Remote preconditioning protects the heart by activating myocardial PKCe-isoform

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Abstract

Objective: Myocardial protection can be achieved by brief ischemia-reperfusion of remote organs, a phenomenon described as remote preconditioning (RPC). Since the intracellular mechanisms of RPC are not known, we tested the hypothesis that RPC might activate myocardial PKCe, an essential mediator of classical ischemic preconditioning. Furthermore, we tried to delineate the mechanisms by which RPC is transduced to the heart with respect to the possible contribution of kinins and neuronal reflexes.

Methods: Anesthetized rats were randomised to undergo either 30 min of waiting (controls) or RPC (brief mesenteric artery occlusion followed by reperfusion) in the absence or presence of chelerythrine (5 mg kg\(^{-1}\)), a specific PKC inhibitor. Myocardial infarct size was measured by TTC staining after 30 min of coronary artery occlusion followed by 150 min of reperfusion. In separate sets of experiments RPC was performed with or without pretreatment with HOE140, a selective B\(_2\)-antagonist or hexamethonium was used to explore the influence of ganglion blockade on RPC. Translocation of PKCe from cytosol to the particulate fraction was measured by quantitative immunoblotting.

Results: RPC significantly reduced infarct size which was completely blocked by the PKC inhibitor. RPC shifted the ratio between cytosolic and particulate PKCe, an indicator for PKC-activation, from 0.95±0.06 in controls to 0.41±0.09 (\(P<0.05\)), and this effect was abolished by HOE140. Activation of PKCe could not be achieved after pretreatment with HEX (0.69±0.06 in HEX vs. 0.78±0.06 in HEX+RPC).

Conclusions: RPC activates myocardial PKCe through a neuronal and bradykinin-dependent pathway. We assume that activation of PKCe is an important step in cardioprotection induced by remote preconditioning.

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Keywords: Endothelial receptors; Infarction; Neurotransmitters; Preconditioning; Protein kinases

1. Introduction

Ischemic preconditioning (IPC) protects the heart against myocardial infarction, a phenomenon which can also be achieved by brief ischemia and reperfusion of a remote organ [1]. The precise mediators and end-effectors of remote preconditioning (RPC) have not yet been elucidated. Neuronal and humoral mediators such as bradykinin (BK) have been suggested for RPC after mesenteric artery occlusion (MAO) followed by reperfusion [1,2]. In those studies, blockade of B\(_2\)-receptors or neuronal ganglia abolished cardioprotection by RPC using reduction of infarct size as the primary endpoint.

Ischemic preconditioning is associated with a translocation of the myocardial protein kinase C (PKC)-isoforms from the cytosolic to the particulate fraction, a process which is considered to represent its activation [3,4]. Although activated PKC is commonly thought to be localized in sarcolemmal and cytoskeletal structures, opening of mitochondrial potassium channels and induction of gene expression were recently demonstrated as potential targets for activated PKC after translocation to mitochondria and nuclei, respectively [5,6]. Activation of the various isoforms of PKC differs among species and depends on the triggers of preconditioning [7–9], but in

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many models activation of PKC-isoform is thought to play a pivotal role in ischemic preconditioning by initiating a signal cascade that leads to cardioprotection [4,10,11].

The role of PKC in intracellular signal transduction after remote preconditioning has not yet been investigated. The aim of the present study was to evaluate whether activation of myocardial PKCe occurs in RPC as an indicator for a common form of cardioprotection in different modes of preconditioning. Furthermore, we tried to delineate the mechanisms by which RPC is transduced to the heart with respect to the possible contribution of kinins and neuronal reflexes.

2. Methods

2.1. Surgical preparation

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Male Wistar rats (270–310 g body weight, Charles River, Sulzfeld, Germany) were anesthetized with pentobarbital (100 mg kg \(^{-1}\), i.p.), tracheotomized, and ventilated with room air (tidal volume: 10 ml kg \(^{-1}\) h \(^{-1}\), 50 strokes per minute) enriched with oxygen to maintain arterial oxygen tension in the normal range. The left jugular vein was cannulated in order to compensate for fluid loss (NaCl 0.9%, 4 ml kg \(^{-1}\) h \(^{-1}\)), or to inject drugs. Another catheter was placed in the left carotid artery to measure mean arterial blood pressure (MAP). Core temperature was continuously monitored and maintained at 37.0–37.7 °C.

The abdominal cavity was opened and the anterior mesenteric artery was freed from surrounding tissue and a loose suture was placed around the artery to facilitate later mesentery artery occlusion (MAO) with an atraumatic clamp.

In rats where infarct size was measured, a lateral thoracotomy was additionally performed, the pericard was opened, and a 6-0 suture was looped under the left descending coronary artery for later induction of coronary artery occlusion. In these experiments anesthesia was maintained with pentobarbital (150 μg kg \(^{-1}\) min \(^{-1}\)). Animals which served as controls underwent the same surgical treatment.

2.2. Experimental protocols

In a first set of experiments the induction of cardioprotection by remote preconditioning and the functional significance of PKC was revealed. After finishing surgery, controls waited for 30 min. A second group underwent RPC that was achieved by 15 min of mesentery artery occlusion (MAO) followed by 15 min of reperfusion. Controls and animals that received RPC were then subjected to 30-min occlusion of the left coronary artery followed by 150 min of coronary reperfusion. In order to explore the contribution of PKC activation to RPC the specific PKC inhibitor chelerythrine was administered in a separate set of experiments as bolus (5 mg kg \(^{-1}\), i.v.) 2 min before RPC was initiated or at the corresponding time point in control animals.

In a second set of experiments the activation of PKC by remote preconditioning and the responsible transduction pathways were studied. Experimental protocols are depicted in Fig. 1. In these experiments hearts were frozen in liquid nitrogen immediately after the waiting period in controls or after the mesentery reperfusion period in rats that achieved RPC. The role of kinins in PKC activation after remote preconditioning was investigated in animals (HOE+RPC), treated with the selective B2-antagonist icatibant (HOE140, 0.5 mg kg \(^{-1}\)), applied as i.v. bolus 2 min before mesentery artery occlusion. In another group that served as a positive control bradykinin (10 nmol kg \(^{-1}\), i.v.) was infused twice over 3 min at 30 and 15 min before finishing the experiments.

Neuronal reflexes were explored in a third experiment. Ganglionic transmission was blocked with hexamethonium (HEX, 10 mg kg \(^{-1}\)) and animals were again either used as controls or subjected to RPC (HEX vs. HEX+RPC).

2.3. Measurement of myocardial infarct size

After the reperfusion period the coronary artery was re-occluded and Chinese ink was injected in situ in the left ventricle. Perfused myocardium stained black and the area at risk (AAR) was delineated. Hearts were quickly excised, atria and the right ventricles were removed, and left ventricles including the septum were cut into slices (1.2-mm thickness). Slices were incubated in 2,3,5-triphenyltetrazolium chloride (1% in 0.1 M phosphate buffer, pH 7.4)
for 30 min at room temperature which stained viable tissue red and so delineated the pale area of infarct size (IS). After slices were weighed, areas of left ventricle (LV), AAR, and IS were measured on both sides of each slice by computer assisted planimetry.

2.4. PKCε translocation

For analysis of PKCε activation, hearts were excised directly after RPC and immediately frozen in liquid nitrogen. Cytosol was separated from the particulate fraction as described previously [7]. In brief, hearts were homogenized, centrifuged (105 000×g, 60 min, 4 °C), and the supernatant was taken as the cytosolic fraction. The pellet was resolubilized in a buffer containing Triton X-100 (1% final concentration), centrifuged under the same conditions and the solubilized proteins in the second supernatant were taken as the particulate fraction including all potential localisations of activated PKC isoforms like sarcolemmal membranes, mitochondria, and nuclei. PKCε was measured by quantitative immunoblotting in the cytosolic and in the particulate fractions. Cytosolic fractions were enriched with Triton X-100 to obtain the same concentrations as in particulate fractions. Proteins (5 μg) from each sample were separated by 7.5% SDS–polyacrylamide gels using standard methods and electrotransferred to polyvinylidene difluoride transfer membranes (Millipore, Eschborn, Germany). Membranes were incubated with monoclonal PKCε-isoenzyme specific antibodies (mouse-anti-PKCε-antibodies, Transduction Laboratories, KY, USA). Detection was performed using a chemiluminescence system (Amersham Pharmacia Biotech, Freiburg, Germany). Immunoblots were digitized using a CCD-camera and quantified using the software Scion Image for densitometry (Scion, MD, USA).

2.5. Drugs

Icatibant (HOE140) was generously supplied by Aventis Pharma (Frankfurt, Germany). 2,3,5-Triphenyltetrazolium chloride, Triton X-100, bradykinin, chelerythrine and hexamethonium were purchased from Sigma (Deisenhofen, Germany) or from Merck (Darmstadt, Germany). Pentobarbital was obtained from the pharmacy of the Medical University of Lübeck.

2.6. Calculations and statistics

Distribution of PKCε is expressed as the ratio between its contents in the cytosolic and particulate fractions. Cardioprotection is expressed as a decrease in the ratio between IS and AAR. All quantitative data are given as means±S.E.M. of six independent experiments. Parameters of infarct size and PKC translocation were compared among the treatment groups using a one-way ANOVA with Bonferroni’s correction. Hemodynamics were tested for differences between the groups by using a two-way ANOVA for repeated measurements. Temporal changes in hemodynamic parameters within one group were tested with a one-way ANOVA for repeated measurements followed by paired t-tests with Bonferroni’s correction when differences were significant. Differences were considered as being statistically significant at an error level of P<0.05.

3. Results

3.1. Hemodynamics

Data for MAP and heart rate (HR) are listed in Table 1. There were no differences in MAP or HR amongst all groups (n=6) after finishing the preparation. Pretreatment with HOE140 did not alter MAP or HR, while HEX and BK significantly decreased MAP without affecting HR. In all conditions employing RPC, MAP was significantly increased during MAO, and a tendency to depressed blood pressure was evident throughout mesenteric reperfusion. MAP returned to control levels before the experiment was terminated.

3.2. Reduction of infarct size after RPC

RPC lead to a marked reduction of myocardial infarct size by 48% (n=6) from 54±5% of AAR in controls to 26±2% (Fig. 2). The AAR in the treatment group (50±6% of the left ventricle) showed no significant difference to that in the control group (47±6% of the left ventricle), indicating a constant placement of the coronary ligature. Blockade of PKC during RPC with chelerythrine completely abolished cardioprotection by RPC while chelerythrine did not itself alter infarct size (51±2% in chelerythrine controls vs. 49±7% in chelerythrine plus RPC). Again, there were no significant differences between the AAR (49±6% vs. 54±7% of the left ventricle, respectively; n=6).

3.3. Activation of PKCε after RPC

Fig. 3 shows original immunoblottings, representative for the performed experiments. Under control conditions 52±4% of total PKCε was found in the activated form in the particulate fraction which was increased by RPC to 72±5%. Quantitative analysis of all experiments are shown as bars. RPC significantly shifted the ratio of the cytosolic to the particulate fraction from 0.95±0.06 in controls to 0.41±0.09 (P<0.05; n=6).

3.4. Contribution of BK and neuronal pathways in activation of PKCε after RPC

The selective B₂-antagonist icatibant (HOE140) was...
used to test the role of bradykinin in translocating PKCe after RPC (Fig. 3). Application of bradykinin served as a positive control and reduced the ratio between cytosolic and particulate fraction to 0.35±0.05 (P<0.05, n=6). Pretreatment with HOE140 (HOE+RPC) completely abolished the effects of remote preconditioning on PKCe-translocation (1.00±0.07, n=6).

To evaluate the significance of nerve activation by RPC for PKCe translocation, ganglia were blocked with hexamethonium (HEX). The ratios of PKCe between the cytosolic and particulate fractions did not differ significantly between HEX treated rats (0.69±0.06, n=6) and controls (0.9±0.06). After pretreatment with HEX, remote preconditioning (HEX+RPC) did not lead to a further translocation of PKCe (0.78±0.06, P>0.05 vs. HEX, n=6).

4. Discussion

This study provides important new insights into the pathways by which remote preconditioning leads to cardioprotection. Several studies have demonstrated that regional ischemia not only elicits a local preconditioning effect but that it can also protect remote, virgin myocardium from later, prolonged ischemia. This phenomenon of preconditioning the myocardium 'at a distance' can be initiated by ischemia in a remote coronary vascular bed [12] or by intermittent ischemia and reperfusion in non-cardiac tissues including the kidney and mesentery [1]. At first we evaluated whether RPC induced by 15 min of mesentery artery occlusion (MAO) followed by 15 min of reperfusion protects the heart from ischemia in our model. Consistent with previously published data, RPC produced a 48% reduction in myocardial infarct size after 30 min of left coronary artery occlusion and 150 min of reperfusion.

Secondly our aim was to investigate the myocardial intracellular signal cascade which is activated by remote preconditioning. Strong evidence indicates that PKC plays a key role in the signalling pathways of ischemic preconditioning (IPC) [4,13]. PKC activation by various agonists of G protein coupled receptors is thought to open mitochondrial potassium channels which might be beneficial in a subsequent ischemia [14,15]. However, PKC activation in the setting of remote preconditioning has not been

Table 1
Systemic hemodynamics in rats

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<th></th>
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Mean arterial blood pressure (MAP) and heart rate (HR) during the experimental protocol. After surgical preparation, control animals underwent 30 min of waiting. RPC (remote preconditioning) was achieved by 15 min of mesenteric artery occlusion (MAO) followed by 15 min of mesentery reperfusion. HEX indicates a pretreatment with the ganglion blocker hexamethonium. BK indicates infusion of bradykinin at the corresponding timepoints of MAO and reperfusion in the RPC group. There were no significant differences in MAP or HR between baseline and final hemodynamics of all groups.

*P<0.05 versus corresponding baseline; †P<0.05 versus corresponding values before MAO (n=6).
studied until now. Inhibition of PKC in our model, using the selective inhibitor chelerythrine, completely abolished cardioprotection achieved by RPC. Activation of PKC may therefore be considered as a common mediator for both the classical ischemic and the remote form of preconditioning. PKC is a family of at least 12 isoforms of serin/threonine kinases, many of which are present in the heart [7]. One of the prerequisites for activation of PKC is its translocation from the cytosolic to the particulate fraction. Measurement of PKC isoforms in those different compartments is a useful means to detect a preconditioned state of the heart. PKC isoforms are commonly thought to bind to specific receptors of activated C kinase (RACKs) localized in membranes [16]. Translocation of specific PKC isoforms is not only directed to sarcolemmal membranes but also to mitochondrial as well as to nuclear fractions where it is supposed to participate in the opening of mitochondrial potassium channels or the induction of gene expression, respectively [5,6,17]. However, the precise implication of PKC translocation to such compartments has not been fully elucidated, although they contain many potential target proteins for PKC [17]. In our study involvement of PKC translocation in the transduction of remote preconditioning to the heart was explored. Therefore, we separated cytosol from a particulate fraction which includes membranes and cytoskeletal structures as well as mitochondria and nuclei.

PKC isoforms are differentially activated by ischemic preconditioning and the spectrum varies with different models. In rabbits, activation of PKCε- and PKCη-isoforms has been suggested to participate in IPC [7,18] whereas PKCε- and PKCδ-isoforms are involved in rats [8,19,20]. Furthermore, changes in the subcellular distribution of PKC isoforms also depend on the stimuli of preconditioning. Whereas ischemic preconditioning translocates PKCε- and PKCδ-isoforms, in the same rat model phenylephrine led to a translocation of PKCδ- and PKCε-isoforms [9], and PKCδ has been shown to be activated in rats by a pharmacological preconditioning with δ1 opioid receptor agonists [21]. Because PKCε is thought to play a pivotal role in most models of IPC and induction of PKCε translocation by specific peptides has been shown to be cardioprotective [22], in the present study we focussed on this isoform and its importance for mediating RPC-induced cardioprotection.

Therefore, we analysed PKCε translocation in hearts preconditioned by mesenteric artery occlusion and reperfusion. Consistent with the results obtained from the experiments with chelerythrine, remote preconditioning markedly distributed PKCε-isoform to the particulate fraction. Previous studies in rabbit hearts have detected most PKC protein (65%) in the cytosolic fraction under control conditions [7]. Using the same method in our rat model, only 52% of PKC was found in the cytosol. This difference might depend on the species observed or on the time frame which is required to excise the hearts and store them in liquid nitrogen. Duration of this procedure was between 15 and 25 s in our experiments.

Although translocation of PKCε also occurs after myocardial ischemia without preconditioning [23], the observed effects in this study depend fully on precursory remote preconditioning, since the hearts were not subjected to ischemia in our protocol.

Transmission of RPC to the heart has not been completely elucidated, but it is known that MAO without reperfusion fails to protect the heart [1]. Furthermore, mediators of preconditioning can be transmitted in perfu-
sion effluent from one isolated organ to another [24]. For this reason, humoral factors are thought to be involved in RPC. As a result of our study, bradykinin essentially contributes to those humoral factors, since antagonism of endogenous bradykinin with HOE140 fully prevents activation of PKCe after RPC. This activation seems to represent the decisive step in the induction of cardioprotection, since blockade of B2-receptors by HOE140 has also been demonstrated to abolish infarct size reduction by RPC [2]. In a previous work we showed that in the absence of RPC basal levels of endogenous BK do not reduce infarct size in rats [25]. Therefore, an increase in kinin levels, which has recently been shown to occur after ischemia/reperfusion of the mesentery [26], seems to be a prerequisite for the cardioprotective action of RPC.

In addition to humoral mediators of RPC, a neuronal pathway has also been described. Blockade of autonomic ganglia with hexamethonium has been suggested to prevent the infarct-size-reducing effect of RPC [1,2]. The neuronal pathway is unique for remote preconditioning and could not be demonstrated with classical ischemic preconditioning [1]. In this light we investigated whether neuronal transmission is also essential for PKCe-activation after RPC using hexamethonium (HEX). Ganglion blockade alone did not influence the ratios between cytosolic and particulate fractions. However, remote preconditioning was no longer able to activate PKCe after pretreatment with HEX. Consequently, two different pathways are involved in mediating remote preconditioning to the heart, a bradykinin dependent pathway and the neuronal transduction, which are both required to achieve the essential intracellular signalling, e.g. translocation of the PKCe isoform.

It remains to be elucidated which additional mediators and intracellular signal molecules are involved in cardioprotection by remote preconditioning. In analogy to classical ischemic preconditioning, it might be speculated that oxygen radicals might also participate in this phenomenon. It is known that ischemia/reperfusion of the mesentery leads to an increased release of oxygen radicals in rats [27]; and it has been reported for rabbits that forming an oxygen radical-generating system with hypoxanthine and xanthine oxidase mimics preconditioning by a PKC dependent mechanism [28]. Furthermore, stimulation of B2-receptors activates the myocardial production of ROS which appear to be essential for the induction of preconditioning [29]. In the latter study oxygen radicals also participated in the opening of mitochondrial potassium channels.

From our data we conclude that remote preconditioning leads to a marked reduction of infarct size which is associated with the activation of PKC in rat myocardium. To delineate the mechanisms by which remote preconditioning is transduced to the heart we explored two different pathways of RPC. Both the B2-antagonist HOE140 and blockade of neuronal ganglia with hexamethonium prevented the activation of PKCe-isoform. We suggest that remote preconditioning protects the heart by activating myocardial PKCe via a neuronal and bradykinin-dependent pathway.

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