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Original article

Roles of phospho-GSK-3β in myocardial protection afforded by activation of the mitochondrial K_{ATP} channel

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ABSTRACT

The aim of this study was to determine the roles of glycogen synthase kinase-3β (GSK-3β) in cardioprotection by activation of the mitochondrial ATP-sensitive K^+ channel (mK_{ATP} channel). In isolated rat hearts, an mK_{ATP} activator, diazoxide, and a GSK-3β inhibitor, SB216763, similarly limited infarct size and the combination of these agents did not afford further protection. The protection by pre-ischemic treatment with diazoxide was abolished by inhibition of protein kinase C-ε (PKC-ε) or phosphatidylinositol-3-kinase (PI3K) upon reperfusion. Infusion of a GSK-3β inhibitor (LiCl), but not diazoxide, during reperfusion limited infarct size. Inhibition of PKC-ε or PI3K did not affect the protection by LiCl. Diazoxide infusion alone did not induce GSK-3β phosphorylation. However, diazoxide infusion before ischemia increased mitochondrial phospho-GSK-3β level and reduced cyclophilin-D (CypD) binding to adenine nucleotide translocase (ANT) at 10 min after reperfusion. This diazoxide-induced GSK-3β phosphorylation was inhibited by blockade of the mK_{ATP} channel before ischemia and by blockade of PKC-ε, PI3K or the adenosine A2b receptor at the time of reperfusion. Inhibition of GSK-3β by LiCl during reperfusion increased phospho-GSK-3β but had no significant effect on CypD–ANT binding. These results suggest that GSK-3β phosphorylation at the time of reperfusion by a PKC-ε, PI3K- and A2b receptor-dependent mechanism contributes to prevention of myocardial necrosis by pre-ischemic activation of the mK_{ATP} channel. Inhibition of CypD–ANT interaction may contribute to mK_{ATP}-induced myocardial protection, though it is not the sole mechanism of phospho-GSK-3β-mediated cytoprotection.

1. Introduction

Although the molecular structure of the mitochondrial ATP-sensitive K^+ channel (mK_{ATP} channel) remains unclear [1,2], significant roles of this channel in ischemic preconditioning (IPC) have been suggested by a number of studies to date [3–6]. Activation of the mK_{ATP} channel by signaling derived from the opioid or bradykinin receptor induces mitochondrial production of reactive oxygen species (ROS), which transmit signals downstream to achieve cardiomyocyte protection [3,4,7,8]. Another putative role of the mK_{ATP} channel is attenuation of mitochondrial Ca^{2+} overload during ischemia/reperfusion, suppressing oxidant stress and preserving mitochondrial functions via matrix volume regulation [2,9,10]. On the other hand, phospho-GSK-3β has recently received attention as a common mediator in protective mechanisms activated by different interventions, including IPC and ischemic postconditioning (IPost-C) [3,4,11,12]. Several pro-survival signaling pathways converge at the step of GSK-3β phosphorylation at Ser9, which inactivates this kinase, and inactivation of GSK-3β has been shown to increase myocardial resistance to infarction [11,12]. However, the relationship between activated mK_{ATP} channels and GSK-3β in cardiomyocytes has not been examined in detail.

The aim of the present study was two-fold. First, we aimed to determine the functional relationship between activation of the mK_{ATP} channel and GSK-3β phosphorylation in myocardial protection. GSK-3β is constitutively active, and an agent that can reverse phosphorylation-induced inactivation of GSK-3β is currently unavailable. Thus, we assessed the effects of mK_{ATP} channel activation on the level of phospho-Ser9-GSK-3β and myocardial tolerance against infarction in comparison with the effects of pharmacological inhibitors of GSK-3β. Diazoxide and 5-hydroxydecanoate (5-HD) were used to activate and inhibit the mK_{ATP} channel, respectively, though these agents have limitations in specificity as tools [13,14]. The second aim of this study was to examine whether cytoprotection by mK_{ATP} channel activation is achieved by modulating regulatory proteins of the mitochondrial permeability transition pore (mPTP). Earlier studies have shown that opening of mPTPs, which leads to cell necrosis, is significantly suppressed by activation of the mK_{ATP} channel in the myocardium [15,16], but its mechanism remains unclear. In the present study, we assessed the effects of mK_{ATP} channel activation on interaction of cyclophilin-D (CypD) with adenine nucleotide translocase (ANT), a major regulatory protein in the mPTP complex, at the time of reperfusion. Binding of CypD to ANT is known to sensitize the mPTP to opening stimuli, and we

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recently found that phosphorylation of GSK-3β by IPC with or without combined activation of the erythropoietin (EPO) receptor was associated with more than 50% reduction of CypD–ANT interaction upon reperfusion in rat hearts [17,18]. The results of the present study indicate that activation of the mK_{ATP} channel before ischemia enhances GSK-3β phosphorylation upon reperfusion by a PKC-ε, PI3K-Akt and adenosine A2b receptor-dependent mechanism. It was also shown that mK_{ATP} channel activation inhibits CypD–ANT interaction upon reperfusion, though inhibited CypD–ANT interaction is unlikely to be the sole mechanism of protection afforded by GSK-3β inactivation.

2. Methods

This study was approved by the Committee for Animal Research, Sapporo Medical University and was conducted in strict accordance with the Guidelines of Sapporo Medical University for Animal Use in Research.

2.1. Isolated perfused rat heart preparation

Isolation of the hearts from Sprague–Dawley rats at 10–12 weeks of age and perfusion with physiological buffer were performed as reported previously [17,19]. In brief, rats were anesthetized with pentobarbital and mechanically ventilated with oxygen supplement. The hearts were isolated after heparinization and mounted onto a Langendorff apparatus and perfused with modified Krebs–Henseleit buffer at 75 mmHg and 37 °C. A saline-filled balloon was placed in the left ventricle (LV) via the left atrium and connected to a pressure transducer to determine LV pressure. The hearts were excluded from the study if LV systolic pressure was less than 80 mmHg or heart rate was less than 250 beats per minute after a 20-min stabilization period.

2.2. Infarct size experiments

2.2.1. Experimental protocols

2.2.1.1. Protocol 1. Infarction was induced by 20-min global ischemia and 2-h reperfusion. Before ischemia, the hearts received no treatment or 10-min infusion of 100 μM diazoxide, 1 μM SB216763 (a GSK-3β inhibitor) [20] or the combination of diaxozide and SB216763 (Fig. 1(A)). After 2-h reperfusion, the hearts were frozen for infarct size determination.

2.2.1.2. Protocol 2. Infarction was induced by 35-min coronary occlusion and 2-h reperfusion. Regional ischemia was selected in this protocol to deliver pharmacological agents to the ischemic myocardium via collateral flow before reperfusion. Since regional ischemia *in vitro* tends to induce smaller infarct than global ischemia of the same duration due to oxygen supply to the ischemic region via collateral flow and intraluminal diffusion, we extended the duration of ischemia to 35 min in this protocol. As shown in Fig. 1(B), the hearts received one of seven treatments: no treatment (control), diazoxide (100 μM) pretreatment, diazoxide pretreatment plus infusion of a PKC-ε inhibitor (100 nM PKC-ε translocation inhibitory peptide [PKC-ε-TIP]) upon reperfusion, diazoxide pretreatment plus infusion of an inhibitor of phosphatidylinositol-3-kinase (PI3K) (15 μM LY294002) upon reperfusion, infusion of LY294002 upon reperfusion alone, diazoxide treatment upon reperfusion, and infusion of a GSK-3β inhibitor (3 mM lithium chloride [LiCl]) [20,21] upon reperfusion. Since a number of previous studies, including ours, have confirmed that 5-HD abrogates the infarct size-limiting effect of diazoxide in the rat hearts [22–24], we did not include study groups for reconfirmation of this finding. The coronary artery was re-occluded at the end of 2-h reperfusion, and fluorescent polymer microspheres (Duke Scientific Co., Palo Alto, CA) suspended in saline were injected into the aorta to negatively mark areas at risk. The hearts were then frozen for subsequent processes.

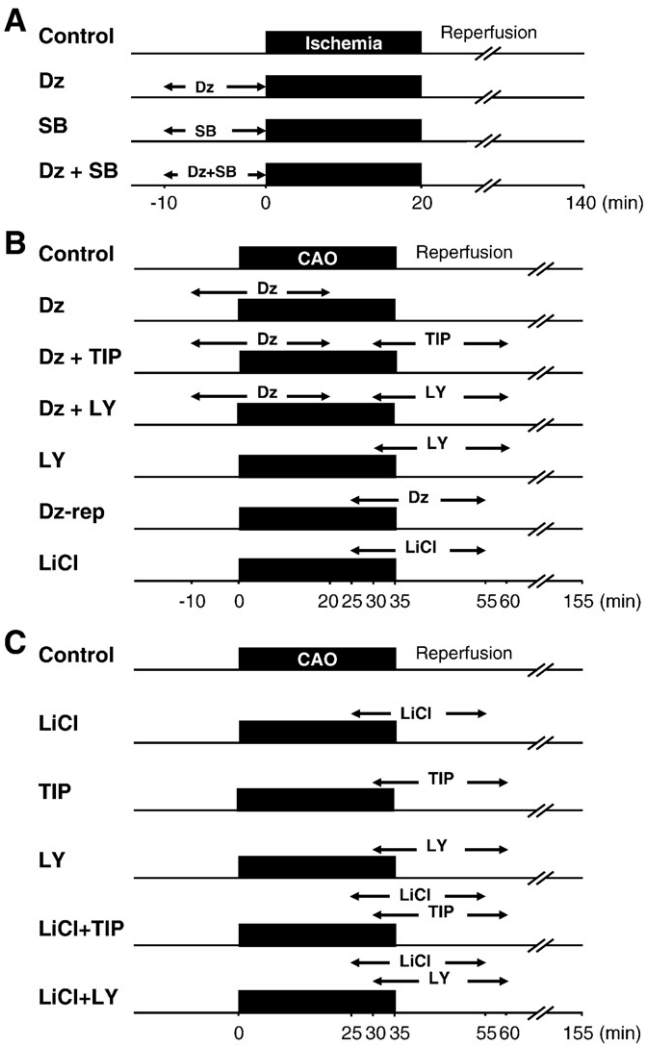

Fig. 1. Experimental protocols in infarct size experiments. (A): Protocol 1 (global ischemia protocol), (B): Protocol 2 (regional ischemia protocol), (C): Protocol 3 (regional ischemia protocol). CAO = coronary artery occlusion. Time intervals of test agent infusion are shown by arrows. Dz = diazoxide, SB = SB216763, TIP = PKC-ε-TIP, LY = LY294002.

2.2.1.3. Protocol 3. The coronary artery was occluded for 35 min and reperfused for 2 h. As shown in Fig. 1(C), the hearts received no treatment or infusion of LiCl, PKCε-TIP, LY294002, combination of LiCl and PKCε-TIP or combination of LiCl and LY294002 upon reperfusion. Doses of LiCl, PKC-ε-TIP and LY294002 and the procedure to mark areas at risk were the same as those in Protocol 2.

2.2.2. Infarct size determination

Frozen hearts were sliced into 2-mm sections and incubated in phosphate buffer containing 1% triphenyltetrazolium chloride. Photographs of heart slices with visualized infarcts and those of areas at risk were taken under room light and under UV light, respectively. Infarct areas, areas of the left ventricle and areas at risk in the photographs were determined by image analysis software.

2.3. Immunoblotting experiments

2.3.1. Tissue sampling

In the first series of experiments, the hearts received 35-min regional ischemia, and each heart was assigned to untreated control,