Drug Development Targeting the Glycogen Synthase Kinase-3β (GSK-3β)-Mediated Signal Transduction Pathway: Role of GSK-3β in Myocardial Protection Against Ischemia/Reperfusion Injury

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Abstract. Although reperfusion is required to salvage ischemic myocardium from necrosis, reperfusion per se induces myocardial necrosis. In this “lethal reperfusion injury”, opening of the mitochondrial permeability transition pore (mPTP) upon reperfusion is crucially involved. The mPTP primarily consists of adenine nucleotide translocator (ANT) and voltage-dependent anion channel, and its opening is triggered by binding of cyclophilin-D (CyP-D) to ANT, which increases Ca2+ sensitivity of the mPTP. Recent studies have shown that inactivation of glycogen synthase kinase-3β (GSK-3β) suppresses mPTP opening and protects cardiomyocytes. Multiple intracellular signals relevant to cardiomyocyte protection converge to GSK-3β and inactivate this kinase by phosphorylation. Although the effect of GSK-3β phosphorylation on mPTP structure and function remains unclear, suppression of ANT–CyP-D interaction by binding of phospho-GSK-3β to ANT and reduction in GSK-3β–mediated phosphorylation of p53 may contribute to elevation of the threshold for mPTP opening. Furthermore, a significant inverse correlation was observed between level of phospho-GSK-3β at the time of reperfusion and the extent of myocardium infarction in heart. Together with the infarct size–limiting effect of GSK-3β inhibitors, this finding indicates that phospho-GSK-3β is a determinant of myocardial tolerance against reperfusion-induced necrosis. Thus, GSK-3β appears to be a target of novel therapy for cardioprotection upon reperfusion.

Keywords: glycogen synthase kinase-3β (GSK-3β), myocardial infarction, signal transduction, mitochondria, mitochondrial permeability transition pore

Introduction

Glycogen synthase kinase-3β (GSK-3β) is a constitutively active Ser/Thr protein kinase, the function of which is regulated by phosphorylation, intracellular translocation, and complex formation with other proteins (1, 2). Roles of GSK-3β in cardiac glycogen metabolism and its function in counteracting ventricular hypertrophy have been well characterized (3, 4). Recently, accumulating evidence suggests that GSK-3β is also critically involved in the fate of cells subjected to extracellular stress, including ischemia/reperfusion (5–10). In this article, we review the current understanding of mechanisms of ischemia/reperfusion injury in cardiomyocytes and roles of GSK-3β in myocardial protection.

Mechanisms underlying cardiomyocyte necrosis after ischemia/reperfusion

After the coronary artery has been occluded, ischemic myocytes lose high-energy phosphates and “starve to death” unless blood flow is restored. However, salvage of ischemic myocardium by restoration of blood flow is compromised by reperfusion-induced injury. Recent animal studies (9, 11–16) have shown that pharmacological intervention (for example, administration of a
Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange inhibitor or an adenosine A\textsubscript{2A} receptor agonist) or non-pharmacological intervention (for example, ischemic postconditioning) at the time of reperfusion can reduce infarct size from 50\% of the ischemic zone to approximately 30\%. Thus, it is clear that a substantial mass of myocardium that could have contributed to functional recovery after reperfusion is lost by “lethal reperfusion injury”.

Reperfusion injury is a result of “priming” the myocardium with ischemic injury, which results in intracellular acidosis, Na\textsuperscript{+} overload (by Na\textsuperscript{+}-H\textsuperscript{+} exchanger, un-inactivated Na\textsuperscript{+} channels, and inhibited Na\textsuperscript{+}-K\textsuperscript{+}-ATPase), depletion of ATP (mainly by mitochondrial ATPase), mitochondrial Ca\textsuperscript{2+} overload, and osmotic loading (11, 12, 17). Ca\textsuperscript{2+} influx via Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange is inhibited by acidosis after sustained ischemia, but abrupt reperfusion washes out extracellular protons and provides oxygen to injured mitochondria. As a result, rapid intracellular Ca\textsuperscript{2+} overload caused by reverse-mode Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange, burst production of reactive oxygen species (ROS) from mitochondria, marked cell edema, and hypercontracture of myocytes occur shortly after reperfusion (11, 12, 17, 18). Intracellular Ca\textsuperscript{2+} overload both activates Ca\textsuperscript{2+}-activated proteases, including calpain (19), and augments mitochondrial Ca\textsuperscript{2+} overload. ROS induce oxidation of lipids and proteins, and hypercontracture of myocytes induces disruption of fragile sarcolemma. All of these reperfusion-triggered changes are detrimental to cardiomyocytes and contribute to cell necrosis. However, the fate of cardiomyocytes after reperfusion appears to heavily depend on mitochondrial function. Capacity of mitochondrial ATP production after reperfusion is crucial to quickly restore intracellular Na\textsuperscript{+} level by Na\textsuperscript{+}-K\textsuperscript{+} ATPase, which is necessary for prevention of catastrophic Ca\textsuperscript{2+} overload and rigor contracture (11, 12). An important mechanism of irreversible impairment of mitochondrial function after ischemia/reperfusion injury is opening of the mitochondrial permeability transition pore (mPTP).

### mPTP and cardiomyocyte necrosis

The molecular structure of the mPTP has not yet been unequivocally determined. However, in a current model, the mPTP basically consists of a voltage-dependent anion channel (VDAC) in the outer membrane and adenine nucleotide translocator (ANT) in the inner membrane (20, 21). The mPTP is closed under physiological conditions but opens in response to cellular stress, allowing passage of substances less than 1,500 Da. Unless it is transient and reversible, opening of the mPTP abolishes transmembrane potential in the mitochondria and thus ATP generation. In experimental preparations, opening of the mPTP has been assessed by uptake of radioactive 2-deoxyglucose (2-DG) into mitochondria, leakage of calcein trapped in mitochondria, loss of mitochondrial membrane potential, or irreversible Ca\textsuperscript{2+} release from isolated mitochondria after repetitive loading of Ca\textsuperscript{2+} (22 – 26). All of these techniques indicate that the mPTP in the cardiomyocyte opens after ischemia/reperfusion or hypoxia/reoxygenation. Timing of mPTP opening was found to be within 3 – 10 min after reperfusion, but not during ischemia, in buffer-perfused rat hearts (20, 22).

Several factors have been shown to contribute to opening of the mPTP (20, 21). The most important factor is mitochondrial Ca\textsuperscript{2+}, and loading of a large amount of Ca\textsuperscript{2+} to isolated mitochondria alone can induce opening of the mPTP. Depletion of ATP, elevation of inorganic phosphate level, and ROS are also known to facilitate opening of the mPTP. However, an important trigger of mPTP opening is a matrix protein, cyclophilin-D (CyP-D). Binding of this protein to ANT increases Ca\textsuperscript{2+} sensitivity of ANT and thus reduces the threshold for opening of the mPTP. Treatments with inhibitors of CyP-D (cyclosporine A and sangliferin A) and deletion of Ppif, a gene coding for mitochondrial CyP-D, significantly increased mitochondrial resistance to mPTP opening in response to Ca\textsuperscript{2+} overload and oxidant stress, indicating that CyP-D plays a major role in mPTP opening (27 – 32).

Contribution of CyP-D–mediated opening of the mPTP to myocardial necrosis after ischemia/reperfusion has been indicated by results of infarct size experiments. Infarct size was significantly reduced by pharmacological inhibitors of CyP-D and by knock-out of the mitochondrial CyP-D gene (20, 31, 33). Furthermore, cyclosporine A did not afford further infarct size limitation in CyP-D knock-out mice (33).

### GSK-3\(\beta\) as a regulator of mPTP opening threshold

GSK-3\(\beta\) has recently received attention as a possible regulator of mPTP opening since this kinase is a common target of multiple signal pathways that lead to myocardial protection from infarction. In fact, myocardial infarct size is reduced by ischemic preconditioning and by treatment with \(\delta\)-opioid, insulin, insulin-like growth factor, erythropoietin, or isoflurane; and all of these interventions induce Ser\(\beta\)-phosphorylation of GSK-3\(\beta\) (6 – 13, 16, 34, 35). However, different protein kinases (i.e., protein kinase C [PKC], Akt, ERK1/2) are responsible, depending on the intervention, for direct phosphorylation of GSK-3\(\beta\) in the cardioprotection.

Evidence for a regulatory role of GSK-3\(\beta\) in mPTP opening was first reported by Juhaszova et al. (5). They
determined the threshold for opening of the mPTP by monitoring mitochondrial membrane potential in isolated cardiomyocytes and used ROS generated by laser irradiation as a trigger of mPTP opening. The threshold for mPTP opening was significantly elevated by inhibitory phosphorylation of GSK-3β at Ser9, transfection of a dominant negative mutant of GSK-3β, or reduction of GSK-3β expression by siRNA. Recently Gomez et al. (8) assessed the involvement of GSK-3β in inhibition of mPTP opening by ischemic postconditioning. They isolated mitochondria from the myocardium after ischemia/reperfusion and determined the threshold for mPTP opening as the amount of loading Ca2+ required to induce irreversible Ca2+ release from the mitochondria. Postconditioning significantly elevated the threshold of mPTP opening in cardiac mitochondria from wild-type mice, but such a protective effect was not detected in mitochondria from transgenic mice expressing GSK-S9A, which cannot be inactivated by phosphorylation at Ser9. Taken together, these findings support the notion that phospho-GSK-3β regulates the threshold for mPTP opening in response to ROS and/or Ca2+ overloading.

To get an insight into the molecular mechanism by which phospho-GSK-3β regulates mPTP opening, we examined intracellular localization of GSK-3β and interaction of GSK-3β with mPTP before and after ischemia/reperfusion in isolated perfused rat hearts (10). GSK-3β was predominantly found in the cytosolic fraction under baseline conditions, but ischemia/reperfusion significantly increased GSK-3β level in the mitochondrial fraction. Reperfusion also increased phospho-GSK-3β level in all cell fractions. GSK-3β was co-immunoprecipitated with ANT and with VDAC before ischemia, but levels of these complexes were increased after reperfusion by approximately 50%, indicating that GSK-3β translocated after reperfusion binds to mPTP.

Based on the finding of reperfusion-induced GSK-3β interaction with ANT and VDAC, we hypothesized that elevation of the threshold for mPTP opening by phosphorylation of GSK-3β is achieved by direct interaction of phospho-GSK-3β with mPTP subunit proteins (10). To test this hypothesis, GSK-3β phosphorylation at Ser9 was induced by ischemic preconditioning and erythropoietin-receptor activation in rat hearts. Phospho-GSK-3β in the reperfused myocardium was increased by preconditioning and erythropoietin-receptor activation in a PKC- and Akt-dependent manner, and the increased phospho-GSK-3β was co-immunoprecipitated with ANT but not with VDAC. Furthermore, the level of CyP-D co-immunoprecipitated with ANT was significantly reduced to 40% in association with a 50% increase in phospho-GSK-3β ANT binding (10). These results suggest that interaction of phospho-GSK-3β with ANT inhibits CyP-D–ANT interaction, resulting in prevention of mPTP opening.

In addition to ANT, p53 may be involved in suppression of mPTP opening by phosphorylation of GSK-3β. p53 is one of more than 20 substrates of GSK-3β, and its phosphorylation enhances functional activity and translocation of p53 to the nucleus and mitochondria (36). A recent study by Venkatapuram et al. (37) showed that an inhibitor of p53, pifithrin-α, lowered the threshold of isoflurane-induced limitation of infarct size in rabbit hearts. Interestingly, this beneficial effect of a p53 inhibitor was abolished by an activator of mPTP, atractylloside. Thus, inhibition of GSK-3β–mediated p53 phosphorylation may also contribute to suppression of myocardial necrosis by GSK-3β phosphorylation.

Impairment of cell signaling upstream of GSK-3β by co-existing diseases

There are redundant signal pathways that induce phosphorylation of GSK-3β in cardiomyocytes (6), and more than one pathway is frequently activated by cardioprotective receptor agonists and ischemic preconditioning (9, 16). However, some of the cytoprotective signal pathways suffer from impairment by concurrent diseases associated with coronary artery diseases (such as diabetes mellitus, hypercholesterolemia, and post-infarct ventricular remodeling) (38 – 42). We have found that post-infarct remodeling impairs activation of PKC-ε after ischemic preconditioning and Jak2–PI3K–Akt signaling by erythropoietin-receptor activation (39–41). Erythropoietin-receptor activation also failed to activate the Jak2–PI3K–Akt pathway in the myocardium of an animal model of type 2 diabetes (42). Thus, from the viewpoint of clinical application, a strategy to directly inhibit GSK-3β would be more preferable to indirect GSK-3β inhibition by use of its up-stream signaling pathways.

Level of phospho-GSK-3β as a determinant of infarct size in hearts in situ

Physiological parameters that determine the extent of myocardial necrosis (i.e., infarct size) after ischemia/reperfusion have been fully characterized, and duration of ischemia, size of the ischemic region, residual blood flow in the ischemic region, and myocardial oxygen consumption are major determinants of infarct size in hearts in situ (43, 44). However, a molecule that ultimately determines the level of tolerance of the cardiomyocyte against necrosis at the time of reperfusion has not been identified. GSK-3β is actually a candidate for such a molecule since multiple cytoprotec-
tive signal pathways converge to this protein kinase (6, 9, 16) as discussed above.

Using a rat model of myocardial infarction, we examined the possibility that level of phospho-GSK-3β at the time of reperfusion, when the mPTP opens, is a determinant of infarct size in hearts in situ (9). To prepare different levels of GSK-3β phosphorylation by activating different signal pathways, we employed ischemic preconditioning, erythropoietin pretreatment, their combination, and pretreatments with protein kinase inhibitors. Infarct size after 20-min coronary artery occlusion/reperfusion in rat hearts in situ was significantly reduced by ischemic preconditioning and by erythropoietin pretreatment, and their combination afforded additive limitation of infarct size. Such an additive effect of ischemic preconditioning and erythropoietin pretreatment was observed for GSK-3β phosphorylation upon reperfusion but not for Akt phosphorylation or STAT3 phosphorylation. A PKC inhibitor (chelerythrine) and a PI3K inhibitor (wortmannin) partly inhibited both GSK-3β phosphorylation and infarct size limitation by preconditioning and erythropoietin. Interestingly, there was a tight inverse relationship between phospho-GSK-3β level upon reperfusion and infarct size 2 h after reperfusion (9). Furthermore, administration of an inhibitor of GSK-3β before reperfusion significantly limited infarct size as previously reported (6, 7). Taken together, these findings suggest that the level of phospho-GSK-3β at the time of reperfusion in the myocardium is indeed a determinant of infarct size in situ.

Is a GSK-3β inhibitor a promising infarct size-limiting agent for clinical application?

Introduction of reperfusion therapy has markedly improved prognosis of patients with acute myocardial infarction (AMI). However, current reperfusion therapy cannot afford sufficient myocardial salvage in approximately 25% of patients, who subsequently develop severe heart failure (45). Thus, there is a clinical need for novel therapy to protect cardiomyocytes from ischemia/reperfusion-induced necrosis. An advantage of a GSK-3β inhibitor for clinical use is its efficiency even when administered immediately before reperfusion. Another favorable profile of GSK-3β inhibitors is that their protective effect would not be impaired by common co-morbidities in AMI patients that modify signaling to cytoprotective kinases such as PKC-ε and Akt (10, 41, 42, 46). Although chronic inhibition of GSK-3β raises concern about risks of cancer development and ventricular hypertrophy (1, 4), such risks would be negligible for single injection of a GSK-3β inhibitor before reperfusion therapy in AMI patients.

Conclusion

Phospho-Ser9-GSK-3β plays a critical role in intracellular signal–mediated interventions that protect cardiomyocytes from ischemia/reperfusion-induced necrosis. The mechanism of GSK-3β phosphorylation differs depending on the receptor activated by the intervention, but inactivation of GSK-3β by phosphorylation at Ser9 elevates the threshold for mPTP opening, which reduces myocyte necrosis. Although the mechanism by which phospho-Ser9-GSK-3β elevates the threshold for mPTP opening is unclear, suppression of ANT–CyP-D interaction by binding of phospho-Ser9-GSK-3β to ANT and reduction in GSK-3β–mediated phosphorylation of p53 may be involved. The level of phospho-Ser9-GSK-3β at the time of reperfusion is a determinant of infarct size, and GSK-3β inhibitors are promising as agents for myocardial salvage in patients with AMI.

References


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GSK-3β in Cardioprotection


