Role of microtubules in ischemic preconditioning against myocardial infarction

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Abstract

Objective: The role of microtubules in ischemic preconditioning (PC) was investigated in isolated perfused rabbit hearts.

Methods: Myocardial infarction was induced by 30-min global ischemia and 2-h reperfusion, and infarct size was expressed as a percentage of the left ventricle (%IS/LV). Using separate groups of rabbits, ventricular biopsies were taken before and after PC for determination of protein kinase C (PKC) translocation and p38-mitogen-activated protein kinase (p38MAP kinase) activation. To depolymerize microtubules, we used two structurally different agents, colchicine (50 μM) and nocodazole (1 μM).

Results: PC with two cycles of 5-min ischemia/5-min reperfusion significantly reduced infarct size from 60.1±5.0% to 20.0±5.0%. Although neither colchicine nor nocodazole modified infarct size in nonpreconditioned hearts, these agents abolished the infarct size-limiting effects of PC (%IS/LV=56.1±6.0% and 53.5±2.5%, respectively). Colchicine prevented translocation of PKC-ε and p38MAP kinase activation by PC. PKC translocation by infusion of 1-oleyl-2-acetyl-sn-glycerol in nonischemic hearts was also prevented by colchicine.

Conclusion: Microtubules play a crucial role in the development of anti-infarct tolerance by PC as a mechanism supporting translocation of activated PKC.

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Keywords: Preconditioning; Microtubule; Protein kinase C; Infarct size

1. Introduction

A transient brief period of ischemia markedly enhances myocardial tolerance against cell necrosis during subsequent ischemic insult, and this phenomenon, termed ischemic preconditioning (PC), has been a subject of intense investigation for more than a decade [1,2]. PC has been shown to be primarily triggered by activation of adenosine A1/A3, bradykinin B2 and δ-opioid receptors, and signals elicited from these Gi/Gq-coupled receptors (GPCR) appear to be conveyed by multiple pathways that include (1) phospholipase C- and D-mediated activation of a protein kinase C (PKC) and its downstream protein kinases [1,2], (2) transactivation of EGF receptors [3] and (3) activation of ADAMs and subsequent release of TNF-α [4]. Recently, various functions of cytoskeletal proteins (i.e., actin filaments, microtubules and intermediate filaments) in intracellular signaling have been characterized in noncardiac cells [5,6]. Cytoskeletal proteins bind a number of enzymes, protein kinases and G proteins, providing platforms for their interaction and also participating in their modification. Earlier studies [7,8] have shown that administration of colchicine, a microtubule depolymerizer, 45 or 30 min before PC abolished PC protection in rabbit and rat hearts. Infarct size limitation by preischemic activation of angiotensin II receptors was also shown to be sensitive to colchicine [9]. However, the mechanism of this action of colchicine has not yet been clarified.

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The present study was designed to determine whether microtubules play a crucial role in PC against infarction by three series of experiments. First, we assessed effects of two structurally different microtubule depolymerizers (colchicine and nocodazole) on the infarct size-limiting effects of PC. In the second series of experiments, we examined the effects of colchicine on PC-induced activation of PKC and on p38-mitogen-activated protein kinase (p38MAP kinase), which are important steps of signal transduction in PC [10–14]. Finally, the effectiveness of colchicine for depolymerization of microtubules in the present preparation was confirmed by determination of free and polymerized tubulins and by an immunohistochemical method. In the present study, we used buffer-perfused rabbit hearts to strictly control the concentrations of inhibitors, and we obtained results showing the importance of microtubules in activation of PKC and p38MAP kinase by PC and cardioprotection of PC.

2. Methods

This study was conducted in accordance with The Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and The Guide for Animal Use for Research in Sapporo Medical University.

2.1. Experiment 1: Infarct size experiments

2.1.1. Surgical preparation and perfusion of isolated rabbit hearts

Rabbit hearts were isolated from male albino rabbits (Japanese White) and perfused as in previous studies [4,11,13,14]. In brief, rabbits were anesthetized with pentobarbital and mechanically ventilated with oxygen supplement. The heart of each rabbit was quickly excised via left thoracotomy, mounted onto a Langendorff apparatus, and perfused with modified Krebs–Henseleit buffer (in mM: NaCl 118.5, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 24.8, CaCl2 2.5, and glucose 10) at pressure of 75 mm Hg and temperature of 38 °C. The buffer was continuously gassed with 95% O2–5% CO2. To measure left ventricular (LV) pressure, a fluid-filled latex balloon was placed into the LV lumen. Baseline LV end-diastolic pressure was adjusted to <5 mm Hg, and hearts were paced at 200 bpm before ischemia when the heart rate was below this level. Coronary flow was measured by timed collection of effluent from the heart. The heart was excluded from the study if LV systolic pressure was <70 mm Hg or if arrhythmias persisted after a 20-min stabilization period.

2.1.2. Experimental protocols

As shown in Fig. 1, all hearts were subjected to 30-min global ischemia and 2-h reperfusion. Prior to ischemia, each heart received no pretreatment (untreated controls), PC, colchicine (50 μM), nocodazole (1 μM), PC plus colchicine or PC plus nocodazole. PC was performed with two cycles of 5-min ischemia/5-min reperfusion. Colchicine and nocodazole were infused for 5 min before the onset of global ischemia so that these agents were retained in the myocardium until the onset of reperfusion.

2.1.3. Infarct size measurement

After 2 h of reperfusion, each heart was frozen and sliced into 2-mm-thick sections from base to apex. The uppermost slices containing large vessels were excluded from infarct size analysis. Infarcts were visualized by triphenyltetrazolium staining, and their sizes were measured by NIH Image, as previously reported [4,11,13,14].

2.2. Experiment 2: Western blotting for PKC and in vitro kinase assay of p38MAP kinase

2.2.1. Perfusion of isolated rabbit hearts

Rabbit hearts were isolated and perfused as in experiment 1. Exclusion criteria in experiment 1 were also applied for the hearts in this series of experiments.

2.2.2. Tissue sampling

2.2.2.1. Protocol 1.

As shown in Fig. 2A, rabbit hearts were perfused and subjected to one of five pretreatments before 10-min global ischemia: no pretreatment (controls), PC with two cycles of 5-min ischemia/5-min reperfusion, colchicine infusion for 25 min, PC plus colchicine infusion for 25 min, PC plus colchicine infusion for 5 min...
before ischemia and colchicine infusion for 25 min plus PC. Ventricular myocardium (0.5–1.0 g) was sampled under baseline conditions, immediately before ischemia, and after 10-min ischemia by using precooled ophthalmology scissors. Immediately after sampling, tissues were frozen in liquid nitrogen and stored at −80 °C until used for Western blotting of PKC.

2.2.2.2. Protocol 2. In this protocol, hearts received 1-oleyl-2-acetyl-sn-glycerol (OAG), a PKC activator, alone before 10-min ischemia or treatment with colchicine plus OAG, as shown in Fig. 2B. OAG was infused at 50 nM for 5 min commencing 10 min before ischemia, and colchicine (50 µM) was infused from 5 min before the onset of OAG infusion until the onset of ischemia. This dose of OAG was selected on the basis of results of an earlier study showing that the same dose of OAG mimicked the infarct size-limiting effect of PC in rabbit hearts [10]. Duration of colchicine infusion in the colchicine control in protocol 1 and that in protocol 2 were made longer than that in the PC plus colchicine group in protocol 1, since we assumed that microtubules in the nonischemic myocardium were more resistant to colchicine than were those in the myocardium subjected to ischemia and reperfusion. OAG solution was prepared according to the method described by Ytrehus et al. [10] and infused into the side arm of the aortic cannule. Colchicine was dissolved in normal perfusing buffer as in protocol 1. Ventricular tissues were sampled before treatment, immediately before ischemia and at 10 min after ischemia and were stored, as in protocol 1, until used for PKC analysis.

2.2.2.3. Protocol 3. This protocol was set up to sample tissues for p38MAP kinase assays. Perfused hearts received one of three treatments: PC with two cycles of 5-min ischemia/5-min reperfusion, 25-min colchicine infusion or PC plus colchicine (Fig. 2A). Ventricular samples were taken before (i.e., under baseline conditions) and at the end of treatment, frozen in liquid nitrogen, and stored at −80 °C.

2.2.3. Western blotting of PKC

Cytosolic and particulate fractions were prepared as previously reported [13,14]. In brief, frozen tissues were homogenized in ice-cold buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 50 µg/ml phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, and 0.3% β-mercaptoethanol. The homogenate was centrifuged at 1000×g for 10 min, and the supernatant was centrifuged at 100,000×g for 60 min. The 100,000-g supernatant was used as the cytosolic fraction, and the 100,000-g pellet was recentrifuged at 10,000×g after treatment with 0.3% Triton-X to obtain the particulate fraction as supernatant. Protein concentration was determined by using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA).

Samples were electrophoresed on a 12.5% polyacrylamide gel and then blotted onto a PVDF membrane (Milipore, Bedford, MA). After blocking with a buffer containing 5% nonfat dry milk, 100 mM NaCl, 10 mM Tris–HCl (pH 7.4), and 0.1% Tween 20, the blots were incubated with 1000-fold diluted antibodies against PKC-ε or PKC-α (Transduction Laboratories, Lexington, KY). PKC was visualized by using an ECL Western blotting detection kit (Amersham, Buckinghamshire, UK), and PKC levels were quantified by using SigmaGel, a gel analysis software (SPSS, Chicago, IL).

2.2.4. Assay of p38MAP kinase activity

Frozen samples were homogenized in ice-cold lysis buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin and 1 mM PMSF. The homogenate was centrifuged at 1000×g for 10 min, and the supernatant was diluted with lysis buffer to prepare samples containing 2 mg protein/ml.

p38MAP kinase activity in samples was determined by using an assay kit from Cell Signaling Technology (p38 MAP Kinase Assay Kit, Cell Signaling Technology, Beverly, MA). In brief, samples were incubated with immobilized antidual phospho-p38MAPK monoclonal antibodies, and immunoprecipitated phospho-p38MAP kinase was washed and suspended in 50 µl of buffer containing 25 mM Tris–HCl (pH 7.4), 5 mM β-glycerophosphate, 2 mM diithio-threitol (DTT), 0.1 mM Na3VO4 and 10 mM MgCl2. Two hundred µM ATP and 2 µg ATP-2 fusion protein were added to this suspension, and the mixture was incubated for 30 min at 30 °C. After 30-min incubation, the reaction was terminated with a buffer containing 187.5 mM Tris–HCl.
Measurements were made under baseline condition (Baseline), 1 min before global ischemia (Treatment) and 120 min after the onset of reperfusion.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>HR (beats/min)</th>
<th>CF (ml/min)</th>
<th>LVDP (mm Hg)</th>
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<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>220±8</td>
<td>67±3</td>
<td>111±7</td>
</tr>
<tr>
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<td>6</td>
<td>215±14</td>
<td>69±7</td>
<td>122±8</td>
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<td>58±5</td>
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<tr>
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<tr>
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<td>71±4</td>
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<table>
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<tr>
<th>Treatment</th>
<th>N</th>
<th>HR (beats/min)</th>
<th>CF (ml/min)</th>
<th>LVDP (mm Hg)</th>
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</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Treatment</td>
<td></td>
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<td></td>
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<tr>
<td>Reperfusion</td>
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</tbody>
</table>

Mean±S.E.M.  
HR, heart rate; CF, coronary flow; LVDP, left ventricular developed pressure. Measurements were made under baseline condition (Baseline), 1 min before global ischemia (Treatment) and 120 min after the onset of reperfusion (Reperfusion).  

* p<0.05 vs. Control.
recovery of LVDP after reperfusion, but such an effect of PC on LV function was not observed in the groups that received colchicine or nocodazole together with PC. Neither colchicine alone nor nocodazole alone modified recovery of the hemodynamic parameters.

3.2.2. Infarct size data

Heart weights in the study groups were comparable (data not shown). Infarct size as a percentage of the left ventricle (%IS/LV) is shown for each heart in Fig. 3. PC significantly reduced %IS/LV from 60.1±5.0% to 20.0±5.0% as was found in our previous studies using the same model of infarction [13,14]. Although colchicine and nocodazole did not modify %IS/LV in non-preconditioned hearts (%IS/LV=59.5±2.5% and 55.5±2.1%, respectively), these microtubule depolymerizers abolished the infarct size-limiting effect of PC (%IS/LV=56.1±6.0%, 55.3±2.5%, both p=n.s. vs. controls).

3.3. Experiment 2

3.3.1. Protocol 1

Fig. 4 shows alteration of PKC-ε levels in the cytosolic and particulate fractions in each study group. In untreated controls, PKC-ε translocation from the cytosol to the particulate fraction was observed 10 min after ischemia but not during the time control period. However, such ischemia-induced translocation of PKC-ε was not detected in colchicine-treated hearts (Fig. 4A). PC alone induced translocation of PKC-ε and also enhanced the PKC-ε translocation during sustained ischemia. Infusion of colchicine immediately after PC ischemia and its infusion commenced before PC similarly abolished translocation of PKC-ε by PC (Fig. 4B). As found in our previous studies [13,14], PKC-α was not translocated by PC or by 10-min ischemia (data not shown).
3.3.2. Protocol 2
Infusion of OAG, an activator of PKC, induced significant translocation of both PKC-ε and PKC-α, as shown in Fig. 5. Colchicine prevented this OAG-induced translocation of PKC-ε and blunted that of PKC-α.

3.3.3. Protocol 3
The level of phospho-ATF-2 after PC was consistently higher than that under baseline conditions, and p38MAP kinase activity normalized by the baseline value was 124.4±7.6% in PC samples. Such increase in p38MAP kinase activity was not detected after PC plus colchicine infusion and after colchicine infusion alone (97.4±5.5% and 96.6±8.6%, respectively, both p<0.05 vs. PC by one-way ANOVA).

3.4. Experiment 3

Data on free and polymerized tubulin levels are summarized in Table 2. Infusion of colchicine for 5 min did not change levels of free and polymerized tubulin in the nonischemic myocardium, and there was a slight trend for increase in free tubulin level after 10-min ischemia. PC alone did not significantly increase free tubulin level. However, there was a significant elevation of free tubulin level and a parallel decrease in polymerized tubulin level after PC plus colchicine infusion. These findings suggest that repetitive episodes of ischemia and reperfusion increased sensitivity of the microtubules to colchicine and that colchicine could depolymerize microtubules in preconditioned hearts. The results of Western blot analysis for tubulin were consistent with observations in immunohisto-

<table>
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<th>Treatment</th>
<th>N</th>
<th>Free tubulin (%baseline)</th>
<th>Polymerized tubulin (%baseline)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
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</tr>
<tr>
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<td>100</td>
<td>107±9</td>
</tr>
<tr>
<td>PC</td>
<td>5</td>
<td>100</td>
<td>138±12</td>
</tr>
<tr>
<td>PC+colchicine</td>
<td>5</td>
<td>100</td>
<td>216±49*</td>
</tr>
</tbody>
</table>

Mean±S.E.M.
Tx, treatment; 1-10, 10 min after ischemia.
Colchicine was infused for 5 min in the colchicine controls and in PC+colchicine-treated hearts.
* p<0.05 vs. baseline.
chemistry of microtubules. As shown in Fig. 6, in the untreated myocardium, networks of microtubules, which were mostly perpendicular to the striated pattern of myofibrils, were observed (Fig. 6A). PC protocol alone did not eliminate microtubules in the myocardium (Fig. 6B), although there appeared to be a trend for reduction compared with that in untreated controls. However, microtubules were not detected in the myocardium subjected to PC and colchicine infusion (Fig. 6C).

4. Discussion

The present study demonstrated that two structurally different microtubule depolymerizers abolished the infarct size-limiting effects of PC. Furthermore, colchicine prevented PC-induced translocation of PKC-ε, a key isoform in PC, and p38MAP kinase activation. Translocations of PKC-α and PKC-ε that were directly activated by OAG were also inhibited by colchicine pretreatment. The same dose of colchicine was shown to dissipate microtubules in the myocardium. These results indicate that integrity of microtubules is important for PC to activate a PKC-mediated pathway in the cardioprotective mechanism of PC.

Colchicine and nocodazole are structurally different agents that are frequently used to depolymerize microtubules or to inhibit dynamic assembly of microtubules. Colchicine binds a free tubulin to form a tubulin–colchicine complex, and this complex inhibits the addition of new tubulin to microtubules [16,17]. Nocodazole is thought to bind tubulin at sites similar to colchicine binding sites, but the effect of this agent is reversible and appears much faster after its application than the irreversible effect of colchicine [17,18]. Blockade of PC-induced protection by colchicine applied for a relatively short incubation period in the present experiments (Fig. 3) may appear surprising, because up to 1 h of incubation is frequently used for colchicine to depolymerize microtubules in noncardiac cells in vitro. One possible explanation for these differences is that PC (i.e., repetitive episodes of ischemia/reperfusion) may have made microtubules more susceptible to colchicine, because reperfusion following sublethal ischemia has been shown to accelerate microtubule disruption presumably by Ca\textsuperscript{2+} overload [19,20]. This notion was supported by the findings that 5-min infusion of colchicine significantly increased the level of free tubulin in preconditioned hearts but not in non-preconditioned hearts (Table 2). It is also notable that colchicine administered 25–30 min before isoflurane-induced PC [21] and angiotensin II-induced PC [9] abolished cardioprotection afforded by these nonischemic and pharmacological PCs. These pharmacological PCs share important mechanisms, including PKC, with ischemic PC [9,21]. Furthermore, the effect of colchicine on PC was completely mimicked by nucodazole in the present study. Taken together, these findings indicate that microtubules play an important role in the PC mechanism.

To obtain an insight into the mechanism by which microtubule depolymerizers abolish PC, we examined the effects of colchicine on translocation of PKC-ε after PC. Activation of PKC induces a conformational change in this kinase, resulting in exposure of substrate binding sites, which makes binding with RACK (the receptor for activated C-kinase) possible [22,23]. It is still not clear how activated PKCs find their way to RACKs anchored in intracellular and surface membranes [22,24–27]. Contribution of microtubules to Ca\textsuperscript{2+} handling has been shown by recent studies [28,29], but interaction of microtubules and PKC-ε in cardiomyocytes remains unclear. However, four studies have shown that translocation of PKC-α in smooth muscle cells is dependent on microtubules [25–27,30]. In the present study, colchicine infusion before PC and its infusion commenced immediately after the second PC ischemia similarly prevented translocation of PKC-ε to the membrane (Fig. 4B). These results suggest the importance of integrity of microtubules shortly after PC ischemia in induction of PKC translocation in cardiomyocytes. Why a period after PC ischemia was crucial for PKC activation was not specifically explored in this study, but a possible explanation is contribution of free radicals generated during reperfusion following PC ischemia. This explanation is consistent with earlier findings that infusion of free radical scavengers during PC abolished PC-induced cardioprotection and that transient infusion of free radicals induced PC-like protection that was sensitive to PKC inhibitors [31,32].

The role of microtubules in PKC translocation is further supported by results showing that OAG-induced translocation of both PKC-ε and PKC-α was also inhibited by colchicine. In the OAG experiments, we infused colchicine for 15 min because we assumed that 5-min infusion of colchicine would not be sufficient to depolymerize microtubules in the nonischemic myocardium, and this assumption was confirmed by the results of experiment 3 (Table 2). Thus, the findings in the OAG experiments may not be directly extrapolated to the mechanism by which colchicine abolishes PC-induced protection. However, the inhibitory effects of colchicine on OAG-induced PKC translocation indicate that the integrity of microtubules is important for transport of activated PKC to at least some RACKs, presumably including those relevant to PC.

A crucial role of PKC-ε in rabbit hearts has been demonstrated by findings of a correlation of translocation of this isoform with PC protection [33] and elimination of the PC effect by a PKC-ε-specific RACK inhibitor [34]. Thus, inhibition of PKC-ε translocation can explain the blockade of PC by colchicine and nocodazole. However, we cannot exclude the possibility that microtubule depolymerization interrupted some other steps in PC-induced signal transduction during sustained ischemia, resulting in loss of cardioprotection. It is unlikely that depolymerization of microtubules per se reduces tolerance of cardiomyocytes to ischemic injury, because neither colchicine alone nor nocodazole alone enlarged infarct size.
In contrast with PKC, the role of p38MAP kinase in PC is still controversial as recently reviewed by Steenbergen [35]. The time courses of p38MAP kinase activation after PC and sustained ischemia in earlier studies using various preparations of myocardial ischemia are not consistent, and it is difficult to reconcile all reported data. However, PC induced activation of p38MAP kinase in most of studies using isolated buffer-perfused rabbit and rat hearts [36–39], and Nakano et al. [12] demonstrated that MAPKAPK2, a downstream kinase of p38MAP kinase, was activated by PC in rabbit hearts. Furthermore, a recent study by Schulz et al. [40] showed that there is a close correlation between p38MAP kinase activity and anti-infarct tolerance of the preconditioned swine myocardium. Although the relationship between PKC and p38MAP kinase in acute PC is not clear, a study by Dana et al. [41] suggested that p38MAP kinase activation is PKC dependent in delayed PC. Although the extent of p38MAP kinase activation was modest, the present study confirmed activation of this kinase by PC, and elimination of both p38MAP kinase activation and PKC translocation by colchicine is consistent with the notion that there is a link between these two protein kinases in the PC mechanism [2,12].

In conclusion, the results of the present study suggest that microtubules play an important role in translocation of activated PKC-ε to RACKs that transmit signals to achieve cardioprotection afforded by PC.

Acknowledgements

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