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European Journal of Pharmacology 535 (2006) 212–219
www.elsevier.com/locate/ejphar

Allopurinol modulates reactive oxygen species generation and Ca^{2+} overload in ischemia-reperfused heart and hypoxia-reoxygenated cardiomyocytes

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Received 16 June 2005; received in revised form 22 December 2005; accepted 10 January 2006
Available online 6 March 2006

Abstract

Myocardial oxidative stress and Ca^{2+} overload induced by ischemia-reperfusion may be involved in the development and progression of myocardial dysfunction in heart failure. Xanthine oxidase, which is capable of producing reactive oxygen species, is considered as a culprit regarding ischemia-reperfusion injury of cardiomyocytes. Even though inhibition of xanthine oxidase by allopurinol in failing hearts improves cardiac performance, the regulatory mechanisms are not known in detail. We therefore hypothesized that allopurinol may prevent the xanthine oxidase-induced reactive oxygen species production and Ca^{2+} overload, leading to decreased calcium-responsive signaling in myocardial dysfunction. Allopurinol reversed the increased xanthine oxidase activity in ischemia-reperfusion injury of neonatal rat hearts. Hypoxia-reoxygenation injury, which simulates ischemia-reperfusion injury, of neonatal rat cardiomyocytes resulted in activation of xanthine oxidase relative to that of the control, indicating that intracellular xanthine oxidase exists in neonatal rat cardiomyocytes and that hypoxia-reoxygenation induces xanthine oxidase activity. Allopurinol (10 μM) treatment suppressed xanthine oxidase activity induced by hypoxia-reoxygenation injury and the production of reactive oxygen species. Allopurinol also decreased the concentration of intracellular Ca^{2+} increased by enhanced xanthine oxidase activity. Enhanced xanthine oxidase activity resulted in decreased expression of protein kinase C and sarcoendoplasmic reticulum calcium ATPase and increased the phosphorylation of extracellular signal-regulated protein kinase and p38 kinase. Xanthine oxidase activity was increased in both ischemia-reperfusion-injured rat hearts and hypoxia-reoxygenation-injured cardiomyocytes, leading to reactive oxygen species production and intracellular Ca^{2+} overload through mechanisms involving p38 kinase and extracellular signal-regulated protein kinase (ERK) via sarcoendoplasmic reticulum calcium ATPase (SERCA) and protein kinase C (PKC). Xanthine oxidase inhibition with allopurinol modulates reactive oxygen species production and intracellular Ca^{2+} overload in hypoxia-reoxygenation-injured neonatal rat cardiomyocytes.

Keywords: Allopurinol; Xanthine oxidase; Ischemia-reperfusion; Reactive oxygen species production; Ca^{2+} overload

1. Introduction

Increased myocardial oxidative stress and altered Ca^{2+} homeostasis play important roles in the myocardial cell injury induced by ischemia-reperfusion or hypoxia-reoxygenation, which leads to cardiomyopathy and heart failure (Tani, 1990; Griffiths et al., 1998). Oxidative stress may promote the entry of Ca^{2+} into vascular myocytes and thus stimulate neointimal

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doi:10.1016/j.ejphar.2006.01.013

hyperplasia, leading to atherosclerosis, vasoconstriction and development of hypertension (Dhalla et al., 2000). An excessive increase in the intracellular concentration of Ca^{2+} would result in Ca^{2+} overload and thus produce myocardial cell damage as seen in cardiomyopathic or ischemic-reperfused hearts. The intracellular Ca^{2+} overload as a consequence of oxidative stress may also play a crucial role in the transition of cardiac hypertrophy to heart failure. Reactive oxygen species can be generated in the heart by several mechanisms such as xanthine oxidase (Wolin, 2000), NAD(P)H (Thannickal and Fanburg, 1995) and cytochrome p450 (Fleming et al., 2001). Xanthine oxidase appears to be an important source of reactive oxygen species production in ischemia-reperfusion (Granger, 1998, 1999) and hypercholesterolemia (Ohara and Peterson, 1993). Thus, xanthine oxidase may have the potential to be an important source of reactive oxygen species production under certain pathophysiological conditions.

Both reactive oxygen species and intracellular Ca^{2+} overload modulate the contractility of cardiomyocytes through phosphorylation of various kinases. Especially, protein kinase C and p38 kinase are involved in the pathophysiology of ischemic contractile dysfunction (Wang et al., 2001; Liao et al., 2002), although mechanisms by which these kinases regulate contractile function are poorly defined. Reactive oxygen species decrease the activity of the sarcoplasmic reticulum Ca^{2+} ATPase, a membrane calcium pump that plays an important role in cardiac calcium handling and which is a determinant of myocardial contractility (Kaneko et al., 1989a, b). The p38 kinase activated by hypoxia mediates phosphorylation of xanthine dehydrogenase/oxidase (Kayyali et al., 2001). Elevated calcium would act upstream of the activator of extracellular signal-regulated protein kinase related with enhanced hypoxia inducible factor 1 transcriptional activity in hypoxia-related signal transduction pathways (Mottet et al., 2002).

Allopurinol is a xanthine oxidase inhibitor that blocks the superoxide production generated in the xanthine oxidase system. Allopurinol has been noted to attenuate reperfusion injury in various organs, including the heart (Tan et al., 1993), liver (Granger et al., 1991), kidney (Terada et al., 1992), and small intestine (Grisham et al., 1986). In ischemia-reperfused heart, generation of reactive oxygen species and Ca^{2+} overload may correlate cardiac dysfunction (Ferrari et al., 1990; Temsah et al., 1999; Bolli and Marbán, 1999).

In this study, we investigated whether allopurinol as a xanthine oxidase inhibitor could rescue reactive oxygen species production and Ca^{2+} overload in hypoxia-reoxygenated cardiomyocytes. Furthermore, we investigated the signaling targets implicated in the inhibitory effect of allopurinol on the xanthine oxidase systems.

2. Materials and methods

2.1. Myocardial ischemia-reperfusion protocol

All experimental procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine and were performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Myocardial infarction was produced in male Sprague–Dawley rats (200±30 g) by surgical occlusion of left anterior descending coronary artery, according to the method described previously with minor modifications (Hwang et al., 2004). Briefly, rats were anesthetized by intraperitoneal injection of ketamine (10 mg/kg) and xylazine (5 mg/kg) and the chest was opened by cutting the third and fourth ribs. The heart was exteriorized through the intercostal space and the left coronary artery was ligated 2–3 mm from its origin with a 5-0 prolene suture (ETHICON, UK). Coronary occlusion was maintained for 1 h, followed by removal of the suture and reperfusion for 3 days. Throughout the operation, the animals were ventilated with 95% O_2 and 5% CO_2, using a Harvard ventilator. Sham-operated animals were treated similarly, except that the coronary suture was not tied. Operative mortality was 10% within 3 days. At different times after surgery, the isolated hearts were immersed in 2% triphenyltetrazolium chloride (TTC) stain for 20 min at 37 °C to determine the infarct size. The infarct myocardium, which does not take up TTC stain when the dehydrogenase enzymes are drained off, remains pale in color. The hearts were sliced and photographed, and the percentage of necrotic tissue was determined using NIH image, version 1.61.

2.2. Primary culture of cardiomyocytes and in vitro hypoxia-reoxygenation treatment

Neonatal rat cardiomyocytes were prepared as described previously (Lipsic et al., 2004). Briefly, hearts of 1- to 2-day-old Sprague–Dawley rat pups were dissected, minced, enzymatically dispersed with 10 ml of collagenase I (0.8 mg/ml, 262 U/mg, Gibco BRL), and centrifuged differentially to yield 5×10^5 cells/mL. After incubation for 4–6 h, the cells were rinsed twice with cell culture medium and 0.1 mM BrdU was added. Cells were then cultured in a CO_2 incubator at 37 °C for 3–5 days. For the treatment of H/R injury, the cells were anaerobically incubated for 1 h using deoxygenated α-MEM containing 1% fetal bovine serum in an anaerobic chamber (Thermo Forma Anaerobic System Model 1025, USA), followed by aerobic culture for different times.

2.3. Xanthine oxidase activity measurements

Xanthine oxidase activity was assayed using the Amplex® Red Xanthine/Xanthine oxidase kit (Molecular Probes Inc., USA). The principle of the assay involves the production of resorufin, a red-fluorescent oxidation product, by H_2O_2 spontaneously degraded from superoxide through oxidation of xanthine catalyzed by xanthine oxidase. In brief, cells were lysed by adding SDS and proteinase K and each reaction mixture containing 50 mM Amplex Red reagent, 0.2 U/ml horseradish peroxidase, 0.1 mM xanthine and the appropriate amount of cell lysates in 0.1 M Tris–HCl buffer (pH 7.5). The reactions were incubated for 30 min at 37 °C with protection