Life Sciences 90 (2012) 649–656

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

The role of K_{ATP} channels on ischemia-reperfusion injury in the rat testis

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ARTICLE INFO

Article history:
Received 7 October 2011
Accepted 28 February 2012

Keywords:
Ischemia-reperfusion injury
KATP channels
Apoptosis
Caspase-3
Testis

ABSTRACT

Aims: To investigate the participation of K_{ATP} channels on the ischemia-reperfusion (IR)-induced apoptosis in the rat testis.
Main methods: Eight-week-old male Sprague–Dawley rats were divided into three groups: control and IR rats without or with cromakalim (300 μg/kg intraperitoneally), 30 min before the induction of ischemia. The right testicular artery and vein were clamped to induce ischemia in the testis. Sixty minutes after the ischemia, a 24 h period of reperfusion followed. Then, expressions of K_{IR}6.1, K_{IR}6.2, caspase-3, PARP, Fas, FasL, and K_{IR}6.1 and K_{IR}6.2 mRNAs were investigated by Western blot analyses and real-time PCR methods, respectively. Furthermore, testicular tissues were processed for histological evaluation and TUNEL staining.
Key findings: Expressions of K_{IR}6.1 protein and mRNA were more than 10-fold of those of K_{IR}6.2 protein and mRNA in the testis. IR significantly increased the expressions of K_{IR}6.1 protein and mRNA as well as K_{IR}6.2 mRNA, caspase-3, and TUNEL index in the testis compared to the control. PARP expressions were significantly lower in the IR group than those of the control. Histologically, severe acute germ cell damage was observed in the IR testis. Treatment with cromakalim ameliorated these parameters compared to the non-treated IR group. There were no significant differences on Fas, FasL and protein level of K_{IR}6.2 expressions between any of the groups.
Significance: Treatment with cromakalim has a protective effect against IR-induced testicular damage via activating K_{ATP} channels. This is the first study to give evidence for the advantageous effect of cromakalim in the germ cell-specific apoptosis induced by testicular IR.
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Introduction

Torsion of the spermatic cord is a medical emergency which occurs primarily in adolescent males and young men and often leads to testicular injury (Anderson and Williamson, 1986). It has been estimated to affect one out of 4000 males younger than 25 years per annum (Anderson and Williamson, 1986). Testicular torsion requires immediate surgical intervention to counter-rotate the testis and spermatic cord in order to restore the blood flow and avoid the loss of germ cells as well as orchiectomy (Ringdahl and Teague, 2006; Workman and Kogan, 1988; Williamson, 1985). Testicular torsion followed by repair is an event involving ischemia-reperfusion (IR), similar to other IR incidents such as stroke, myocardial infarct, and kidney injury (Lysiak et al., 2007). IR of the testis can result in severe dysfunction of the organ due to the generation and accumulation of reactive oxygen species (ROS). Many research groups have demonstrated the loss of spermatogenesis after IR of the testis in spite of the return of the blood flow upon reperfusion. This loss of spermatogenesis both in the rat (Turner et al., 1997) and the mouse (Lysiak et al., 2001) has been attributed to germ cell-specific apoptosis (GCA) with other cells in the testis remaining rather unaffected.

Apoptosis, or programmed cell death, is the result of the activation of an intracellular program that leads to cell death via nuclear degradation without induction of an inflammatory response. It is well established that IR of the testis results in GCA (Turner et al., 1997). The downstream central component of the apoptotic mechanism involves a family of aspartic acid-directed cysteine proteases known as caspases. Caspases are expressed as inactive proenzymes and participate in a cascade triggered in response to pro-apoptotic signals (Yagmurdur et al., 2008). Among these, caspase-3 and -9 are considered to be the major executioner proteases.

Ischemic preconditioning (IPreC) is a mechanism by which brief periods of ischemia produce protection against subsequent longer ischemic periods (Murry et al., 1986). The end-effector molecules targeted by these pathways remain equivocal. ROS play a double edge role in the process of IPreC, and those produced during the preconditioning phase is thought to be protective.

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0024-3205/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2012.03.006

The ATP-sensitive potassium (K_{ATP}) channels, originally discovered in cardiac muscle (Noma, 1983) are non-voltage dependent, potassium-selective channels gated by the intracellular nucleotides ATP and ADP. There are three types of K_{ATP} channels: the sarcolemmal (sarcK_{ATP}), the mitochondrial (mitoK_{ATP}), and the nuclear (nucK_{ATP}) (Zhuo et al., 2005). According to recent data, it seems that K_{ATP} channels provide ischemic protection (Ardehali and O'Rourke, 2005).

The aim of our study was to shed light in the apoptotic mechanism that occurs following IR injury in the testis and to investigate the possible roles of K_{ATP} channels in this mechanism through the use of cromakalim, a K_{ATP} channel opener.

Materials and methods

Animal model

All animal experiments were performed according to the Tottori University Committee for Animal Experimentation guidelines. For the present study we used eight-week-old male Sprague–Dawley rats weighing 250–300 g. The animals were randomly separated in three aged matched groups. One group was used as control (group Cont; n = 6), one group represented the IR group with no treatment (group IR; n = 6) and the third group was administered with cromakalim dissolved in dimethyl sulfoxide (DMSO), a non-selective K_{ATP} channel opening vasodilator, 300 μg/kg intraperitoneal injection (i.p.), 30 min before the induction of ischemia (group Crom; n = 6). Preliminary experiments were performed in order to decide the most effective dose for cromakalim (100 μg/kg (n = 3), 300 μg/kg (n = 3), and 1000 μg/kg (n = 3); non-treated IR (n = 3); IR + DMSO (n = 3); Cont (n = 3)). Data we obtained from the histopathology score and apoptotic index indicated that cromakalim 1000 μg/kg results were very similar to the respective of cromakalim 300 μg/kg, and also that the results from cromakalim 100 μg/kg, non-treated IR and IR + DMSO groups were very similar. According to our preliminary findings we chose for our study the cromakalim dose of 300 μg/kg, and not to include a group treated with the vehicle. Additionally, preliminary experiments and published work from our laboratory (Shimizu et al., 2011a) indicated that cromakalim should be administered before the induction of ischemia.

All animals were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). Control animals were sham operated. The IR model in the rat testis was performed according to our previous publication by Shimizu et al. (2011b). In the animals of groups IR and Crom through a right inguinal incision the right spermatic cord was identified and the right testicular artery and vein were clamped for 60 min with a small Sugita standard aneurysm clip (Mizuho Ikakogyo, Tokyo, Japan) with a holding force of 145 g. After 60 min of ischemia, a 24 h period of reperfusion followed. At the completion of the reperfusion period all animals were sacrificed and ipsilateral testes from all animals were collected. A testicular sample was fixed in Bouin's solution whereas another testicular sample was immediately frozen and stored at −80 °C until used.

Real-time polymerase chain reaction (PCR) of $K_{IR}6.1$ and $K_{IR}6.2$ messenger RNAs

Real-time PCR was performed according to our previous reports (Saito et al., 2007). $K_{IR}6.1$ and $K_{IR}6.2$ mRNAs in the testis were measured by real-time PCR method. The mRNAs were purified using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The reverse transcriptase (RT) mixture (20 μl) containing 1 μg of total RNA was prepared and incubated at 37 °C for 60 min. Fifteen microliters of the mixture was used for real-time PCR, which was carried out using a Light Cycler system with a LightCycler-FastStart Hybridization Probe kit (Roche Diagnostics, Tokyo, Japan). The primers and probe sequences specific to the genes of $K_{IR}6.1$ (GeneBank Accession: NM_017099), $K_{IR}6.2$ (GeneBank Accession: NM_031358) and β-actin (GeneBank Accession: NM_031144) were used according to our previous report (Saito et al., 2007). The primer and probe of the β-actin used were from the LightCycler-Primer/Probe Set (rat), and was used as the internal standard. A total of 5 μl of cDNA solution was used for the sample.

Protein extraction for Western blot analysis

Briefly, testicular samples (100 mg) were homogenized in 150 μl lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 50 mM Tris/HCl, pH 7.5, 2.0 mM EDTA, 1 mM Na3VO4, 2 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin, and 5 mg/ml aprotinin). The homogenate was subjected to centrifugation at 10,000 g for 10 min. The supernatant was collected and used for colometric detection and quantitation of total protein (Pierce® BCA Protein Assay Kit, Thermo Scientific, Loughborough, UK).

Determination of $K_{IR}6.1$, $K_{IR}6.2$, caspase-3, poly (ADP-ribose) polymerase (PARP), FasL Fas, and β-actin by Western blot analysis

The protein samples (50 μg) were subjected to SDS-polyacrylamide gel electrophoresis (12% gradient gels or 10% gels). Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with PBS, 0.1% Tween 20 (PBS-T) containing 5% nonfat dried milk, washed with PBS-T, and incubated overnight at 4 °C on a shaker with antibodies for cleaved $K_{IR}6.1$ (1:200), $K_{IR}6.2$ (1:200), caspase-3 (1:1000), PARP (1:2000), FasL (1:200), Fas (1:200), and anti-β-actin (1:500) in PBS-T containing 5% nonfat dried milk. The blots were washed with PBS-T (6 × 10 min) and incubated for 1 h at 4 °C on a shaker with secondary antibody conjugated with horseradish peroxidase (1:5000) in PBS-T containing 5% nonfat dried milk. After thorough washing with PBS-T (6 × 10 times) the detection was performed using enhanced chemiluminescence reagent. The procedure in order to determine $K_{IR}6.2$ was performed as above with the only difference that 5% nonfat dried milk was replaced by 5% BSA.

The antibodies used were as follows: rabbit polyclonal antibody for $K_{IR}6.1$ (H-80: sc-20808, Santa Cruz Biotechnology, Inc., CA), rabbit polyclonal antibody for $K_{IR}6.2$ (H-55: sc-20809 Santa Cruz Biotechnology, Inc., CA), rabbit monoclonal antibody, which detects the endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175 (#9664, Cell Signaling TECHNOLOGY, Inc., Danvers, MA), rabbit polyclonal antibody for PARP (H-250: sc-7150 Santa Cruz Biotechnology, Inc., CA), rabbit polyclonal antibody for FasL (N-20: sc-834 Santa Cruz Biotechnology, Inc., CA), rabbit polyclonal antibody for Fas (A-20: sc-1023 Santa Cruz Biotechnology, Inc., CA), and rabbit polyclonal antibody for anti-β-actin (Catalog No: 54590, AnaSpec, Inc., San Jose, CA). Anti-β-actin was used as a loading control for normalization.

Myeloperoxidase activity assay

Myeloperoxidase (MPO) activity in testicular tissue of ischemic testes was detected by a spectrophotometric method using a MPO ELISA kit (HyCult Biotechnology, Uden, the Netherlands), which reflects the number of polymorphonuclear neutrophils in tissue.

Hematoxylin–eosin staining

After fixation, the tissues were embedded in paraffin. Tissue sections (5 μm) were cut from the paraffin blocks. Sections were deparaffinized, gradually hydrated and examined by hematoxylin and eosin staining. Each section was viewed under light microscope at ×400 magnification. Histological examinations were performed by a pathologist (K.S.) blinded to the experiment as previously described (Shimizu et al., 2011a, 2011b). The testicular tissues were

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