
Heracell 150 (incubator)	Heraeus Holding GmbH, Hanau
Heraeus functionline (warming incubator)	Heraeus Holding GmbH, Hanau
Immobilon™-P Transfer Membrane	Millipore Corporation, USA
KS 501 digital	IKA® Werke GmbH 6 Co. KG, Staufen
Labofuge 400R	Heraeus Holding GmbH, Hanau
Laminarbox Herasafe	Heraeus Holding GmbH, Hanau

M6 Lauda (water bath)	MS Laborgeräte, Wiesloch
Magnetic stirrer	R3T GmbH, Taufkirchen
MC1 Laboratory LC4200 (Waage)	Sartorius AG, Göttingen
mc6® (fume hood)	Waldner Laboreinrichtungen GmbH & Co. KG
Medap (Vacuum vessel)	Medizinische Apparate, Med.-Techn. Gasanlagenbau, Austria
Minisart® (Sterilfilter)	Sartorius AG, Göttingen
Monoject™ Blunt Cannula	Covidien, Mansfield, USA
MP20/System II (Membrane vacuum pump)	Biometra® biomedizinische Analytik GmbH, Göttingen
Nanodrop one	Thermo Scientific Inc., USA
Nikon TMS-F (Mikroskop)	Nikon Corporation, Tokio, Japan
OV3 (warming incubator)	Biometra® biomedizinische Analytik GmbH
InoLab® pH meter (7110)	Thermo Scientific Inc., USA
Parafilm	American Can Company, Greenwich
Pharmacia LKB MultiTemp II (Cooling system - electrophoresis)	GE Healthcare UK Limited, UK
Photofilm	Fujifilm Corporation, Japan
Power Pac 1000 (Electrophoresis)	Bio-Rad Laboratories Inc., USA
Power Pac 200 (Blotting)	Bio-Rad Laboratories Inc., USA
Heating plate, Type PZ 35	Störk-Tronic, Störk GmbH & Co. KG, Stuttgart
Protean® II xi Cell (Electrophoresis)	Bio-Rad Laboratories Inc., USA
Quantstudio 3	Thermo Scientific Inc., USA
Shaker	Eppendorf AG, Hamburg
Sunrise™ Tecan	Tecan Deutschland GmbH, Crailsheim
Thermomixer 5437	Eppendorf AG, Hamburg
Thermostat Julabo	JULABO Labortechnik GmbH, Seelbach
Trans-Blot™ Cell (Blotting)	Bio-Rad Laboratories Inc., USA
UVIKON spectrophotometer 930	Tresser instruments, UK
Vortex	IKA® Werke GmbH 6 Co. KG, Staufen
Wallac 1414 Win Spectral Liquid Scintillation Counter	Perkin Elmer, Waltham, USA

WT17 (tumbling shaker)	Biometra® biomedizinische Analytik GmbH, Göttingen

2.2 Methods

2.2.1 Cell biological methods

2.2.1.1 Preparation of human endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords anonymously acquired from healthy donors according to the Declaration of Helsinki, "Ethical principles for Medical Research Involving Human Subjects" (1964). Donors were informed and gave written consent. The study was approved by the Jena University Hospital Ethics Committee (no. 3463-06/12).

Fresh umbilical cords were collected in sterile containers and then surface sterilized by using 70% ethanol. The veins were perfused with sterile wash buffer (table 9). After perfusion, the veins were filled with freshly prepared collagenase-II (0.01% in M199) and cords were incubated in a water bath at 37 °C for 3 min with gentle massaging upon which the endothelial cells were detached. The cell suspension was collected in a falcon tube containing stop medium to inactivate the action of collagenase-II (table 9). The veins were then rinsed with stop medium, which was collected in the same falcon tube. Thereafter, the tube was centrifuged at 500 x g for 6 min. The cell pellet was resuspended in stop medium and seeded in a 75 cm^2 culture flask pre-coated with 0.2% gelatin. Cells were incubated under standard growth conditions (37 °C, 5% CO_2, 95% humidity) overnight. After the cells had firmly adhered, the flask was washed twice with phosphate buffer-saline (PBS), to remove debris and non-adherent cells. Complete growth medium supplemented with 0.5% ciprofloxacin was added and the cells were cultured under standard growth conditions.

Table 9: Components and composition of solutions and media used for HUVEC preparation and cultivation

Medium/Solution	Components	Final concentration
Wash buffer	NaCl, sterile	0.9%

0.01% collagenase-II solution	M199 Collagenase-II	 0.1 mg/ml
Stop medium	M199 FCS Penicillin, Streptomycin (for overnight culture)	 10% 100 U/ml, 100 µg/ml
Complete growth medium	M199 FCS Human serum Penicillin, Streptomycin L-glutamine Heparin Endothelial mitogen or ECGS Vitamin C	80% 17.5% 2.5% 100 U/ml, 100 µg/ml 680 µM 7.5 U/ml 7.5 µg/ml or 3 mg/ml 100 µM
20 mM vitamin C stock solution	M199 Vitamin C	 3.5 mg / 800 µl M199 (Neutralized with 200 µl 0.1 N NaOH)
Gelatin solution	Gelatin	0.2% in autoclaved water, dissolved at 56 °C
PBS (pH 7.4)	NaCl KCl KH_2PO_4 Na_2HPO_4 x 2 H_2O	145 mM 2.7 mM 1.5 mM 8 mM

2.2.1.2 Cultivation and seeding of HUVEC

After reaching confluency, cells were washed twice with PBS and detached with Trypsin-EDTA (0.05%/0.02%) at 37 °C for 2-3 min. Trypsin was deactivated by addition of stop medium (Table 9). Cells were centrifuged (500 x g, 6 min), pellets were resuspended in growth medium, supplemented with 0.5% ciprofloxacin and seeded in 2-3 new gelatin-coated 75 cm² cell culture flasks. For seeding, HUVEC of the first passage were detached as described and cell number was determined using a Neubauer counting chamber. Cells were plated at densities listed in table 10 in complete growth medium where ciprofloxacin was replaced by penicillin/streptomycin, and used for experiments after 2-3 days in culture, on reaching confluence. Experiments were generally performed in HUVEC of the second passage. HUVEC obtained and prepared from different donors were considered as biological replicates.

Table 10: Cell number and medium volume

Cell culture equipment	Cell number	Volume of growth or (+) medium

24-well plate	70,000 / well	0.5 ml / well
12-well plate	100,000 / well	1 ml / well
30 mm dish	240,000 / dish	2 ml / dish
60 mm dish	360,000 / dish	4 ml / dish
90 mm dish	1,200,000 / dish	8 ml / dish

2.2.1.3 Preparation of MLEC

MLEC were prepared from lungs obtained from either wild type or knockout mice (C57BL/6). All animals were sacrificed by cervical dislocation and the whole-body surface was immediately disinfected with 70% ethanol. The lungs were excised and transferred into ice-cold DMEM and processed immediately. Sterile instruments such as scissors and tweezers were used to remove the hilar region and then to mince the organ and obtain a homogeneous mass. The minced tissue was collected in a falcon tube and incubated with 0.2% collagenase-II in DMEM and incubated at 37 °C, 5% CO_2 for 1 h. During the incubation period, gentle mixing of the minced tissue was performed every 10 min. Next, the contents were titrated 20 times using a blunt edge cannula and filtered through a 40 µM cell strainer, to remove undigested tissue particles. The cell strainer was then washed with minus (-) medium (MLEC growth medium without endothelial growth supplement, table 11) to stop the activity of collagenase-II. The solution was centrifuged at 500 x g for 6 min. The cell pellet was resuspended in plus (+) medium (MLEC growth medium with endothelial growth supplement, table 11) and seeded into a 75 cm^2 culture flask pre-coated with 0.2% gelatin. Cells were incubated under standard growth conditions overnight. The next day, the mixed cultures were washed vigorously with 2% FCS/PBS, (+) medium was added and cells were incubated under standard growth conditions. To obtain endothelial cells, two antibody-based selections were carried out. The first positive selection of endothelial cells was performed before the mixed culture reached full confluency to prevent overgrowth of fibroblasts. Magnetic beads coupled to sheep anti-rat IgG (3 x 10^6 beads per 75 cm^2 flask) were washed three times with 2% FCS/PBS using a magnet and then conjugated to rat anti-mouse CD102 antibody (4.5 µg per 75 cm^2 flask) for 2 h at 4 °C on a rotator machine. Antibody-conjugated beads were washed thrice with 2% FCS/PBS solution, then added to the mixed cultures (kept on ice 5 min prior) and incubated at 4 °C for 1 h. After binding of the conjugated beads to endothelial cells, culture flasks were first washed twice with warm PBS and rinsed once with trypsin-EDTA. Cells were then detached by adding trypsin-EDTA and keeping the flask at 37 °C for 4-5 min. The action of trypsin-EDTA was stopped by the addition of

(-) medium and the cell solution transferred into a 15 ml tube. A second 15 ml tube was placed in a magnet and the suspension was very slowly pipetted into this tube at the magnet contact side. This allowed only endothelial cells bound to magnetic beads to be retained at the wall of the tube. After 10 min of incubation, the solution containing bead-free cells was discarded and the magnet was removed leading to detachment of bead-bound endothelial cells. The tube wall was rinsed with (+) medium and the cells were resuspended and seeded into new culture flask. A second selection procedure was performed after 3- 4 days in culture to increase the purity of the cell population. The latter was assessed by CD31 staining (a specific endothelial cell marker) using flow cytometry and varied from 85% - 95%. After the second selection, endothelial cells were seeded for experiments.

Table 11: Components and composition of solutions and media used for MLEC preparation and cultivation

Media/Solution	Components	Concentration
0.02% Collagenase-II solution	M199 Collagenase	 0.2 mg/ml
(-) medium	DMEM F12 high glucose FCS Penicillin Streptomycin L-glutamine	80% 20% 100 U/ml 100 µg/ml 2 mM
(+) medium	DMEM F12 high Glucose FCS Penicillin Streptomycin L-glutamine Heparin Endothelial mitogen or ECGS	80% 20% 100 U/ml 100 µg/ml 2 mM 25 g/ml 75 µg/ml or 3 mg/ml

2.2.1.4 Seeding of MLEC

For seeding, confluent MLEC cultures were washed twice with PBS and detached using Trypsin-EDTA as described for HUVEC, and cell number was determined using a Neubauer counting chamber. Cells were plated at densities as described in table 10, cultured under standard growth conditions and used for experiments after 2-3 days, on reaching confluence.

2.2.1.5 Cultivation and seeding of Vero cells

GMK or Vero cells were used as reporter cells for viral titration experiments. The cells are highly susceptible to virus infections as they lack interferon response production [117]. Adherently growing cells were cultured in Eagle's Minimum Essential Medium (EMEM), glycine free supplemented with 25 µM Hepes and 10% FCS in 75 cm^2 or 175 cm^2 flasks at 37 °C and 5% CO2 and 95% humidity. For detachment, cells were washed twice with PBS and after addition of trypsin-EDTA to the flasks (3 ml for 75 cm^2 or 6 ml for 175 cm^2 flask) incubated at 37 °C for 10 -12 min. The flasks were vigorously tapped, and the cells were then mechanically detached. After transferring cells into EMEM medium containing 10% FCS, cells were mixed well, and the cell number was determined using a Neubauer counting chamber. 100,000 cells/well were seeded into each well of a 96-well plate and cultured for 1 h before the Median Tissue Culture Infectious Dose ($TCID_{50}$) method was performed to determine viral replication. Remaining cells were seeded into new flasks.

2.2.2 Viral infection experiments

MLEC or HUVEC to be infected were seeded in 30 mm or 60 mm dishes (for western blotting experiments) or 24-well plates (for titration experiments) according to the cell numbers mentioned in table 10 with an extra well or extra dish to calculate cell number before infection. Confluent cultures were infected with a multiplicity of infection (m. o. i.) of 5 with the HSV-1 Kos strain. Cell number was calculated again to determine the total infection volume (virus + medium), required for each experiment by the following formula:

$$\text{Infection volume (ml)} = \frac{\text{Cell count (per ml)} * \text{total volume (ml)} * \text{m.o.i.}}{\text{Virus titer } (TCID_{50} \text{ per ml})}$$

For infection, endothelial cells were incubated for 1 h with the virus-containing medium. Uninfected controls were included in every experiment. After 1 h, the infection medium was replaced by fresh growth medium and cells were incubated for 24 h *post infectionem* (p. i.). Then, samples were processed for western blots or titration experiments. To obtain samples for $TCID_{50}$ titrations, the 24-well plates containing infected cells and medium were stored at -20 °C for one day and thereafter thawed at room temperature. This freeze-thaw cycle caused the cells to burst and release the

intracellular virus into the medium. The samples containing intra- and extracellular virus (probes) were then used to determine virus concentration as described in 2.2.3. For titration experiments, each condition was tested in triplicates.

Table 12: Infection volume and media volume

Cell culture equipment	Infection volume	Growth medium
24-well plate	0.2 ml	0.5 ml
30 mm dish	0.5 ml	2 ml
60 mm dish	1 ml	4 ml
90 mm dish	2 ml	8 ml

2.2.3 Determination of viral concentration by $TCID_{50}$ method

The $TCID_{50}$ method quantifies the amount of virus in endothelial samples required to produce a cytopathic effect (CPE) in 50% of the Vero reporter cells (figure 9).

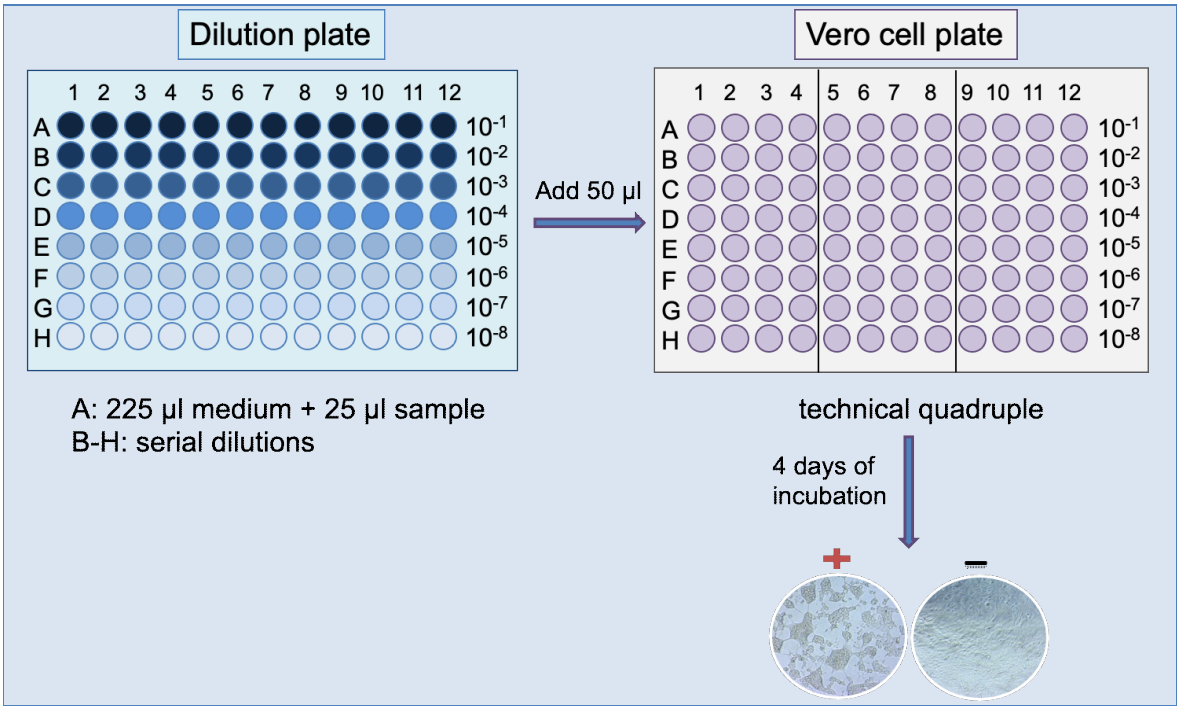


Figure 9: Determination of viral concentration by $TCID_{50}$ method
Each sample was serially diluted in a dilution plate and added to the Vero cell plate in technical quadruple. The plate was incubated for 4 days and observed for viral cytopathic effects. The viral titer was then determined by the Reed and Muench method [118].

It requires seeding of the reporter cells on one hand and dilution of the virus-containing samples from endothelial cells on the other hand before the reporter cells can be infected. Each determination of virus-induced cytopathic effects was performed in

technical quadruplicates. For the Vero cell plate, 100 µl of cell suspension in EMEM medium was seeded in a 96-well plate (100,000 cells/well) and cells were allowed to attach for 1 h at 37 °C. During this time, the virus dilution plate was prepared. For this, 225 µl of EMEM medium was added to each well in a second 96-well plate. Probes containing the intra + extracellular virus were thawed and centrifuged at high speed shortly to precipitate the debris. Then, 25 µl of each sample probe was added to each well of the top row and serially diluted in the rows below up to 10^{-8}. From this dilution plate, 50 µl of each well were then added to the corresponding well of the Vero cell plate. The plates were incubated at 37 °C, 5% CO_2 for 4 days. Cytopathic effects were then documented for each sample. Unaffected Vero cell layers were considered 'cytopathic-negative' while Vero cells showing destruction of the cell monolayer, clumping or rounding of cells were defined as 'cytopathic-positive'. The virus titer was calculated using the endpoint method determined by Reed and Muench [118] by the following formula:

$$\text{Proportional distance (PD)} = \frac{A - 50\%}{A - B}$$

$$TCID_{50} = (\text{critical dilution}) + (PD * \log \text{ of critical dilution})$$

Where, A = %CPE above 50%, B = %CPE below 50%, critical dilution = dilution above 50% CPE

2.2.4 Transfection with siRNA

To study the role of different proteins in mediating viral replication, proteins were downregulated using the siRNA technology (siRNAs enlisted in table 6). HUVEC were seeded in 24-well plates one day prior to transfection (50 - 70% confluency). The cells were transfected with 0,5 µg/ml non-targeting or specific siRNA using the SAINT-sRNA transfection kit (Synvolux Therapeutics B.V.). For each well in a 24-well plate, siRNA was diluted in 19.6 µl PBS and 4 µl Saint-sRNA were diluted in 16 µl PBS separately. Both solutions were combined and 160 µl M199 supplemented with 0.25% HSA were added. This transfection solution (200 µl) was added to the well after having washed cells twice with pre-warmed HBSS. After 4 h, 500 µl of growth medium was added and the cells were used for experiments after 72 h of transfection. Downregulation efficiency was verified by western blotting.

2.2.5 Cell stimulation and inhibition studies

To study the role of AMPK upon viral infection different AMPK activators (AICAR, A769662, SC4) were used. In addition, inhibitors of the Akt/mTOR pathway were employed (Akt inhibitor VIII, Torin 2). Autophagy inhibitors (bafilomycin A1, 3-methyladenine) were used to investigate functions of autophagy upon viral infection and metabolic inhibitors (2-deoxyglucose, simvastatin) were applied to study role of metabolism in viral replication. The respective compounds were dissolved in DMSO (A769662, SC4, Torin 2, bafilomycin A1, simvastatin) or medium (AICAR, Akt inhibitor VIII, 3-methyadenine, 2-deoxyglucose).
Unless specified otherwise, HUVEC were pretreated with the respective compounds according to concentrations mentioned in the table 4 for 1 h in growth medium. Equal amounts of the respective solvents were added as controls. Cells were either analyzed after addition of AMPK activators (signaling, lipid synthesis) or infected with the virus after addition of activators or inhibitors as described in section 2.2.2. Medium was refreshed 1 h p.i. and compounds were re-added. All infection experiments were performed for 24 h, unless specified otherwise. A769662 and SC4 were re-added to the cells after 12 h p.i.

2.2.6 Protein analytics

2.2.6.1 Western blotting

For western blot experiments, 30 or 60 mm-dishes were used, and cell numbers as mentioned in table 10 were seeded. All employed buffers and constituents are shown in table 13. After experimental incubation periods, whole cell lysates were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The entire lysis procedure was performed on ice. First, the cells were washed twice with ice-cold wash buffer and then they were incubated with lysis buffer for 15 min (60 µl for 30 mm and 80 µl for 60 mm dishes). Thereafter, the cells were scraped from the dish and collected in respective Eppendorf tubes. The tubes were then centrifuged at 700 x g for 6 min at 4 °C to remove the cell debris. The supernatant (lysates) was then transferred to new

respective safe lock Eppendorf tubes. 2 µl of each sample was used for protein determination in duplicates. Laemmli buffer was added to the remaining sample in a 1:3 dilution. The samples were boiled for 5 min at 95 °C, with gentle shaking to allow protein denaturation and stored at -20 °C until further use. Protein concentrations was determined using the standard LOWRY method with the BIO-RAD DC Protein Assay. The absorbance was measured at 750 nm using a spectrophotometer (Sunrise™) and the Magellan™ software.

Proteins were separated using SDS-PAGE. Most commonly 5% stacking gels and 7.5% resolving gels were used. For LC3B detection, gradient gels (4-20%) were employed. An equal amount of proteins was loaded for each sample. The gels were placed into a water-cooled electrophoresis chamber filled with the electrophoresis buffer and the proteins were separated based on their molecular weights. The separated proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes using the BioRad Trans-Blot water-cooled tank transfer system, which allows the negatively charged proteins to move from the gel towards the positively charged electrode, thus getting immobilized on the PVDF membrane.

After blotting the membranes were washed with TN-buffer and blocked with 5% non-fat dry milk in TN-Tween buffer for 1 h at room temperature (RT). Next after washing off the residual blocking milk with TN-Tween, the membranes were incubated overnight with the respective primary antibodies (enlisted in table 5) at 4 °C. The next day, the unbound antibody was washed away with TN-Tween and the membranes were incubated with peroxidase-coupled secondary antibody at RT for 1 h. After one more round of washing, the proteins on the membranes were visualized using Amersham™ ECL/ ECL+ Western Blotting Detection Reagents and developed on films using Kodak GBX developer and fixing reagents. The films were then scanned and quantified using the Image J program (National Institutes of Health, Bethesda, Maryland, USA).

If the same membrane had to be probed with another antibody, it was stripped to remove the previously bound antibody at 54 °C for 35 min with gentle shaking. Then, the membrane washed with TN buffer and blocked with milk following the same steps as mentioned above.

Table 13: Buffer and gel constituents for western blotting

All buffers used for western blotting were prepared in ultrapure water and are enlisted below.

Buffer/ gel	components	Concentration/ Volume

Wash buffer (pH 7.4)	Tris NaF $Na_4P_2O_7$ EDTA EGTA Na_3VO_4 DTT	50 mM 50 mM 10 mM 2 mM 1 mM 1 mM 1 mM
Lysis buffer	wash buffer (pH 7.4) with: Triton X-100 SDS freshly added: PMSF PIC	 1% 0.1% 1 mM 10 µl/ml
Laemmli buffer (pH 6.8)	Tris EDTA Glycerol SDS β-Mercaptoethanol bromophenol blue	186 mM 10 mM 15% 9% 6% 0.03%
Protein standard (Lowry)	BSA	10 mg (in 10 ml 0.9% NaCl solution)
Tris/HCl buffer (pH 8.8) For resolving gel	Tris	1.875 M
Tris/HCl buffer (pH 6.8) for stacking gel	Tris	1.25 M
Resolving gel 7.5%	Water Tris/HCl (pH 8.8) Acrylamide 10% SDS APS (0.5 g in 1.5 ml water) TEMED	16 ml 6 ml 7.6 ml 300 µl 100 µl 15 µl
Stacking gel 5%	Water Tris/HCl (pH 6.8) Acrylamide 10% SDS APS TEMED	3.6 ml 0.5 ml 0.8 ml 50 µl 17 µl 5 µl
Gradient gel 4%	Water Tris/HCl (pH 8.8) Acrylamide 10% SDS APS (0.5 g in 1.5 ml water) TEMED	4.57 ml 1.4 ml 935 µl 70 µl 24 µl 3.5 ml
Gradient gel 20%	Water	830 µl

	Tris/HCl (pH 8.8) Acrylamide 10% SDS APS (0.5 g in 1.5 ml water) TEMED	1.4 ml 4.67 ml 70 µl 24 ml 3.5 µl
Stacking gel 3% for gradient gel	Water Tris/HCl (pH 6.8) Acrylamide 10% SDS APS TEMED	4.62 ml 0.6 ml 0.7 ml 60 µl 20 µl 6 µl
Electrode buffer	Tris Glycine SDS	25 mM 192 mM 1%
Blotting buffer	Tris Glycine Methanol	25 mM 192 nM 10%
TN buffer (pH 7.6)	Tris NaCl	10 mM 1.5 M
TN-Tween buffer	TN buffer with: Tween® 20	 0.1%
Stripping buffer (pH 6.7)	Tris SDS β-Mercaptoethanol	62.5 mM 2% 100 mM

2.2.7 Molecular biology techniques

2.2.7.1 RNA extraction and reverse transcription polymerase chain reaction (RevT-PCR)

To study the expression of genes involved in inflammatory and interferon pathways against HSV-1 infection, cells were seeded in 30 mm-dishes at densities mentioned in table 10 and grown to confluence. Cells were incubated with AMPK activators for the indicated times and/or infected. After treatments, cells were lysed, and RNA was isolated using the column-based method by the Macherey-Nagel RNA isolation kit according to the manufacturer's protocol.

After isolation, the RNA concentrations and purity were determined using the Nanodrop spectrophotometer. Equal RNA concentrations (500-800 ng) from each sample were converted to cDNA by RevT-PCR using the First Strand cDNA Synthesis Kit by Thermo Fischer Scientific. Respective RNA amounts were brought to a final volume of 10 µl with RNase-free water and 1 µl of Oligo(dT)-Primer was added to each

sample. The samples were then incubated at 65 °C for 5 min. 9 µl of master mix (containing 4 µl 5x reaction buffer, 1 µl RiboLock RI, 2 µl dNTP-Mix and 2 µl reverse transcriptase for each sample) was added. Reverse transcription was performed by incubating the samples at 37 °C for 60 min followed by 5 min at 70 °C to inactivate the enzymes. The cDNA was then stored at -20 °C until further use.

2.2.7.2 Real-time PCR

The prepared cDNA samples were used for real-time PCR using the SYBR™ Green qPCR Master mix by Thermo Fischer Scientific. The master mix consists of buffer, thermostable DNA polymerase, deoxy-ribonucleotide triphosphates (dNTP's) and the SYBR™ Green dye.

Each sample was measured in triplicates for each specific primer in a 96-well plate (the reaction mixture consisted of 10 µl of SYBR™ Green master mix, cDNA and water + 2 µl of the specific primer pair in each well). All the primers used are enlisted in table 7. PCR was performed, where the increased emission of the SYBR™ Green bound to the double-stranded DNA product of interest, amplified using the specific primer, was recorded. Mean value of the triplicates per sample was normalized to the expression of the house-keeping gene (β-actin) and represented as graphs.

2.2.8 Flow cytometry

2.2.8.1 Cytokine measurement

To measure the protein concentration of cytokines involved in inflammation and interferon response, the LEGENDplex™ Human Inflammation Panel (13-plex or 13-analytes) was used. Cells were seeded onto 24-well plates at densities mentioned in table 10 and grown until confluence. Cells were incubated with AMPK activators for the indicated times and/or infected. After the treatments, cell supernatants were collected in Eppendorf tubes and PIC was added to each tube to inhibit proteases. Each supernatant was mixed well, divided into three Eppendorf tubes, snap-freezed in liquid nitrogen and stored at -80 °C until further use.

On the day of the assay, one tube of each sample was thawed and centrifuged to precipitate cell debris. The manufacturer's protocol for V-bottom plate for cell culture supernatants was followed (displayed in figure 10), after which the beads were analyzed by flow cytometry.

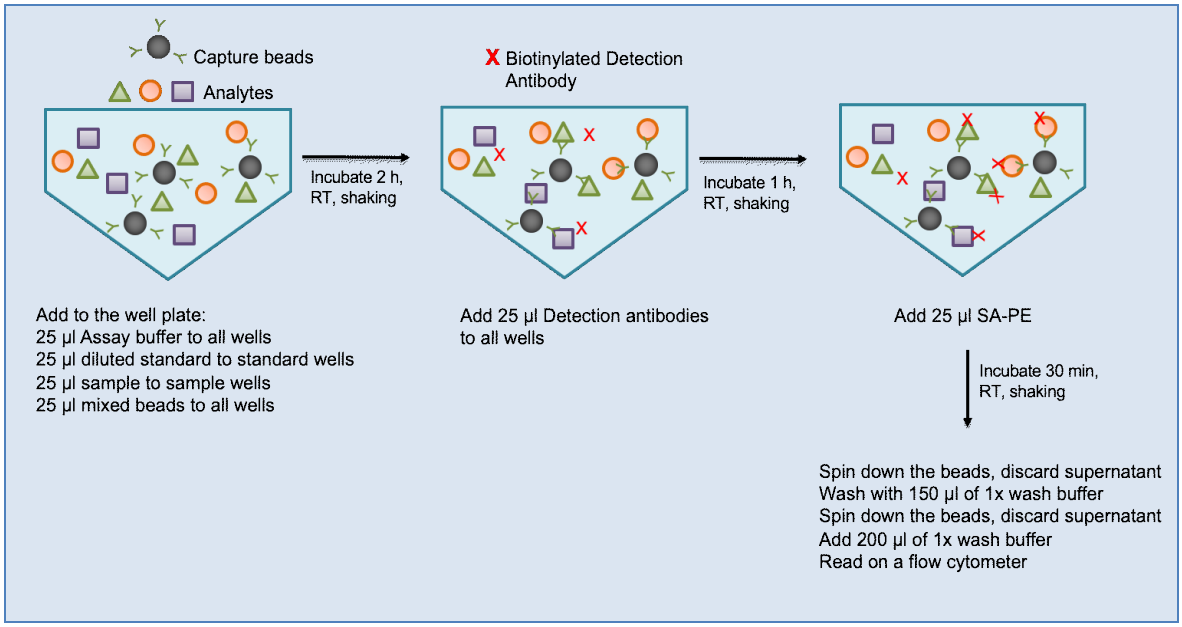


Figure 10: LEGENDplex™ assay protocol for V- bottom plate
Each sample was measure in duplicates in a V-bottom plate. Assay protocol as shown in the figure was followed for each sample. (Modified from BioLegend.com)

The principle of the assay as shown in figure 11 involves segregation and capturing analyte-specific populations based on size and phycoerythrin (PE) fluorescent signal of the bound beads. The concentration of each analyte is then determined using the standard curve run in the same assay by using the LEGENDplex™ software.

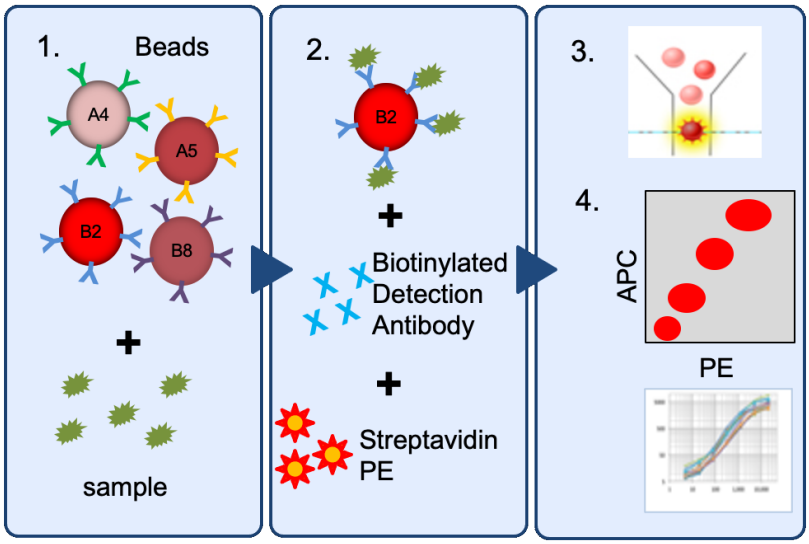


Figure 11: Principle of LEGENDplex™ assay
The principle for the assay is based on segregation and capturing analyte-specific populations based on size and PE fluorescent signal of the bound beads. 1: sample and beads are mixed. 2: samples bound to the beads are then mixed with biotinylated Detection Antibody and Streptavidin-PE. 3. The samples are then acquired on a flow cytometer. 4. Acquired samples

are run through the LEGENDplex™ software and the concentration of each analyte is determined by using standard curves (Modified from BioLegend.com).

2.2.9 Metabolism

2.2.9.1 Incorporation of ^{14}C-acetate

To investigate the role of AMPK in lipid synthesis, the incorporation of radiolabeled acetate (^{14}C-sodium acetate) was measured. Since acetate is incorporated into FA and cholesterol, the values primarily point to FA and cholesterol synthesis in cells. Cells were seeded in 12-well plates at densities mentioned in table 10. Each condition was measured in duplicates. Cells were treated with A76 and SC4 for 12 or 24 h (with re-addition at 12 h) and 4 h prior to the end of the stipulated time, the growth medium was replaced with acetate medium (table 14). Finally, the medium was removed, and the cells were washed thrice with 0.5 ml Hepes/$CaCl_2$ on ice. Then, 0.2 ml of 50 mM Tris-buffer was added, and cells were scraped and transferred to Eppendorf tubes. The wells were washed with 0.25 ml methanol, which was also transferred to the respective Eppendorf tubes. Subsequently, 0.25 ml of methanol and chloroform was added to every tube. Samples were vortexed for 1 min, incubated for 15 min on ice and vortexed intermittently. To achieve organic and aqueous phase separation, 0.25 ml chloroform, 0.25 ml of 0.1 M KCL and 2 µl citric acid to decrease pH and enhance extraction, were added to each tube. Samples were vortexed for 1 min and incubated for 5 min on ice and then centrifuged at 500 x g for 5 min. The lower organic phase was transferred to a 10 ml vial. The chloroform was vaporized and 0.25 ml of 1% Triton was added to solubilize the lipids. The vials were vortexed and left at room temperature for about 1 h before 5 ml of scintillation cocktail was added. After mixing the vials the radioactivity was measured and the incorporation of ^{14}C-acetate was determined. In parallel, samples under same experimental procedure were lysed using solubilization buffer to determine the protein content using LOWRY method. The radioactivity values were then normalized to protein content.

Table 14: Medium and buffers for incorporation of ^{14}C-acetate

Medium/Buffer	Components	Concentration
Acetate containing medium	M199	
	fatty acid free BSA	0.25%
	Biotin	500 pM
	L-Carnitine	50 µM
	L-Glutamine	680 µM

	ECGS ^{14}C-acetate	2.5 µg/ml 0.5 µCi/ml
Solubilization buffer	NaOH Na_2CO_3 SDS	100 mM 1.9 M 1%
Hepes Buffer (pH 7.4)	NaCl KCl $MgSO_4$ Hepes-buffer substance Glucose	145 mM 5 mM 1 mM 10 mM 10 mM
Hepes/$CaCl_2$	Hepes (pH7.4) $CaCl_2$	10 mM 1.5 mM
Tris-buffer (pH 7.5)	Tris	50 mM
KCl	KCl	0.1 M (7.45 g) in 1 liter Millipore water
Citric acid	Citric acid	1 M (1.92 g) in 10 ml Millipore water
Triton-X-100	Trition-X-100	1%

2.2.10 Statistical Analysis

Information regarding number of subjects used for each experiment is given respectively in the figure legends. Paired and unpaired (for MLEC) two-tailed Student's t-test and one-way or two-way repeated measurements analysis of variance (ANOVA) were used for significance calculation after variance analysis with Holm-Sidak correction for multiple comparisons. $p<0.05$ was considered as significant. Data in the figures are represented as mean ± SEM of independent experiments performed with cells from different donors.

3. RESULTS

3.1 HSV-1 replicates in endothelial cells

The first aim of our study was to characterize virus replication in HUVEC and MLEC. We used the $TCID_{50}$ method to determine the viral spreads at different time points post infection in the cells. Figure 12 shows a time-dependent increase in HSV-1 concentration in HUVEC (A) and MLEC (B) confirming that HSV-1 replication does occur in endothelial cells.

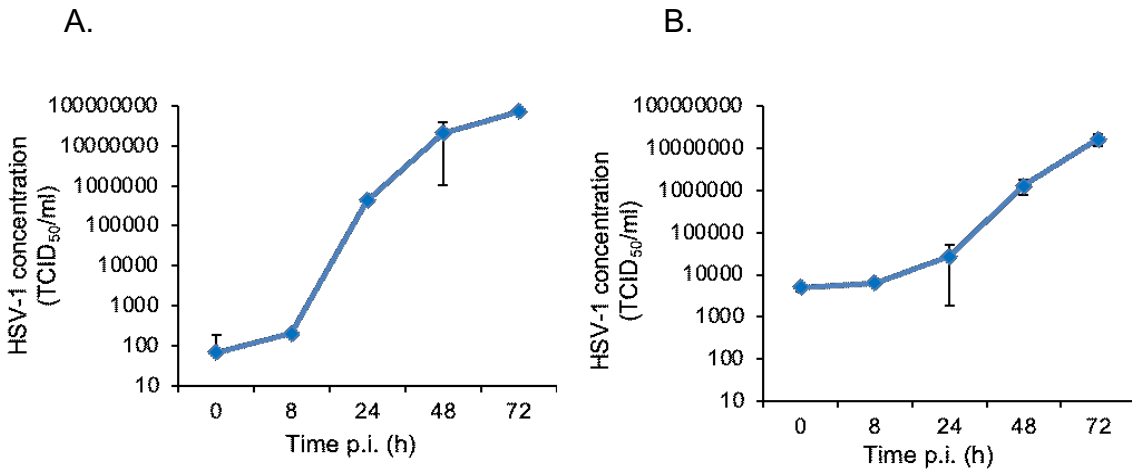


Figure 12: HSV-1 replicates in endothelial cells
HUVEC (A) and MLEC (B) were infected with HSV-1 at the dose of 5 m.o.i. and samples were studied from 0-72 h p.i. Combined intra- and extracellular virus titers are demonstrated as mean ± SEM generated from three different cell batches.

3.2 HSV-1 infection induces alteration of cellular signaling pathways in endothelial cells

Conventionally, viral replication relies on intense interactions with host cellular signaling pathways. To understand whether HSV-1 infection of endothelial cells affects major pathways related to proliferation, survival, metabolism and stress adaptation, HUVEC were lysed after 2 h or 24 h of HSV-1 infection and subjected to western blot analyses for different cellular proteins (figure 13-15).

3.2.1 Akt and mTORC1

The protein kinase Akt is a major regulator of survival and growth. Figure 13 A shows that infection of endothelial cell with HSV-1 for 24 h led to an increased Akt

phosphorylation at S473 while short-term infection of 2 h was not sufficient to induce this response. The expression of Akt was not altered in both conditions. The activation of Akt was paralleled by an enhanced phosphorylation of p70S6 kinase, a well-known downstream target of mTORC1 suggesting that HSV-1 infection activates the Akt/mTORC1 pathway (figure 13 B). Raptor, a component of the mTORC1 complex, showed a moderately increased phosphorylation at S792, an inhibitory site, after 2 h suggesting an inhibition by HSV-1 infection at this time while no significant changes were seen after 24 h (figure 13 C).

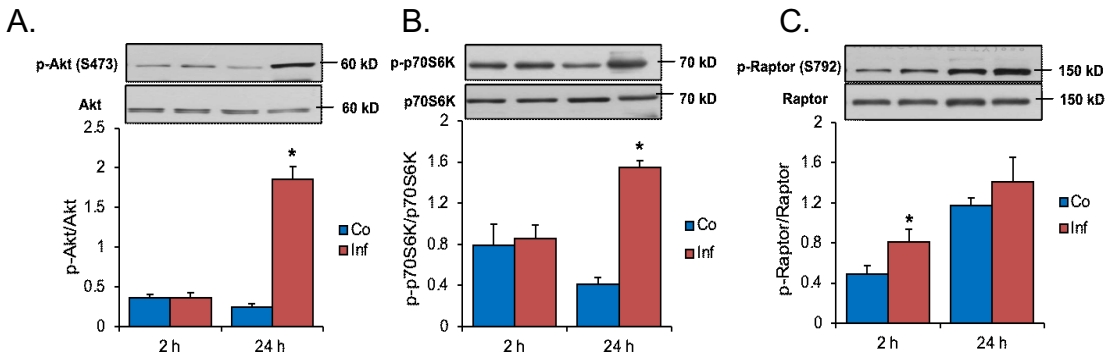


Figure 13: HSV-1 infection stimulates the Akt/mTOR pathway
HUVEC were infected with HSV-1 for 2 or 24 hours, lysed and compared to non-infected control cells in western blot analyses. Representative immunoblots and densitometry analysis for phospho-Akt, phospho-p70S6K and phospho-raptor normalized to protein expression are shown as mean + SEM from three different cell batches. * p value < 0.05 versus uninfected controls of the respective times.

3.2.2 ERK and JNK

We next checked for changes in the expression and activation of ERK and JNK, major protein kinases involved in growth and stress response. While the expression of these proteins in endothelial cells was not altered by HSV-1 infection and no changes in phosphorylation occurred after 2 h of infection, an increase in JNK phosphorylation at Thr183/Tyr185 was observed after 24 h (figure 14 B). In contrast, ERK phosphorylation at T202/T204 was decreased after 24 h of HSV-1 (figure 14 A). These data point to an activation of the JNK pathway while the mitogenic ERK pathway was inhibited.

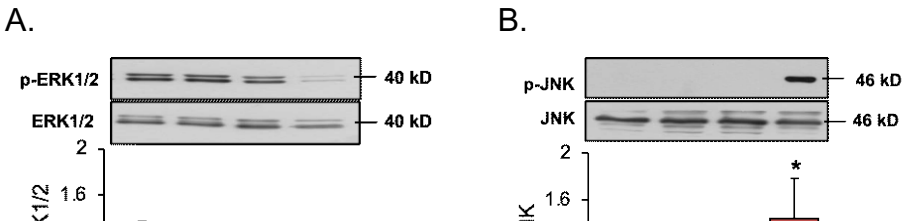
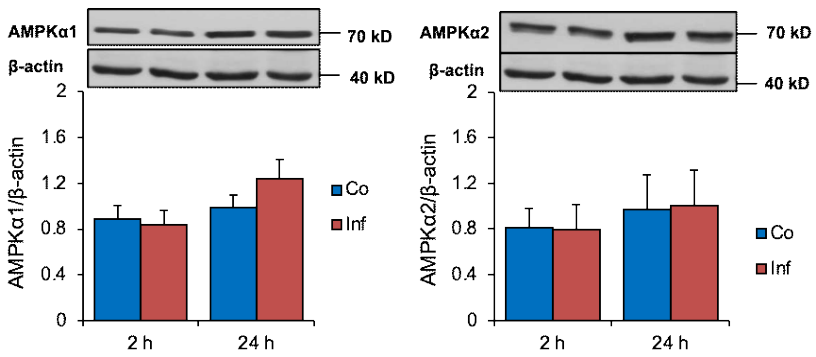


Figure 14: HSV-1 infection stimulates the JNK pathways while ERK activation is inhibited
HUVEC were infected with HSV-1 for 2 or 24 hours, lysed and compared to non-infected control cells in western blot analyses. Representative immunoblots and densitometry analysis for phospho-ERK (A) and phospho-JNK (B) normalized to protein expression are shown as mean + SEM from three different cell batches. * p value < 0.05 versus uninfected controls of the respective times.

3.2.3 AMPK and ACC

We further checked whether HSV-1 infection modifies the expression and/or activity of AMPK. Figure 15 A shows that expression of AMPKα1 or α2 catalytic subunits were not altered in HSV-1-infected cells. In addition, no changes in the phosphorylation of AMPKα at the activation site T172 or the inhibitory site S485 were seen. In addition, phosphorylation of ACC at S79, a well-known AMPK site, remained unchanged (figure 15 B) indicating that HSV-1 infection did not stimulate the AMPK pathway.

A.



B.

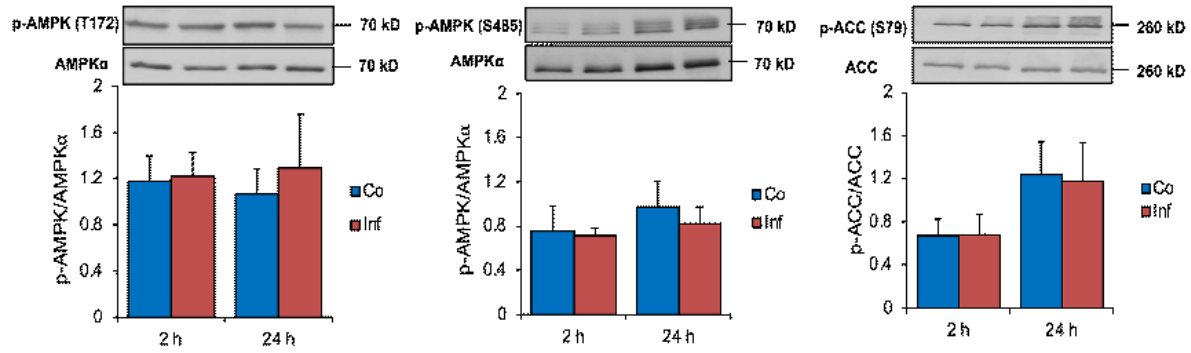


Figure 15: HSV-1 infection does not alter AMPK expression or activation of the AMPK/ACC pathway in endothelial cells
HUVEC were infected with HSV-1 for 2 or 24 hours, lysed and compared to non-infected control cells in western blot analyses. Representative immunoblots and densitometry analysis are shown. (A). AMPKα1 and AMPKα2 expression normalized to β-actin from three different cell batches. (B). phospho-AMPK (T172), phospho-AMPK (S485) and phospho-ACC (S79) normalized to protein expression are shown as mean + SEM from six different cell batches.

3.3 The Akt/mTORC1 pathway is required for virus replication

Since HSV-1 infection led to an activation of the Akt/mTORC1 pathway (figure 13), we investigated whether this pathway is required for the replication of the virus. To this end, we treated endothelial cells with Akt inhibitor VIII (figure 16 A), which prevents activation of Akt and with Torin 2, a potent and selective ATP-competitive inhibitor of mTOR (figure 16 B).

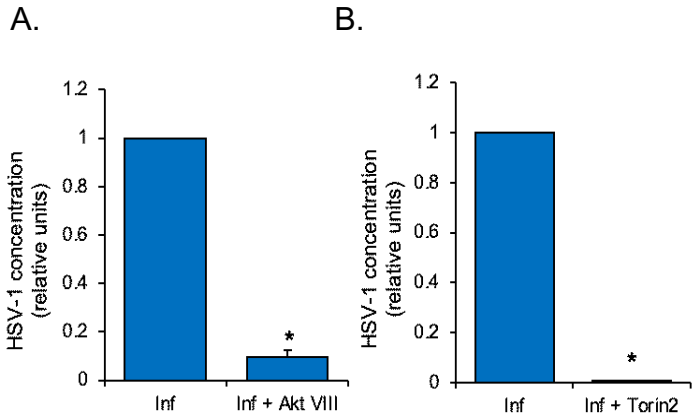


Figure 16: The Akt/mTORC1 pathway is required for HSV-1 replication in endothelial cells
HUVEC were pretreated with Akt inhibitor VIII (20μM) (A) or Torin 2 (100 nM) (B) for 1 hour and thereafter infected with HSV-1 for 24 h. Relative HSV-1 concentrations compared to untreated controls are demonstrated as mean + SEM generated from four different cell batches by the $TCID_{50}$ method. * $p < 0.05$ versus untreated cells.

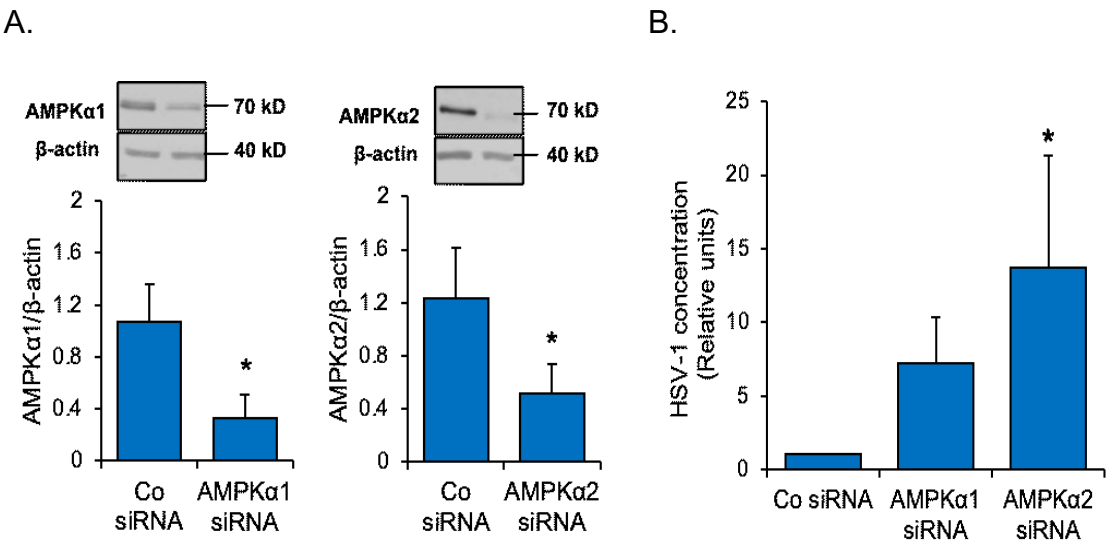
Both compounds strongly suppressed HSV-1 replication in endothelial cells indicating that the Akt/mTORC1 pathway plays an essential role in HSV-1 progeny production (figure 16).

3.4 AMPK inhibits virus replication in endothelial cells

Although no changes were seen in AMPK signaling upon HSV-1 infection (figure 15), we checked whether viral replication in endothelial cells was affected by changes in with AMPK expression or activation (figure 17-19).

3.4.1 AMPK downregulation promotes viral replication in endothelial cells

As a first approach, we applied the siRNA technology to downregulate the catalytic subunits AMPKα1 or AMPKα2 in HUVEC. Efficient downregulation of AMPK isoforms by siRNA technology is shown in figure 17 A. HSV-1 replication was higher upon downregulation of either AMPK isoform and was significantly enhanced in cells where AMPKα2 was downregulated (figure 17 B). Secondly, we validated these results in MLEC prepared from wild type and AMPKα2-/- mice and found an increased HSV-1 replication in AMPKα2-/- MLEC when compared to wild type cells (figure 17 C). These results hint at a protective role of AMPK against HSV-1 replication in endothelial cells.



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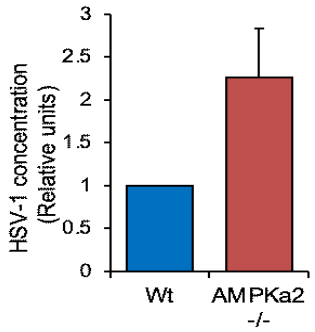


Figure 17: AMPK down regulation promotes HSV-1 replication in endothelial cells
HUVEC were transfected with control siRNA or AMPKα1 or AMPKα2 siRNA (0, 5 µg/ml, 72 h). After 72 h, transfected cells were infected with HSV-1 (B) for 24 h. Relative HSV-1 concentrations to control siRNA are demonstrated as mean + SEM generated from five different cell batches by the $TCID_{50}$ method. * $p < 0.05$ vs. control siRNA-treated cells. (C). MLEC obtained from wild type (Wt) and AMPKα2-/- mice were infected with HSV-1 for 24 hours. Relative HSV-1 concentrations to Wt are demonstrated as mean + SEM generated from six different mice by the $TCID_{50}$ method. (A). Cell lysates were analyzed for the indicated proteins in western blots and representative blots and densitometric evaluation indicate efficient transfection of the AMPK isoforms demonstrated as mean + SEM generated five different cell batches. * $p < 0.05$ versus control siRNA-treated cells.

3.4.2 Viral replication in endothelial cells is controlled by AICAR

We next addressed the question of how activation of AMPK, a cellular master regulator, affects viral replication and progeny production in endothelial cells. We used a pharmacological AMPK activator, AICAR, which activates AMPKα isoforms by mimicking the action of AMP. We found a significant dose-dependent decrease in HSV-1 replication in HUVEC after 24 h of infection (figure 18).

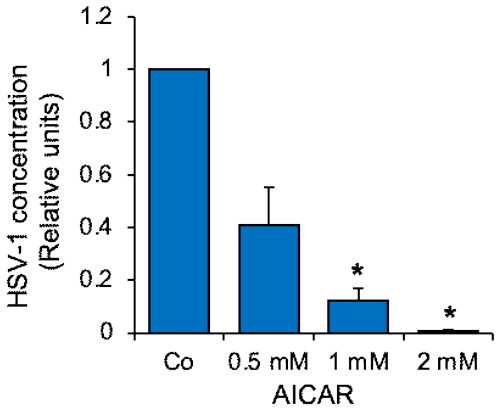


Figure 18: HSV-1 replication in endothelial cells is controlled by AICAR
HUVEC were pretreated with AICAR at the indicated concentrations for 1 hour and then infected with HSV-1. Relative viral concentrations to untreated control are demonstrated as mean + SEM generated from three different cell batches. * *p* value < 0.05 versus untreated control.

3.4.3 Pharmacological AMPK activators inhibit HSV-1 replication in endothelial cells

Since AICAR is an indirect AMPK activator, which mimics the action of AMP and may have off-target effects, we additionally tested the influence of the direct AMPK activators A769662 (A76) and SC4 on HSV-1 replication in endothelial cells. A76 displays selectivity towards β1 subunit-containing AMPK heterotrimers [10], while SC4 has a higher affinity to heterotrimers containing the catalytic subunit α2 [11]. Preincubation of cells for 1 h with A76 (10 µM or 50 µM) or SC4 (0.1, 1 or 10 µM) prior to infection and infection of HSV-1 for 24 h did not alter HSV-1 replication in endothelial cells although AMPK activation was observed under these conditions (data not shown). However, 1 h preincubation of the compounds plus a re-addition after 12 h of infection resulted in a significant inhibition of HSV-1 (Figure 19 A, B), which seemed not to be related to the dose of the compounds. Together, these data support the finding that AMPK activation is able to counteract HSV-1 replication in endothelial cells.

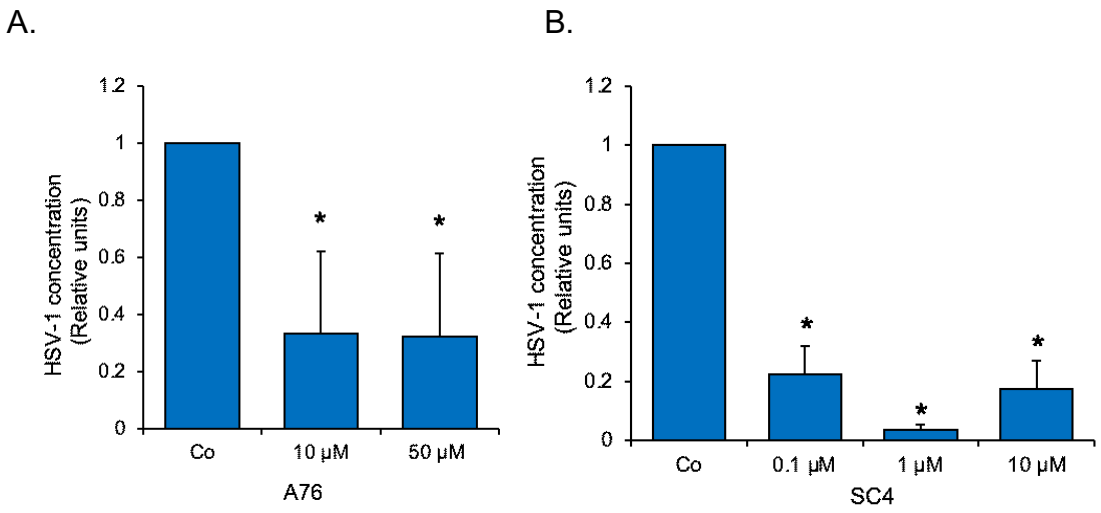


Figure 19: Pharmacological activation of AMPK inhibits HSV-1 replication in endothelial cells
HUVEC were pretreated with A76 (A) or SC4 (B) at the indicated doses for 1 hour and thereafter infected with HSV-1. The compounds were not re-added. Relative HSV-1 concentrations to untreated controls are demonstrated as mean + SEM generated from five different cell batches for A76 (A) and one batch for SC4 (B). HUVEC were pretreated with A76 (C) or SC4 (D) at the indicated doses for 1 hour and thereafter infected with HSV-1. The compounds were re-added after 12 h after infection. Relative HSV-1 concentrations to untreated controls are demonstrated as mean + SEM generated from five different cell batches. * p value < 0.05 versus untreated control.

3.4.4 Pharmacological AMPK activators inhibit mTORC1

AMPK is known to negatively regulate mTORC1. We thus tested whether treatment of endothelial cells with A76 and SC4 leads to alterations of mTORC1 activation. Figure 20 shows that both compounds show a tendency to increase the phosphorylation of raptor (figure 20 A), a component of mTORC1, at its inhibitory site, and decrease the phosphorylation of p70S6 kinase (figure 20 B), a well-known downstream target of mTORC1 after 12 h of stimulation as compared to untreated cells. The downregulation of p70S6K reached significance with 12 h of SC4 treatment. These data confirm that activation of AMPK inhibits the mTORC1 pathway and suggest that this is one of the mechanisms underlying the inhibition of HSV-1 replication by AMPK activators.

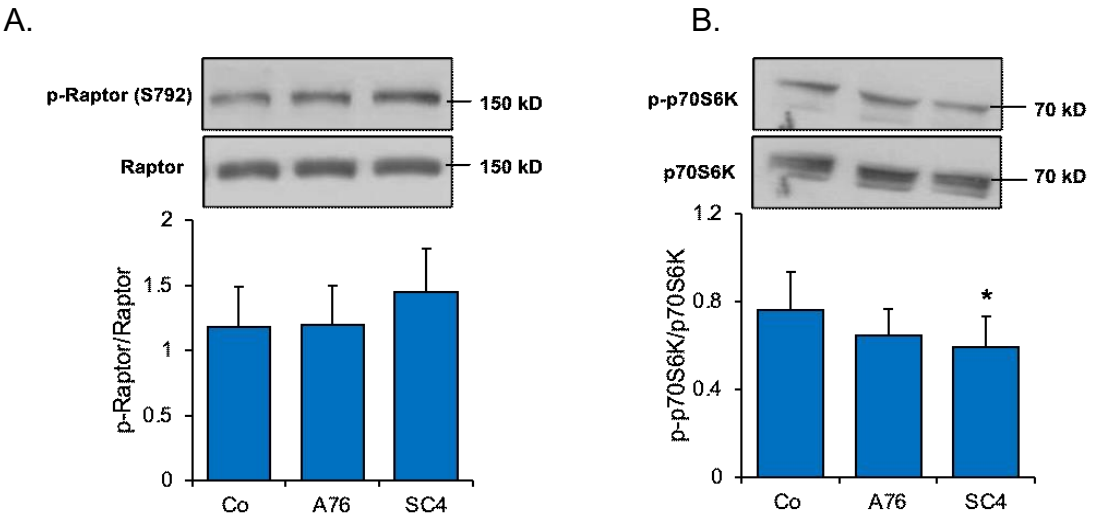


Figure 20: Pharmacological AMPK activators inhibit mTORC1 in endothelial cells
HUVEC were pretreated with 10 µM of A76 or SC4 for 1 hour and the compounds were left for 12 h. After incubation with the compound's cells were lysed and compared to non-treated control cells in western blot analyses. Representative immunoblots and densitometry analysis for phospho-raptor (A) and phospho-p70S6K (B) normalized to protein expression are shown as mean + SEM from five different cell batches. * p value < 0.05 versus untreated control.

3.5 HSV-1 infection alters the autophagy pathway in endothelial cells

Since mTORC1 and AMPK were shown to affect HSV-1 replication in an opposite way and both are involved in the negative or positive regulation of autophagy, respectively, we decided to explore whether virus infection affects autophagy. HUVEC were lysed after 2 h or 24 h of HSV-1 infection and subjected to western blot analyses for different autophagy proteins (figure 21-23).

3.5.1 ULK1/ATG14

The protein kinase ULK1 plays a central role in initiating autophagy by integrating signals from mTOR and AMPK and transducing them to the downstream autophagy pathway. Consistent with the findings of unchanged AMPK and increased mTOR activities upon HSV-1 infection, we found almost no changes in the phosphorylation of ULK1 at S556 (figure 21 A), the AMPK phosphorylation site, but a slightly increased phosphorylation at S757 (figure 21 B), the mTOR phosphorylation site, after 24 h of HSV-1 infection. The latter is known to mediate inhibition of ULK1 and accordingly, the phosphorylation of ATG14, an ULK-1 substrate and member of the VPS34/PI3K complex involved in autophagosome nucleation, was decreased (figure 21 C). Thus, HSV-1 infection seems to interfere with autophagy induction [79,93].

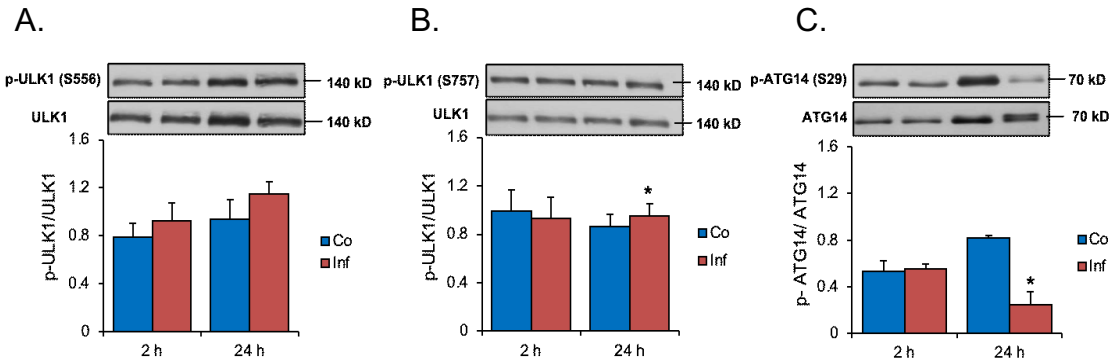


Figure 21: HSV-1 infection inhibits ULK1
HUVEC were infected with HSV-1 for 2 or 24 hours, lysed and compared to non-infected control cells in western blot analyses. Representative immunoblots and densitometry analysis for phospho-ULK1 S556 (A) and S757 (B) and phospho-ATG (S29) (C) normalized to protein expression are shown as mean + SEM from three to five different cell batches. * p value < 0.05 versus uninfected controls of the respective times.

3.5.2 Beclin1

We also looked at beclin1, another member of the VPS34/PI3K complex involved in autophagy induction. Figure 22 shows that HSV-1 infection of endothelial cells resulted in phosphorylation of beclin1 at S15 (ULK1 phosphosite) (A) and at S93 (AMPK phosphosite) (B), which are both indicative of autophagy induction. This contradicts the observed inhibition of ULK1 in response to HSV-1 infection, which suggested reduced autophagy. These data may point to a role of beclin1 in HSV-1 replication independent of autophagy and controlled by kinases other than ULK1.

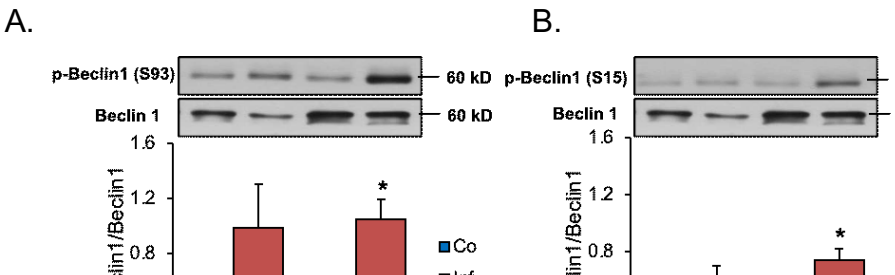
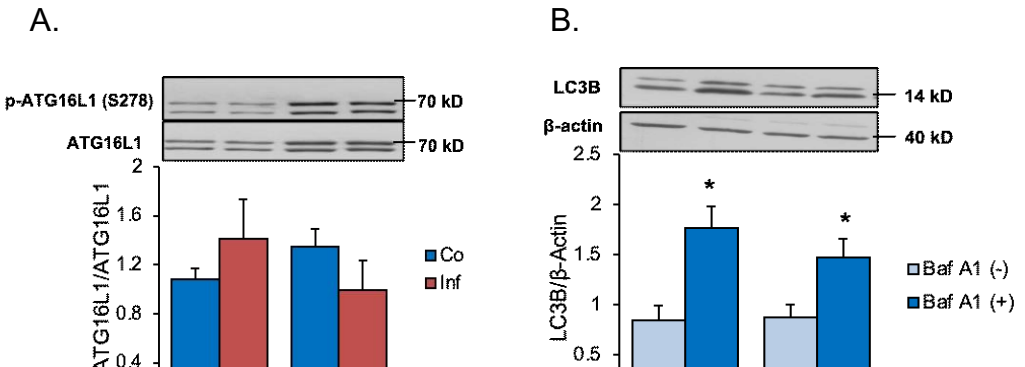


Figure 22: HSV-1 infection stimulates beclin1
HUVEC were infected with HSV-1 for 2 or 24 hours, lysed and compared to non-infected control cells in western blot analyses. Representative immunoblots and densitometry analysis for phospho-beclin1 (S15) (A) and phospho-beclin1 (S93) (B) normalized to protein expression are shown as mean + SEM from three different cell batches. * p value < 0.05 versus uninfected controls of the respective times.

3.5.3 Autophagy markers

To verify whether and how the alterations in early autophagy signaling in HSV-1-infected cells, i.e., ULK1 inhibition and beclin1 activation, affect the process of autophagy, we looked at phosphorylation of ATG16L1 at S278, which is indicative of autophagosome maturation. We observed only tendencies to higher ATG16L1 phosphorylation after 2 h and to lower values after 24 h of HSV-1 infection in endothelial cells (figure 23 A). Next, we checked for LC3B conjugation as an indicator of autophagosome formation. To increase the visibility of possible effects, we inhibited lysosomal degradation of LC3B by employing bafilomycin A1. This treatment led to enhanced accumulation of conjugated LC3B within 24 h in both control and HSV-1-infected cells with moderately lower values in the latter (figure 23 B). Finally, we performed measurements of autophagic flux via time-dependent monitoring of LC3B conjugation in the presence of bafilomycin A1. We found that accumulation of conjugated LC3B occurred with a comparable kinetics in control cells and HSV-1-infected cells up to 90 min but was lower at 120 min (figure 23 C). Together, these data indicate that the net impact of HSV-1 infection, which had opposing effects on the activities of the autophagy initiators ULK1 and beclin1, is small and may be moderately inhibitory.



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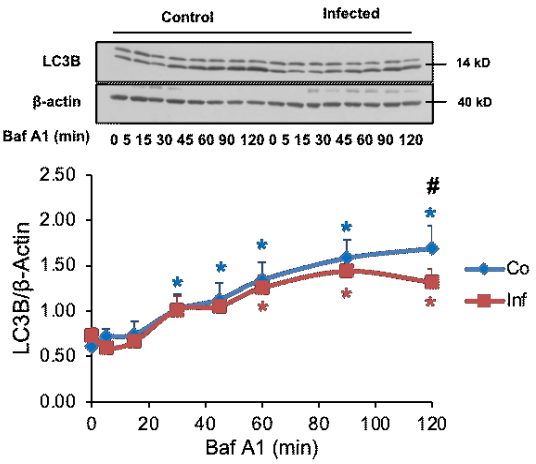

Figure 23: HSV-1 infection interferes with the autophagy pathway
HUVEC were infected with HSV-1 for respective times, lysed and compared to non-infected control cells in western blot analyses. For long-term LC3B monitoring (A), cells were preincubated with 50 nM bafilomycin A1 (Baf A1) for 1 hour. For short-term flux measurement (C), cells were treated with 50 nM bafilomycin A1 (Baf A1) for the indicated times. Representative immunoblots and densitometry analysis for phospho-ATG16L1 (S278) (A) and LC3B normalized to β-Actin are shown as mean + SEM from three - five different cell batches. * *p* value < 0.05 versus uninfected control/untreated control, # *p* value < 0.05 versus uninfected controls for the respective times.

3.6 ULK1 and beclin1 protect endothelial cells from HSV-1 replication

Although autophagy was little affected by HSV-1 infection, it still could play a role in mediating or inhibiting virus replication. To explore this, we performed experiments in cells, in which ULK1 or beclin1 were downregulated by siRNA technology. Previous work from our group had revealed that under these conditions autophagy was blocked [77]. Figure 24 A shows that downregulation of both kinases was successful. In parallel, an enhanced virus production was observed in cells lacking ULK1 and beclin1, which reached statistical significance in cells with ULK1 downregulation (figure 24 B). These findings indicate that ULK1 and beclin1 restrain HSV-1 replication in endothelial cells

and suggest that autophagy may protect from virus replication. However, additional or alternative mechanisms through which ULK1 and beclin1 may act, cannot be excluded.

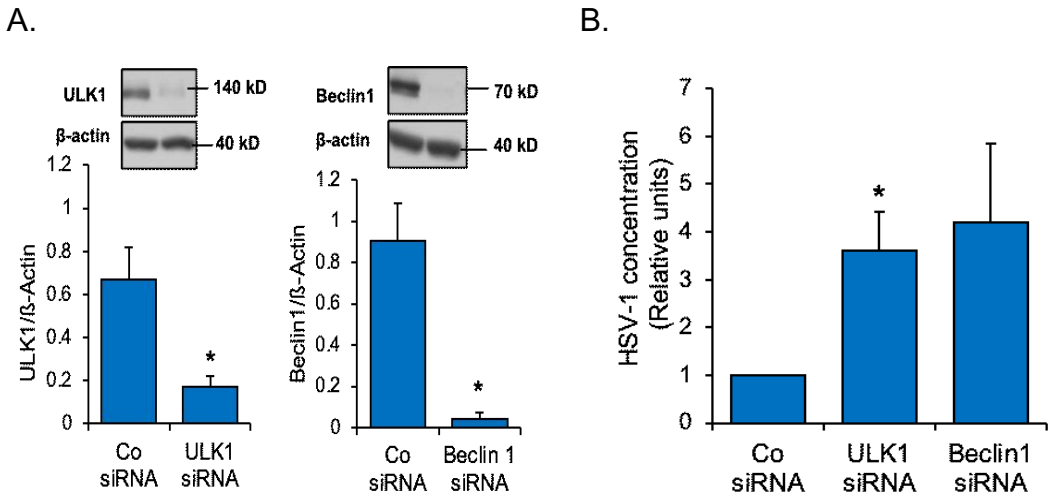

Figure 24: ULK1 and beclin1 play a protective role against HSV-1 replication in endothelial cells
HUVEC were pretreated with control siRNA, ULK1 or beclin1 (0, 5 µg/ml, 72 h) and thereafter infected with HSV-1 for 24 h. (A). Cell lysates were analyzed for the indicated proteins in western blots. Representative blots and densitometric evaluation indicate efficient transfection demonstrated as mean + SEM generated from four different cell batches. * $p < 0.05$ vs. control siRNA. (B). Relative HSV-1 concentrations to control siRNA are demonstrated as mean + SEM generated from six different cell batches by the $TCID_{50}$ method. * $p < 0.05$ versus control siRNA-treated cells.

3.7 AMPK inhibits HSV-1 replication in the absence of ULK1 or beclin1

Since AMPK is known to stimulate autophagy via phosphorylating ULK1 and beclin1, we wanted to check whether the protective effects of these three proteins on HSV-1 replication were linked. We asked whether the inhibition of HSV-1 replication by pharmacological AMPK activators was dependent on the presence of ULK1 and Beclin1 and therefore on initiation of autophagy by AMPK. To this end, the AMPK activators A76 and SC4, which had been shown to inhibit HSV-1 replication (see 3.4.3), were added to endothelial cells, in which ULK1 and beclin1 were downregulated by specific siRNAs. We found that the inhibition of HSV-1 replication by AMPK activators was still significant in the absence of ULK1 and beclin1 (figure 25), even though ULK1 and beclin1 depletion caused enhanced virus replication. Thus, the protective role of AMPK may not be mediated via ULK1 or beclin1-triggered autophagy and both proteins may have AMPK-independent anti-viral effects.

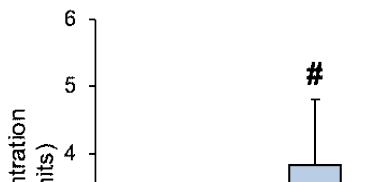

Figure 25: Inhibition of HSV-1 replication by AMPK is not mediated by ULK1 and/or beclin1
HUVEC were treated with siRNA directed against ULK1 or beclin1 (0, 5 µg/ml, 72 h). The cells were then pretreated with 10 µM of A76 or SC4 for 1 hour and thereafter infected with HSV-1 in the presence of the compounds for 24 h. A76 or SC4 were readded at 12 hours after infection. Relative HSV-1 concentrations to untreated cells obtained by the $TCID_{50}$ method are demonstrated as mean + SEM generated from three different cell batches. * p value < 0.05 versus untreated control of respective siRNA. # p value < 0.05 versus control siRNA-treated cells.

3.8 Autophagy is required for HSV-1 replication in endothelial cells

Next, we wanted to better understand whether the anti-viral effects of ULK1 and beclin1, although independent of AMPK, depend on autophagy and thus whether autophagy may protect from HSV-1 replication. This we blocked autophagy downstream from ULK1 or beclin1 by targeting LC3B, which is essentially involved in autophagosome formation. Figure 26 A shows that treatment of endothelial cells with specific siRNA led to an efficient downregulation of LC3B when compared to cells transfected with control siRNA. However, in contrast to what has been observed in cells lacking ULK1 or beclin1, LC3B depletion led to a moderate decrease in virus production (Figure 26 B). This suggested that autophagosome formation is required for virus replication. To substantiate these data, we used pharmacological tools to block autophagy, i.e., 3-methyladenine (Figure 26 C), which inhibits formation of autophagosomes by inhibiting the VPS34/PI3K complex, and bafilomycin A1 (Figure 26 D), which inhibits fusion between autophagosomes and lysosomes as well as lysosomal degradation. Both compounds led to a significant decrease in HSV-1 replication. Together, these results indicate that autophagosome maturation and autophagosome-lysosome fusion are beneficial for HSV-1 replication in endothelial

cells and contradict the suggestion that the anti-viral effects of ULK1 and beclin1 are mediated through autophagy.

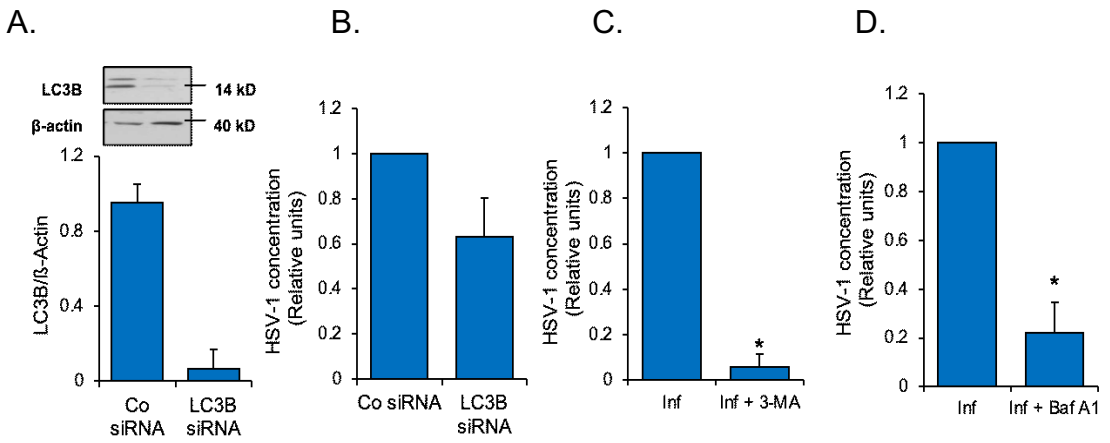

Figure 26: Autophagy is required for HSV-1 replication in endothelial cells
HUVEC were pretreated with control siRNA or LC3B siRNA (0,5 µg/ml, 72 h) (A, B), 3-methyladenine (10 mM) (C) or bafilomycin A1 (50 nM) (D) for 1 h and thereafter infected with HSV-1 for 24 h. (A). Cell lysates were analyzed for the indicated proteins in western blots. Representative blots and densitometric evaluation indicate efficient transfection demonstrated as mean + SEM generated from four different cell batches. (B, C, D). Relative HSV-1 concentrations are demonstrated as mean + SEM generated from three to six different cell batches by the $TCID_{50}$ method. * $p < 0.05$ versus untreated cells.

3.9 HSV-1 replication depends on cellular metabolism

To confirm a role of host cell metabolism in HSV-1 replication in endothelial cells we used 2-deoxy-D-glucose (2-deoxyglucose, 2-DG), a glucose analog which inhibits glycolysis through its action on hexokinase, the rate-determining step in glycolysis. Application of 2-DG to endothelial cells almost completely blocked HSV-1 replication (figure 27 A). We also applied simvastatin, a specific inhibitor of HMG-CoA reductase, the enzyme converts HMG-CoA to mevalonate, an early step in cholesterol biosynthesis. Endothelial cells pretreated with simvastatin showed a significant decrease in HSV-1 replication, thus hinting that cholesterol biosynthesis is essential for HSV-1 replication in endothelial cells (figure 27 B).

A. B.

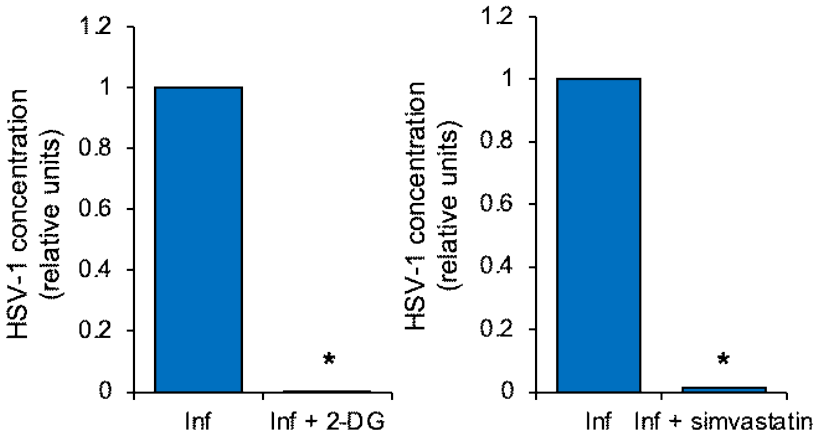

Figure 27: Metabolic inhibitors significantly decrease HSV-1 replication
HUVEC were pretreated with 20 mM 2-DG (A) or 10 µM simvastatin (B) for 1 hour and thereafter infected with HSV-1 for 24 h. Relative HSV-1 concentrations to untreated cells obtained by the $TCID_{50}$ method are demonstrated as mean + SEM generated from four (A) and three (B) different cell batches. * p value < 0.05 versus untreated cells.

3.10 ACC1 is required for HSV-1 replication

Cellular fatty acid metabolism is under the control of AMPK, which is known to target acetyl CoA carboxylase isoforms 1 and 2 (ACC1 and ACC2). ACC1 regulates fatty acid synthesis and ACC2 controls oxidation of fatty acids. AMPK activation inhibits activity of both ACC isoforms leading to inhibition of fatty acid synthesis and stimulation of fatty acid oxidation [52]. To clarify a possible role of the AMPK/ACC1 in HSV-1 replication in endothelial cells, we performed experiments in cells, in which ACC1 was downregulated using specific siRNA (figure 28 A). ACC1 depletion significantly inhibited virus replication in endothelial cells suggesting that HSV-1 replication requires fatty acid synthesis (figure 28 B), and that AMPK may protect from virus replication, at least in part, by inhibiting ACC1.

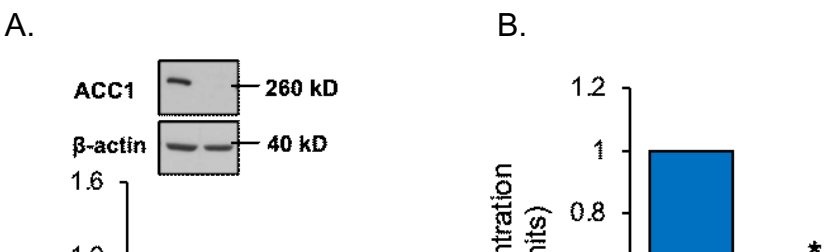

Figure 28: ACC1 is required for HSV-1 replication
HUVEC were pretreated with siRNA directed against Acetyl CoA carboxylase 1 (ACC1) (0, 5 µg/ml, 72 h) before infection with HSV-1. (A). Cell lysates were analyzed for the indicated proteins in western blots and representative blots and densitometric evaluation indicate efficient transfection demonstrated as mean + SEM generated from four different cell batches. (B). Relative HSV-1 concentrations to control siRNA obtained by the $TCID_{50}$ method are demonstrated as mean + SEM generated from six different cell batches. * $p < 0.05$ versus control siRNA-treated cells.

3.11 AMPK activators lead to reduced acetate incorporation in endothelial cells

To further substantiate that AMPK activation inhibits fatty acid synthesis, which could be one of the mechanisms restricting HSV-1 replication in endothelial cells, we analyzed the influence of AMPK activators on the incorporation of radiolabeled acetate into cells as a measure for lipid synthesis. Upon treatment with SC4 at 12 h, we saw significantly decreased acetate incorporation as compared to the control, thus confirming inhibition of fatty acid synthesis via AMPK activation as an antiviral response. Treatment of endothelial cells with A76 showed a tendency to lower acetate incorporation (figure 29).

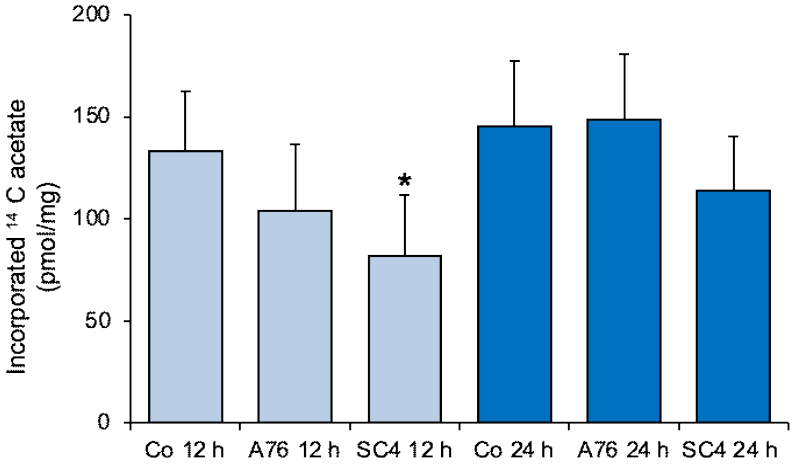

Figure 29: AMPK activation reduces acetate incorporation in endothelial cells
HUVEC were pretreated with 10 µM of A76 or SC4 for 1 h. After 12 or 24 h (with 12 h re-addition) of incubation with the compounds, ^{14}C-acetate containing medium was added to the cells for 4 h along with the compounds. Lipids were extracted via chloroform-methanol extraction. Incorporated ^{14}C-acetate is demonstrated as mean + SEM generated from six different cell batches. * p value < 0.05 versus 12 h untreated control.

3.12 HSV-1-infected endothelial cells exhibit an interferon response and increased inflammation

3.12.1 HSV-1 infection leads to increased inflammation in endothelial cells

Since virus infection of endothelial cells may induce an inflammatory response, we looked at the expression of inflammatory cytokines in endothelial cells infected with HSV-1 in a time dependent experiment. After infection of HUVEC with HSV-1 for 3, 6, 12 and 24 h, we observed a trend of an increased mRNA expression of IL-1β, TNFα, IL-6 and IL-8 upon HSV-1 infection. The expression of the genes was highest after 24 h of infection (figure 30 A-D).

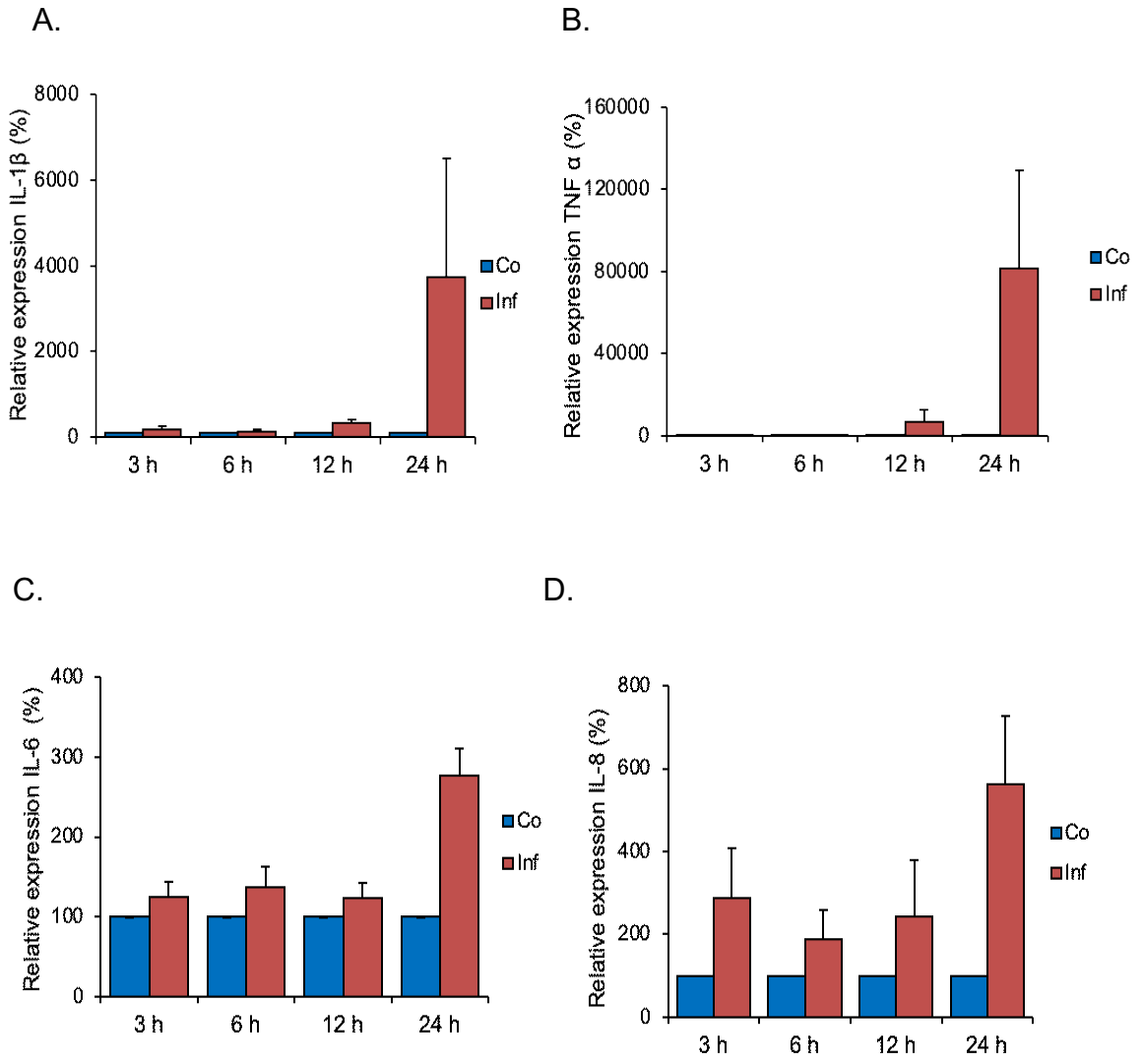

Figure 30: HSV-1 infection induces expression of inflammatory genes in endothelial cells
HUVEC were infected with HSV-1 for 3, 6, 12 and 24 h with corresponding non-infected controls. After respective times of incubation, the RNA was extracted, cDNA was prepared with equal concentrations of RNA. The cDNA's to be tested were then used to put up RT-PCR for Interferon IL-1β (A), TNFα (B), IL-6 (C) and IL-8 (D) genes with β-Actin as a housekeeping gene. Relative gene expression to uninfected control cells, normalized to β-Actin expression, for each time point are demonstrated as mean + SEM generated from four different cell batches.

To verify mRNA data on the protein level, we analyzed supernatants of infected cells for their cytokine content by flow cytometry using the LEGENDplex™ Human Inflammation Panel. We found a moderate time-dependent increase of IL-1β, IL-6 and IL-8 secretion under basal conditions, which was potentiated by HSV-1 infection of endothelial cells (figure 31 A, B, C), however reaching saturation after 12 h. The data also did not reach statistical significance. TNFα remained undetected.

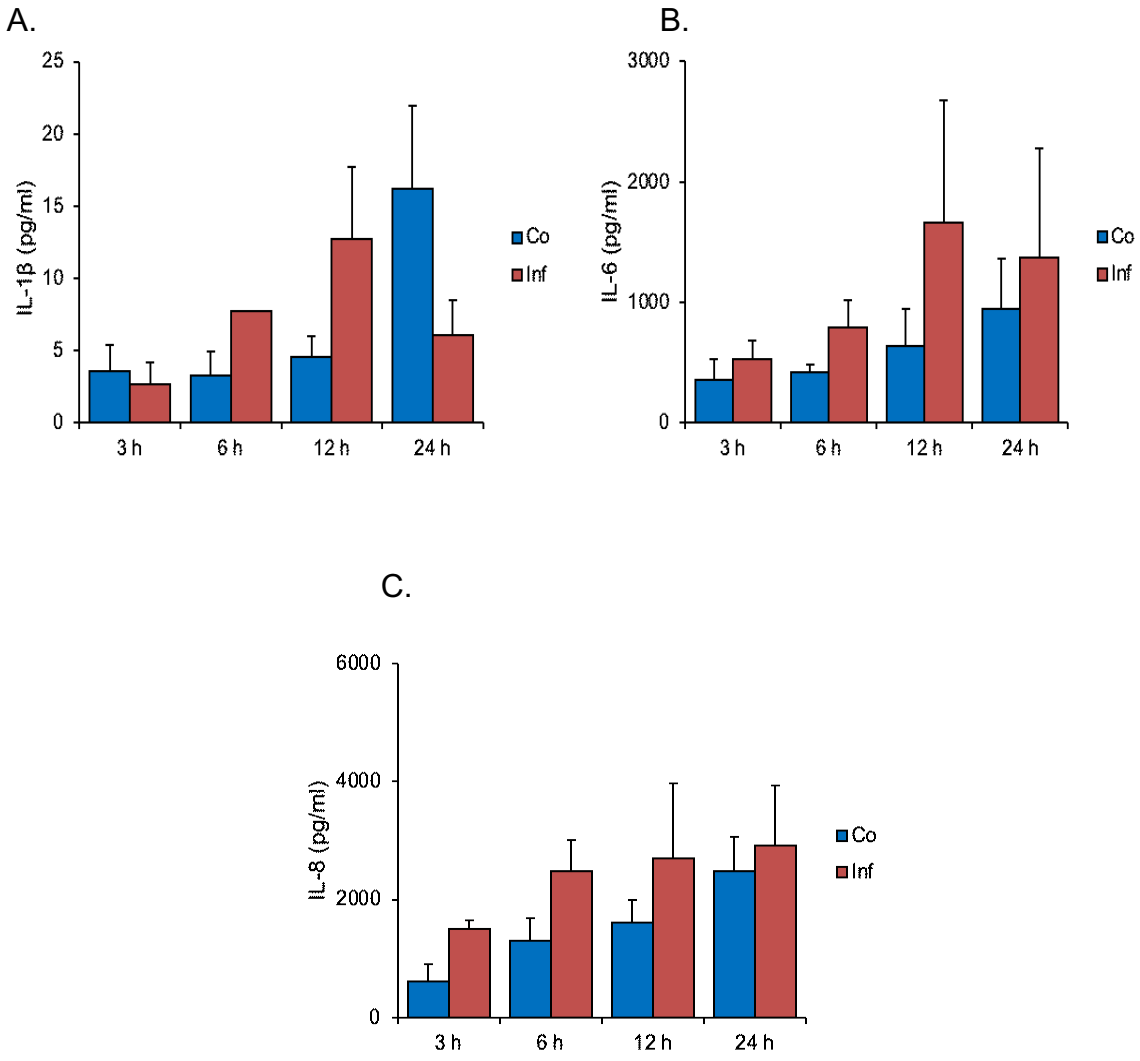

Figure 31: HSV-1 leads to increased inflammatory cytokines concentrations in endothelial cells
HUVEC were infected with HSV-1 for 3, 6, 12 and 24 h with corresponding non-infected controls. After respective times of incubation, supernatants were collected and frozen until use. The LEGENDplex™ assay was performed with the supernatants and concentrations were determined using a standard curve. IL-1β (A), (C), IL-6 (B) and IL-8 (C) concentrations for each time point are demonstrated as mean + SEM generated for control and HSV-1 infected cells from three different cell batches for 3 and 6 h and from seven different cell batches for 12 and 24 h.

3.12.2 HSV-1 infection leads to increased interferon expression in endothelial cells

Since cells are able to activate antiviral defense mechanisms such as the production of interferons (IFNs), we checked whether endothelial cells respond to HSV-1 infection with increased expression of various interferons. Evident from figure 32 A, B and C, we observed a trend to a time-dependent increase in the mRNA expression of IFN α2,

β1 and γ in HSV-1 infected cells, respectively. This response was, however, not detectable at the protein level (data not shown).

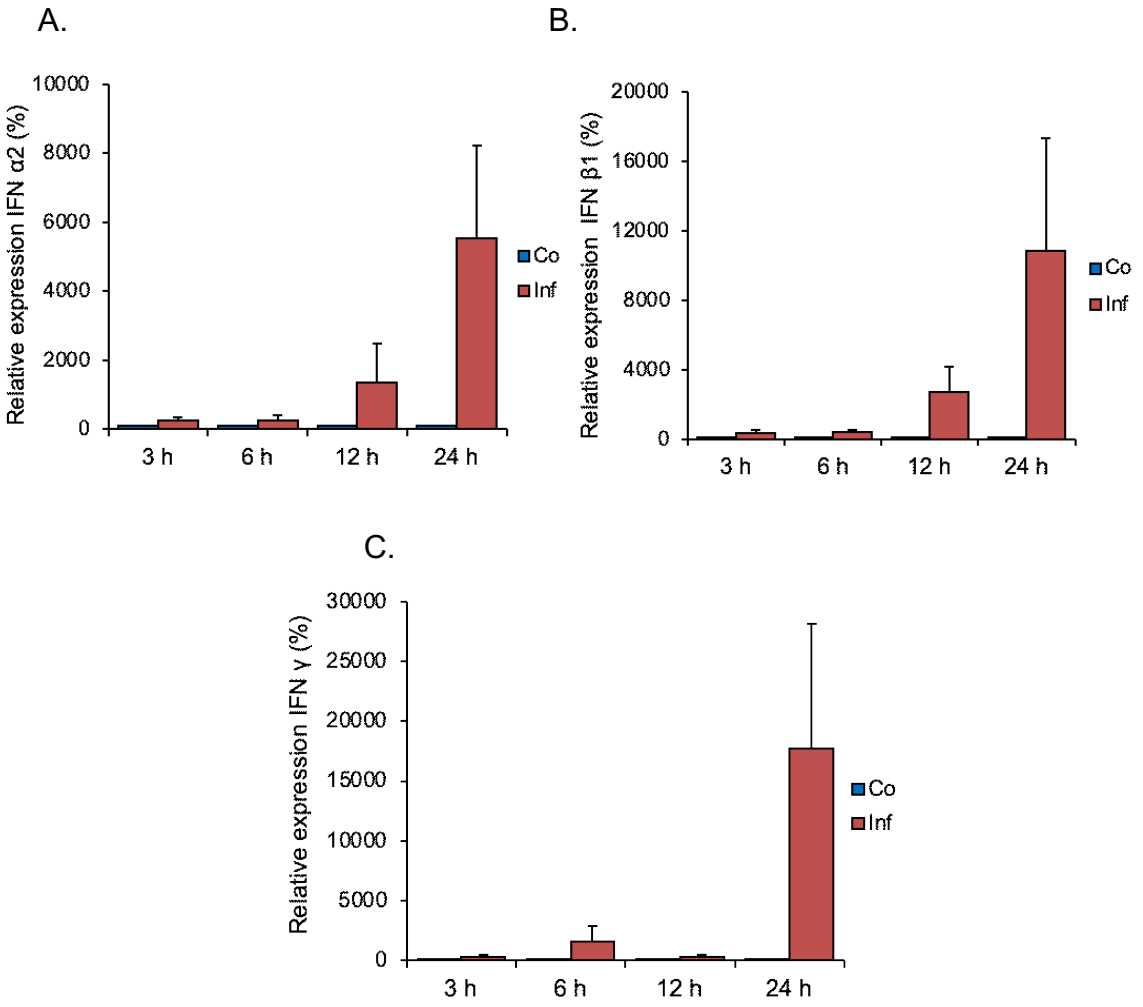

Figure 32: HSV-1 infection leads to increased interferon expression in endothelial cells HUVEC were infected with HSV-1 for 3, 6, 12 and 24 h with corresponding non-infected controls. After respective times of incubation, the RNA was extracted, cDNA was prepared with equal concentrations of RNA. The cDNA's to be tested were then used to put up RT-PCR for IFN α2 (A), β1 (B) and γ (C) genes with β-Actin as a housekeeping gene. Relative gene expression to uninfected control cells, normalized to β-Actin expression, for each time point are demonstrated as mean + SEM generated from four different cell batches.

3.13 AMPK activators and interferon response

Since the inflammatory response had batch to batch variations and seemed to be only transient and the IFN response being more important against viral infections, to understand whether AMPK activators influence the IFN response, we analyzed interferon mRNA expression in control and HSV-1-infected cells in the presence and absence of A76 or SC4. We found no significant effects of the compounds on IFN α2

or β1 expression in non-infected or infected cells. However, under certain conditions (for IFN α2 after 24 h of SC4 stimulation and for IFN β1 after 12 h of A76 and SC4 stimulation) displayed a tendency to a potentiate the interferon response, which needs to be clarified in further studies.

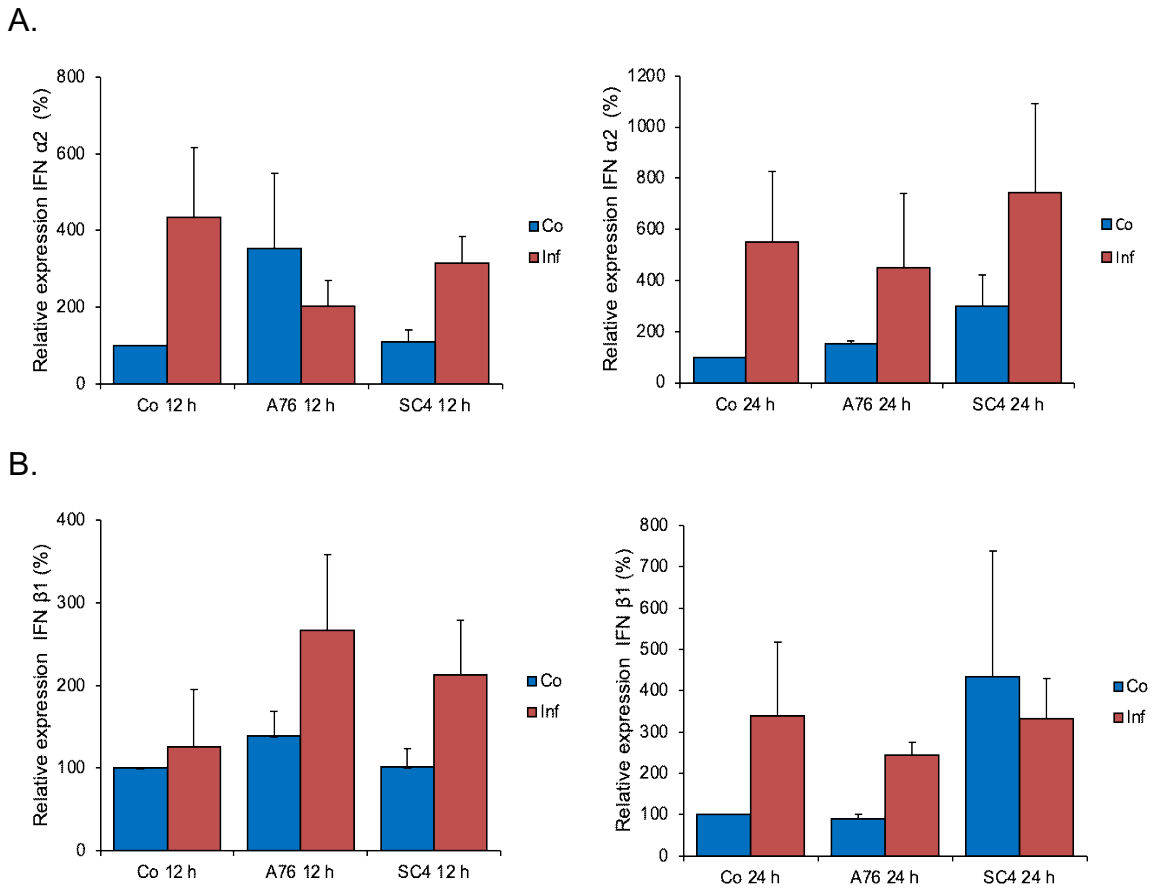

Figure 33: AMPK activators show a tendency to induce interferon expression upon infection

HUVEC were pretreated with 10 µM of A76 or SC4 for 1 h and thereafter infected with HSV-1 for 12 or 24 h along with the compounds for 12 or 24 h (with 12 h re-addition) with corresponding non-infected controls and untreated controls. After respective times of incubation, the RNA was extracted, cDNA was prepared with equal concentrations of RNA. The cDNA's to be tested were then used to put up RT-PCR for IFN α2 (A) and β1 (B) genes with β-Actin as a housekeeping gene. Relative gene expression to uninfected control cells, normalized to β-Actin expression, for each time point are demonstrated as mean + SEM generated from five-six different cell batches.

4. DISCUSSION

HSV-1 is a highly infectious and ubiquitous pathogen. Along with its primary site of infection, the epithelium, HSV-1 is known to infect the endothelium, which may be a site for viral latency and may promote atherosclerosis [50,51]. In line with this, HSV-1 has been detected in atherosclerotic plaques [119]. Various studies have been conducted on the HSV-1 virus and its replication in various cell types. However, molecular processes involved in virus replication are still incompletely understood although a better understanding of these mechanisms would open new strategies to interfere with virus replication. In line with this, it becomes increasingly important to study the role of host cell proteins as candidates for restricting HSV-1 infection and replication. AMPK, known to be a master regulator of cellular metabolism, could be one such candidate.

Since the mechanism by which HSV-1 replicates in vascular endothelial cells remains unclear, this project was aimed to understand the replication of HSV-1 in the endothelium and the role of AMPK in this process. To this end, primary human endothelial cells were infected with HSV-1 and different aspects of cellular signaling, autophagy, lipid metabolism, antiviral defense and the role of AMPK in these aspects were examined. Our study confirmed the infection and replication of HSV-1 in human and mice endothelial cells. Various proteins important in cellular proliferation, survival, and stress response were affected by HSV-1 infection. We show that AMPK plays a protective role against HSV-1 replication in endothelial cells. This effect was a consequence of inhibiting the mTOR pathway, which is required for HSV-1 replication, of interfering with lipid synthesis and, possibly, of promoting the antiviral interferon response. In addition, our results extend the knowledge about the role of the autophagy pathway in HSV-1 replication in endothelial cells. Collectively, our data provide new insights to a regulatory pathway for HSV-1 replication via AMPK in endothelial cells.

4.1 HSV-1 infects and replicates in endothelial cells

HSV-1 replication was studied after infection of HUVEC and MLEC with HSV-1 and monitoring viral replication up to 72 p.i. Maximal titers were observed at 24 - 48 h p.i. This observation is in line with previous reports showing that, in addition to its primary infection sites of the skin and mucosal epithelium, HSV-1 can replicate in cells of the immune system such as monocytes [120], macrophages [121] and in endothelial cells

of different origins. For instance, HSV-1 has been shown to infect corneal endothelial cells and establish latency in these cells [122,123]. Vascular endothelial cells are also known to be a site of infection and latency of the HSV-1 virus [50,124,125]. Of note, infection of HSV-1 in endothelial cells is thought to be an underlying factor in endothelium dysfunction leading to vascular complications such as atherosclerosis. In addition, HSV-1-mediated endothelial dysfunction may be involved in the damage of the blood-brain barrier, which is thought to be one of the mechanisms for Herpes simplex encephalitis (HSE), a severe complication caused by HSV-1 infection [126]. Thus, studies on the impact of HSV-1 infection in endothelial cells are of clinical relevance and can offer significant insights into viral replication and antiviral strategy research.

4.2 HSV-1 requires and alters cellular signaling pathways in endothelial cells

4.2.1 The Akt/mTORC1 pathway is required for HSV-1 replication in endothelial cells

Infection of HSV-1 leads to a series of changes in the cell as well as utilization of the cell's own machinery to the advantage of the virus. To characterize alterations in cellular signaling routes in endothelial cells, we performed western blotting experiments of major cellular signaling proteins after 2 or 24 h of HSV-1 infection. No striking changes of the Akt/mTORC1 pathway were observed after 2 h of HSV-1 infection except for an increase in the activation of raptor. In contrast, 24 h of infection brought about an increase in the activation of Akt and of p70S6K, an mTORC1 target. Since this suggested that the Akt/mTORC1 pathway was important for HSV-1 replication, we treated HUVEC with Akt VIII, an Akt inhibitor, and Torin 2, an inhibitor of mTOR and monitored HSV-1 replication under these conditions. As compared to untreated cells, HSV-1 replication was strongly diminished in cells treated with these inhibitors indicating that the Akt/mTORC1 pathway indeed plays a significant role in HSV-1 replication in HUVEC.

Our results are in line with previous studies showing that HSV-1 led to phosphorylation and activation of Akt in various cell lines such as oral carcinoma cells [36], human cervical epithelial cells, human neuroblastoma cells, human keratinocyte cells as well as in primary genital tract epithelial cells [127]. These studies point to a role of Akt in

HSV-1 entry into the cells and in regulating the expression of viral IE, E and L genes and viral DNA replication via its effects on cell survival and growth [36,127,128]. This could also apply to endothelial cells. The activation of p70S6K, a downstream target of mTORC1, by HSV-1 infection, which points to the role of the mTOR pathway, has also been observed in other cells. In fibroblasts, the HSV-1 kinase Us3 mimicking Akt is thought to bring about this activation, stimulating viral translation and replication [129]. In neurons, mTOR is also thought to be important in controlling viral latency [130]. In endothelial cells, mTOR activation could be a consequence of Akt activation and could help HSV-1 in protein synthesis and replication. The importance of this pathway is emphasized by the almost complete inhibition of HSV-1 replication by inhibitors of the respective kinase, which was shown in our study.

4.2.2 HSV-1 upregulates JNK and downregulates ERK in endothelial cells

We next looked at JNK and ERK proteins by western blot analysis after lysis of HUVEC following 2 or 24 h of HSV-1 infection. We found an increase in the activation of JNK after 24 h but on the other hand a decrease in ERK phosphorylation.
HSV-1 had earlier been shown to activate JNK in Baby Hamster kidney cells via VP16 [131]. Since activation of JNK is a coping mechanism of cells to environmental stress, it could be a defense mechanism of endothelial cells against HSV-1. Alternatively, p38 is required in part to activate pathways leading to viral protein synthesis via eIF4E phosphorylation [132]. Decreased ERK phosphorylation in response to HSV-1 indicates suppression of ERK activity, which, in turn, may be involved in preventing cell death and reducing antiviral interferon responses [133,134]. Suppression of ERK activity was also observed in human dermal fibroblasts after HSV-1 infection and was dependent on the virus-encoded Us3 Ser/Thr protein kinase [135]. Oppositely, in HEp-2 cells, an increased ERK phosphorylation was seen after HSV-1 infection [136]. These variations might be an indication of cell-specific effects and also due to different cell culture conditions.

4.2.3 AMPK signaling is not affected by HSV-1 infection in endothelial cells

To elucidate the role of AMPK in HSV-1 replication, we first looked at alterations in AMPK signaling after 2 or 24 h of HSV-1 infection and subjected lysates of infected cells to western blot analyses. No changes were observed in expression of AMPKα1 or AMPKα2, the catalytic subunits of AMPK. The phosphorylation of AMPK at T172

also remained unchanged as compared to control cells indicating that AMPK was not activated. In addition, the S485 site, the inhibitory phosphorylation site of AMPK did not vary between control and HSV-1 infected cells. The AMPK substrate, ACC also remained unaltered, thus confirming that HSV-1 infection did not alter AMPK activity. This is in contrast to a study in neurons where HSV-1 transiently decreased AMPK phosphorylation at 2 h p.i. [137]. In addition, AMPK signaling was decreased upon Zika virus infection in HUVEC cell line and primary human retinal vascular endothelial cells (HRvEC) [138] and upon Hepatitis C virus infection in a liver carcinoma cell line [101]. So far, however, the evidence for a possible role of AMPK in HSV-1 replication arose from two studies, in which AMPK activity was modulated by extracellular added agents or by activation of intracellular pathways, and not by showing effects of virus on AMPK pathways. A study conducted on neurons showed inhibition of HSV-1 replication and protection of cells via nutraceutical AMPK/SIRT1 activators [139]. In contrast, a recently discovered interferon-stimulated viral restriction factor, Tudor domain-containing 7 (TDRD7), was shown to suppress HSV-1 replication via AMPK inhibition in different cell lines such as HT1080, ARPE19, HeLa, HEK293T, NuFF, L929 and MEF's [140], indicating that AMPK may be required for virus replication. From these data we concluded that, probably, AMPK modulation could be a better way to assess its role in viral replication rather than just to check the effect of virus on basal AMPK signaling.

4.3 AMPK has a protective role against HSV-1 replication in endothelial cells

Following the above described line, we first downregulated the catalytic subunits of AMPK, AMPKα1 or AMPKα2, in HUVEC using the siRNA technology and infected these cells with HSV-1. Downregulation was confirmed via western blot analysis. We observed an increase in HSV-1 replication in cells, in which either AMPKα isoform was downregulated, even if the increase was only significant with AMPKα2 downregulation. In accordance with the results derived from HUVEC, we also saw an increase in HSV-1 replication in endothelial cells obtained from the lungs of AMPKα2 knockout mice as compared to cells from wild type mice. We next checked for the effect of pharmacological AMPK activators in HSV-1 replication. We used AICAR, an AMP mimetic, and the specific activators A76 and SC4 and found that all three compounds

significantly reduced HSV-1 replication in endothelial cells. The effect of AICAR was dose-dependent, however, A76 and SC4 did not display a dose-dependent effect.
Our results are in line with the study in neuronal cell line HT22 as stated in the previous chapter but also extend these findings [139]. Leyton et al. employed compound C, which is an unspecific AMPK inhibitor, and the activators AICAR, resveratrol and quercetin, which are not AMPK-specific. Compound C showed an increase in the viral protein expression (ICP8) at 4 h p.i., however the expression of the late viral transcript ICP5 as well as the HSV-1 titer were inhibited suggesting that it only worked in the early stage of infection. AICAR, resveratrol and quercetin inhibited viral proteins and genome abundance in neuronal cells, however, the effect on viral titer was not significantly changed [139]. In our study, downregulation of AMPKα1 or AMPKα2 and the applied compounds A76 and SC4 represented specific and efficient approaches and substantiated the previous study. Importantly, we were able to show the effect of AMPK modulation on progeny virion production in primary endothelial cells and clearly demonstrated the protective role of AMPK against HSV-1 infection in endothelial cells. In contrast, Subramanian et al. suggested a pro-viral role of AMPK in HSV-1 replication in different cell lines, which does not match our results. The reason is not clear but may be related to the employed cellular systems and to the fact that most of the results were based on the use of compound C [140]. The effects of AMPK modulation on virus replication may also depend on the type of virus. In HepG2 cells, AMPK has been shown to promote Dengue virus (DENV) replication by stimulating lipophagy and not basal autophagy [141]. In contrast, AMPK was shown to protect against HBV virus replication in liver cell lines via activation of autophagy [142] and against CVB3 virus replication in HeLa cells by inhibition of lipid accumulation [143]. In addition, an antiviral role of AMPK against West Nile virus (WNV) has been suggested in neuroblastoma cells, since WNV inhibited autophagy via AMPK degradation, which led to accumulation of protein aggregates [144]. However, our study is the first to demonstrate the protective role of AMPK against HSV-1 in primary endothelial cells with the use of new age, specific activators. We also show that in addition to the AMPKα1 isoform AMPKα2 could be of importance in this protective role.

4.4 AMPK could protect endothelial cells against HSV-1 via inhibition of the mTORC1 pathway

Having seen that AMPK displays a protective role against HSV-1 and that the mTORC1 pathway is essential for HSV-1 replication (see 4.2.1) and knowing that AMPK can phosphorylate raptor which in turn inhibits the mTORC1 complex [91], we checked if the latter applies to our conditions. We added the AMPK activators, A76 and SC4 to HUVEC for 12 h, lysed the cells and subjected the lysates to western blot analysis of raptor and p70S6K, a target of activated mTORC1. Both AMPK activators led to moderate phosphorylation of raptor and decreased phosphorylation of p70S6K as compared to control cells. The effect was more pronounced with SC4 and reached significance for p70S6K. These data indicate that one of the mechanisms by which AMPK restricts HSV-1 is by mTORC1 inhibition. The relatively modest changes might be due to the presence of full growth medium during incubation, i.e., the same medium used for studying virus replication. This medium contains 20% serum growth factors that may counteract mTOR inhibition. In line with this, mTOR inhibition was found to be much stronger, when experiments were performed in low serum medium (data not shown).

The cross-talk between AMPK and mTORC1 plays an important role in regulating the energy balance in the cell [92]. As mentioned in section 4.2.1, functional mTOR is essential for the synthesis of HSV-1 proteins in the cell [129]. In addition, since mTOR activation inhibitory phosphorylates ULK1 (the kinase, which may trigger the induction of autophagy), it may reduce autophagy, a potentially antiviral response [93]. mTOR activity may be inhibited by AMPK thus contributing to AMPK-mediated protection against HSV-1 replication.

As far as we know, our study is the first describing the role of the AMPK-mTORC1 axis in HSV-1 replication in vascular endothelial cells. It demonstrates that one of the possible mechanisms of protection of endothelial cell against HSV-1 by AMPK is by inhibiting the mTORC1 complex, which in turn would reduce viral protein synthesis. The latter remains to be confirmed in HSV-1 infected cells.

4.5 The autophagy pathway may have a pro-viral role in endothelial cells

4.5.1 The autophagy pathway is moderately altered in HSV-1 infected endothelial cells

Our next focus was to understand the role of autophagy in HSV-1 replication. Autophagy, being an important homeostatic process in cells, is also one of the major

pathways used by the cell against infecting pathogens. Firstly, we wanted to understand whether this process itself is altered by HSV-1. To this end, HUVEC were lysed after 2 and 24 h of infection and subjected to western blot analysis of proteins involved in various stages of autophagy.

No dramatic changes were seen after 2 h of infection. However, after 24 h of infection, phosphorylation of ULK1, a central player of autophagy initiation, was increased at the ULK S757 site (the mTOR site). This phosphorylation inhibits ULK1 and accordingly, a significantly decreased phosphorylation of ATG14, the substrate of ULK1, was observed. Furthermore, we saw a moderate decrease of ATG16L1 phosphorylation, a marker for autophagosome maturation, after 24 h of infection. We also checked for accumulation of conjugated LC3B, a marker for autophagosomes, after HSV-1 infection in the presence of bafilomycin A1, an inhibitor of lysosomal degradation. After 24 h of HSV-1 infection, less conjugated LC3B was seen, and short-term measurements revealed a slightly reduced autophagic flux in infected cells. All these data point to a moderate inhibition of autophagy in HSV-1-infected HUVEC although these observations may be of little physiological relevance. Of note, beclin1, a component of the PI3K complex, which is also involved in autophagy induction, was activated by HSV-1 infection after 24 h as shown by phosphorylation of both, S15 (ULK1 phosphosite) and S93 (AMPK phosphosite). This may counteract mechanisms initiated by mTOR-mediated ULK1 inhibition and contribute to the low net effect of HSV-1 infection on autophagy. Alternatively, beclin1 activation could have other functions than autophagy induction and represent a defense response of the host cell as discussed below. Still, it remains to be clarified why phosphorylation at S15 and S93 are increased although the respective upstream kinases are not altered (AMPK) or even inhibited (ULK1).

According to literature, the effect of HSV-1 on autophagy is highly based on the cell type. It seems that HSV-1 has evolved mechanisms to evade it. A study in HEK293, MCF7 and SK-N-SH cells showed that ICP34.5, an HSV-1 protein, binds to beclin1 and inhibits autophagy. In viruses lacking this protein, autophagy is triggered by the eukaryotic translation initiation factor 2-kinase 2/protein kinase RNA-activated pathway (eIF2AK2/PKR pathway) [145]. In HeLa cells, Us11, a viral protein, which is expressed later than ICP34.5, directly binds to PKR and inhibits autophagy [146]. Furthermore, HSV-1-miR-H1 is thought to target ATG16L1 and reduce its expression in lower esophageal sphincter (LES) in esophageal achalasia patients [147]. In contrast to

these findings, which suggest autophagy inhibition by HSV-1, autophagy is induced by HSV-1 infection in THP-1 cells via MyD88 and supports HSV-1 replication [148]. Our study in endothelial cells shows evidence for both, inhibition and activation of autophagy proteins, but the net effect is a modest inhibition of autophagy by HSV-1. These data underline the complexity of autophagy regulation and suggest that individual autophagy proteins may crosstalk to pathways other than autophagy.

4.5.2 ULK1 and beclin1 protect against HSV-1 but LC3B could be pro-viral in endothelial cells

Since we observed inhibition of ULK1 but activation of beclin1 as well as a tendency to lower LC3B conjugation in infected cells, we went ahead to dissect the role of each protein in detail. To this end, we used siRNA technology against the three proteins and infected these cells with HSV-1. We observed increased viral replication in cells, in which ULK1 or beclin1 were downregulated. The increase reached significance with ULK1 downregulation as compared to control siRNA-treated cells. however, in cells lacking LC3B, decreased HSV-1 replication was observed. These data suggest that ULK1 and beclin1 protects from HSV-1, while LC3B, an important player of functional autophagy, supports viral replication.

To further validate the LC3B data, we used two pharmacological compounds known to block autophagy: 3-MA, which inhibits autophagosome formation and bafilomycin A1, which inhibits the autophagic pathway by preventing autophagosome lysosome fusion and lysosomal degradation. We observed significantly diminished HSV-1 replication in cells treated with either compound as compared to untreated cells. Thus, functional autophagy seems to be required for HSV-1 replication. On the other hand, the effects of ULK1 and beclin1, which protect from virus replication despite their role in autophagy induction, may be independent from the autophagic pathway.

As mentioned in section 4.5.1, the interplay between HSV-1 and autophagy is vastly dependent on cell types and seems to be complex and fluctuating between the different stages of autophagy. Our data indicate that autophagy seems to be advantageous for HSV-1 replication in endothelial cells and may not have an anti-viral function in these cells. In contrast, murine TG neurons containing large autophagosomes were resistant to productive HSV-1 gene expression [149]. Even though autophagy was shown to be required for HSV-1 replication in several studies, the full proteolytic process is probably not needed. In human neuroblastoma cells, for instance, HSV-1 induced autophagy

but blocks autophagosome lysosome fusion by triggering autophagosome accumulation [150]. In SIRC corneal cell line, HSV-1 triggers LC3B lipidation and cytoplasmic acidification but inhibits autophagosome lysosome fusion as a means to inhibit apoptosis in the infected cells [151]. Looking at the host cell side, it seems to be the choice of the cell whether to use autophagy or interferon signaling against invading pathogens. A study by Yordy et al. showed that mouse primary neuronal cells prefer to use autophagy as a defense mechanism to HSV-1 as compared to MEFs and mouse keratinocytes [152]. Endothelial cells may belong to those cells, in which autophagy is not involved in HSV-1 removal but rather in virus replication. In addition, the autophagosome could be a site that HSV-1 could use for evasion from the other host cell responses.

The protective effect of ULK1 and beclin1 could be attributed to their role in antiviral defense independent of autophagy. In addition to being initiators of the autophagy pathway, more and more evidence proposes the role of ULK1 and beclin1 in immune signaling and interferon responses [153–157]. Liang et al. indicated cGAS-beclin1 interaction balances the IFN response by regulation of cGAMP and autophagy in different cell types [156]. Saleiro et al. showed a dual regulatory role of ULK1 in IFN signaling: ULK1 regulates early induction of type 1 IFN response and later suppresses excessive IFN production via STING suppression in various malignant hematopoietic cell lines [157]. These findings could hint at the role of ULK1 and beclin1 in IFN response against HSV-1 in endothelial cells independent of autophagy as well, however, this needs to be confirmed.

4.6 Protective effects of AMPK against HSV-1 are not related to autophagy in endothelial cells

AMPK is known to be a positive regulator of autophagy via direct phosphorylation at the S556 residue of ULK1 [93]. It is also known to activate beclin1 [158,159]. Since AMPK, ULK1 and beclin1 all seem to be protective against HSV-1 we decided to check whether these effects are linked. We, therefore, applied the AMPK activators A76 and SC4 in cells lacking ULK1, beclin1 and infected these cells with HSV-1. The activators significantly reduced HSV-1 replication in endothelial cells even in cells lacking either ULK1 or beclin1. These data confirm that AMPK exerts its anti-viral function

independent from activating ULK1 or beclin1 and thus, most probably independent from autophagy. This is in line with the finding that autophagy rather supports HSV-1 replication than counteracting it as discussed above. The role of autophagy as discussed seems to be complex and multilayered in response to HSV-1 replication in endothelial cells and it needs to be further investigated through which mechanism it could be beneficial to the virus.

4.7 AMPK could prevent HSV-1 replication via modulation of metabolic pathways in endothelial cells

To further dissect the means by which AMPK protects endothelial cells against viral replication, we next focused on metabolic targets, in particular ACC1, which is the key enzyme in FA synthesis. AMPK activation induces an inhibitory phosphorylation on ACC1, which leads to inhibition of FA synthesis [64]. Upon efficient downregulation of ACC1 in HUVEC by siRNA technology, we observed a significant decrease in HSV-1 replication as compared to control siRNA-treated cells. This result suggests that ACC1 and, in turn, FA synthesis play an important role in viral replication and that the anti-viral effect of AMPK may, in part, be mediated via inhibition of ACC1. Moreover, inhibition of cholesterol synthesis by simvastatin, an inhibitor of the HMGCR, also blocked HSV-1 replication. Since AMPK is known to impair this enzyme as well [65], HMGCR could be a second metabolic target through which AMPK interferes with HSV-1 replication. Accordingly, we demonstrated that treatment of endothelial cells with the AMPK activators A76 and SC4 reduced incorporation of radio-labeled acetate into the neutral lipid fraction of cells confirming that AMPK is able to control FA and cholesterol synthesis in endothelial cells. Our data show that HSV-1 replication strongly depended on the metabolization of glucose since virus production was almost completely blocked if glycolysis was inhibited by 2-DG. Glucose is the major energy source for endothelial cells, which produce most of their energy via aerobic glycolysis [102]. Although AMPK is known to stimulate glycolysis in several cell types, it may have opposite effects in endothelial cells (preliminary data of the group). Thus, further studies will investigate whether inhibition of glycolysis by AMPK may contribute to its anti-viral effect.

Viral replication undeniably needs functioning metabolic pathways for the synthesis of viral lipids and proteins and more necessarily for fulfilling the energy demands. HSV-1 is no exception and several studies have related HSV-1 replication to metabolic

alterations and requirements. Crespillo et al. have shown that valproic acid, a short-chain fatty acid, which inhibits HDACs and glycogen synthase kinase 3 and alters lipid metabolism, drastically reduced HSV-1 in a human oligodendroglioma cell line [160]. Higher accumulation of lipids in HSV-1-infected human fetal and bovine adult arterial smooth muscle cells was observed in another study, which suggested that this may contribute to HSV-1-induced atherosclerosis [119]. Wudiri and Nicola showed that HSV-1 replication in MEFs at different stages from viral entry to virion release was dependent on cellular cholesterol [161]. In addition, depleting HSV-1 envelope cholesterol by methyl beta-cyclodextrin inhibited viral entry and infectivity in various cell types dramatically [162]. HSV-1 has been shown to induce glycolysis in Vero cells via PFK-1, demonstrating high ATP requirements in the cell for efficient viral replication [163]. Together, these studies point to the importance of cholesterol, FAs and glycolysis in HSV-1 replication, which was confirmed in our study in endothelial cells. Moreover, we were able to show a regulatory role of AMPK in this context and the efficiency of pharmacological AMPK activators in reducing HSV-1 replication. Our results demonstrate that the anti-HSV-1 effect of AMPK in endothelial cells may, in part, work via inhibition of FA and cholesterol biosynthesis. Further studies on the effects of AMPK activation on glycolysis and FAO will extend this data.

4.8 HSV-1 infection leads to an interferon response and moderately increased inflammation in endothelial cells

The innate immune system is the first line of defense against invading pathogens of which the IFN response is important in establishing an anti-viral state. Upon virus infection cells secrete interferons mainly type I IFNs and, in turn, activate several ISGs. To confirm if endothelial cells produce IFNs in response to HSV-1, we checked for the expression of IFN α2, β1 and γ by RT-PCR in HUVEC after 3, 6, 12 and 24 h of infection. We observed a time-dependent increase in the expression of all three IFNs as compared to uninfected control cells indicating the activation of an antiviral response in endothelial cells. These data are in line with several studies characterizing the IFN response as a defense mechanism against HSV-1 across various cell types [164–169]. The antiviral effects against HSV-1, a DNA virus, are mostly mediated in a cGAS-STING-dependent manner. Reinert et al. showed that the cGAS-STING pathway in microglia causes antiviral defense in the central nervous system against HSV-1 [170].

A study in HUVEC showed decreased reproduction of HSV-1 and interference with the production of pro-inflammatory factors on application of human recombinant IFNα, β and γ [124]. Thus, our results add to these findings and suggest the production of IFNs as a means of restricting viral replication.

In addition to monitoring the interferon response, we also looked at the expression and secretion of the inflammatory cytokines IL-1β, TNFα, CCL2, IL-6 and IL-8 in HUVEC after 3, 6, 12 and 24 h of HSV-1 infection. Inflammation is also a line of defense against invasive pathogens and plays a critical role in tissue repair, regeneration and restoring homeostasis. Endothelial cells are known to be active mediators of the inflammatory response [109,110]. We observed an increased mRNA expression of the cytokines with time upon HSV-1 infection as compared to uninfected cells. This was paralleled by a modest and transient increase of cytokine secretion from infected endothelial cells with highest values measured at 12 h for IL-1β, IL-6 and IL-8. This may indicate a timely resolution of the inflammatory response. In addition, the highest average increase of cytokine release was about 2.5-fold and showed large variations, and TNFα levels were even not detectable in supernatants of HSV-1-infected cells. Thus, the inflammatory response may support antiviral defense but may probably play a minor role.

4.9 AMPK activators augment the interferon response in endothelial cells

After confirming the antiviral response by endothelial cells, we wanted to understand, if AMPK activation, in addition to inhibiting mTORC1 and metabolic pathways, offers protection against HSV-1 by increasing the cell's IFN response. We therefore applied the AMPK activators A76 or SC4 to HUVEC for 12 h or 24 h along with HSV-1 infection and checked for the expression of IFNs α2 and β1 by RT-PCR. Both activators led to a modest increase in IFN expression in control cells although the effects differed with respect to time of treatment and the individual cytokines. While A76 tended to induce IFN α2 expression at 12 h, SC4 enhanced the expression of IFN α2 and IFN β1 at 24 h. In addition, both compounds showed a trend to amplify the IFN β1 response at 12 h. These may point to a role of AMPK in regulating the interferon response. However, the results did not reach significance due to variations among the different cell batches and more studies are needed to clarify AMPK-mediated and/or metabolic regulation of the IFN response. In a previous paper, the IFN-induced protein TDRD7 has been

shown to inhibit AMPK in different cell lines [140], which seems to be contradictory to a potential regulatory role of AMPK in the IFN response but could also represent a negative feedback regulation. Another study in plasmacytoid dendritic cells (pDCs) showed that infection with the influenza or HSV virus activates AMPK via TLR7/9 thus potentiating IFN α production [171]. Yu et al. showed that metformin via activation of AMPK induced Type I IFN signaling and protected against HCV in Huh7.5.1 cells [172]. Thus, further experiments to dissect the function of AMPK in IFN signaling in endothelial cells and protection against HSV-1 would to be an attractive area to supplement the knowledge currently available in this field.

5. CONCLUSION AND OUTLOOK

The data obtained in this study confirm the infection and replication of HSV-1 in human and mice vascular endothelial cells and demonstrate a protective role of the cellular master regulator, AMPK, against HSV-1. AMPK interferes with HSV-1 replication via inhibiting the mTORC1 pathway, lipid synthesis and probably via promoting an antiviral response. In addition to the AMPKα1 isoform, the importance of the AMPKα2 isoform against HSV-1 replication in endothelial cells is also highlighted. We illustrate alterations of important cellular signaling pathways i.e., the activation of Akt/mTORC1, JNK and downregulation of ERK upon HSV-1 infection of endothelial cells. The autophagy pathway, although moderately inhibited in HSV-1-infected cells, appears to support virus replication. In contrast, ULK1 and beclin1, which are known to trigger autophagy, protect from HSV-1 but in an autophagy-independent manner. Taken together, our study emphasizes the regulatory role of AMPK against HSV-1 replication in endothelial cells using new age, specific activators. AMPK activation could be exploited as an effective antiviral strategy, given that no viral-specific vaccines have been developed against HSV-1 and that targeting host cell proteins would inhibit virus replication but prevent undesired complications in the infected individual as opposed to antiviral drugs.

Future studies should address certain important questions. We need to unravel the pro-viral function of autophagy and to demonstrate the mechanism through which ULK1 and beclin1 display a protective role against HSV-1 in endothelial cells. Further studies on anti-viral metabolic activities of AMPK including glycolysis and FAO are required to expand the current data. Lastly, additional experiments strengthening the role of AMPK in potentiating an antiviral IFN response of endothelial cells will further increase the value of the current study.

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7. LIST OF ABBREVIATIONS

%	Percent
º C	degree Celsius
α-CTD	C-terminal domain
2-DG	2-Deoxyglucose
3-MA	3-Methyadenine
4EBP1	eukaryotic initiation factor4E binding protein
ACC1/2	Acetyl-CoA Carboxylase 1/2
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AID	auto inhibitory domain
Akt	Protein kinase B (PKB)
AMP	Adenosine monophosphate
AMPK	5' Adenosine monophosphate (AMP)-activated protein kinase
ATG	autophagy-related gene
ATP	Adenosine triphosphate
Baf	Bafilomycin A1
Beclin1	B-cell lymphoma-interacting protein 1
Ca^{2+}	Calcium ions
CAMKK2	Ca^{2+} /calmodulin-activated protein kinase kinase 2/β
CBM	Carbohydrate-binding module
CBS	Cystathionine β-synthase repeats
cGAMP	cyclic GMP–AMP
cGAS	cyclic GMP-AMP Synthase
cm^2	square centimeter
CPE	cytopathic effects
CVB3	Coxsackievirus B3
DAMPs	danger-associated molecular patterns
E	early gene
EC	endothelial cell

eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FA	fatty acid
FAO	fatty acid oxidation
FOXO	fork head box protein O1
G6P	Glucose-6-phosphatase
GLUT1/4	Glucose transporter 1/4
GMK	African Green Monkey Kidney cells
GP	Glycogen phosphorylase
GS	Glycogen synthase
h	hour
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A-reductase
HSV-1	Herpes simplex virus 1
HUVEC	human umbilical vein endothelial cells
ICAM	Intracellular adhesion molecule
ICP	Infected Cell Protein
ICTV	International Committee on Taxonomy of Viruses
IE	immediate-early gene
IFN	Interferon
IL	Interleukin
ISG	Interferon-stimulated genes
JNK	c-Jun N-terminal kinase
KD	serine/threonine kinase domain
kD	kilodalton
L	late gene
LAT	latency-associated transcript
LC3B	microtubule-associated protein 1 light chain 3 beta

LKB1	liver kinase B1
M	molar
MAPK	mitogen-activated protein kinase
MEF	mouse embryo fibroblast
mg	milligram
min	minute
ml	milliliter
MLEC	mouse lung endothelial cells
mM	millimolar
m.o.i.	Multiplicity of infection
mTORC	mechanistic target of rapamycin
mTORC1	mTORC complex 1
mTORC2	mTORC complex 2
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
nm	Nanometer
NO	Nitric oxide
NTD	N-terminal domain
ORF	open reading frames
p	phospho
p70S6K	p70 ribosomal protein S6 kinase
p.i.	post infectionem
PAMPs	pathogen-associated molecular patterns
PDK1	Phosphoinositide-dependent kinase-1
PFK1/2	6-Phosphofructo-1/2-Kinase
PGC1α	peroxisome proliferator activated receptor gamma coactivator 1α
PI3K	Phosphoinositide-3-Kinase
PI3P	Phosphatidylinositol-3-Phosphate
PKR	protein kinase RNA-activated
PP2C	Protein phosphatase 2C
PPRs	Pattern-recognition receptors
ROS	reactive oxygen species

S	Serine
SIRT1	sirtuin 1
SOD	Superoxide dismutase
STING	stimulator of interferon genes
T	Threonine
$TCID_{50}$	Median Tissue Culture Infectious Dose 50
TDRD7	Tudor domain-containing 7
TG	trigeminal ganglia
TG	triglyceride
TK	thymidine kinase
TNFα	Tumor necrosis factor alpha
TSC1/2	tuberous sclerosis complex 1/2
ULK1	Unc-51-like kinase 1
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VP	viral protein
VPS34	vacuolar protein sorting 34
Y	Tyrosine
ZMP	AICAR 5'-monophosphate
µg	microgram
µl	microliter
µM	micromolar

8. LIST OF FIGURES

9. LIST OF TABLES

10. ACKNOWLEDGEMENTS

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