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# Oxidative activation of CaMKIIδ in acute myocardial ischemia/reperfusion injury: A role of angiotensin $AT_1$ receptor-NOX2 signaling axis

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ABSTRACT

During ischemia/reperfusion (IR), increased activation of angiotensin $AT_1$ receptors recruits NADPH oxidase 2 (NOX2) which contributes to oxidative stress. It is unknown whether this stimulus can induce oxidative activation of $Ca^{2+}$/calmodulin-dependent protein kinase IIδ (CaMKIIδ) leading into the aggravation of cardiac function and whether these effects can be prevented by angiotensin $AT_1$ receptors blockade. Losartan, a selective $AT_1$ blocker, was used. Its effects were compared with effects of KN-93, an inhibitor of CaMKIIδ. Global IR was induced in Langendorff-perfused rat hearts. Protein expression was evaluated by immunoblotting and lipoperoxidation was measured by TBARS assay. Losartan improved LVDP recovery by 25%; however, it did not reduce reperfusion arrhythmias. Oxidized CaMKIIδ (oxCaMKIIδ) was downregulated at the end of reperfusion compared to before ischemia and losartan did not change these levels. Phosphorylation of CaMKIIδ mirrored the pattern of changes in oxCaMKIIδ levels. Losartan did not prevent the higher lipoperoxidation due to IR and did not influence NOX2 expression. Inhibition of CaMKII ameliorated cardiac IR injury; however, this was not accompanied with changes in the levels of either active form of CaMKIIδ in comparison to the angiotensin $AT_1$ receptor blockade. In spite of no changes of oxCaMKIIδ, increased cardiac recovery of either therapy was abolished when combined together. This study showed that oxidative activation of CaMKIIδ is not elevated at the end of R phase. NOX2-oxCAMKIIδ signaling is unlikely to be involved in cardioprotective action of angiotensin $AT_1$ receptor blockade which is partially abolished by concomitant CaMKII inhibition.



## 1. Introduction

The capability of $Ca^{2+}$/calmodulin-dependent protein kinase IIδ (CaMKIIδ) to interconnect cellular $Ca^{2+}$ and redox signaling (Luczak and Anderson, 2014), which are significantly altered in myocardial ischemia/reperfusion injury (MIRI) (Dhalla et al., 2000; Braunwald and Kloner, 1985), has attracted research attention. It has been demonstrated that the excessive activity and/or the up-regulated expression of CaMKIIδ underlie the signaling pathways promoting cardiac contractile dysfunction (Adameova et al., 2012; Zhang et al., 2003), arrhythmogenesis (Adameova et al., 2012; Purohit et al., 2013), cardiomyocyte death through apoptosis, necrosis (Vila-Petroff et al., 2007) and/or necroptosis (Szobi et al., 2014). The activity of CaMKIIδ is low at basal cytosolic $Ca^{2+}$ levels; however, upon binding of the $Ca^{2+}$/calmodulin complex ($Ca^{2+}$/CaM) its enzymatic action is greatly increased (Hudmon and Schulman, 2002). This initial $Ca^{2+}$/CaM-induced activation of the kinase can be further promoted by post-translational modifications, such as autophosphorylation across neighboring subunits at threonine 287 ($Thr^{287}$) and oxidative modification of a pair of methionines 281 and 282 ($Met^{281/282}$) (Erickson et al., 2008). In comparison to autophosphorylation, knowledge about the role of oxCaMKIIδ in the heart is limited. One of the important sources of reactive oxygen species (ROS) in the heart, which may lead to CaMKIIδ oxidation, are the angiotensin $AT_1$ receptor-activated NADPH oxidases (NOX), in particular NOX2 (Kleikers et al., 2012).

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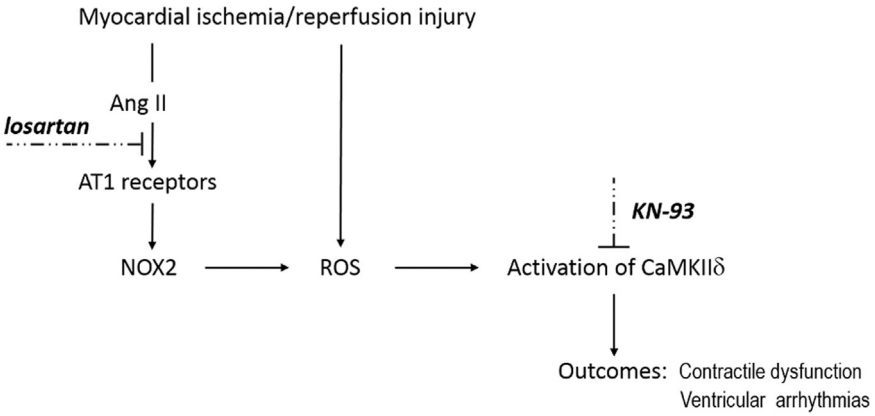


**Fig. 1.** Hypothesis suggesting the involvement of angiotensin $AT_1$ receptor-NOX2-oxCaMKIIδ axis in acute myocardial ischemia-reperfusion injury and the capability of an angiotensin $AT_1$ receptor blockade by losartan to prevent cardiac damage by modifying this signaling.

In support, knockout or pharmacological inhibition of NOX2 decreased CaMKIIδ oxidation which was associated with prevention of cardiac remodeling (He et al., 2011), attenuation of sinus node dysfunction (Swaminathan et al., 2011) and cardiomyocyte death (Erickson et al., 2008; Palomeque et al., 2009). In settings of MIRI, enhanced angiotensin $AT_1$ receptor signaling (Yang et al., 1998a; Yang et al., 1998b), increased ROS production (Dhalla et al., 2000) and depletion of energy (Docherty et al., 1999) contribute to disturbances in excitation-contraction coupling (ECC) and cellular injury (Braunwald and Kloner, 1985). In view of the critical role of these pathomechanisms in MIRI and the existence of a cross-talk between angiotensin $AT_1$ receptor stimulation leading into enhanced ROS production via NOX2 and the activation of CaMKIIδ producing deleterious effects, we tested the hypothesis that a selective $AT_1$blocker, losartan, can attenuate MIRI-induced ventricular arrhythmias and contractile dysfunction by limiting oxidative activation of CaMKIIδ (Fig. 1). The effects of losartan were compared with the action of a direct inhibition of CaMKII by selective inhibitor KN-93.

## 2. Materials and methods

### 2.1. Animals

All studies were in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010) and Directive 2010/63/EU implemented in the Slovak parliament act no. 39/2007. All animal experiments were approved by the Ethics Committee of the Faculty of Pharmacy, Comenius University and the Animal Care and Use Committee of the Slovak Republic, act no. Ro-2500/10-221. Male Wistar rats (250–300 g, $n=52$), housed under constant temperature of $22\pm2$ °C with a constant 12:12 h light/dark cycle, were used in the study. Animals were fed a standard pelleted diet and water ad libitum.

### 2.2. Heart perfusion and experimental protocol

After anesthesia induction (sodium pentobarbitone, 60 mg/kg, i.p.), the hearts were rapidly excised and perfused by the Langendorff technique at constant perfusion pressure and temperature of 37 °C. The perfusion solution (a modified Krebs–Henseleit buffer with pH 7.4) containing 118 mM NaCl, 3.2 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.18 mM $KH_2PO_4$, 2.5 mM $CaCl_2$ and 5.5 mM glucose was gassed with 95% $O_2$ and 5% $CO_2$. For measuring the left ventricular pressure, a latex balloon connected to a pressure transducer (AD Instruments, Germany), was inserted into the left ventricle (LV). The balloon was filled with the perfusion solution and the left ventricular end-diastolic pressure was adjusted to 5–7 mm Hg. Values for the left ventricular developed pressure (LVDP) and the indices of cardiac contraction and relaxation ($+dP/dt$, $-dP/dt$), and other baseline hemodynamic parameters were obtained by using PowerLab/8SP software (AD Instruments, Germany).

All hearts were perfused with the oxygenated perfusion solution for 30 min. Global ischemia (I) was induced by the clamping of aortic inflow and was confirmed by the absence of coronary flow, fast decline of contractile activity of the heart, drop of LV pressure and gradual diminution of cardiac electrical activity. By the end of I, the heart did not reveal any signs of functional activity. After 30 min of I, 40 min reperfusion (R) was induced by releasing the clamp. Thereupon, the hearts were removed for further biochemical analysis. Non-ischemic groups underwent the same protocol without the ischemia/reperfusion procedure (Fig. 2).

An epicardial electrogram was registered by means of two electrodes attached to the apex of the heart and the aortic cannula. Ventricular arrhythmias developed during reperfusion of previously ischemic hearts were defined according to the Lambeth Convention and the severity of arrhythmias was quantified using a 5-point scoring system (Curtis et al., 2013) as described elsewhere (Adameova et al., 2012).

### 2.3. Pharmacological interventions

To block angiotensin $AT_1$ receptors, losartan (*p.o.*, 20 mg/kg/day, 14 days) kindly provided by Zentiva (Slovakia), was used. CaMKIIδ inhibition was achieved by KN-93 (Sigma-Aldrich, USA) which was present in the perfusion solution 10 min before I and during the first 10 min of R at the concentration of 0.5 μmol/dm$^3$. The doses of the angiotensin $AT_1$ receptor antagonist and the CaMKII inhibitor were selected according to our and other's previous data (Adameova et al., 2012; Dent et al., 2006; Guo et al., 2003; Szobi et al. 2014). It is worthy to note that a higher concentration of KN-93, unlike the concentration used in this study, is known to influence the baseline hemodynamic parameters by inhibiting other protein kinases and channels (Adameova et al., 2012; Bell et al., 2012; Sumi et al., 1991). To further characterize the potentially cardioprotective effects of KN-93, we performed a preliminary study. In the presence of KN-93, all studied parameters were unchanged in the non-ischemic hearts (data not shown).