Ischemic preconditioning triggers the activation of MAP kinases and MAPKAP kinase 2 in rat hearts

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Abstract While much is known about the beneficial effects of myocardial stress adaptation, relatively less information is available about the adaptive mechanisms. To explore the signaling pathways of stress adaptation, isolated working rat hearts were divided into three groups. Group I was adapted to stress by conventional technique of repeated ischemia and reperfusion consisting of 5 min of ischemia followed by 10 min of reperfusion, repeated four times. Group II was treated with 100 μM of genistein, a tyrosine kinase inhibitor, followed by preconditioning as described for group I. The third group, perfused with buffer only for 60 min, served as control. All hearts were subjected to 30 min of ischemia followed by 30 min of reperfusion. The results of our study demonstrated better postischemic myocardial functions in the preconditioned hearts as evidenced by increased aortic flow, coronary flow, developed pressure and lesser amount of tissue injury as evidenced by the decreased creatine kinase release. The preconditioning effects were associated with enhancement of phospholipase D activity in the heart. The preconditioning effect was almost abolished by the genistein treatment which also prevented the enhancement of phospholipase D activities. Additionally, preconditioning of the rat hearts stimulated protein kinase C, MAP kinase, and MAPKAP kinase 2 activities which were inhibited by genistein. The results identifies for the first time tyrosine kinase-phospholipase D as potential signaling pathway for ischemic preconditioning, and implicates the involvement of multiple protein kinases in myocardial adaptation to ischemia.

Key words: Ischemia/reperfusion; Stress adaptation; Tyrosine kinase; MAP kinase; MAPKAP kinase 2; Phospholipase D; Signal transduction; Heart

1. Introduction

Myocardial adaptation to ischemic stress, which is the manifestation of the earlier stress response that occurs during repeated episodes of brief ischemia and reperfusion, can render the myocardium more tolerant to a subsequent potentially lethal ischemic injury [1]. This transient adaptive response has been demonstrated to be associated with decreased reperfusion-induced arrhythmias [2], increased recovery of postischemic contraction functions [3], and reduction of the infarct size [4]. The adaptive protection has been found to be mediated by gene expression and their transcriptional regulation [5]. It has been demonstrated that cellular protein kinase C (PKC) activation is an important step in the mechanism of adaptive protection of the heart [6]. However, the intracellular signaling events, which mediate stress responses of the myocardium, are not fully understood yet. Recently, many studies have indicated that mitogen-activated protein (MAP) kinases, a novel serine/threonine protein kinase family, play an essential role in mediating intracellular signal transduction events [7,8]. In response to extracellular stimulation, MAP kinases are rapidly activated and in turn regulate cellular functions by inducing the phosphorylation of proteins, such as an oncogene product c-*jun*, S6 ribosomal protein kinase, and MAP kinase activated protein (MAPKAP) kinase 2 [9,10]. MAPKAP kinase 2 has been implicated in a novel mammalian stress activated signal transduction pathway initiated by a variety of mitogens, pro-inflammatory cytokines, or environmental stresses, where it regulates its substrate molecules by serine/threonine phosphorylation [11,12]. Stimulation of cultured cardiomyocytes with A_1 selective adrenergic analogues, endothelin 1, fibroblast growth factors, and mechanical stress activates the MAP kinase signaling cascade [13]. In addition, our previous studies indicate that environmental stresses, including heat shock and oxidative stress, and phorbol ester treatment of cultured cardiac myoblast cells resulted in a rapid increase in cellular MAPKAP kinase 2 activity (Zu, Ai, Gilchrist, Sha'afi, Das and Huang, personal communication). To understand signaling events that mediate in vivo the ischemic response of the myocardium, changes in enzymatic activities of tissue MAP kinases and MAPKAP kinase 2 derived from ischemic preconditioning-treated hearts were detected. Effects of tyrosine protein kinases on the activation of the myocardial MAP kinases/MAPKAP kinase 2 cascade were examined using a specific kinase inhibitor. Moreover, changes in myocardial phospholipase D activity in response to ischemic preconditioning were determined.

2. Materials and methods

2.1. Isolated perfused rat heart preparation and experimental protocol

Sprague-Dawley male rats of approximately 350 g body weight were anesthetized with intraperitoneal pentobarbital (80 mg/kg). After intravenous administration of heparin (500 IU/kg), the chests were opened, and the hearts were rapidly excised and mounted on a non-recirculating Langendorff perfusion apparatus. Retrograde perfusion was established at a pressure of 100 cm H_2O with oxygenated normothermic Krebs-Henseleit bicarbonate (KHB) buffer with the following ion concentrations (in mM): 118.0 NaCl, 24.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.7 $CaCl_2$, and 10.0 glucose. The KHB buffer had been previously equilibrated with 95% O_2/5% CO_2, pH 7.4 at 37°C. The hearts were preconditioned by subjecting them to 5 min ischemia by terminating the coronary flow followed by 10 min of reperfusion. The process was repeated four times [14]. The pulmonary vein was then cannulated and the Langendorff perfusion discontinued for subsequent working heart perfusion as described previously [15]. It was essentially a left-heart preparation in which oxygenated KHB at 37°C enters the cannulated pulmonary vein and left atrium at

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a filling pressure of 17 cm H_2O. The perfusion fluid then passes to the left ventricle from which it is spontaneously ejected through the aortic cannula against a pressure of 100 cm H_2O. The working hearts were perfused for 5 min for stabilization. Normothermic ischemia was induced for 30 min by terminating the left atrial flow which was followed by 30 min of reperfusion. To examine the effects of myocardial tyrosine protein kinases on the preconditioning-induced kinase activation, isolated rat hearts were perfused with 100 μM genistein (Sigma Chemical, St. Louis, MO), a specific tyrosine kinase inhibitor, supplemented with KHB buffer for 10 min, while the control group was perfused with buffer alone. Control experiments were performed by perfusing the hearts in the presence and absence of 100 μM genistein for 60 min without ischemia and reperfusion. The experiments were terminated prior to genistein treatment (baseline), after preconditioning, and after ischemia and reperfusion. Heart biopsies were frozen in liquid nitrogen for subsequent analysis of phospholipase D, protein kinase C, MAP kinases, and MAPKAP kinase 2. The release of creatine kinase (CK) was estimated in the perfusate buffer.

To estimate phospholipase D activity, hearts were perfused with the buffer containing 250 mCi [1-^{14}C]butanol (NEN, Boston, MA; 4 mCi/mmol) (final concentration of butanol was 20 mM) prior to any treatment and preconditioning [16]. Biopsies were frozen in liquid nitrogen for subsequent assay for [^{14}C]phosphatidylbutanol.

To examine the effects of genistein on myocardial functions, aortic flow and developed pressure were measured. The aortic flow was monitored using a calibrated rotameter while the developed pressure was determined as the difference between aortic end-systolic and aortic end-diastolic pressure measured through an on-line aortic pressure transducer. The data were recorded and analyzed in real time using the Cordat II data acquisition, analysis, and presentation system (Data Integrated Scientific Systems, Pinckney, MI; Triton Technologies, Inc., San Diego, CA) [15].

Inhibition of tyrosine kinase by genistein was confirmed by treating the samples with SDS-PAGE followed by Western blotting using a monoclonal antiphosphotyrosine antibody (1 μg/ml) obtained from Upstate Biotechnology Inc., Lake Placid, NY.

2.2. Estimation of phospholipase D

The frozen biopsies were homogenized and extracted with chloroform/methanol mixture [16]. Phospholipids were separated by thin layer chromatography on silica gel K6 plates using the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/H_2O (6:11:2:9, v/v) as solvent. The phosphatidylbutanol band was identified by co-chromatography of authentic standard. The band was scraped off into a tube and radioactivity quantitated using a liquid scintillation counter.

2.3. Enzymatic assay of protein kinase C, MAP kinases, and MAPKAP kinase 2

Approximately 0.3–0.4 g of heart biopsy was placed in a measured volume of ice-cold Tris-sucrose buffer that contained 0.35 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM DTT and 0.1 mM PMSF. The tissue was homogenized with a Polytron homogenizer, and the resulting homogenate was centrifuged at 15000×*g* for 20 min. The supernatant was used for the estimation of MAP kinases and MAPKAP kinase 2. To estimate protein kinase C (PKC), hearts were homogenized in a buffer containing 20 mM Tris-HCl, 250 mM sucrose, 2 mM EGTA, 4.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 250 mg/ml trypsin inhibitor, 1 mM benzamidine, 0.005% leupeptin, pH 7.4. Homogenates were centrifuged at 14000×*g* for 20 min. The resulting supernatants are centrifuged again at 105000×*g* for 90 min. The final supernatant was used as cytosolic fraction. The pellets from 14000×*g* centrifugation were rehomogenized in the same buffer and centrifuged at 14000×*g* for 20 min. The pellets obtained were discarded. The supernatants were centrifuged again at 105000×*g* for 90 min. The supernatants were discarded, and the pellets from the two 105000×*g* centrifugations were combined and resuspended in a homogenizing buffer containing 0.3% Triton X-100 (v/v) using a hand-held homogenizer. This fraction was again centrifuged at 105000×*g* for 45 min. This final supernatant serves as particulate fraction. Protein concentration was determined with a BCA protein assay kit (Pierce, Rockville, IL).

To examine MAPKAP kinase 2, a synthetic peptide substrate derived from the glycogen synthase N-terminus (KKPLNRTLSVASLPG-amide) was used [17]. The kinase assay was initiated by adding 15 μg of supernatant protein to a 40 μl reaction mixture containing 20 mM HEPES, pH 7.3, 10 mM $MgCl_2$, 1 mM EGTA, 5 μM sodium *ortho*-vanadate, 5 μM okadaic acid, 2 mM DTT, 40 μM [γ-^{32}P]ATP (4.4×10^3 cpm/pmol), and 40 μM substrate peptide. The reaction was allowed to proceed for 10 min at 30°C. The amount of ^{32}P incorporation into the peptide was analyzed by a liquid scintillation counter. MAP kinase activity was assessed using myelin basic protein as substrate under the same conditions as the MAPKAP kinase 2 assay [18]. For the protein kinase C assay, the resulting supernatant (solubilized membrane) and the cytosolic fractions were subjected to ion-exchange chromatography using DEAE columns (bed volume 2 ml). After application of the samples to the column by quantity, the columns were washed with 10 ml of the above buffer. PKC is eluted with the same buffer but with the inclusion of 400 mM NaCl. Protein concentration is measured in the elute using BCA reagent from Pierce. PKC activity was determined according to the method described by Hannun et al. [19]. Aliquots containing 10–15 μg of total protein were used in the PKC reaction. The reaction mixture contains in a final volume of 100 μl: 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 15 mM Mg-acetate, 150 μM ATP, 1 μCi [γ-^{32}P]ATP. Reactions are divided and carried out with 4 mM Ca^{2+} and 1 μM phorbol-12 myristate-13 acetate and 65 μg/ml L-α-phosphatidyl-L-serine (PS), PS only, and with 4 mM EDTA but no PS. 250–300 μM epidermal growth factor receptor peptide (EGFR) is used in our reactions as substrate (specific for PKC). Reactions were carried out at 25°C for 30 min and terminated by adding 100 μl of 75 mM *ortho*-phosphoric acid. 150 μl of this reaction mixture is spotted onto a P-81 phosphocellulose paper, washed with 75 mM orthophosphoric acid and radioactivity counted in a liquid scintillation counter. The specific activity is calculated by subtracting kinase activity in the presence of 4 mM EDTA and no PS from Ca^{2+} plus PS-stimulated kinase activity.

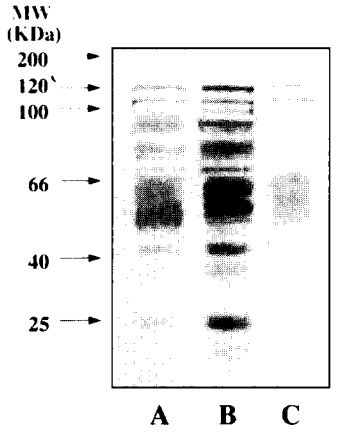

Fig. 1. Effects of preconditioning on tyrosine phosphorylation and its inhibition by genistein. Isolated hearts were preconditioned (4×PC) by repeated ischemia and reperfusion. Control hearts were perfused under identical conditions without preconditioning protocol. Another group of hearts were preperfused with genistein prior to preconditioning. Hearts were immediately frozen after the experiments. Phosphorylation of tyrosine kinase was examined by treating the samples with SDS-PAGE followed by Western blotting using a monoclonal anti-phosphotyrosine antibody (1 μg/ml). Results are representative of four experiments per group. Each experiment was repeated at least three times with identical results. Lane A: control; lane B: preconditioned; lane C: genistein followed by preconditioning.

3. Results

3.1. Effects of genistein on tyrosine kinase phosphorylation

As shown in Fig. 1, tyrosine phosphorylation was enhanced after preconditioning. Genistein blocked the phosphorylation of specific cellular proteins including 98 kDa, 80 kDa, 77 kDa, 70 kDa, 40 kDa, 38 kDa, 25 kDa and 20 kDa proteins.

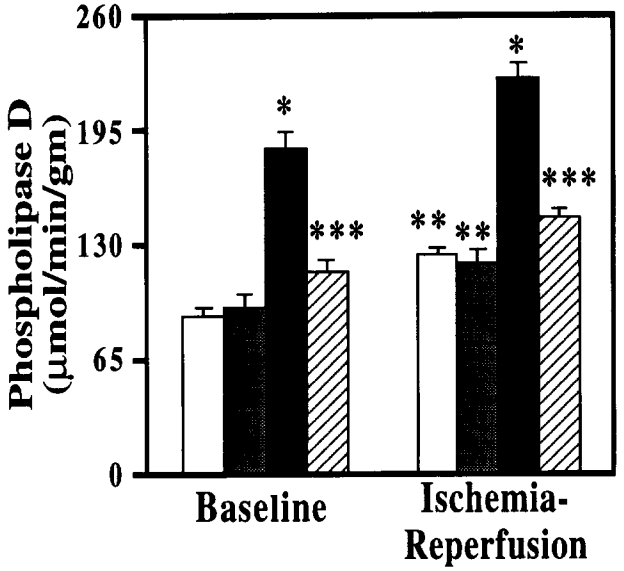

Fig. 2. Effects of preconditioning and genistein on phospholipase D in rat heart. Isolated rat hearts were preperfused with 100 μM genistein for 10 min prior to preconditioning by repeated ischemia and reperfusion. The control group did not receive any genistein. After preconditioning, hearts were subjected to 30 min of ischemia followed by 30 min of reperfusion. Results are expressed as means ± S.E.M. of six animals per group. Each assay was run in duplicate. $^{*}P < 0.05$ compared to control; $^{**}P < 0.05$ compared to baseline control; $^{***}P < 0.05$ compared to preconditioned group. White columns, control; dark-hatched columns, control + genistein; black columns, preconditioned; light-hatched columns, preconditioned + genistein.

3.2. *Effects of genistein on myocardial phospholipase D*

Phospholipase D activity was increased by 50% after ischemic preconditioning (Fig. 2). The enzyme activity was further increased after 30 min of ischemia followed by 30 min of reperfusion. Genistein had no effect on phospholipase D in the normal heart, but it inhibited the preconditioning and ischemia/reperfusion-mediated enhancement of the activities.

3.3. *Effects of genistein on protein C kinase, MAP kinase and MAPKAP kinase*

The activities of MAP kinase as well as MAPKAP kinase were enhanced significantly after preconditioning (Fig. 3). They were further activated after ischemia and reperfusion. The tyrosine kinase inhibitor, genistein, inhibited the activation of kinases. After preconditioning PKC was translocated from the cytosol to the particulate fraction and stimulated by 113%. Genistein inhibited this translocation/activation by 42% (Fig. 3). After ischemia/reperfusion, PKC activity in the particulate fraction of the control group was increased by 46% compared to the baseline value (Fig. 4). The preconditioned group showed a 35% increase in PKC activity after ischemia/reperfusion. Genistein inhibited this increased activity by 77%. Genistein by itself had no effects on any of the kinase activities.

3.4. *Effects of genistein on postischemic myocardial performance*

We also studied the left ventricular performances of the normal hearts after preconditioning and ischemia/reperfusion, and compared the results with those of genistein-treated hearts. As shown in Table 1, heart rate was not affected by preconditioning or by ischemia/reperfusion. Genistein had no effect on the heart rate. DP, dp/dt_{max}, AF, CF – all remained unchanged after preconditioning. These parameters were significantly lowered after ischemia/reperfusion in both groups. However, the values were significantly higher compared to the non-preconditioned control group. The beneficial effects of preconditioning were completely abolished in genistein-treated hearts. The left ventricular functions of control hearts showed no effects after genistein treatment.

4. Discussion

This study demonstrates for the first time that ischemic preconditioning triggers protein tyrosine kinase activation of phospholipase D in rat heart, because preconditioning-mediated enhancement of phospholipase D was inhibited by the blocker of tyrosine kinase, genistein. Inhibition of tyrosine kinase by genistein also attenuated the activation of MAP kinases, MAPKAP kinase 2 and protein kinase C further suggesting for the first time the existence of a phospholipase D-mediated signaling pathway in the ischemic myocardium.

The results supports our previous hypothesis that phospholipase D plays a major role in cell signaling in the ischemic heart [20]. Phospholipase D hydrolyzes the phosphate ester bond of the head group that converts the phospholipids, especially phosphatidylcholine, into phosphatidic acid and free head group alcohol generating the second messenger, diacylglycerol. The intermediate compound, phosphatidic acid, may itself be a second messenger product, but its precise role in signal transduction is not known. Diacylglycerol serves as the major co-factor for the translocation and activation of protein kinase C. In turn, protein kinase C may trigger the MAP kinase pathway by activating MAP kinase kinase kinases (MAP KKK).

Table 1
Effects of genistein on myocardial functions

	Control		Preconditioned		Genistein	
	Baseline	I/R	Baseline	I/R	Baseline	I/R
Heart rate	305 ± 3.2	295 ± 4.7	294 ± 4.9	285 ± 9.0	302 ± 11.3	289 ± 9.1
DP	73 ± 1.5	35.4 ± 1.8^{+}	74 ± 3.1	45.4 ± 1.4*	74 ± 0.8	37.3 ± 3.5
dp/dt_{max}	2983 ± 74	1520 ± 63^{+}	3064 ± 111	2055 ± 89*	2875 ± 134	1650 ± 120
AF	43.5 ± 0.9	14.4 ± 0.7^{+}	44.0 ± 0.9	24.3 ± 1.2*	43.0 ± 0.8	15.3 ± 1.6
CF	24.1 ± 0.6	17.1 ± 0.4^{+}	24.3 ± 0.4	19.6 ± 0.5*	23.4 ± 0.5	17.8 ± 0.5

$^{+}P < 0.05$ compared to baseline; $^{*}P < 0.05$ compared to control.
DP, developed pressure; dp/dt_{max}, maximum first derivative of developed pressure; AF, aortic flow; CF, coronary flow; I/R, after 30 min ischemia followed by 30 min reperfusion.

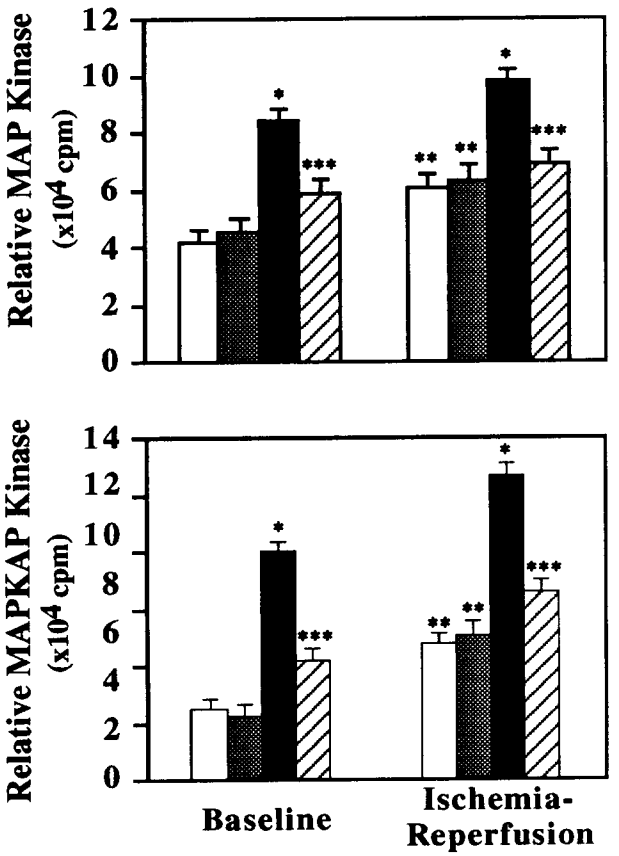

Fig. 3. Effects of preconditioning and genistein on MAP kinases and MAPKAP kiase 2 in rat heart. Isolated rat hearts were preperfused with 100 μM genistein for 10 min prior to preconditioning by repeated ischemia and reperfusion. The control group did not receive any genistein. After preconditioning, hearts were subjected to 30 min of ischemia followed by 30 min of reperfusion. Results are expressed as means ± S.E.M. of six animals per group. Each assay was run in duplicate. $^{*}P<0.05$ compared to control; $^{**}P<0.05$ compared to baseline control; $^{***}P<0.05$ compared to preconditioned group. White columns, control; dark-hatched columns, control + genistein; black columns, preconditioned; light-hatched columns, preconditioned + genistein.

We have used genistein to inhibit tyrosine kinase activity. This fungicide has been found to inhibit several tyrosine kinases such as c-*src* and v-*abl* with negligible inhibitory effect towards serine/threonine kinases [21]. The results of our study thus suggest that genistein inhibited protein kinase C, MAP kinases and MAPKAP kinase 2 activities through tyrosine kinase and secondary to the inhibition of phospholipase D activity in the heart. However, any non-specific effects of genistein on rat heart cannot be precluded from this experiment.

In this study, the detected enzymatic activity in the in vitro kinase assay using MBP as a substrate resulted from the activation of the tissue Erk and/or p38 MAP kinases. In in vitro studies, both Erk and p38 MAP kinases can phosphorylate and activate MAPKAP kinase 2 [17,22]. To detect tissue/cellular MAPKAP kinase 2 activity, a synthetic peptide derived from the N-terminus of glycogen synthase [22] is widely used as a specific substrate in in vitro kinase assays. In the present study, 20 μM H-7 was utilized in the kinase assay to inhibit enzyme activities mediated through other cellular kinases, including cAMP-dependent protein kinase ($K_i = 3.0$ μM), protein kinase C ($K_i = 6.0$ μM), and protein kinase G ($K_i = 5.8$ μM). The specificity of the kinase assay using whole tissue lysate and the peptide substrate has already been demonstrated from the observations that induced MAPKAP kinase 2 activity, detected using the in vitro kinase assay with whole tissue lysates and synthetic peptide as substrate, was inhibited by the presence of the competitive inhibitory peptide for MAPKAP kinase 2 [23]. The MAP kinase activity thus truly reflects the total MAP kinase activity irrespective of whether it is derived from ERK, JNK or p38 MAP kinases. It should be noted that a recent study demonstrated that p38 MAP kinase, and not ERK MAP kinase, leads to the activation of MAPKAP kinase 2 in vivo [24].

A growing body of evidence indicates that in response to an external stress, an intracellular kinase cascade becomes rapidly activated. This kinase cascade includes mitogen-activated protein (MAP) kinases, MAP kinase kinases and MAP kinase kinase kinases. Three distinct MAP kinases have been identified: the ERK group, the JNK/SAPK group, and the p38 MAP kinase isoform, of which the latter can activate MAPKAP kinase 2 by phosphorylation [22]. The precise physiological role of MAPKAP kinase 2 is not known, but this kinase has been implicated as a downstream molecule of the stress-activated protein kinase cascade. It has been shown that except for MAPKAP kinase 2, no other kinases including protein kinase C are capable of inducing phosphorylation of HSP 27 directly [24]. Induction of the expression of HSP 27 in response to diverse stresses has been demonstrated. For example, both ischemia/reperfusion and oxidative stress can induce the expression of HSP 27 in mammalian hearts [25]. The facts that phosphorylation of HSP 27 precedes its

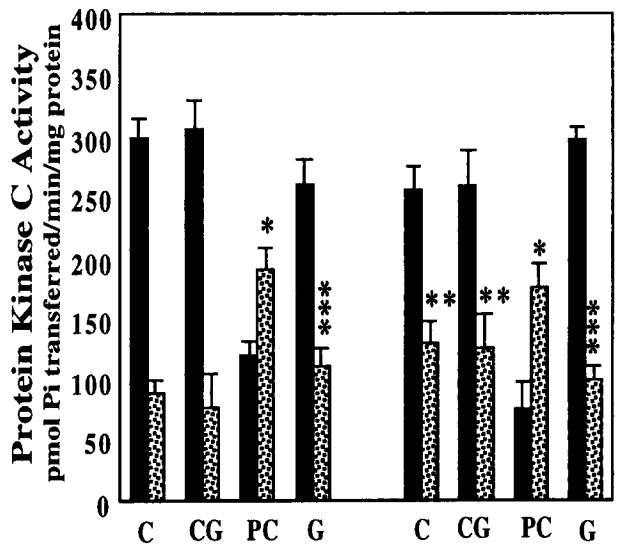

Fig. 4. Effects of preconditioning and genistein on PKC in rat heart. Isolated hearts were perfused with 100 μM genistein for 10 min prior to preconditioning by repeated ischemia and reperfusion. The control group did not receive any genistein. After preconditioning, hearts were subjected to 30 min of ischemia followed by 30 min of reperfusion. Hearts were homogenized and particulate and cytosolic fractions were prepared by differential centrifugation. PKC was assayed in both particulate and cytosolic fractions. Results are expressed as means ± S.E.M. of six animals per group. Each assay was run in duplicate. $^{*}P<0.05$ compared to control; $^{**}P<0.05$ compared to baseline control; $^{***}P<0.05$ compared to preconditioned group. C: control; CG: control + genistein; PC: preconditioned; G: preconditioned + genistein. Black columns, cytosolic fraction; stippled columns, particulate fraction.

activation [26] and HSP 27 is induced in response to stress including ischemic preconditioning and implicated in myocardial preservation [14], suggest a role of MAPKAP kinase 2 in ischemic preconditioning and myocardial adaptation.

Although our understanding of myocardial adaptation to stress is far from complete, it is believed that such adaptation occurs in two distinct phases: early adaptation also known as preconditioning, a rapid short-lived phenomenon likely to be mediated by the signal transduction process; and late adaptation, believed to occur by the regulation of gene expression [27]. The results of this study indicate that ischemic preconditioning rapidly activates *the tyrosine kinase-phospholipase D signaling pathway resulting in the activation of protein kinase C, MAP kinases and MAPKAP kinase 2*. Very recently, we have demonstrated that a variety of stresses can rapidly activate MAPKAP kinase 2 which in turn can induce heat shock proteins in myocardial cells (Zu, Ai, Gilchrist, Sha'afi, Das and Huang, personal communication). Activation of heat shock proteins has been shown to be instrumental for the adaptation of the heart to ischemic stress [25]. It is tempting to speculate that activation of MAPKAP kinase 2 may be the cellular link between preconditioning (early adaptation) and ultimate adaptation.

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