7. PI3K-Akt regulation as a molecular mechanism of the stress response during aerobic dormancy

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Abstract. The biochemical mechanisms that direct intracellular signal transduction networks have been extensively studied in a variety of organisms spanning invertebrates to vertebrates. Depending on environmental conditions, animals may adjust these cellular pathways in favour of growth signals or, at the opposite extreme, initiate death signals. Animals living in extreme environments are often faced with stresses (e.g. oxygen, water and/or fuel limitation, temperature extremes) and as a result have evolved adaptations that ensure safe transitions into hypometabolic states (e.g. dormancy, hibernation, aestivation, diapauses, etc.) when conditions are unfavourable for normal life. Metabolic rate may be reduced by as much as 70-99% as compared with active states and a variety of preservation strategies may be activated, all relying on the restructuring of evolutionarily conserved metabolic pathways. Akt/PKB is a pivotal stress kinase which exerts control over pathways including glucose metabolism.
(through GSK-3), protein synthesis (through mTOR), cell cycle regulation (through p27KIP1 and MDM2), and apoptosis (through BAD and caspases). In addition, via cross-talk with the NF-κB, Wnt, and MAPK signalling pathways, Akt/PKB is placed in a unique regulatory position in the stress response. By evaluating the natural responses of Akt/PKB signalling in diverse animal groups facing a range of stress challenges, researchers will discover the cellular plasticity of central metabolic pathways, how these pathways may be altered depending on the needs of the organism, and finally unravel important regulatory secrets involved in hundreds of stress and disease pathologies.

**List of symbols and abbreviations**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4EBP</td>
<td>eIF4E binding protein</td>
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<tr>
<td>Apaf-1</td>
<td>apoptotic protease activating factor 1</td>
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<tr>
<td>ASK1</td>
<td>apoptotic signal-regulated kinase 1</td>
<td>(also called MAPKKK5)</td>
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<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter</td>
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<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<td>c-Myc</td>
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<td>CDK</td>
<td>cyclin dependent kinase</td>
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<td>CDKI</td>
<td>cyclin-dependent kinase inhibitor</td>
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<td>checkpoint kinase 1</td>
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<td>CREB</td>
<td>cAMP response element-binding</td>
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<tr>
<td>eEF</td>
<td>eukaryotic elongation factor</td>
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<tr>
<td>eIF</td>
<td>eukaryotic initiation factor</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated protein kinase</td>
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<td>FOXO</td>
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<td>FRP</td>
<td>frizzled related protein</td>
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<td>GβL</td>
<td>G-protein β-subunit like protein (also called LST8)</td>
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<td>HM</td>
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<tr>
<td>I-R</td>
<td>ischemia-reperfusion</td>
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<td>Abbreviation</td>
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<tr>
<td>IGF-1</td>
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<td>INK4</td>
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<td>IRS</td>
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<td>c-Jun N-terminal kinase</td>
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<td>Kip</td>
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<td>MAPKK</td>
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<td>MAPKKK</td>
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<td>MDM2</td>
<td>murine double minute 2</td>
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<td>MK2</td>
<td>MAPK-activated protein kinase-2</td>
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<td>MPF</td>
<td>M-phase promoting factor</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>mTORC1</td>
<td>mTOR complex 1</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>NFAT</td>
<td>nuclear factor of activated T-cells</td>
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<td>Nrf2</td>
<td>NFE2L2, or nuclear factor (erythroid-derived 2)-like 2</td>
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<td>p70-S6K</td>
<td>ribosomal protein S6 kinase, 70 kDa</td>
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<td>platelet-derived growth factor</td>
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<td>PDK1</td>
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<td>PGC-1α</td>
<td>peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
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<td>PH</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PIF</td>
<td>PDK1-interacting fragment</td>
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<tr>
<td>PKB</td>
<td>protein kinase B</td>
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<td>PRAS40</td>
<td>Proline-rich Akt Substrate 40 kDa</td>
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<td>PTEN</td>
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<td>Raptor</td>
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<tr>
<td>Rictor</td>
<td>rapamycin-insensitive companion of mTOR</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
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<tr>
<td>SGK</td>
<td>Serum/glucocorticoid regulated kinase</td>
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<tr>
<td>S6K</td>
<td>ribosomal protein S6 kinase</td>
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1. Introduction

All living organisms rely on sequences of biological events which interpret external stimuli and trigger a network of intracellular signalling cascades that restructure the cellular environment. Signal transduction is the key event for launching the stress response. External stress signals trigger changes to the existing protein/enzyme machinery (e.g. via posttranslational modifications such as protein phosphorylation) as well as changes in gene expression (e.g. via transcription factor regulation) in order to modulate the type and/or amount of regulatory factors. Therefore, when a stress occurs, it is converted into various biological signals following cell signalling pathways and leading to both modification of the properties of existing enzymes/proteins and gene transcriptional/translational events that change the numbers and types of cell proteins. The framework for these cellular changes depends on balancing contrasting signals that break down organic matter in order to harvest energy in the form of ATP (catabolic pathways), versus those that build biomolecules utilizing energy to promote growth (anabolic pathways). Therefore, the “decision” to promote growth, suspend life, or initiate death signals is regulated by signal transduction pathways and the harmonization of antagonistic signals that equilibrate an organism with its environment.

Studies that evaluate the relationship between stress factors and the natural responses of organisms are extremely informative as they illustrate how signalling pathways may be altered in a context dependent manner. Using a comparative biochemical approach that analyzes multiple systems of aerobic dormancy, our lab has documented the connections between selected signalling pathways and the biochemical adjustments that regulate the ability to enter a hypometabolic state [1-3]. Hypometabolism is quite common across phylogeny and allows organisms to suspend normal life and conserve fuel/energy by transitioning into a dormant/torpid state until stressful conditions are alleviated [4]. Hypometabolism contributes to many phenomena including hibernation in cold weather, aestivation in arid/hot environments, anaerobiosis when oxygen is limiting, anhydrobiosis under ultra-dry conditions, and others such as diapause or dauer state that allow a stress-responsive pause in the normal life cycle of a species. As such,
entrance into a hypometabolic state can essentially rescue life by broadening the range of stressors that an organism can tangibly cope with. Through alterations of metabolic pathways which inhibit the majority of biological functions, life can be put on hold and, subsequently, continue unscathed upon reversal to the active lifestyle.

Using examples from several different animal groups, this chapter focuses on aerobic dormancies, such as aestivation and hibernation, as a strategy for dealing with extreme environments. Aestivation allows organisms to survive the dry season through adaptations which improve water retention and provide sufficient fuel reserves to survive the long summer months [2]. Hibernation allows small mammals to escape the massive costs for thermogenesis that would otherwise be needed for winter survival [5]. While these strategies each possess unique sets of adaptations, all share one common feature: metabolic rate depression. By entering into a hypometabolic state where metabolic rate may be reduced by as much as 70-99% compared with active states, animals greatly extend the time that they can survive under stressful conditions [4].

The regulatory mechanisms that ensure entrance into, long-term maintenance, and arousal from the hypometabolic state depend on innumerable regulatory circuits which converge in an intricate fashion. These mechanisms may include the complete suppression, reversal or maintenance of central metabolic pathways such as glucose metabolism, protein synthesis, cell cycle, and apoptosis [6]. The regulation of carbohydrate metabolism is the first and most direct path towards survival, providing both the building blocks and the energy for anabolic functions. Matched with signals that increase rates of protein synthesis and promote cell cycle progression, the cell can grow and proliferate in its environment. By contrast, there is constant competition from catabolic processes and the pathways which degrade cellular machinery and initiate programmed cell death. Adding yet another layer of regulation, these key pathways often demonstrate extensive cross-talk, relaying messages in order to ultimately arrive at a concerted response. A common denominator in all of these metabolic pathways is the regulation of the serine/threonine protein kinase Akt (also known as protein kinase B, PKB) and the multitude of downstream effects associated with this pivotal stress kinase [7-8].

Since the first reports about Akt in 1991, this enzyme has been considered to be one of the most important players in cell signalling [9-11]. Akt is mainly involved in growth factor regulated pathways under the upstream control of PI3K (phosphoinositide 3-kinase) and has come to prominence because of its abundant downstream actions including controls
on glucose metabolism, protein synthesis, cell proliferation (related to cell cycle), cell survival (related to apoptosis), etc., as well as its involvement in human disease pathologies [7-8]. Furthermore, Akt participates widely in crosstalk with other signalling pathways (e.g. those controlled by Nf-κB, ERK, Wnt), indicating a critical position of PI3K-Akt in cell signalling [12-16]. Most importantly, the PI3K-Akt signalling cascade responds robustly to stress signals [17].

Whereas an overwhelming body of research is dedicated to unravelling the regulatory secrets of cell survival and metabolism, many aspects of even the most well-defined signalling pathways remain puzzling. Therefore, the aim of this chapter is (1) to present an overview of the PI3K-Akt signalling pathway, (2) highlight the mechanisms of Akt regulation in glucose metabolism, protein synthesis, cell cycle and apoptosis, and finally (3) outline the role of Akt in the regulation of hypometabolism with examples ranging from invertebrates to vertebrates.

2. Akt and the PI3K-Akt pathway

2.1. Akt/PKB family

Akt is a 56 kDa serine/threonine protein kinase that is also known as PKB or as RAC-PK (Related to the A and C Protein Kinase). It belongs to the AGC protein kinase family that includes over 60 members among them protein kinases A, G and C as well as S6K (ribosomal protein S6 kinase), SGK (Serum/glucocorticoid regulated kinase), PDK1 (phosphoinositide-dependent kinase-1), etc. [18]. Members in this kinase family share a similar structure of their catalytic domain, as well as the mechanism by which they are activated. The growth factor and insulin responsive AGC kinases are upstream of a variety of biological events, including the regulation of metabolism, cell growth, proliferation, and survival [19]. Multiple upstream factors affect Akt including epidermal growth factor, insulin, IGF-1 (insulin-like growth factor-1) and PDGF (platelet-derived growth factor) [20]. In addition, Akt can be regulated in response to environmental stresses including heat shock and hypoxia, among others [20-27].

Three isoforms of Akt have been identified to date in mammalian tissues: the original Akt form (also known as Akt1, PKBα, or RAC-PKα), Akt2 (PKBβ or PAC-PKβ) and Akt3 (PKBγ or PAC-PKγ). The three isoforms are widely and differentially expressed, each tissue in mammals having at least one isoform. The expression pattern of Akt seems related to the differentiation level of cells with a higher expression level in terminally differentiated cells [28-29]. The amino acid sequences of the three isoforms have a high degree of
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similarity. Like most of the AGC kinases, all three Akt isoforms have a PH (pleckstrin homology) domain in the N terminus, a central kinase/catalytic activity domain, and a C terminal HM (hydrophobic motif) domain [18]. All three Akt isoforms follow the same pattern of regulation [28-29].

2.2. Upstream regulatory signals and Akt activation

The full expression of Akt activity requires phosphorylation at multiple sites and includes translocation to the plasma membrane where the enzyme is influenced by phospholipid signalling molecules (Fig. 1). In the case of Akt1, two core sites must be phosphorylated to stimulate kinase activity: S473 in the HM domain and T308 in the catalytic domain T-loop [30]. T308 has long been known to be phosphorylated by the upstream kinase PDK1 that also phosphorylates the catalytic domain of most AGC kinases [30-31] whereas, more recently, the kinase that phosphorylates S473 was determined to be mTORC2 (mammalian Target of Rapamycin Complex 2) [32-33]. To summarize the regulation of Akt, it is easiest to start with upstream signalling events occurring at the plasma membrane.

Figure 1. Upstream regulatory signals and Akt activation. (A) Recruitment of Akt to plasma membrane by PIP$_2$/PIP$_3$; (B) phosphorylation of Akt by mTORC2; (C) interaction between Akt and PDK1 via the Akt PIF pocket; (D) the release of PDK1, the active Akt, and stabilization of Akt via an activation loop.
Multiple upstream factors are involved in Akt/PKB signalling at the plasma membrane including membrane receptors, adapter proteins, class I PI3K, PTEN (phosphatase and tensin homologue), and SHIP (SH2 Domain-containing Inositol 5'-Phosphatase). PI3K is the main direct factor involved in Akt regulation and addition of PI3K inhibitors prevents phosphorylation of Akt [34]. PI3K can be activated by either receptor tyrosine kinases (Class IA), such as IGF-1R (IGF-1 receptor), or G-protein coupled receptors (Class Ib), such as members of the Ras superfamily of small GTPases [35]. For example, IGF-1 binds specifically to the receptor tyrosine kinase IGF-1R which, following receptor autophosphorylation and activation of receptor tyrosine kinases, phosphorylates and activates PI3K (Fig. 1A) [24].

At the plasma membrane, PI3K produces the phosphatidylinositol-3,4,5-trisphosphate (PI[3,4,5]P3 or PIP3) from phosphatidylinositol-3,4-bisphosphate (PI[3,4]P2 or PIP2) [35]. This reaction is opposed by at least two different phosphatases; the phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase PTEN and the inositol 5-phosphatase SHIP [36]. PTEN dephosphorylates the 3-position of the inositol ring to convert PIP3 to PI[4, 5]P2 [37] and the inhibition of PI3K under conditions where PTEN was activated caused a rapid drop in PIP3 levels [38]. By contrast, SHIP specifically recognizes and removes phosphate at the 5-position on PIP3 to produce PI[3, 4]P2 [36] and SHIP-null mice show increased PIP3 levels [39]. Although signalling downstream of PI3K is weakened through the dephosphorylation of PIP3 by SHIP, PI[3, 4]P2 may also activate downstream responses which are both PI3K-dependent and PIP3-independent [36, 40-41].

Both PIP3 and PI[3,4]P2 are responsible for recruitment of Akt and PDK1 to the plasma membrane through interactions with their PH domains (Fig.1A) [40-43]. Andjelkovic et al. [44] showed that after treatment with IGF-1, native Akt was almost immediately localized to the plasma membrane whereas a mutant Akt with a deleted PH domain did not translocate. However, Akt1 constructed without the PH domain could still be activated and/or phosphorylated even though no plasma membrane translocation was detected [44]. Studies also showed that the PH domain-dependent interaction between Akt and PIP3 failed to stimulate Akt activity in vitro, and treatment with PI[3,4]P2 could not activate Akt catalytic activity dramatically in vitro [40-41]. Therefore, it is hypothesized that the PH domain does not directly stimulate catalytic activity, but rather creates a greater chance for interaction of the PDK1 catalytic domain with Akt at the plasma membrane.

As mentioned above, mTORC2 is now known to be the kinase that phosphorylates Akt/PKB at S473 (S474 in Akt2; S472 in Akt3) [32-33]. This is a large multi-subunit complex consisting of mTOR, Rictor (Rapamycin-insensitive companion of mTOR) and GβL (G-protein β-subunit like protein).
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(Fig. 1B). In contrast to mTORC1 (mTOR complex 1) (discussed below under *protein synthesis regulation*), mTORC2 is not rapamycin sensitive and its cellular functions are only beginning to be discovered [33]. Whereas S473 phosphorylation plays a regulatory role in the activation event, it has little effect on kinase activity when presented alone [45]. Similarly, other phosphorylation events in the HM domain such as Y474 were also reported to contribute to the conformational change occurring during Akt activation [46].

Following S473 phosphorylation, Akt1 is next phosphorylated on T308 (T309 in Akt2; T305 in Akt3) by PDK1 which also phosphorylates the catalytic domain of most AGC kinases (Fig. 1C) [30-31]. PDK1 is considered to be a special AGC kinase that contains a PH domain and catalytic domain, but lacks a HM domain [31]. Due to the role of the PH domain in recruitment to the plasma membrane, PDK1 is only able to activate Akt in a PIP3/PI[3,4]P2-dependent manner [40] and deletion of the PH domain attenuates PDK1-dependent Akt activation [47-48]. In addition, PDK1 interacts with Akt partially through the PIF (PDK1-interacting fragment) pocket in the Akt HM domain as also occurs for the PDK1-dependent phosphorylation of other AGC kinases [31, 49]. One possible explanation for this interaction is that the PIF pocket with phospho-S473 provides PDK1 with a docking site to create a physical interaction for exerting its kinase activity on Akt [31, 49].

Finally, following phosphorylation by PDK1 at T308, Akt1 is in a stabilized conformation and is free to move away from the plasma membrane (Fig. 1D). Once free, several additional phosphorylation sites have been reported to play a role in Akt activation. For example, tyrosine phosphorylations at Y315 and Y326 within the catalytic domain appear to occur after activation by Akt upstream signals and mutations on these two sites hindered Akt activation [50]. Finally, Akt achieves a stabilized conformation and is primed to initiate interactions with a multitude of downstream targets.

3. Downstream effects of Akt signaling

Akt acts on a variety of proteins and phosphorylates Ser/Thr residues that occur in a characteristic sequence, Arg-X-Arg-X-(Ser/Thr), on the substrate protein [51]. Yang *et al.* (2002) identified the structural basis for Akt activation and the substrate motif via analysis of the crystal structure of purified Akt. Proteins presenting this structural motif can be considered as potential Akt targets. Therefore, a wide range of putative targets downstream of PI3K-Akt signalling have been revealed [52]. They are involved in regulating various vital biological processes (Fig. 2).
Figure 2. The involvement of Akt in regulating various vital biological processes including: (A) glucose metabolism, (B) protein synthesis, (C) cell cycle, and (D) apoptosis. Akt exerts its regulatory effects via the phosphorylation of downstream proteins. Akt regulates glucose metabolism through GSK-3, CREB, NFAT, and GLUT1/4; protein synthesis through TSC2 and PRAS40; cell cycle through p27Kip1, FOXO, MDM2, Wee1 and Myt1, and Chk1; apoptosis through Bad, Caspase-9, and FOXO. Arrowheads denote positive regulatory effects while blunt-ended lines denote negative regulatory effects. An "X" denotes the action is inhibited.

3.1. Regulation of glucose metabolism

PI3K-Akt signalling exerts control over glucose metabolism in two central ways; (1) release of the inhibitory effects of GSK-3 (glycogen synthase kinase 3), and (2) altered expression levels of glucose transporters [53-54]. GSK-3 is a serine/threonine protein kinase encoded by two isoforms in mammals, GSK-3α and GSK-3β, and has been implicated in a wide range of cellular functions (e.g. metabolism, differentiation, cell fate determination) [6, 53, 55]. In general, GSK-3 phosphorylation has an inhibitory effect on its downstream targets thereby causing them to be recognized and degraded by ubiquitin proteosomal pathways [56]. These inhibitory effects change the ability of substrate enzymes to catalyze chemical reactions or substrate transcription factors to localize to the nucleus to initiate gene transcription. Akt lies upstream of GSK-3 and phosphorylates S21 on GSK-3α or S9 on GSK-3β, thus inactivating GSK-3 and preventing its inhibitory downstream action (Fig. 2A) [53].
GSK-3 is so named because glycogen synthase was the first downstream target of the kinase to be recognized [55]. GSK-3 inactivates glycogen synthase by the sequential phosphorylation of residues S652, S648, S644 and S640 and this blocks glycogen synthesis, particularly at times when glycogen catabolism is required [57]. GSK-3 also inhibits various transcription factors including CREB (cAMP response element-binding), c-Jun (cellular-Jun), c-Myc (cellular-Myc), HSF-1 (heat shock factor-1), NFAT (nuclear factor of activated T-cells), and GATA-4 [53, 58-59]. In addition, GSK-3 regulates the Wnt (wingless int) signalling pathway (see Cross-talk section).

Akt promotes glycolysis through its positive effect on glucose transporters (GLUT) [54, 60-61]. Akt up-regulates the expression of GLUT1 (responsible for basal glucose uptake in cells) through the activation of the mTORC1 pathway (see Protein synthesis regulation) [62]. Activation of mTORC1 promotes the expression of the transcription factor HIF-1α (Hypoxia Inducible Factor-1α), which lies upstream of GLUT1 [63]. In addition, the expression level of GLUT1 can be up-regulated through a CAP-dependent translational activation (mediated by eIF4E) which relies on the inhibition of 4EBP (eIF4E binding protein) by mTORC1 [62]. Akt also plays a role in the regulation of GLUT4 (the insulin-regulated transporter) by promoting its translocation to the plasma membrane [26]. Moreover, it has been reported that the Akt–dependent inhibition/phosphorylation of both FOXO1 (see Apoptosis regulation) and PGC-1α (peroxisome proliferator-activated receptor gamma coactivator-1α) [65] has the potential to modulate hepatic glucose levels [64].

3.2. Regulation of protein synthesis

A central action of PI3K-Akt signalling is the regulation of protein synthesis through the control of the mTOR (mammalian target of rapamycin) pathway. mTORC1 is formed with mTOR, Raptor (Regulatory associated protein of mTOR) and GβL (G-protein β-subunit like protein, also called LST8) (Fig. 2B). One of the main inhibitors of mTORC1 is the Tuberous sclerosis complex (TSC1/TSC2) which exerts its control through RHEB. The inhibitory mechanism depends on the GTPase activity of TSC2 which converts the GTP-bound, active RHEB (Ras homologue enriched in brain) into the GDP-bound, inactive form [66]. The PI3K-Akt pathway plays an upstream regulatory role by inhibiting TSC2 activity and therefore indirectly promoting mTORC1 activity. TSC2 can be phosphorylated by active Akt on three sites (S939, S981 and T1462) [67-69] where it is then recognized by 14-3-3 and prevented from forming a complex with TSC1 [70].
A newly discovered protein, PRAS40 (Proline-rich Akt Substrate 40 kDa), is believed to be another negative regulator of mTORC1 [71-72]. PRAS40 inhibits the kinase activity of mTORC1 by a physical interaction with Raptor [73]. As indicated by its name, PRAS40 is a major target protein of Akt. Active Akt phosphorylates PRAS40 at T246 to trigger a 14-3-3 protein interaction which sequesters PRAS40 from binding to mTORC1 [71].

Active mTORC1 promotes protein synthesis by phosphorylating targets including 4EBP and p70-S6K (ribosomal protein S6 kinase, 70 kDa). 4EBP is a negative regulator of ribosomal eIF4E (eukaryotic Initiation Factor 4E). The hypo-phosphorylated form of 4EBP binds to eIF4E to prevent it from interacting with 5’ cap-containing mRNAs to form the pre-initiation complex [69, 72-73]. Active mTORC1 hyper-phosphorylates 4EBP at multiple sites, thereby blocking its inhibitory interaction with eIF4E and allowing mRNA transcripts to be brought into assembling ribosomes. For the AGC kinase p70-S6K, on the other hand, phosphorylation on T389 by mTORC1 is a prerequisite for its PDK1-dependent full activation [74-75]. Active p70-S6K then phosphorylates eIF4B which in turn contributes to the helicase activity of eIF4A to promote mRNA translation [76-78].

Hence, in general, the PI3K-Akt pathway acts as an activator of mTORC1, and thereby promotes protein synthesis. However, a negative feedback loop regulating the process has also been proposed. After stimulation by insulin, the activated insulin receptor phosphorylates IRS (insulin receptor substrate) at a tyrosine site, and the phospho-IRS then activates PI3K to produce PIP3 and P[3, 4]IP2 which leads to Akt activation. Activated Akt then stimulates mTORC1 which phosphorylates p70-S6K. Subsequently, activated p70-S6K phosphorylates IRS at a serine site to promote its degradation and thereby terminates the signalling cascade [79-81]. In addition, it has been reported that PTEN can also regulate Akt in the negative feedback loop, albeit in an indirect fashion [82].

PI3K-Akt signalling may also promote protein synthesis in an mTORC1-independent mechanism. GSK3β can also phosphorylate eIF2B (eukaryotic initiation factor 2B) at S540, causing inhibition of its guanine nucleotide exchange activity and preventing it from recharging the GTP residue on eIF2α [83]. Insulin treatment released this inhibition via inactivation of GSK3β [83]. Therefore, Akt-dependent inhibition of GSK3β may be involved as an upstream factor in this event.

3.3. Cell cycle control

Another aspect of PI3K-Akt action is the regulation of cell cycle progression [84]. The cell cycle has four main phases: G1 (growth), S (DNA
replication), G2 (preparation for mitosis), and M (mitosis). The cell cycle can be regulated by various factors and events in each phase, the crucial regulatory events being mediated by a group of dimeric proteins that each consist of one cyclin dependent kinase (CDK) (the catalytic subunit) and one cyclin (the regulatory subunit). The two most important regulatory periods are the G1/S phase transition and the G2/M phase transition. For the G1/S transition in eukaryotic cells, CDK2, -4, and -6 along with cyclins A, -D1, -D2, -D3 and -E play crucial roles, whereas CDK1 (also known as Cdc2) and cyclins A, -B1, -B2 are vital to the G2/M phase transition. The CDK1/Cyclin A (-B) complex is also called MPF (M-phase promoting factor), with the function of driving the cell cycle into M phase [85].

The activity of most regulatory CDK/cyclin complexes is related to their phosphorylation state. For example, for the G2/M phase transition, a set of protein kinases are of great importance including Cdc25, Wee1, and Myt1 [86] and these are opposed by CDKIs (CDK inhibitors). Comparable effectors that act at the G1/S phase transition include members of the INK4 (inhibitors of CDK4) family (p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, p19^{INK4D}) that are specific to CDK 4, 6; and the Cip/Kip (CDK-interacting protein/Kinase inhibition protein) family (p21^{Cip1}, p27^{Kip1}, p57^{Kip2}) that regulate CDK2: Cyclin E1/E2 [87]. The family of 14-3-3 proteins is also key in cell cycle control. Various cell cycle regulatory factors can be sequestered away from the nucleus by 14-3-3 proteins in response to different signals, thereby limiting their function [85].

The positive regulatory effects of the PI3K-Akt cascade on the cell cycle come largely from the inhibition of CDKIs (Fig. 2C). For example, phosphorylation of p27^{Kip1} at T157 by Akt contributes to its sequestration by 14-3-3 protein, which prevents p27^{Kip1} from localizing into the nucleus. In addition, Akt inhibits the specific FOXO transcription factor that regulates p27^{Kip1} to suppress expression of this protein [88]. Another CDKI, p21^{Cip1}, is also inhibited by Akt in an indirect fashion: Akt activates MDM2 (murine double minute 2) by phosphorylation which in turn promotes the degradation of the transcription factor p53 that controls p21^{Cip1} expression [89-92]. The phosphorylation of MDM2 by Akt at sites of S166 and S188 specifically stabilizes MDM2 and inhibits its self-ubiquitination, thus preventing it from degradation by proteases [93].

Akt also has negative effects on G2/M phase transition regulatory kinases, including Wee1 and Myt1 [94-96], both of which contribute to inhibitory phosphorylation of CDK1. Inactivation of Wee1 and Myt1 by Akt phosphorylation ensures that CDK1 remains in its activated, non-phosphorylated form. In addition, direct phosphorylation of the DNA damage Chk1 (checkpoint kinase 1) by Akt at S280 blocks its translocation into the
nucleus, thereby preventing Chk1 from phosphorylating Cdc25. Thus, Cdc25 is activated and via its phosphatase activity may dephosphorylate/activate p-CDK1 and allow cells to enter M phase [97]. As mentioned above, the PI3K-Akt pathway activates mRNA translation by the Akt-TSC-mTOR-4EBP pathway and this activation promotes the translation of many genes, including cyclin D1, which is required for cell cycle progression [98]. Furthermore, the first identified PI3K-Akt pathway substrate, GSK3, is also an important negative regulator of Cyclin D and E (via protease degradation) [99-100].

3.4. Apoptosis regulation

The starting event in apoptosis is the release of cytochrome C from mitochondria into the cytoplasm under the control of the antiapoptotic Bcl family member, Bcl2. Cytochrome C forms a so-called apoptosome in conjunction with the adapter protein Apaf-1 (Apoptotic protease activating factor 1), caspase 9 and dATP. This interaction activates caspase 9 which in turn initiates the upcoming proteolysis by activating other caspase members, including caspase 3 and 7 [101-102]. PI3K-Akt signalling contributes to the regulation of programmed cell death by controlling several key factors including BAD (Bcl-2-associated death promoter), caspase 9, GSK3β, and the FOXO family of transcription factors. In general, the PI3K-Akt pathway has a prosurvival role and is active when nutrients and energy are plentiful; hence, Akt action typically inhibits apoptosis (Fig 2D).

Unphosphorylated BAD can bind and inhibit the prosurvival Bcl-2 family protein, Bcl-XL (B-cell lymphoma-extra large), which interacts with Apaf-1 to prevent the activation of caspase 9 and thereby suppresses apoptosis [103]. Akt exerts inhibitory effects on the proapoptotic protein BAD by phosphorylating the protein at S136 and facilitating a subsequent 14-3-3 protein-dependent sequestration event [104-105]. Evidence of the importance of Akt-mediated BAD phosphorylation, is demonstrated by increased apoptosis rates when a wild type BAD is replaced with a mutant at S136 [104]. Furthermore, caspase 9 can be directly phosphorylated by Akt at S196 [106] resulting in a decrease in its proteolytic activity. Indeed, when S196 was replaced by A196, caspase 9 showed greater proapoptotic activity than the wild type in the presence of active Akt.

In addition to regulating factors that are directly involved in the apoptosis process, Akt also controls the FOXO transcription factors that contribute to the expression of apoptosis-requiring components. This includes up-regulation of the death signal factor cytokine Fas ligand. The Fas ligand then binds to the Fas receptor on the cell surface to induce apoptosis by activating caspases in an indirect manner. Akt directly phosphorylates
FOXO1, FOXO3a and FOXO4 in the nucleus, each at three sites. These phospho-FOXOs can then be sequestered by 14-3-3 proteins in a similar manner to the Akt-dependent regulation of BAD and other targets mentioned above [reviewed in 107]. Therefore, Akt inhibits FOXO transactivation activity by preventing it from localizing into the nucleus and, thereby, indirectly suppresses apoptosis.

Akt may also control apoptosis via GSK3β. The prosurvival factor, MCL-1 (myeloid cell leukemia sequence 1), of the Bcl-2 family is reported to be directly inhibited by GSK3β [108]. Therefore, an inhibition of GSK3β by Akt has potential effects on the GSK3β-dependent inhibition of MCL-1. Crosstalk between PI3K-Akt and other signalling pathways (NF-κB, JNK/p38 pathway, etc.) also provides some lines of evidence for Akt-dependent promotion of cell survival (see Crosstalk section).

4. Akt and crosstalk with other signalling pathways

In addition to the direct effects of PI3K-Akt signalling described above, the pathway also interacts with other major signalling pathways. This crosstalk extends its regulatory importance in terms of the stress response, especially the interactions with the NF-κB, Wnt, and MAPK pathways.

4.1. Akt and NF-κB

The NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) signalling pathway regulates functions including innate immunity, cell proliferation, and apoptosis, among others [109-111]. A wide range of signal proteins including TNF (tumor necrosis factor), IL-1 (interleukin-1), and upstream signalling pathways such as JNK/p38 signalling are able to activate the NF-κB pathway [112-114].

NF-κB features five members sharing a Rel homology domain: p65 Rel A, C-Rel, Rel B, p52 and p50. The first three have a C-terminal transactivation domain, whereas the other two (p52 and p50) only have an inhibitor binding region (ankyrin repeats) shared by the inhibitory factor IκB [115]. Because they lack the transactivation domain, p52 and p50 do not have transactivation activity unless they form dimers with p65 RelA, RelB or C-Rel. In most cases, the active NF-κB is a heterodimer composed of p65 and p50, but another common form of active NF-κB is the p52/Rel B dimer [115]. Activation of NF-κB by its upstream pathways all lead to the formation of the NF-κB heterodimer, either via protease degradation of the inhibitory factor IκB or the processing of the regulatory heterodimer subunit p105 to make the active p50 that forms a dimer with p65. The ubiquitination-dependent protease degradation of IκB
requires phosphorylation. The upstream kinases for such inhibitory phosphorylation include IKKs (IκB kinase) and CK2 (Casein kinase 2) which is regulated through JNK/p38 signalling [115]. IKKs also require a phosphorylation-dependent activation to exert their catalytic activity on IκB [115]. It has been reported that under PDGF stimulation, TNF can induce the activity of PI3K which in turn activates Akt, eventually leading to an increase in NF-κB DNA binding activity [12]. Also, Akt can phosphorylate/activate IKKα at T23, triggering IKK-dependent NF-κB activation [116-117].

4.2. Akt and WNT signalling through GSK3β

Although originally identified for its involvement in embryogenesis, the Wnt pathway is now known to be involved in regulating many different biological events [118]. The Akt downstream target, GSK3β, plays a key role in Wnt signalling. The first signal protein that was identified in this pathway was the glycoprotein Wnt which is recognized by a receptor complex composed of seven transmembrane frizzled proteins and FRPs (frizzled related proteins). However, besides Wnt, it has been reported that the Wnt signalling pathway can also be activated by multiple other signals, such as insulin, IGF-1, PDGF, cAMP, and protein kinase A [119]. β-Catenin is the core functional component of the pathway which goes into the nucleus and contributes to target gene expression when activated. Otherwise, β-catenin is tightly held by a so-called “destruction” complex containing GSK3β, APC and Axin. GSK3β phosphorylates and destabilizes β−catenin at three sites (S33, S37, T41), leading to subsequent protease degradation [120]. When the pathway is stimulated, β-catenin is released from the “destruction” complex and translocates into the nucleus. The nuclear β-catenin interacts with the co-factor TCF4 to become a dual transcription factor. Also, β-catenin can act as a co-factor of the transcription factor FOXO contributing to its transactivation activity [121].

As mentioned above, the phosphorylation of GSK3β at S9 by Akt attenuates its catalytic activity. Therefore, Akt may contribute to Wnt signalling activation, although studies have shown contrary results in different cell types [16, 122-123]. It has been reported that in intestinal cells, the translation level of Cyclin D1 and c-Myc genes downstream to Wnt signalling were stimulated in an Akt/mTOR-sensitive manner [16]. This would suggest a potential regulatory role of PI3K-Akt on Wnt signalling activation. Furthermore, the well-defined Akt downstream target, mTOR, can be effectively activated by Wnt signalling via GSK3β [124]. According to these findings, it appears PI3K-Akt signalling and its downstream targets have the potential to activate the Wnt pathway and vice versa.
4.3. Akt and MAPK pathways

In addition to NF-κB and Wnt signalling, Akt also interacts with MAPK (mitogen-activated protein kinase) signalling pathways, including the JNK/SAPK (c-Jun N-terminal kinases or stress-activated protein kinases), p38 family kinases, and ERKs (extracellular signal-regulated protein kinases) [125]. MAPKs are serine/threonine protein kinases that respond to a wide range of extracellular stimuli (for example, growth factors, cytokines, stress) and have been identified as regulators of many cellular activities (e.g. oxidative and osmotic stress, apoptosis, mitosis, cell differentiation and proliferation, immune response, memory, etc.) [1]. A comprehensive description of MAPK regulation is beyond the scope of the present article but the reader is directed to Cowan & Storey (2003) for more information. Briefly, all MAPK related pathways follow a common pattern of activation which begins with an extracellular signal that interacts with cell surface receptors, GTP-binding proteins or with other kinases [1]. These messages are transmitted through the cytosol to the nucleus by the sequential phosphorylation of MAPKKK (MAPK kinase kinases), MAPKK (MAPK kinases), and MAPKs. The MAPKs then phosphorylate their target proteins (e.g. transcription factors) which alter gene expression and modulate the cellular environment.

The stress-responsive JNK/p38 pathway can be inhibited by Akt via phosphorylation of the JNK/p38 upstream factor ASK1 (Apoptotic signal-regulated kinase 1, or MAPKKK5) at S83, which prevents ASK1 from being activated by apoptotic stimuli [14]. In addition, p38 regulates the activation of Akt through a complex with other factors including HSP27 (heat shock protein 27) and MK2 (MAPK-activated protein kinase-2) in human neutrophils [15, 126]. Therefore, Akt can promote cell survival through both inhibitory effects on proapoptotic factors and the apoptotic signalling pathways. Akt, as an oncoprotein, inhibits the ERK pathway by directly phosphorylating c-Raf at T259 in a human breast cancer cell line, thereby bypassing the normal cell cycle arrest function of ERK signaling [13]. Another study from the same group showed that the interaction between the PI3K-Akt pathway and ERK signalling only occurred in certain stages of muscle development [127].

5. Hypometabolism and survival in extreme environments

All organisms must deal with variation in their environment involving a wide range of factors, both physical and biological, and occurring on time scales from minutes to years. As a result, organisms require adaptive strategies that allow them to take advantage of favourable conditions (and
quickly promote growth and development) and alleviate stresses associated with unfavourable ones. One of the more dramatic ways of dealing with severe stress is to conserve energy reserves by strongly suppressing metabolic rate and entering a hypometabolic state. Hypometabolism (also known as dormancy, torpor) characterizes a wide variety of survival strategies among animals including hibernation, aestivation, anaerobiosis, anhydrobiosis, diapause, and dauer state, among others [4]. Studies that have analyzed the intracellular signalling pathways involved in mediating oscillations in stress-responsive metabolic rate have been pivotal in uncovering Akt regulatory mechanisms as well as identifying similarities in these pathways, conserved from nematodes to mammals [6, 128-129]. For example, the nematode, *Caenorhabditis elegans*, enters a resting or dauer state between the second and third stage of larval development and the regulation of the dauer state has been studied extensively [6]. In the dauer condition, a dramatic suppression of metabolic activity produces energy savings that can sustain the nematode through periods of limited food, fluctuating temperatures, and overcrowding. Many insects also exhibit a period of dormancy, or diapause, during at least one stage of their life cycle whether it be during egg, pupal, larval or adult life stages [128]. This includes model organisms such as the common fruit fly, *Drosophila melanogaster*, and the silkworm, *Bombyx mori*, and in both cases, links have been made to Akt regulation [128].

The Akt pathway in *C. elegans* and *D. melanogaster* is regulated by insulin receptors, the phosphorylation of PI[3, 4]P2 by PI3K, the dephosphorylation of PIP3 by PTEN, and recruitment of PDK and Akt to the cell membrane [6, 128]. In addition, the downstream effects of Akt activation parallel those seen in mammalian model organisms exerting control over glycogen biosynthesis, protein synthesis, cell cycle, and apoptosis [6, 128]. Studies with nematodes and fruit flies have shed light on multiple facets of PI3K-Akt signalling and several review articles have already described the activation, regulation, and downstream effects of Akt networks during dauer and diapause states [130-133]. Thus, the following sections focus on other forms of aerobic dormancies, such as aestivation and hibernation, not yet highlighted in the literature.

Even though individual species may experience unique environmental challenges, all strategies that deal with extreme environments contain parallels in the mechanisms by which hypometabolism is achieved. For example, the controlled suppression of metabolic rate is matched with adaptations which reprioritize ATP use by many different cellular processes. This cellular reorganization relies on the concerted effects of signal transduction pathways that adjust the amount/type of biomolecules in order to
support hypometabolism. Therefore, by comparing and contrasting Akt related signalling pathways and the stress response over a range of stress-responsive strategies, we will unravel the regulatory mechanisms of this central metabolic pathway and potentially shed light on its involvement in hundreds of disease pathologies. The following sections begin with a brief description of some of the common forms of aerobic dormancies and, subsequently, outline the secrets uncovered about Akt regulation in nature.

5.1. Aestivation

Aestivation is a state of aerobic torpor which is triggered primarily by arid conditions (low water stress) but is also often associated with high temperatures and low food availability [2]. Suppression of physiological functions such as breathing and heart rate as well as reduced muscle activity contribute to energy savings in the hypometabolic state [134]. In addition, multiple biochemical adjustments mediated by mechanisms such as reversible protein phosphorylation, manipulation of signal transduction networks, and differential gene expression, ensure that aestivators are able to achieve both coordinated entry/exit from the torpid state and also sustain long term viability while hypometabolic [2]. Although utilized by many different species, research on the molecular mechanisms of aestivation has focused mainly on a few model species including various pulmonate land snails (Otala lactea, Helix aspersa and others), lungfish, and desert toads and frogs [2,134].

As a first line of defense, aestivators seek refuge in locations adequately sheltered from the external environment [136]. In addition, mechanisms that minimize water loss are key and include the acquisition of large body water stores prior to aestivation and a switch to apnoic breathing patterns that minimize water loss during breathing [137]. Evaporation of water from the body can also be suppressed by either a physical barrier (e.g. snails secrete a mucus epiphragm over the opening of the shell) [138] or biochemical means (e.g. extreme elevation of solutes such as urea to retard water loss in a colligative manner) [134]. Nonetheless, aestivators do lose substantial amounts of body fluids over what can be 9-10 months of dormancy and, thus, they typically also exhibit improved tolerances for tissue desiccation. For example, spadefoot toads (Scaphiopus couchii) readily survive the loss of as much as 60% of their total body water [134].

5.2. Mammalian hibernation

Hibernation allows many small mammals to survive deep cold and limited food availability over the winter months. By strongly suppressing
metabolic rate, often to <5% of normal resting rate, and letting body temperature fall to ambient, many small mammals can achieve energy savings of about 90% compared with the costs of remaining euthermic over the winter months [139,140]. The hibernation season consists of long bouts of deep cold torpor (days to weeks) interspersed with brief arousals back to euthermia (usually < 24 hours) [141]. While torpid, animals experience greatly reduced organ perfusion rate (<10%), heart rate (from 350-400 to 5-10 beats/min), and breathing rate (from >40 to <1 breath/min) as well as a generalized suppression of all other physiological functions [142]. Between hibernation bouts, animals rewarm themselves using heat produced from non-shivering thermogenesis in brown adipose tissue and shivering thermogenesis by skeletal muscle [3]. Interbout arousals are the most energetically costly part of hibernation and while their purpose is not fully understood, they may be important for several reasons including (1) refresh the immune system, (2) restore neural circuits, and (3) renew essential biomolecules [142].

6. Stress response by PI3K-Akt during hypometabolism

6.1. Evidence of PI3K-Akt regulation during aerobic dormancy

A recent study has shown that aestivating land snails, *Otala lactea*, show unique PI3K-Akt signalling patterns [143]. A significant increase in total Akt protein and in phospho-Akt (S473) content occurred in both foot muscle and hepatopancreas in aestivating compared with active snails (Fig. 3). Kinetic assays also showed that the activity of Akt increased in both tissues, as well as affinity for a model peptide substrate. Several downstream targets of Akt were also investigated in the same study. The amount of phospho-FOXO3a (S253) and phospho-BAD (S136) rose significantly in both tissues during aestivation (total protein did not change) (Fig. 4A) whereas mTOR showed no change in either S2481 or S2448 phospho-forms. Activity of GSK3 (both α and β isoforms) increased in both tissues during aestivation but total GSK3β protein did not change and the amount of phosphorylated GSK3β (S9) decreased significantly (Fig. 4B).

The results for FOXO3a and BAD correlated well with the observed activation of Akt. During aestivation, increased levels of phospho-Akt as well as the subsequent inactivation of FOXO3a and BAD by Akt phosphorylation, suppresses pathways that induce apoptosis and, thus, contribute to cellular survival in the hypometabolic state. Despite the involvement of Akt in FOXO3a and BAD regulation, the activity and expression levels of the growth and metabolism related factors (mTOR and GSK3β, respectively) did not show consistent connections with the upstream Akt data. This suggests
that different downstream targets of PI3K-Akt signalling may be selectively regulated during aestivation. It may be hypothesized that mTOR was not phosphorylated and activated because the energetic costs associated with high rates of protein synthesis are not conducive to conserving limited ATP resources during hypometabolism. Indeed, additional studies in the foot muscle and hepatopancreas of *O. lactea* confirmed that rates of protein synthesis are dramatically reduced by about 80% during aestivation, the phosphorylation of eIF2α and eEF2 are significantly increased, and 4EBP1 demonstrated decreased phosphorylation levels [135]. Similarly, the inhibitory action of GSK3 on anabolic processes, such as glycogen synthesis, as well as protein synthesis, through eIF2B, results in energy savings which promote the hypometabolic state.

Another study examined PI3K-Akt in an anoxia-tolerant marine snail, the common periwinkle *Littorina littorea*. As opposed to the situation of aerobic hypometabolism in the land snail, entry into a state of anoxic hypometabolism in the marine snail had no effect on total Akt protein or phospho-Akt (S473) content [144]. These results emphasize a stress-dependent pattern of Akt regulation during hypometabolism. It is possible that the periwinkle may utilize other intracellular mechanisms to promote survival

**Figure 3.** Protein levels and phosphorylation state of Akt in foot muscle and hepatopancreas of land snails *Otala lactea*. Top: Representative bands at 60 kDa from three independent samples in each condition showing Akt and phospho-Akt level. Bottom: Histogram shows the ratio of normalized mean band intensity (± s.e.m., n=3) for aestivated versus active snails. a – values for aestivation significantly different from active snails (P <0.05) [From 143].
Figure 4. The protein and phosphorylation levels of selected Akt downstream targets in foot muscle and hepatopancreas of land snails, Otala lactea. (A) Levels of FOXO3a and BAD. Top: Representative bands showing total and phosphorylated levels of FOXO3a and BAD in foot muscle and hepatopancreas of active and 10 days aestivated O. lactea. FOXO3a was detected as the bands at 110 kDa and BAD at 20 kDa; phospho-antibodies detected p-FOXO3a (S253) and BAD (S136). Bottom: Histograms showing normalized mean EST:ACT ratios (± s.e.m., n=5). (B) GSK-3 protein levels in foot muscle and hepatopancreas of active and 10 days aestivated O. lactea. Top: Representative bands showing total protein levels of both GSK3 subunits (GSK-3β and GSK-3α) and the levels of phospho-GSK-3β (S9). Bottom: Histograms showing normalized mean EST:ACT ratios (± s.e.m, n=5). a – values for aestivation are significantly different from active snails (P<0.05) [From 143].
and/or limit cellular damage under anoxia such as heightened antioxidant defenses or upregulation of stress-related heat shock proteins.

Akt is also proving to be important in mammalian hibernation with a role for Akt signalling identified in two distinct hibernator groups, bats and rodents. Data from two different species of hibernating bats, *Myotis lucifugus* and *Murina leucogaster*, revealed specific expression and activation patterns of Akt [145, 146]. The findings in both species were comparable to an earlier study carried out with the greater horseshoe bat, *Rhinolophus ferrumequinum* [147]. Studies with little brown bats, *M. lucifugus*, examined the relative expression level of Akt and p-Akt (S473) in seven tissues via immunoblotting.

**Figure 5.** Total and phosphorylated levels of Akt in little brown bats, *Myotis lucifugus*. Western blotting was used to analyze total Akt protein and phospho-Akt levels (S473) in aroused (A) vs torpid (T) bats. Top: Representative bands (60 kDa) show total Akt protein levels (left) and phosphorylated Akt levels (right). Bottom: Histograms show normalized band intensities, mean ± s.e.m., n=3. * -Values for torpid bats are significantly different from aroused animals (P <0.05) [From 145].
Differential expression patterns were identified in selected tissues during cold torpor as compared with aroused animals (i.e. euthermic controls). In brain, heart, kidney, liver, and skeletal muscle, no significant differences in total Akt were seen during the torpid state, whereas white adipose tissue showed a significant reduction in total Akt. Heart and skeletal muscle registered no change in active Akt (phospho-S473) but the amount of p-Akt decreased significantly in brain, kidney, liver, and white adipose tissue. However, brown adipose tissue showed a unique response: total Akt protein was enhanced as well as the amount of the active form (phospho-S473) during hibernation (Fig. 5). In general, the data agreed with the general metabolic pattern in hibernating species whereby an overall decrease in Akt activity is consistent with reduced insulin signalling during torpor [5, 145]. However, brown adipose tissue showed an unexpected up-regulation of Akt requiring further investigation. Similar to the response seen in the land snail, Akt may selectively regulate downstream signalling cascades suggesting that only certain branches of the Akt network are activated in brown adipose tissue of bats.

Studies of the pectoral muscle of the greater tube-nosed bat (*M. leucogaster*) delineated the role of Akt-mTOR/FOXO1 signalling in muscle mass retention during hibernation [146]. The researchers hypothesized that intermittent periods of arousal are a mechanism whereby hibernators may replenish muscle proteins depleted during hibernation. Therefore, arousal may be viewed as a period of rejuvenation which contributes to reducing the deleterious effects of extended periods of disuse. The ratio of p-Akt/Akt, p-mTOR/mTOR, and p-FOXO1/FOXO1 were measured in summer active versus hibernating bats and also assessed over a time course of torpor (1-7 days) and arousal. Levels of total Akt and mTOR did not change between summer active and hibernating conditions whereas p-Akt and p-mTOR levels decreased significantly. In addition, the p-Akt/Akt ratio did not vary significantly over the time course whereas the p-mTOR/mTOR ratio increased slightly after 1 day spent in torpor, decreased after 7 days in deep torpor, and returned to control levels during arousal. The overall levels of FOXO1 matched Akt expression, demonstrating no change when comparing summer versus winter animals or over the time course of torpor and arousal. The oscillation in p-mTOR expression levels indicates the possibility that protein synthesis may briefly be activated at different points over torpor-arousal in order to help maintain muscle mass. However, the activated p-mTOR expression pattern does not match the activation profile of Akt which remains unchanged throughout the time course. Further investigation is required; however, it is possible that while Akt phosphorylation levels have decreased, the enzymatic activity of the kinase...
matches the activation profile of p-mTOR. Nonetheless, it may be concluded that whereas Akt-mTOR regulation may play a role in maintaining muscle mass during hibernation, other pathways may also be involved.

In another recent study, total protein levels, enzymatic activity, kinetic properties, structural stability, and differential isozyme regulation of Akt were evaluated in Richardson’s ground squirrels, *Spermophilus richardsonii* [129]. Immunoblotting data showed no significant change in total Akt in muscle and liver but the amount of active, phospho-S473 Akt decreased significantly in both tissues during torpor (Fig. 6). In addition, the activity of Akt, measured with a specific substrate peptide, was reduced by 60-66% during torpor as compared to euthermic controls in muscle and liver. The role of reversible phosphorylation in changing the activity state of Akt was also confirmed with *in vitro* incubation studies. Akt from the euthermic muscle tissue showed a dramatic decrease in activity after treatment of alkaline phosphatase, which showed that euthermic Akt was the high phosphate active form and indicated that the activity change during hibernation was due to

![Figure 6](image)

**Figure 6.** Effect of hibernation on total and phosphorylated levels of Akt in muscle and liver of Richardson's ground squirrels, *Spermophilus richardsonii*. The upper panel shows representative Western blots. Akt was detected as the dominant band at 60 kDa. Histograms in the lower panel show the normalized band intensities from both conditions (mean ± s.e.m., n=5). * - Significantly different from the corresponding value for euthermia (P<0.05) [From 129].
dephosphorylation of the enzyme. In accordance with these data, studies of 13-lined ground squirrels, *Spermophilus tridecemlineatus*, also demonstrated that Akt phosphorylation and kinase activity were suppressed in liver during hibernation [148]. In summary, Akt enzymatic activity and relative protein expression patterns in ground squirrels agree with data collected from aestivators and hibernating bats described above. Thus, evidence suggests that a pivotal function of Akt regulatory mechanisms during hypometabolism is the inhibition of biosynthetic pathways.

Additional analysis of *S. richardsonii* muscle Akt showed that the properties of this protein kinase changed significantly between euthermic and hibernating states [129]. In assays at 22°C, $S_{0.5}$ ATP values (the substrate concentration producing half-maximal enzyme activity) increased by 3.3-fold as compared to the corresponding values for the enzyme from euthermic muscle. The data indicate that Akt undergoes significant changes in its protein-substrate relationships during torpor. This was particularly pronounced for ATP affinity and could have a major effect on suppressing Akt function *in vivo* during torpor because reduced affinity for ATP during torpor occurs at the same time that ATP availability is also reduced (the total adenylate pool falls by about 30% during torpor). Thus, it may be concluded that there is a reduced enzymatic potential for ATP-dependent phosphorylation of Akt targets during hibernation.

In addition to changes in Akt kinetic properties, the structural stability of the enzyme was analyzed as a possible regulatory mechanism of Akt activity in euthermic versus hibernating states. To evaluate possible differences in structural stability, Akt was subjected to urea denaturation. The $I_{50}$ values, the concentration of inhibitor that reduces activity by 50%, were 3.51 ± 0.26 M for control and 2.99 ± 0.46 M for hibernating conditions, displaying no significant difference. Therefore, urea denaturation experiments concluded that the structural stability of Akt did not change between the two states. Ion exchange chromatography was also used to evaluate the Akt isozyme pattern in muscle. Activity in euthermic control muscle eluted in three peaks indicating the presence of three isozymes: Akt1, Akt2, and Akt3. However, in hibernator muscle, only Akt1 and Akt2 peaks were detected and both of these showed much reduced activity consistent with the general reduction in Akt activity and phosphorylation state described above. Thus, there is evidence that the three Akt isozymes are differentially regulated during hibernation, with Akt3 in particular being strongly suppressed. This may provide a mechanism by which selective regulation of different branches of Akt downstream targets could be achieved when animals enter torpor.

Other studies analyzed Akt responses during the transitory periods of the torpor-arousal cycle (entrance and arousal) rather than deep torpor. During entrance and arousal, which are characterized by high metabolic rates, the
amount of active Akt increased in liver 13-lined ground squirrels as assessed using immunoblotting [149]. This activation of Akt correlated with the phosphorylation states of two Akt substrates; GSK3β (Ser9) and PRAS40 (Thr246). Phospho-GSK3β levels increased 2.5-fold during entrance into torpor and 2.6-fold during arousal, whereas phospho-PRAS40 levels increased by 4.5-fold in entrance and 4.6-fold in arousal (as compared to control animals). In order to further probe the activation of the mTORC1 pathway, the activation levels of p70-S6K as well as 4EBP1 were also measured. The data identified an upregulation of p70-S6K(T389), a direct target of the mTORC1 complex, during early torpor (T_b=5-8°C for a day) and early arousal (T_b=9-12°C) whereas 4EBP1 showed significant increases in relative protein content during entrance and arousal. Similar to the studies with greater tube-nosed bats, the above data lends support to the hypothesis that intermittent arousal periods back to euthermia during the hibernating season may provide a mechanism to renew cellular stores depleted during deep torpor. Activation of both biosynthetic pathways (through GSK3β) as well as protein synthesis (through mTORC1) during entrance and arousal suggests these processes are important in the liver during hibernation.

Studies of pathways central to the stress response in aestivation and hibernation have eloquently demonstrated mechanisms of Akt regulation. Typically, Akt is involved in anabolic processes and, thus, it would be expected that Akt is inactivated in order to contribute to energy conservation in the hypometabolic state. However, activated Akt has the capacity to selectively alter multiple downstream targets, such as GSK-3β, mTOR, BAD and FOXO, depending on the need of the organism and differential action by Akt may alter the relative functionality of different target pathways in a hypometabolic state. For example, the data for aestivating snails indicate that simultaneous inhibition of BAD and FOXO via phosphorylation can occur while leaving GSK-3β inhibition intact, and not stimulating mTOR activity. This could help to ensure that the aestivating land snail can maintain a depressed metabolic state while also inhibiting the detrimental signals that might initiate programmed cell death. In hibernators, Akt is inactivated during deep torpor characterized by low metabolic rates, while transitory phases characterized by higher metabolic rates, such as entrance and interbout arousal, showed activated Akt. Therefore, given the large number of downstream effects observed through Akt signalling; a circumstance-dependent, tissue-specific, and sometimes even organism-specific response is observed.

6.2. Akt and ischemia-reperfusion

In addition to its occurrence as part of several well-studied clinical disorders (e.g. heart attack, stroke), I-R (ischemia-reperfusion) is a major
component of hibernation torpor-arousal cycles. A key challenge for survival of mammalian hibernators is a strong reduction of heart rate and blood flow during torpor, matched with the rapid resurgence of oxygen supplies during arousal. Therefore, hibernating mammals, such as the thirteen-lined and Richardson’s ground squirrels, demonstrate characteristic features of ischemic-reperfusion cycles [150].

Previous studies have shown that the PI3K-Akt pathway plays a critical role in reducing damage during ischemia-reperfusion in multiple tissues. For example, the introduction of a constitutively active mutant form of Akt (myrAkt) protected rat liver tissue from I-R injury through its antiapoptotic effect in phosphorylating BAD [151]. Neuroprotective roles for PI3K-Akt signalling have also been shown in brain, again via antiapoptotic action [152]. PI3K-Akt signalling also appears to exert a similar protective effect in rat heart during ischemic insult [153]. These findings all show the pro-survival role of Akt in protecting organs from I-R induced apoptosis and organ dysfunction.

One major pressure induced by reperfusion is oxidative stress. This occurs due to a surge in reactive oxygen species (ROS) production as a result of a rapid increase in oxygen consumption and mitochondrial activity as hibernating mammals awake from deep torpor. Specifically, the extremely high rates of oxygen uptake and consumption are needed to support nonshivering thermogenesis to rewarm the body. Recent studies have shown that the HSP27 is a co-activator of Akt [126]. HSP27 acts as a scaffold protein for Akt, holding other cofactors to form an activation complex. H_{2}O_{2} treatment enhanced the interaction between HSP27 and Akt as well as S473 phosphorylation [154]. It also has been reported that Akt was activated in H_{2}O_{2}-treated vascular smooth muscle cells under various conditions [155]. These findings showed that ROS can act upstream of PI3K-Akt activation, indicating a possible protective effect from Akt action in response to oxidative damage.

Transcription factors under the control of Akt regulation may also play a role in the detoxification of ROS. For example, the redox-sensitive transcription factors, NFκB and Nrf2 (i.e. NFE2L2, or nuclear factor (erythroid-derived 2)-like 2), defend cells from oxidative stress by the upregulation of antioxidant enzymes. Studies with the hibernating ground squirrels have demonstrated upregulation of multiple antioxidant enzymes during hibernation including peroxidredoxins, manganese superoxide dismutase and hemeoxygenase under NFκB and Nrf2 control [150, 156-157]. Ground squirrels also show upregulation of HIF-1α which is known to respond to changes in available oxygen [158]. Indeed, HIF-1α protein levels were elevated by 60-70% in the two organs responsive for thermogenesis (brown adipose and skeletal muscle) [158].
Akt and regulation of aerobic dormancy

Akt may also impose other regulatory mechanisms on the ischemic-reperfusion response through cross-talk with MAPK pathways and, thus, Akt signalling may indirectly mediate this response. For example, MAPKKK family kinases, such as MEKK1 (or MAPKKK1), phosphorylate and activate IKK complexes which, in turn, phosphorylate the IkB protein, the inhibitor of NFκB [159]. MAPKs (ERK, JNK and p38) are also involved in the transcriptional activation of Nrf2-mediated gene expression [160], and HIF-1α is regulated by ERK2 signalling [158]. Indeed, a sequential activation of a three tiered MAPK cascade involving MAPKKKs (c-Raf, MEKK), the MAPK kinase (MEK1/2), and finally the MAPK (ERK1/2) was documented in response to dehydration in organs of an aestivating frog, *Xenopus laevis* [161]. In addition, immunoblotting with phospho-specific ERK antibody as well as kinase activity profiles demonstrated that the homologue of p42(ERK2) was increased in snail (*L. littorea*) foot muscle under anoxia [162], whereas ERK1/2 and the ERK-activated kinase, MAPKAPK-1, rose significantly in muscle and brain during hibernation in Richardson's ground squirrels [163]. Furthermore, the MAPKs, p38 and JNK, showed distinct responses to ischemic insults as compared to ERK2 regulation; (1) phospho-p38 MAPK rose in *L. littorea* hepatopancreas during anoxia while JNK exhibited no change [144], (2) phospho-p38 activity increased in muscle and heart while JNK activity rose in muscle, heart, liver and kidney in the Richardson's ground squirrels during hibernation [163], and (3) phospho-p38 was activated in skeletal muscle during hibernation of bats, *M. lucifugus* [164].

7. Conclusion

Due to its multiple downstream effects, PI3K-Akt signalling is considered to be one of the most important regulatory signalling pathways of the stress response. As described in previous sections, Akt is generally associated with cell growth through its regulation of metabolism, protein synthesis, cell proliferation, and anti-apoptosis. Akt controls cell proliferation via the activation of cell cycle promoting factors (such as cyclins) and the suppression of the inhibitory factors (e.g. CDKIs). The pathway contributes to managing protein synthesis either following the mTOR-dependent or the eIF2B-dependent mechanisms. Activated Akt can both directly (e.g. phosphorylation of BAD) or indirectly (e.g. inhibitory effect on FOXO transcription factor and p38 signalling) impose negative effects on apoptosis. Its antiapoptotic and prosurvival effects protect cells from death resulting from metabolic perturbations and/or injuries associated with external stresses. In addition, the positive roles in glucose uptake (via promotion of GLUT4 membrane translocation and GLUT1 expression) and glycogenesis (via the inhibition on
GSK3β and other related factors) make PI3K-Akt signalling pivotal to fuel preservation and energy metabolism. While the full involvement of PI3K-Akt signalling in response to environmental factors still remains to be fully discovered, a firm connection between the stress response and PI3K-Akt has been proposed by studies in aestivating and hibernating species described in this chapter. In summary, it appears that PI3K-Akt signalling is important in all forms of aerobic dormancies, which sheds some light on its regulatory roles under direct exposure of unfavourable conditions. Therefore, based on past achievements, further investigation can be carried out for a more complete understanding of the stress-responsive mechanism of PI3K-Akt signalling. These will bring us new insights into the biochemical mechanisms behind the physiological adaptations to environmental pressure.

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