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5. The role of glycogen synthase kinase-3 in the decision between cell survival and cell death

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Abstract. Glycogen synthase kinase 3 (GSK3), originally recognized for its role in glycogen synthesis, is a multifunctional kinase involved in important biological processes such as insulin sensitivity, hematopoietic stem cell repopulation, survival of tumor cells, cytoprotection in normal and pathological neurons and in cardiomyocytes. Tissue-specific effects and segregated functions within intracellular compartments (mitochondria, nucleus, cytoplasm) further contribute to branch out the activity of GSK3. Due to the extensive properties of this kinase, novel drugs targeting GSK3 are under development for potential therapeutic use in neurological disorders, diabetes and cancer. Emerging evidence indicates that GSK3 is a central regulator of a stress-activated network acting in concert with metabolic sensors of the bioenergetic requirements of the cells. The crosstalk between the PI3K/AKT/mTOR, the p90 RSK/MAP kinase (ERK1/2, JNK, p38) pathways and other key regulators of intracellular homeostasis including the transcription factors hypoxia inducible transcription

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factor-1 (HIF-1), NF κ B, Nrf2 and the LKB1/AMPK/malonyl-CoA fuel sensing pathway set the threshold of tolerance to stress through modification of glucose metabolism and redox balance. Modulation of GSK3 activity thus operates at the crossroad between cell survival and cell death and can arbitrate cell fate by promoting adaptive mechanisms that lead to cytoprotection against mild and transient stress, or to cell death under conditions of severe threat such as acute oxidative damage. This work provides an overview of recent findings on the role of GSK3 in tumor establishment and progression, with a special focus on the regulation of metabolic parameters related with energy and redox homeostasis in response to GSK activation or inhibition, which could be exploited as potential targets for cancer treatment.

Introduction

GSK3 is a ubiquitous serine/threonine kinase that controls a wide range of cellular processes, including insulin sensitivity, glucose metabolism and cell survival through regulation of energy homeostasis. GSK3 is composed of two isoforms, GSK3 α and GSK3 β . GSK3 is a unique kinase normally active (unphosphorylated) in unstimulated cells and is negatively regulated by phosphorylation of GSK3 α at Ser21 and GSK3 β at Ser9 by AKT and other kinases of the AGC (protein kinase A, protein kinase G, protein kinase C) family including p90 ribosomal S6 kinase (p90RSK) [1] upon stimulation by growth factors. Thus, GSK3 activity is kept under control by survival signals propagated by growth factors mainly through the PI3K/AKT pathway upon AKT phosphorylation at Ser473. GSK3 is also regulated by activating phosphorylation of GSK3 α and β at Tyr276 and Tyr216, respectively. Phosphorylation of GSK3 α at Ser21 and GSK3 β at Ser9 by AKT can show non-overlapping and even opposite effects, for example in cardiac hypertrophy [2].

As a downstream target of the PI3K/AKT survival pathway, the inhibition of GSK3 triggered by tyrosine kinase (RTK) and G-protein-coupled receptors (GPCR) can block mitochondrial apoptosis [3,4]. This property is beneficial to protect from damage untransformed cells including neurons [5], cardiomyocytes [6,7] and pancreatic β cells [8]. Similarly, pharmacologic inhibitors of GSK3 can exert cytoprotective effects [9]. The cytoprotective activity of lithium chloride and numerous, more specific GSK3 inhibitors has been extensively studied in pathological neuronal cells [10,11] and in cardiomyocytes under stress [12].

Among other functions, GSK3 inhibition or, more recently, genetic deficiency has been shown to normalize glucose metabolism in animal models of insulin resistance [13] and to support pancreatic β cells survival [8], thus fostering the development of GSK3 inhibitors as antidiabetic agents [14].

In cancer, an important function of GSK3, in particular GSK3 β , is to regulate the central step in the canonical Wnt/ β -catenin signaling pathway. The overactivation of this pathway has been implicated in the pathogenesis of several types of tumors, such as colon and prostate adenocarcinomas [15].

GSK3 phosphorylation stimulated by hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1) through the oncogenic AKT pathway can enhance tumor cell proliferation, motility, invasion and metastatic spread [16].

Since the formulation of the Warburg hypothesis on the generation of excessive lactate in the presence of oxygen by tumor cells, numerous studies have progressively unraveled a specific metabolic signature of epithelial cells in tumors characterized by glucose usage for energy production (aerobic glycolysis) [17]. GSK3 intervenes in key molecular events that regulate glucose metabolism such as binding of overexpressed hexokinase II (HK II) to mitochondria [18] and stabilization of HIF-1 [19].

On the other hand, GSK3 has also been implicated in the inhibition of caspase 8-mediated apoptosis triggered by death receptors, thus GSK3 inhibitors can potentiate cell death propagated through the extrinsic pathway [4,20]. More in general, new data add to mounting evidence that, at least in some cell types, GSK3 inhibition can be detrimental to tumor cell survival [21-23].

This work will focus on seminal and recent studies that have shed light on GSK3 as an important node in a regulatory network involved in the adaptive response to critical extracellular conditions affecting intracellular homeostasis (Fig. 1). In particular we will discuss in which way the metabolic consequences of GSK3 activation or inhibition support cell survival or dictate cell death in cells under stress and, in particular, in transformed cells.

1. GSK3 in cell survival and cell death: A matter of timing

GSK3 inhibition has been reported to induce cytoprotection in neuronal cells and cardiomyocytes. The cytoprotective properties of GSK3 inhibition appear particularly relevant in the context of the biological phenomenon defined as preconditioning [7] or hormesis [24] and in postconditioning, consisting of a series of adaptive responses to mild stress that protect cells against a subsequent, severe injury. These concepts have informed the improvement of therapeutic strategies in neuroprotection (neurohormesis) and cardioprotection to avoid the adverse effects of oxidative stress and necrotic cell death during reperfusion after cerebral or myocardial ischemia [7].

One of the central events in reperfusion is the opening of the mitochondrial permeability transition pore (mPTP). This protein complex is formed, among other less well-identified subunits, by cyclophilin D and the voltage dependent anion channel (VDAC). The involvement of adenine nucleotide translocase (ANT), hexokinase II and creatine kinase has been recently questioned [25]. mPTP controls selective exchanges of ions and metabolites between the cytoplasm and mitochondria. Opening of the mPTP located in the inner mitochondrial membrane is an on-off process that leads to mitochondrial depolarization, ATP depletion and eventually mitochondrial swelling. Mitochondrial damage and mPTP opening at reperfusion is caused by a burst of ROS mostly originating as superoxide anion during mitochondrial respiration (probably at complex 1 and 3 of the mitochondrial respiratory chain) and elevated calcium levels. Hydrogen peroxide derived by the activity of superoxide dismutase (SOD) can in turn be transformed in highly reactive hydroxyl radicals that damage mitochondrial proteins and lipids.

Mild mitochondrial depolarization with limited production of ROS is one of the mechanisms of preconditioning that allows cells to better tolerate oxidative stress during ischemia/reperfusion injury. GSK3 plays a prominent role in preconditioning by elevating the threshold of tolerance to ROS damage, thus protecting mitochondrial membrane integrity against mPTP opening. Proteins forming the mPTP complex and antiapoptotic proteins regulating the susceptibility to the mitochondrial permeability transition such as Bcl2 may be the GSK3 targets involved in the cytoprotective effects [7].

The temporal dynamic of preconditioning is relevant to obtain the cytoprotective effect. Cells must have time to respond to the noxious stimulus by transiently perturbing mitochondrial respiration to produce a limited amount of ROS with prevailing signaling function. Then, following a biphasic kinetics, the adaptive response ensues. Several lines of evidence indicate that GSK3 inhibition is a key event integrating survival signals emerging from the stimulation of different molecular pathways including those mediated by insulin and IGF-1, PKC, PKA.

1.1. The role of GSK3 in cardioprotection

A well-characterized experimental model of preconditioning governed by GSK3 inhibition has been described by Sollott and coworkers in cardiomyocytes [12]. The consequences of ATP depletion caused by impaired mitochondrial oxidative phosphorylation are particularly dangerous in cardiomyocytes that, differently from neurons, cannot rely on glucose as an energy substrate for ATP production and do not tolerate low pH due to lactic acid production by anaerobic glycolysis. The role of GSK3 has been

studied by analyzing the effects of different pharmacological inhibitors of the mPTP. Two pathways leading to inhibition of the mitochondrial pool of GSK3, which differ in their ability to confer transient or stable, memory-associated protection against oxidative stress have been identified by using different pharmacological agents, including lithium chloride, insulin and mitochondrial ATP-dependent K⁺ channel openers [7,12,25]. Phosphorylation of GSK3 β at Ser9 has been proposed to elevate the threshold for mPTP opening by preserving the binding of hexokinase II to the mPTP complex [18]. At present, the exact molecular mechanism through which GSK3 inhibition elevates the threshold for mPTP activation remains undefined. However, based on the encouraging results that indicate GSK inhibition in preconditioning as a valuable tool to limit ischemia/reperfusion injury, this therapeutic approach deserves in depth exploration in further preclinical and clinical studies.

1.2. The role of GSK3 in neuronal cell survival and death

The role of active GSK3 in neuronal cell death has been extensively studied in several models of neurotoxicity and neurodegeneration. Although the adaptations to limit neuronal cell loss and to maintain tissue integrity in the brain differ in acute damage such as ischemic/reperfusion and neurotoxic injury, and in chronic pathological conditions, some common mechanisms include oxidative stress and disruption of calcium homeostasis. These conditions eventually lead to impairment of mitochondrial function followed by apoptotic or necrotic cell death [26].

GSK3 has been found to regulate different molecular activators of the mitochondrial (intrinsic) apoptotic pathway [4]. In neuronal cells, GSK3 has been reported to phosphorylate Bax, favoring its mitochondrial translocation, and the mixed lineage kinase 3 (MLK3), a mitogen-activated protein kinase kinase kinase that activates the proapoptotic c-Jun N-terminal kinase (JNK) pathway [27,28]. The recent finding that Wnt-regulated GSK3 β is implicated in cell death of cerebellar granule neurons induced by trophic factor deprivation assigns a new role to the Wnt GSK3 pool.

GSK3 overexpression or overactivation in the brain has been found in animal models of Alzheimer's and Parkinson's disease [29]. Most neurodegenerative diseases are characterized by impaired mitochondrial function and glucose hypometabolism. Of note, neuroprotection can apparently be achieved by metabolic changes enhancing glucose metabolism in neurons. Two main molecular mechanisms underlying neuroprotection by enhanced glycolysis have been identified: HIF-1 induction boosting glucose flux through glycolysis and the pentose phosphate pathway [30], and

induction of HKII binding to mitochondria induced by GSK3 inhibition [31]. Enhanced glucose metabolism can result in the production of reducing equivalents in the form of NADPH. This HIF-mediated mechanism has been identified as an adaptation to tolerate oxidative stress due to β -amyloid toxicity and to contribute to the selection of a β -amyloid-resistant phenotype [30].

Paradigmatic models of neuronal cell death upon trophic factor withdrawal show that GSK3 inhibition by growth factors is an essential event to preserve neuronal cell survival [32]. In accordance with the activity of other growth factors, NGF neuroprotection is linked to GSK3 inhibition through activation of PI3K/AKT signaling and the effects of NGF deprivation have provided an informative set of data on the mechanisms implicated in GSK-mediated cell death [28].

Lithium chloride has found application in psychiatry as a mood stabilizer in manic-depression illness (bipolar disorders) for over fifty years. In 1996, Woodgett and coworkers discovered that lithium chloride is a pharmacological inhibitor of GSK3 [33]. Since then, numerous GSK3 inhibitors other than lithium, including alsterpaullone, kenpaullone, SB216763 and AR-A014418 have been synthesized and reported to protect neurons against a variety of stressful conditions. The effects of lithium and other GSK3 inhibitors at molecular level have been studied in *in vitro* and *in vivo* experimental models recapitulating oxidative neuronal damage found in Parkinson's and Alzheimer's disease and other injuries including hypoxia-ischemia in the immature brain, oxygen-glucose deprivation, potassium deprivation, glutamate excitotoxicity. Some of the biological effects of lithium cannot be ascribed to GSK3 inhibition in that lithium can also inhibit inositol monophosphatase (IMP) and induce macroautophagy due to free inositol and myo-inositol-1,4,5-triphosphate (IP3) depletion [34]. This biological effect is therapeutically relevant in neurodegenerative disorders induced by intracellular self-aggregation and accumulation of peptides such as β -amyloid, α -synuclein, tau or mutant huntingtin, in neurofibrillary tangles, which are autophagy targets. Nevertheless, the neuroprotective properties of selective GSK3 inhibitors confirm that GSK3 plays a central role in neuronal survival and plasticity. Interestingly, other neuroprotective drugs such as valproic acid indirectly induce GSK3 phosphorylation [11].

GSK3 inhibitors belong to two categories: ATP competitive inhibitors localized in the ATP-binding pocket (CHIR-99021, AR-A014418, SB216763, SB415286) and non ATP competitive compounds (TDZD8, TWS119). Lithium chloride competes with magnesium and causes Ser9 and Ser21 autophosphorylation in GSK3 β and GSK3 α , respectively. Different GSK3

inhibitors including AR-A014418, SB216763, SB415286, do not interfere with GSK3 α/β phosphorylation at Ser21 and Ser9. Thus, the effects of the pharmacological inhibition of GSK3 on downstream targets can be independent of phosphorylation induced by PI3K/AKT and other AGC kinases (Fig. 1).

The question of why dysregulated, hyperactive GSK3 appears particularly damaging in neurons remains to be answered. The identification of oxidative stress as one of the most important event in the pathogenesis of classical models of neuronal injury by neurotoxic agents such as amyloid- β peptide and glutamate excitotoxicity and the use of pharmacological inhibitors of mitochondrial damage to maintain neuronal cell survival has provided important information about the requirements of this highly specialized cell type with unique biological functions.

Several recent reports suggest that, among other factors, neuronal GSK3 activation appears particularly harmful due to the unique set of stress-related GSK substrates in this cell type. As an example, active GSK3 plays a pivotal role in Alzheimer's disease by increasing tau phosphorylation and β -amyloid production [35].

Most of the GSK3 inhibitors, except for CHIR-99021, show low affinity and weak inhibition for other kinases [1]. Nevertheless, GSK3 inhibitors represent a promising category of therapeutic agents in neurodegenerative disorders and, consequently, in recent years the search for more specific GSK3 inhibitors to avoid side effects due to off-site targeting has been intensified [14].

1.3. The opposing roles of GSK3 in tumors

Over-activation of survival signaling pathways mediated by AKT and NF κ B is a common feature of tumor cells. In recent years, great efforts have been made to advance the search for synthetic or naturally occurring antitumor drugs able to selectively inhibit protein kinases regulating cell survival pathways. The clinical utility of highly selective protein kinase inhibitors, however, has been questioned. To overcome the emergence of molecular resistance to specific inhibitors, it has been proposed the alternative strategy of pointing to multi-targeted protein kinase inhibitors or cocktails of selective antagonists [36]. Another possibility is the targeting of multifunctional kinases that act as master regulators of different and cross-talking signaling pathways. This appears to be the case for GSK3. In some tumor cell types active GSK3 mediates mitochondrial apoptosis, while in others it negatively regulates cell death induced by death receptors and

caspase 8, thus GSK3 has been shown to enhance cell death induced by TRAIL [4]. Thus, the effects of GSK3 inhibition on tumor cell survival depend on cell type-specific contexts. The increasing interest in histone deacetylase (HDAC) inhibitors as neuroprotective and cardioprotective drugs [11,37] and, at the same time, as promising anticancer agents underscores the evidence that GSK3 inhibition by lithium chloride, valproate and other HDAC inhibitors can lead to opposite outcomes in different cellular contexts. Evidence from *in vitro* and *in vivo* studies with specific GSK3 inhibitors like AR-A014418 confirm this observation: AR-A014418 has been indicated as a potential therapeutic tool in neurodegenerative disorders, however it potentiates the activity of anticancer drugs in glioblastoma and melanoma cells [38] and other tumor cell types [4]. Similarly, the regulation of NF κ B by GSK3 exerts antiapoptotic effects in pancreatic cancer cells [39], while it induces cell death in astrocytes [40]. We have shown that GSK3 inhibition modulates the threshold of sensitivity to anticancer synthetic triterpenoids in prostate adenocarcinoma cell lines. We observed that GSK3 inhibition or genetic depletion changes the shape of cell death from apoptotic to necrotic by enhancing caspase 8 activity and mitochondrial and nuclear apoptosis mediated by downstream caspase 9 and caspase 3 [22].

The activation of the Wnt/ β catenin pathway can promote tumor progression. Active GSK3 β phosphorylates and promotes β -catenin proteasomal degradation by the destruction complex formed by the adenomatous polyposis coli (APC) and Axin proteins. This process inhibits Wnt-stimulated TCF/LEF-dependent transcription and accumulation of an oncogenic, transcriptionally active form of β -catenin [41]. In those tumor cell types in which the role of active GSK3 β in the Wnt/ β -catenin pathway prevails over other functions, the inhibitory phosphorylation of GSK3 β can thus be functional to tumor progression through the stabilization and activation of β -catenin. Recently, multiple proteins have been identified as potential candidates for GSK3-regulated phosphorylation and Wnt-regulated proteasomal degradation, thus broadening the range of cellular activities controlled by the Wnt/GSK3 destruction complex. These targets, besides β -catenin-mediated transcription, are involved in cellular functions including RNA processing, cytoskeletal dynamics, and cell metabolism [42]. Several recent excellent reviews have exhaustively treated the role of GSK3 and the Wnt- β -catenin pathway in the control of cell fate [43], therefore this topic will not be discussed further in this review.

The paradoxical effects produced by the same compound in different pathological contexts occur despite some neurodegenerative disorders like

Alzheimer's disease, diabetes and cancer show common pathophysiological traits including mitochondrial dysfunction, excessive generation of ROS, a precarious redox equilibrium due to altered expression of antioxidant enzymes and insulin resistance. The first consideration is that pathological neurons, cardiomyocytes and pancreatic β cells are not transformed cells; secondly, they are non-proliferating, post-mitotic cells. These observations could imply that untransformed and rapidly proliferating, transformed cells adopt different metabolic rearrangements to cope with similar pathological manifestations. Here we will illustrate some of the metabolic changes resulting from GSK3 modulation and will discuss several hypothesis on why the attempt to restore intracellular homeostasis can produce diverging effects in untransformed and in transformed cells.

2. Linking the metabolic effects of GSK3 with cytoprotective and anticancer properties

It is now established that the metabolic control of intracellular homeostasis is intimately linked to cell signaling networks integrating information from environmental changes or intracellular pathological events such as peptide or protein aggregation and tangle formation in neurons, endoplasmic reticulum (ER) stress due to glucose deprivation, or oncogene activation in cells living in chronic stressful conditions. In particular AKT, mTOR, GSK3, FoxO and sirtuins (Sirt 1-3) belong to a signaling network acting in concert with energy sensors such as AMP-activated protein kinase (AMPK) and transcription factors including the hypoxia inducible transcription factor 1 (HIF-1) and NF-E2-related factor 2 (Nrf2) capable of producing metabolic and redox adjustments in response to the bioenergetic requirements of the cells. While short-term damage elicits adaptive responses usually functional to cell defense and repair, long-term activation of homeostatic molecules, for example HIF-1, AKT/GSK3 and Nrf2, can help cells to substantially reprogram metabolism in pathological directions (Fig. 1).

2.1 GSK3 supports the glycolytic phenotype

The glycolytic phenotype of most tumor cells is supported by the overactivation of the PI3K/AKT/GSK3 pathway that can confer resistance to apoptosis. Other specific cell types such as immune cells with intense biosynthetic activity show an hypermetabolic state and preferentially use glucose as a primary energy source [44]. However, it is not clear whether the glycolytic switch may have a causative role or be a consequence of transformation. The observation that metabolic diseases characterized by

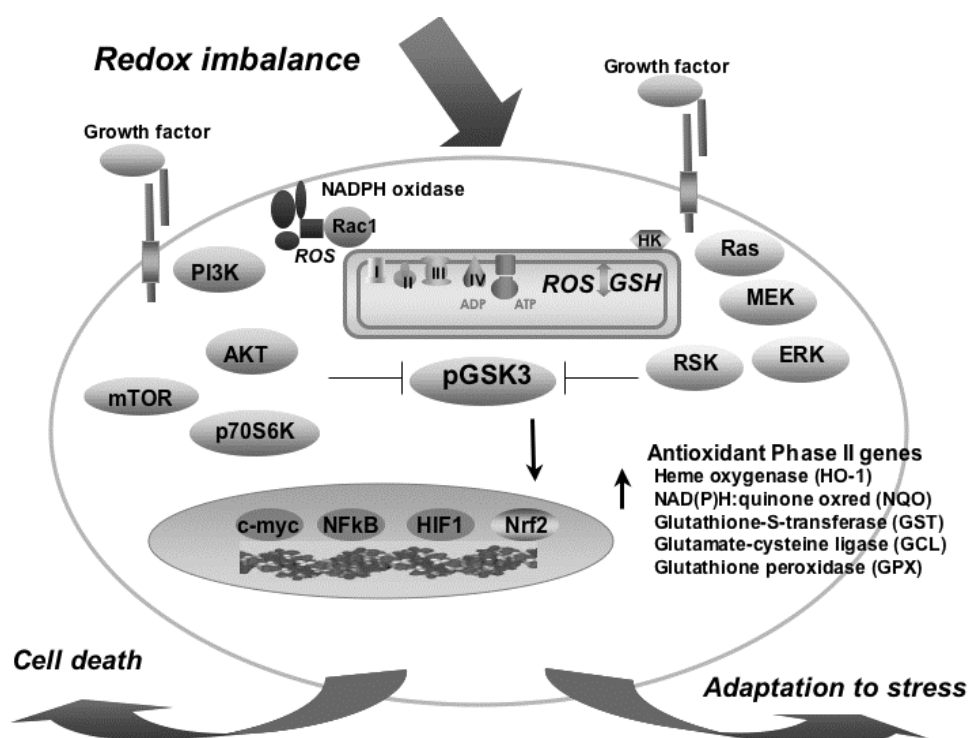


Figure 1. Redox imbalance is a common trait of different pathological conditions including cardiovascular diseases, neurodegeneration, metabolic disorders, diabetes, and cancer. One of the main line of antioxidant defense against oxidative stress involves the activation of cell signaling pathways regulating energy expenditure and adjustment of glucose metabolism. The enhanced transcriptional activity of Nrf2 following GSK3 inhibition provides a battery of antioxidant enzymes, while HIF1 induction of glycolytic enzymes can compensate the impairment of mitochondrial ATP production. This mechanism is effectively involved in neuroprotection. In cardiomyocytes, a central event apparently necessary to protection against ischemia/reperfusion injury is the limitation of mPTP, which can be obtained by GSK3 inhibition. In tumor cells under constitutive oxidative stress, the perturbation of a precarious redox equilibrium by ROS induction or GSH depletion can lead to cell death and represents a promising anticancer strategy. Different redox-active drugs have been shown to enhance GSK3 phosphorylation. Due to the up-regulation of GSH and antioxidant enzymes consequent to GSK3 inhibition, this event could be an attempt to counteract oxidative stress.

altered glucose balance and defective mitochondrial energy production including insulin resistance and obesity are risk factors for some types of cancer suggest that impairment of glucose homeostasis is important in the early phases of cancer development [45]. On the other hand, the hypermetabolic state of frank tumor cells showing activated oncogenes like ras and myc indicate that glycolysis supported by AKT could be an adaptation to cope with the high bioenergetic requirements of tumor cells due to oncogene activation and increased ROS production.

Emerging evidence is elucidating the link between glucose metabolism and inhibition of tumor cell death. GSK3 can regulate glucose metabolism through multiple mechanisms, with varied effects in normal and tumor cells [14,46]. During growth factor deprivation, but in conditions of enhanced glucose metabolism, GSK3 inhibition by protein kinase C can stabilize the antiapoptotic Bcl-2 family protein Mcl-1, which is degraded by a GSK3-mediated mechanism [47].

Overexpression of HKII and enhanced binding to mitochondria promotes glucose consumption and exerts an antiapoptotic effect. It has been reported that active GSK3 can phosphorylate HK II, thus inhibiting its binding to mitochondria, and trigger cell death in tumor cells [48]. As a result of AKT activation by growth factors, the inhibitory phosphorylation of GSK3 promotes binding of HK II to mitochondrial outer membrane, an event that enhances glucose metabolism. [18,49]. This same adaptive mechanism involving HK II overexpression and binding to mitochondria, with the consequent enhancement of glucose metabolism, can be also due to chronic inhibition of GSK3 and plays an opposing, neuroprotective role in neuronal cells treated with the mitochondrial complex I inhibitor rotenone [23,31,48].

GSK has been shown to mediate HIF-1 α stabilization upon ROS-induced RhoB activation in hypoxic conditions [50], or during early hypoxia in other experimental models [51], thus reinforcing glucose utilization through HIF-mediated transcription. Conversely, in prolonged hypoxia GSK3 dephosphorylation and activation contributes to HIF-1 α degradation [19] and probably hypoxic cell death.

2.2 GSK3 in regulation of energy balance

Cross-talk and reciprocal regulation between GSK3 and AMPK, a major molecular mediator of energy balance within the cell [52], plays a key role in cellular and systemic metabolic regulation. AMPK is a heterotrimeric complex that is composed of a catalytic α -subunit, a β -subunit, and a γ -subunit that binds AMP. Low energy levels in the cell due to oxygen or glucose deprivation or enhanced ATP consumption are sensed as a decreased ATP/AMP ratio. Allosteric AMPK activation by AMP binding is accompanied by phosphorylation of the active site by upstream kinases including the tumor suppressor LKB1 and calmodulin-dependent kinase kinase- β (CaMKK β). AMPK then phosphorylates various substrates including metabolic enzymes, transcription factors and co-activators to inhibit energy consuming biosynthetic pathways, first of all protein synthesis promoted by mTOR activation, along with lipid and carbohydrate biosynthesis, while it activates catabolic reactions to preserve ATP. The

metabolic effects of AMPK inhibition by AICAR or metformin, include improved glucose uptake and glycolysis and lipid metabolism [53]. AMPK phosphorylates AcetylCoA carboxylase 1 and 2 (ACC1, ACC2), blocking fatty acid synthesis and improving fatty acid oxidation. It has been recently demonstrated that AMPK is inhibited upon binding of glycogen to its β -subunit [53]. In this way, through the modulation of glycogen binding, AMPK can also sense and control the availability energy stores. AMPK inhibitors such as metformin are in clinical use for the treatment of the metabolic syndrome, dyslipidemia and diabetes. Activation of AMPK triggers a phosphorylation cascade involving the tuberous sclerosis complex proteins (TSC1 or hamartin, TSC2 or tuberin), that inhibit the mTOR activator Rheb, and leading to mTOR inhibition. In conditions of critical energy levels due to low ATP, glucose or oxygen, AMPK and GSK3 cooperate to activate a pro-survival pathway by blocking mTOR activity. In different non-tumorigenic cell lines including HEK293, MEF, 293T, AMPK directly phosphorylates TSC2 on serine residues 29–32 and primes serine residues for subsequent phosphorylation by GSK3 [54]. AMPK has been shown to induce AKT-mediated Ser9 phosphorylation of GSK3 in HepG2 cells that is required for inhibition of cAMP-response element (CRE)-dependent genes, such as phosphoenolpyruvate carboxykinase C (PEPCK-C) [55]. Conversely, in both hippocampal neurons and the neuroblastoma cell line SH-SY5Y both the AMPK inhibitors AICAR and phenformin induced AKT and GSK3 dephosphorylation [56].

AMPK activation not only limits ATP-consuming biosynthetic processes, but also inhibits cell growth and proliferation by inducing the accumulation of cell-cycle inhibitors such as p53, p27 and p21. Based on the evidence of the proapoptotic effects on energy demanding cancer cells, AMPK inhibitors have received attention as potential antineoplastic drugs [53].

In conclusion, GSK3 signaling essentially contributes to the network involving modulation of mTOR and AMPK activation, all integrating at the level of the TSC2 complex, in order to arrange growth control with energy availability within the cell (Fig. 2).

Other major metabolic changes affecting energy balance in tumor cells involve the diversion of lipogenesis by fatty acid synthase (FAS) to enhance cell survival at the expenses of energy storage [57] and myc-stimulated high rate glutamine metabolism to support anabolic growth [58]. Several reports show that GSK3 plays a role in these key tumor-specific metabolic adaptations. Active GSK3 has been reported to down-regulate the transcriptional activity of adipocyte determination and differentiation-dependent factor 1 (ADD1)/SREBP1c that controls FAS expression [59] and to phosphorylate and promote degradation of a *Drosophila* homologue of c-myc upon priming by casein kinase I (CKI) [60].

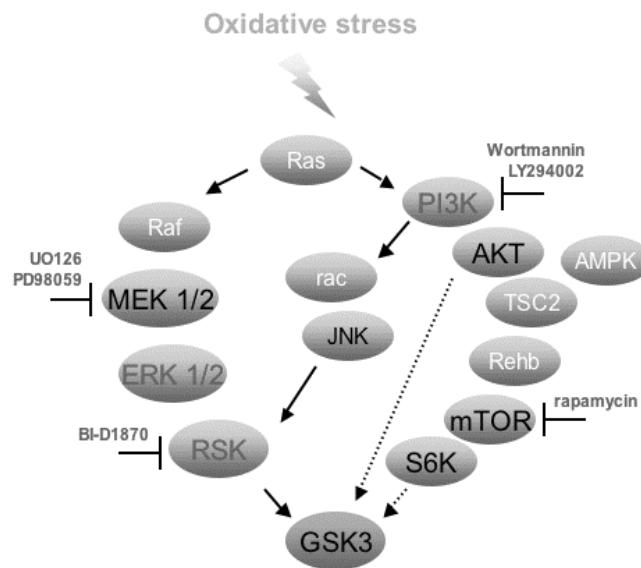


Figure 2. The main intracellular stress-sensitive signaling pathways converge on GSK3 regulation. Dissection of the different branches of the network by the use of pharmacological inhibitors including the MEK inhibitors PD98059 and UO126, the PI3K inhibitors wortmannin and LY294002, the mTOR inhibitor rapamycin and the p90 RSK inhibitor BI-D1870 served to set the hierarchical order of the single molecular components of the network. Although targeted genetic depletion is a straight forward approach to assign a role to the different molecular components of the network, the therapeutic perspectives to modulate the activity of the network foresee the use of protein kinase inhibitors. The reciprocal regulation and feed-back inhibition or activation in the network are not represented for simplification, but contribute to finely tune the duration and the effectiveness of the adaptive signals aimed at preserving cell survival as a first goal. If the intra- and extracellular conditions do not allow the completion of the survival programs, the attempts to balance adverse conditions result in a futile cycle and cells will eventually undergo cell death.

2.3. GSK3 in intracellular redox regulation

One of the most remarkable properties of active GSK3 is to modulate the activity of the transcription factor Nrf2, a major regulator of redox homeostasis [61]. This property directly links the neuroprotective effects of GSK3 inhibition with the antioxidant defenses of the cells. A pool of nuclear Nrf2 is constitutively present in unstressed cells to maintain basal levels of transcription of antioxidant phase II genes regulated by the *cis*-acting element antioxidant response element (ARE). To maintain homeostasis, part of Nrf2 is negatively regulated by Keap1 which promotes its ubiquitylation and degradation in the cytoplasm. In conditions of redox imbalance due to enhanced production of hydrogen peroxide and ROS, or decreased antioxidant defenses including GSH, cytoplasmic Nrf2 is stabilized and,

following translocation, contributes to the nuclear active pool. ARE activation by a transcriptional complex formed by Nrf2 and small Maf proteins induces the expression of antioxidant proteins including heme oxygenase (HO-1), NADPH:quinone oxidoreductase (NQO1), thioredoxin (Trx) and enzymes involved in GSH metabolism such as glutathione S-transferase A1/A2 (GST A1/A2), glutathione peroxidase (GPx), γ -glutamyl cysteine ligase (γ GCL). The induction of this last enzyme causes an adaptive elevation of intracellular GSH. Once normal levels of GSH are reached, a negative feed-back operated by GSH itself inhibits further GSH synthesis by γ GCL. Active GSK3 has been reported to phosphorylate Nrf2 [62]. This post-translational modification excludes Nrf2 from the nucleus, thus limiting the expression of Nrf2-dependent genes. Thus, upon GSK3 inhibition, Nrf2 can activate the transcription of ARE-regulated phase II antioxidant enzymes. As a consequence, GSK3 inhibition can enhance GSH utilization in cells with overall antioxidant effects. Importantly, persistent oxidative stress has been shown to inhibit cytoprotective Nrf2 activation by GSK3 in neuronal cells [63], confirming the neuroprotective effects of GSK3 inhibition.

Nrf2 plays a relevant role in tumorigenesis. Nrf2 promotes detoxification and cytoprotection against oxidative, electrophilic and xenobiotic stress and its activation has been implicated in prevention of tumor development and progression in numerous animal models. Nrf2 is apparently a major molecular target of cancer chemopreventive compounds, in particular of chemopreventive phytochemicals or their synthetic derivatives [64]. In established tumors, Nrf2 targeting by different anticancer agents induce caspase-dependent and independent cell death essentially by glutathione depletion and redox imbalance [65]. In some tumor cell types, Nrf2 activation, as judged by induction of HO-1, occurs in cells undergoing cell death [66]. Therefore, while the cytoprotective and antioxidant response in normal or preneoplastic cells in the early phases of transformation elicits an antitumor effect by preserving cell survival and tissue integrity, the same response may apparently contribute to cell death in established tumor cells.

Several lines of evidence indicate that GSK3 itself is sensitive to redox regulation [62,63,67,68]. Of note, key molecular targets of survival pathways with proinflammatory and proangiogenic activity including the nuclear transcription factors NF- κ B and HIF, AKT and mTOR are redox-sensitive molecules (Fig. 1).

Different redox-active pharmacological agents and intracellular conditions that perturb intracellular redox homeostasis modulate GSK3 activity [67]. The modification of intracellular redox conditions toward a more oxidized state, as already discussed in the case of GSH metabolism regulated by Nrf2, can affect, in particular, GSK3 intracellular localization and activity.

The molecular events following GSK3 inhibition, including induction of phase II genes such as heme oxygenase, elevation of intracellular GSH and of enzymes related with GSH metabolism, indicate that GSK3 inhibition exerts a cytoprotective effect against oxidative stress (Fig.3). This cytoprotective response is apparently evoked by transient, acute stress in untransformed as well as in tumor cells. A different condition seems to be established in chronic degenerative disorders, where oxidative stress becomes a permanent condition that necessitates metabolic adaptations, and active GSK3 accumulates in neuronal cells with detrimental effects on cell survival. Time-dependent effects

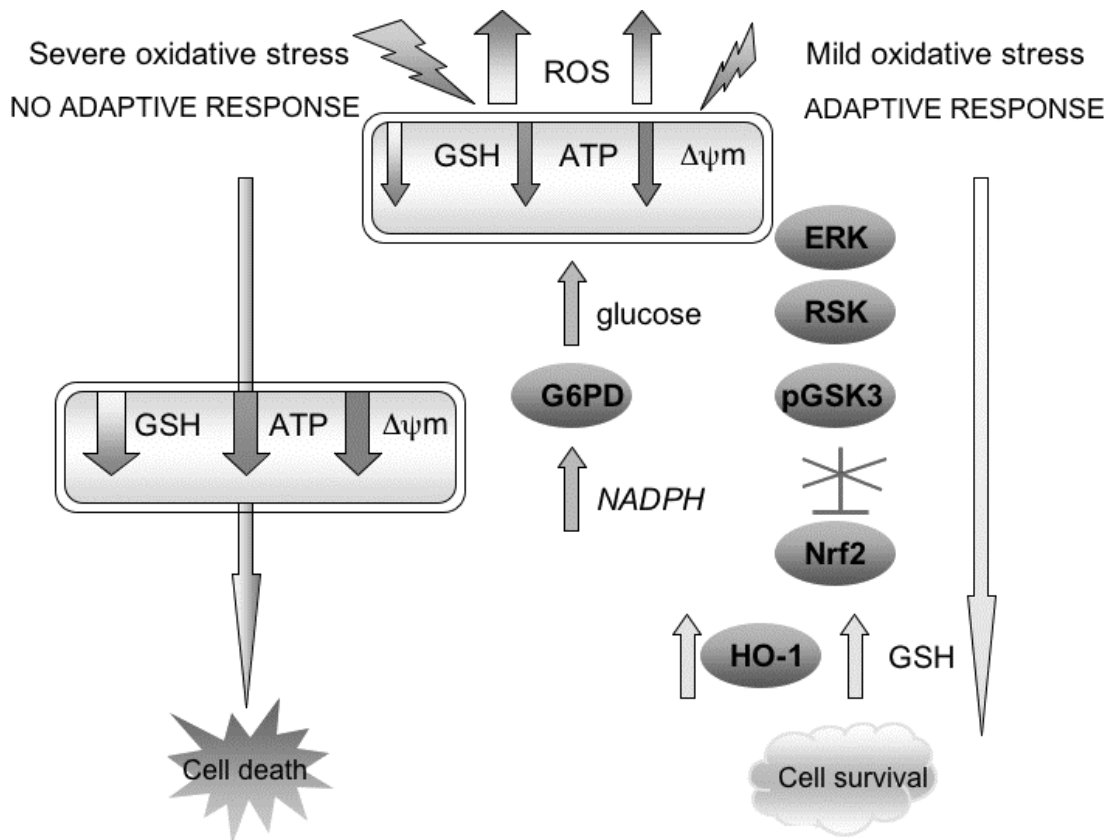


Figure 3. The expression of antioxidant enzymes such as heme oxygenase-1 (HO-1) and a set of stress-inducible Nrf2-regulated genes stimulated by GSK3 inhibition requires the coordinated activation of ancillary pathways providing energy substrates and cofactors necessary to guarantee the proper functioning of the antioxidant machinery. The expression of antioxidant enzymes requires transient up-regulation of G6PD providing reducing equivalents in the form of NADPH, concomitantly with enhanced glucose uptake. The attempt to mount an antioxidant adaptive response against oxidative stress can be elicited in normal and stressed untransformed cells, as well as in cells under selective pressure imposed by tumorigenic conditions such as chronic inflammation, and in frank tumor cells. The final outcome changes depending on the intracellular bioenergetic state and microenvironmental conditions that must be permissive to effectively obtain cell repair and survival.

have been described for ERK1/2. Transient ERK activation can induce an adaptive elevation of intracellular GSH [69]. However, sustained ERK activation can both lead to oncogenic adaptations or to cell death [70]. Several lines of evidence suggest that the consequences of transient versus sustained GSK3 inhibition can have similar effects in some cellular contexts.

2.4. GSK3 as a molecular target of redox-active phytochemicals

Emerging evidence indicates that natural or synthetic derivatives of phytochemicals interfere with key redox-regulated events in tumorigenesis and tumor-predisposing conditions such as metabolic disorders including hyperinsulinemia, obesity and diabetes, and in aging [71]. Remarkably, several redox-active dietary phytochemicals have shown neuroprotective [72] and cardioprotective effects [73] in preclinical studies. It is now established that metabolic disorders, besides defining risk factors for cardiovascular diseases and diabetes, also increase the risk for cancer. Starting from the natural compounds, novel synthetic derivatives are under development for the production of more effective drugs with increased therapeutic efficacy. This class of compounds are presently under intense pre-clinical and clinical scrutiny for potential use in diabetes, neurodegenerative and cardiovascular disorders and in cancer treatment [74]. Nrf2 is a major target of cancer chemopreventive and cytoprotective phytochemicals and their synthetic derivatives [64]. Few data are presently available on the involvement of GSK3-mediated Nrf2 activation in the mechanism of action of these compounds. However, owing to the fact that Nrf2 regulation by GSK3 could explain several of the cytoprotective effects of GSK3 inhibition in different pathophysiological contexts, here we will briefly mention recent reports on the involvement of Nrf2 in the activity of model phytochemicals which appear promising therapeutic agents: resveratrol, sulforaphane and curcumin. Resveratrol, a polyphenol found in red grapes, has shown a range of remarkable biological properties in preclinical models of aging that resemble the beneficial metabolic effects of dietary restriction [75]. Similarly to other phytochemicals with potential application in long-term cancer chemoprevention, resveratrol shows both cytoprotective and anticancer properties. GSK3 inhibitory phosphorylation induced by resveratrol has been shown to protect against mitochondrial damage induced by arachidonic acid [76], and to be implicated in cardioprotection [77]. Induction of Nrf2-regulated antioxidant genes depending on GSK3 inhibition induced by sulforaphane has been shown to protect hippocampal neurons against lethal kainate-induced oxidative stress [78]. Curcumin and sulforaphane activate an antioxidant defensive response mediated by Nrf2-induced GPx expression [79].

3. Effects of GSK3 on stem cells

An unexpected function of GSK3 recently discovered is to regulate embryonic stem cell (ESC) pluripotency and self-renewal. GSK3 inhibition has been shown to sustain the expression of three molecular targets involved in stem cell repopulation: the pluripotent state-specific transcription factors Oct-3/4, Rex-1 and Nanog [80]. GSK3 regulates different signaling pathways implicated in HSC function, including Wnt, Hedgehog and Notch pathways. Interestingly, GSK3 inhibitors were found to modulate genes regulated by Wnt, Hedgehog and Notch pathways selectively in hematopoietic stem cells (HSC) of the primitive hematopoietic compartment, while mature cells were unaffected [81]; most importantly, GSK inhibitors were toxic to MLL leukemia cells [82]. Again, GSK3 can act in opposite ways in cells of the same origin depending on transformation. Similarly to the role of GSK3 in different intracellular compartments and molecular complexes, the partition of GSK3 in subcellular pools appears a regulatory mechanism *per se* in stem cell renewal [83]. The PI3K/AKT pathway and c-myc sustain ESC self-renewal. GSK3 phosphorylates and induce degradation of c-myc, thus antagonizing ESC self-renewal. The mechanism operating in mouse ESC depends on GSK3 phosphorylation by AKT that excludes shuttling of GSK3 into the nucleus and causes phospho-GSK3 accumulation in the cytoplasm. In this way AKT neutralizes the antagonizing effect of GSK3 on ESC self-renewal.

Summary

The regulation of cell survival occurs through the activity of interrelated sensing systems that monitor, among others, nutrient and glucose availability, the level of tissue oxygenation and redox homeostasis. NF- κ B, HIF, FOXO, Sirt1, PI3K/AKT/mTOR/ GSK3 and the MAP kinases ERK1/2, JNK and p38 belong to a signaling network deciphering the overall metabolic state of the cell. The cross-talk between the different branches of the network results in the activation of effector mechanisms that engage both pro-survival and pro-death pathways in adverse environmental conditions. The modulation of these pathways propagates information to effector systems involved in glucose metabolism, energy expenditure and redox homeostasis that allow cells to adapt to transient environmental changes, with an overall positive effect on cell survival. Under conditions of chronic stress, however, the persistent activation of these salvage pathways to which GSK3 belongs can contribute to the transcriptional reprogramming involved in tumor insurgence, stabilization and progression. Active GSK3 phosphorylates and promotes the degradation of substrates involved in molecular pathways

including Wnt/ β catenin and Hedgehog/MYCN in normal cells, thus blunting important oncogenic signaling. However, GSK3 overactivation has apparently detrimental effects on cell survival. Under physiological conditions, GSK3 is kept under negative control by cell survival signaling triggered by trophic factors and supported by adequate energy availability. In different pathological conditions, such as neurodegenerative and cardiovascular disorders, GSK3 shows excessive accumulation and overactivation, thus its inhibition exerts cytoprotective effects. In cancer, GSK3 probably plays a different role during the early phases of tumorigenesis and in established tumor cells. Several lines of evidence indicate that dysregulated GSK3 inhibition, depending on constitutive activation of AKT and other oncogenes, can support tumor-associated metabolic adaptations such as the glycolytic switch and can play an antiapoptotic role by preserving mitochondrial integrity in stressful conditions. On the other hand, Nrf2, which can be activated upon GSK3 inhibition, has been identified as a major target of several cancer chemopreventive drugs, providing a cellular defense against tumorigenic oxidative damage. The up-regulation of Nrf2-regulated antioxidant enzymes following GSK3 inhibition, while accounting for the cytoprotective effects against cell death in brain cells, can confer resistance to anticancer cytotoxic agents. Paradoxically, different drugs inducing GSK3 inhibition, including several phytochemicals or their synthetic derivatives, specific pharmacologic inhibitors and HDAC inhibitors, have shown promising anticancer effects in preclinical models. One hypothesis to explain the opposing roles of GSK3 inhibition is that different effects depend on the bioenergetic requirements of the cells. In cells that rely on oxidative phosphorylation for energy production and in cells that can tolerate the use of glycolysis as an alternate source of ATP, such as neuronal cells, GSK3 inhibition exerts cytoprotective effects. GSK3-mediated cytoprotection can thus be beneficial in cells that are not subjected to oncogenic stimulation, while can promote cell survival in tumorigenic cells. Once the metabolic reprogramming that leads to stabilization of tumor cells, first of all the glycolytic switch, is fully realized, GSK3 inhibition in sensitive cells might perturb a precarious equilibrium characterized by a high metabolic rate fueled by enhanced glucose metabolism and adaptive adjustments of redox balance to tolerate increased ROS production. An emerging therapeutic approach highlights the possibility to exploit specific metabolic adaptations such as enhanced glucose usage, elevated production of reactive oxygen species and antioxidant enzymes to selectively target tumor cells [84]. GSK3 pharmacologic targeting could offer the opportunity to investigate the application in oncology of drugs already in use for the treatment of neurological disorders, which have been proved to be safe upon long-term administration.

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