Cardioprotective Effects of the Novel Na⁺/H⁺ Exchanger-1 Inhibitor KR-32560 in a Perfused Rat Heart Model of Global Ischemia and Reperfusion: Involvement of the Akt-GSK-3β Cell Survival Pathway and Antioxidant Enzyme

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To investigate the cardioprotective effects and mechanism of action of KR-32560 [(5-(2-methoxy-5-fluorophenyl)furan-2-ylcarbonyl)guanidine], a newly synthesized NHE-1 inhibitor, we evaluated the effects of KR-32560 on cardiac function in a rat model of ischemia/reperfusion (I/R)-induced heart injury as well as the role antioxidant enzymes and pro-survival proteins play these observed effects. In isolated rat hearts subjected to 25 min of global ischemia followed by 30 min of reperfusion, KR-32560 (3 and 10 μM) significantly reversed the I/R-induced decrease in left ventricular developed pressure and increase in left ventricular end-diastolic pressure. In rat hearts reperfused for 30 min, KR-32560 (10 μM) significantly decreased the malondialdehyde content while increasing the activities of both glutathione peroxidase and catalase, two important antioxidant enzymes. Western blotting analysis of left ventricles subjected to I/R showed that KR-32560 significantly increased phosphorylation of both Akt and GSK-3β in a dose-dependent manner, with no effect on the phosphorylation of eNOS. These results suggest that KR-32560 exerts potent cardioprotective effects against I/R-induced rat heart injury and that its mechanism involves antioxidant enzymes and the Akt-GSK-3β cell survival pathway.

Key words: KR-32560, Cardioprotection, Na⁺/H⁺ exchanger, Akt, GSK-3β, Reperfusion

INTRODUCTION

The exact molecular mechanisms underlying ischemia and reperfusion (I/R) heart injury remain obscure. However, accumulating evidence indicates that the calcium and free radical hypotheses are not mutually exclusive (Piper et al., 1998; Snabaitis et al., 2002; Mentzer et al., 2003). In the calcium hypothesis, Na⁺/H⁺ exchanger (NHE) plays a key role in regulating intracellular pH and Na⁺ levels in many types of cells, acting as an antiporter and initiating the process leading to intracellular Ca²⁺ overload in cardiomyocytes (Karmazyn et al., 1999; Doggrell and Hancox, 2003; Masereel et al., 2003). Of the nine NHE isoforms thus far identified as NHE-1 to NHE-9, NHE-1 is the most predominant isoform expressed in cardiomyocytes (Fliegel, 2009). During cardiac ischemia, cytosolic acidosis-stimulated NHE-1 causes Na⁺ influx in exchange for H⁺ efflux, resulting in intracellular Na⁺ overload in conjunction with other proteins such as Na⁺-HCO₃ symporter (Leem et al., 1999), which in
turn activates Na⁺/Ca²⁺ exchanger (NCX) in reverse mode and leads to intracellular Ca²⁺ overload (Masereel et al., 2003). Intracellular Ca²⁺ overload is an important pathophysiological factor that produces cardiac dysfunction during I/R injury based on its multiple direct and indirect harmful effects (Avkiran and Marber, 2002; Mentzer et al., 2003). In the same vein, blockade of NHE-1 activity before ischemia or after the onset of reperfusion has been reported as an efficient therapeutic modality for the protection of heart from I/R injury mainly by reducing intracellular Ca²⁺ overload (Avkiran et al., 2008). Accordingly, several classes of NHE-1 inhibitors including cariporide (Scholz et al., 1995), eniporide (Bhattaram et al., 2005), zoniporide (Marala et al., 2002), KR-32570 (Lee et al., 2005a, 2005b, 2005c) and KR-33028 (Jung et al., 2006) have been shown to have potent cardioprotective activities in various in vitro and in vivo experimental models of I/R-induced heart injury.

Studies on the cardioprotective mechanisms of NHE-1 inhibitors suggest that delayed mitochondrial matrix acidification and ATP exhaustion are active processes during ischemia (Ruiz-Meana et al., 2003), and that inhibition of hypoxia-induced mitochondrial cell death pathway occurs in H9c2 cells (Jung et al., 2006). Accumulating evidence also has shown that the mitochondrial permeability transition (MPT), which plays a central role in mitochondria-mediated death pathways, occurs in the heart as a result of I/R injury (Javadov et al., 2008). Quite interestingly, some studies have suggested that a diversity of stimuli may lead to cardioprotection via two different pathways, both of which are independent or dependent on mitochondrial volume and limit the mitochondrial permeability transition (MPT) via inhibition of GSK-3β of the mitochondrial permeability transition pore (MPTP) complex (Juhaszova et al., 2004). Regarding the role of NHE-1, studies have demonstrated that activated NHE-1 can function as a scaffold for the recruitment of proteins such as signalplex (ezrin/radixin/moesin complex), phosphoinositide 3-kinase (PI3K) and Akt (Wu et al., 2004), the latter two of which have antiapoptotic (Wu et al., 2004) and hypertrophic effects (Condorelli et al., 2002; Kilic et al., 2005) when activated in cardiomyocytes. These findings suggest that there are additional complex signaling pathways involved in the regulation of cell survival and death following NHE-1 activation and inhibition.

A previous study demonstrated that KR-32560 ([(5-(2-methoxy-5-fluorophenyl)furan-2-ylcarbonyl]guanidine) (Fig. 1) is significantly more potent in inhibiting intracellular pH recovery in PS120/NHE-1 (h NHE-1 transfected) cells compared to cariporide (Lee et al., 2005c). We also reported that KR-32560 significantly reduces myocardial infarction as well as the incidence of various types of ventricular arrhythmias in an anesthetized rat model of I/R heart injury (Park et al., 2005). It also exerts an inhibitory effect on the aggregation of washed rabbit platelets induced by collagen and arachidonic acid (Lee et al., 2006). KR-32560 appears to mediate its beneficial effects through NHE-1 inhibition, as evidenced by its reduction of Na⁺ propionate-induced, NHE-1-mediated rabbit platelet swelling (Park et al., 2005). In the present study, we evaluated the cardioprotective effects of KR-32560 in globally ischemic isolated rat hearts with special emphasis on elucidating the involvement of prosurvival proteins (Akt, GSK-3β and eNOS) and antioxidant enzymes.

**MATERIALS AND METHODS**

**Animals and chemicals**

Male Sprague-Dawley rats (300-380 g, Samtako Bio Korea) were housed for 2 weeks in an animal storage room under standard conditions (constant temperature and humidity of 22.5 ± 1.0°C and 55 ± 5%, respectively, with a 12 h light/dark cycle) and given free access to standard chow and tap water. KR-32560 was synthesized at the Medical Science Division, Korea Research Institute of Chemical Technology (KRICT) and dissolved in 0.04% dimethylsulfoxide (DMSO) in modified Krebs-Henseleit bicarbonate buffer solution as needed. Sodium pentobarbital was purchased from Hanlim Pharmaceutical Co., heparin from Choongwae Pharmaceutical Co., and all other chemicals from Sigma-Aldrich Korea.

**Measurement of cardiac function in perfused rat hearts subjected to global ischemia and reperfusion**

Experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health. Briefly, rats (male SD rat, 300-380 g) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) (Lee et al., 2005a). Heparin (1000 U/kg) was then injected into tail veins after which their tracheas were