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Role of protein kinase C in the reduction of infarct size by *N*-methyl-1-deoxynojirimycin, an α-1,6-glucosidase inhibitor

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1 Preischaemic treatment with *N*-methyl-1-deoxynojirimycin (MOR-14), an α-1,6-glucosidase inhibitor, attenuates glycogenolysis and lactate accumulation during ischaemia and markedly reduces infarct size in rabbit hearts. In the present study, we have investigated whether protein kinase C (PKC), a principal mediator of ischaemic preconditioning, is also involved in the cardioprotective effect of MOR-14.

2 To assess the effect of PKC inhibition on infarct size in MOR-14-treated hearts, 38 rabbits were subjected to 30 min of ischaemia followed by 48 h of reperfusion. Infarct size, as a per cent of area at risk, was significantly smaller in rabbits administered 100 mg kg^{-1} of MOR-14 10 min before ischaemia ($17 \pm 2\%$, $n=10$), than in a control group ($46 \pm 5\%$, $n=10$). This beneficial effect of MOR-14 was abolished when 5 mg kg^{-1} of chelerythrine, a PKC inhibitor, was given 10 min prior to MOR-14 injection ($39 \pm 4\%$, $n=10$), although chelerythrine alone did not alter infarct size ($43 \pm 4\%$, $n=8$). Further, chelerythrine had no effect on MOR-14-induced attenuation of glycogen breakdown and lactate accumulation in hearts excised at 30 min of ischaemia.

3 Immunoblot analysis of PKC in homogenates of Langendorff-perfused rabbit hearts revealed that MOR-14 significantly increased levels of PKC-ε in the particulate fraction at 20 and 30 min of ischaemia and in the cytosolic fraction at 30 min of ischaemia.

4 Taken as a whole, our data suggest that PKC acts downstream of the inhibition of glycogenolysis by MOR-14 to reduce infarct size. Thus, activation of PKC is a more direct mediator of the cardioprotection afforded by MOR-14 than is inhibition of glycogenolysis.
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Abbreviations: ATP, adenosine 5′-triphosphate; ECL, enhanced chemiluminescence; EGTA, ethylene-glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethane sulphonic acid; NADP, nicotinamide adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PVDF, polyvinylidene difluoride; SDS, sodium dodecylsulphate; TTC, 2,3,5-triphenyltetrazolium chloride

Introduction

A reduction in glycogenolysis is presently considered to be one of mechanisms underlying ischemic preconditioning (Jennings *et al*., 1991; Asimakis *et al*., 1992). We previously tested the extent to which pharmacological inhibition of glycogenolysis during ischaemia would reduce infarct size and found that preischemic administration of *N*-methyl-1-deoxynojirimycin (MOR-14), an α-1,6-glucosidase inhibitor, preserved glycogen levels and markedly reduced infarct size in the rabbit heart (Arai *et al*., 1998). Recently, we also found that similar cardioprotection was achieved by preischaemic treatment with *N*-hydroxyethyl-1-deoxynojirimycin (miglitol) which has been used clinically in the treatment of non-insulin dependent diabetes mellitus (Minatoguchi *et al*., 1999). However, the precise mechanism underlying the protective effect of these drugs remains unknown.

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Protein kinase C (PKC) is an important player in numerous intracellular signal transduction pathways. Activation of PKC itself (Ytrehus *et al*., 1994; Mitchell *et al*., 1995; Li & Kloner, 1995) and PKC-coupled receptors, including adenosine A1 (Liu *et al*., 1991), α-adrenergic (Tsuchida *et al*., 1994), angiotensin AT1 (Liu *et al*., 1995), and bradykinin B2 (Wall *et al*., 1994) receptors, elicits an ischemic preconditioning-like effect. Inhibition of ischaemic preconditioning by pharmacological antagonists of PKC also provides strong evidence that PKC is involved in the protective response (Ytrehus *et al*., 1994; Mitchell *et al*., 1995; Hu & Nattel, 1995).

Recently, Opie's group showed that PKC is also responsible for cardioprotection afforded by metabolic preconditioning (Awan *et al*., 1999). Using Langendorff-perfused rat hearts, they demonstrated that improved functional recovery induced by preischaemic glucose deprivation was abolished by chelerythrine, a PKC inhibitor, suggesting that metabolic inhibition-induced protection is a signaling phenomenon involving PKC activation. Therefore,

the present study was designed to clarify whether the activation of PKC is involved downstream of the glycogenolytic inhibition in the hearts treated with α-1,6-glucosidase inhibitor.

Biochemical analyses have revealed that PKC consists of a family of at least 12 isoforms (Nishizuka; 1988, Asaoka *et al*., 1992). The expression of PKC isoforms and their implications for cellular regulation differ among species; for example, rats might rely on the δ- and α-isoforms for cardioprotection (Mitchell *et al*., 1995; Goldberg *et al*., 1997), while in dogs, PKC-α might be a dominant isoform activated by ischaemic preconditioning (Kitakaze *et al*., 1997). In the current experiment using rabbits, we focused on ε-isoform because it has been recognized as the specific PKC isoform responsible for the development of cardioprotection in rabbit hearts (Armstrong & Ganote, 1994; Qiu *et al*., 1998).

Methods

Materials

MOR-14 was synthesized by Nippon Shinyaku Co., Ltd. (Kyoto, Japan). Chelerythrine was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Antibody raised against PKC-ε was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). All other chemicals were purchased from Sigma Chemical Co. or Wako Pure Chemical Industries Ltd. (Tokyo, Japan) and were of reagent grade.

Assessment of the cardioprotective mechanism of MOR-14

Surgical preparation of the animals Male Japanese white rabbits, weighing between 1.7–2.3 kg, were anaesthetized with intravenous sodium pentobarbital (30 mg kg^{-1}), intubated, and mechanically ventilated with room air. Polyethylene catheters were placed in the internal carotid artery for continuous blood pressure monitoring and in the jugular vein for saline and drug administration. After a left thoracotomy was performed in the third intercostal space, the heart was exposed and a 4-0 silk suture was placed beneath the large arterial branch on the anterolateral surface of the left ventricle as a snare to temporally occlude blood flow. Reperfusion was achieved by loosening the snare around the coronary artery. Rabbits used in the infarct size study were subjected to 30-min coronary occlusion followed by 48-h reperfusion, and rabbits used to assess glycogen and lactate concentrations received 30-min ischaemia without reperfusion. For rabbits receiving 48 h of reperfusion, all surgical procedures were performed aseptically.

Infarct size study Rabbits were randomly assigned to one of four groups: the MOR-14 ($n=10$) and saline ($n=10$) groups received, respectively, 100 mg kg^{-1} of MOR-14 or an equivalent volume of saline 10 min before ischaemia; the chelerythrine + MOR-14 group ($n=11$) received 5 mg kg^{-1} of chelerythrine 10 min prior to MOR-14 injection; and the chelerythrine + saline group ($n=9$) received 5 mg kg^{-1} of chelerythrine 10 min before saline. The chelerythrine dosage was chosen as one that completely blocked the infarct size-reducing effect of ischaemic preconditioning in a previous study (Ping *et al*., 1999). MOR-14 was dissolved in saline. Chelerythrine was dissolved in dimethyl sulphoxide and then further diluted with saline before use.

After the respective treatment protocols, the coronary artery of each rabbit was occluded for 30 min and reperfused for 48 h. Rabbits were then reanaesthetized and the hearts were excised. The coronary artery was then reoccluded at the same site, and monastral blue dye was infused retrogradely into ascending aorta to differentiate the risk area from non-ischaemic myocardium. After the atria, epicardial fat, valvular tissue, and right ventricular free wall were removed, the left ventricle was cut into five transverse slices parallel to the atrioventricular ring. To visualize the infarct area, the left ventricular slices were incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C (Fishbein *et al*., 1981) and photographed. The areas at risk were identified by an absence of monastral blue dye staining, while TTC negative areas, which failed to demonstrate brick red staining, were considered to represent infarcted myocardium. The region of infarcted tissue and the area at risk were then traced. The volume of infarct area and area at risk was calculated by multiplying the planimetred areas by the slice weight.

Myocardial glycogen and lactate This protocol included three groups treated with MOR-14, saline, or chelerythrine + MOR-14 as described above. The hearts were quickly excised after 30 min of ischaemia, and transmural myocardial samples weighing approximately 200 mg were taken from the centre of the ischaemic region, defined by the distribution of cyanosis during ischaemia, and from a non-ischaemic region on the opposite side of the ventricle. The samples were immediately frozen in liquid nitrogen and stored at −80°C.

Prior to assay, samples were homogenized in 6% (wt wt^{-1}) perchloric acid. For myocardial glycogen measurement, the homogenate was incubated for 2 h at 40°C with amyloglucosidase to hydrolyze the glycogen. The resulting glucose residue was then measured using an NADP-linked spectrophotometric method using hexokinase and glucose-6-phosphate dehydrogenase (Keppler & Decker, 1984). Glycogen concentrations were expressed as mg glucose g myocardium wet wt^{-1}. Lactate in the extracts was measured spectrophotometrically by monitoring the H_2O_2 formation resulting from the enzymatic reaction with lactate oxidase (Bergmyer, 1974).

Assessment of the subcellular distribution of PKC-α and -ε

Perfusion of isolated rabbit heart Rabbits were anaesthetized and ventilated as described above. The hearts were excised, immediately arrested in ice-cold buffer, mounted, and perfused at a constant pressure of 100 cm H_2O with Krebs-Henseleit solution gassed with 95% O_2–15% CO_2. After a 20 min stabilization period, the hearts were perfused with buffer containing either MOR-14 or an equivalent volume of saline for 10 min and then rendered globally ischaemic for up to 30 min at 37°C. MOR-14 was administered at a concentration of 2 mmol l^{-1}, which matches the plasma concentration measured 5 min after intravenous injection of 100 mg kg^{-1} in rabbits. At the onset of ischaemia (0 min), and after 10, 20 and 30 min of ischaemia, the ventricles were quickly separated, frozen in liquid nitrogen, and stored at

−80°C. Two samples were obtained from each left ventricle. One sample was used to measure myocardial glycogen and lactate concentrations as already described, and the other sample was used to assess the subcellular distribution of PKC-ε.

Subcellular fractionation Frozen samples were weighed and homogenized in 5 volumes of buffer containing (mM) sucrose 300, HEPES 4, EGTA 2, phenylmethylsulphonyl fluoride (PMSF) 1 and leupeptin 20 μM using a Polytron homogenizer at the maximum speed in five 5-s bursts. The homogenates were centrifuged at $1000 \times g$ for 10 min, after which the supernatant containing the cytosolic and particulate fractions was spun at $105{,}000 \times g$ for 1 h. The resultant supernatant was referred to as the cytosolic fraction. The pellet (particulate fraction) was dispersed in a buffer containing (mM) Tris-HCl 20, 1 EGTA 1, PMSF 1, leupeptin 20 μM, and 0.5% TritonX-100. The protein concentration in the samples was determined by the Bradford method (Bio-Rad protein assay kit).

Immunoblot analysis PKC-ε present in the cytosolic and particulate fractions was assessed using a standard SDS–PAGE Western immunoblotting technique. The proteins (30 μg) present in each sample fraction were separated by SDS–PAGE on 8.0% gels and then electrophoretically transferred to 0.45 μm PDGF membranes using a Semi-Dry Transfer Cell (Bio-Rad). The transfer buffer contained (mM) Tris-HCl 25 and glycine 192 in 20% methanol. The blots were initially blocked overnight with 5% milk in buffer containing (mM) Tris-HCl 20 (pH 7.4), NaCl 137, 0.05% Tween-20, and then incubated for 1.5 h with anti PKC-ε antibody (1 : 100 dilution). The primary antibody was monoclonal raised against peptides corresponding to amino acids 1-175 as mapped to the carboxyterminus of human PKC-ε. After washing, the blots were incubated for 1 h at room temperature with a peroxidase-linked, goat anti-mouse secondary antibody (1 : 5000 dilution). The bound antibody was then visualized using an enhanced chemiluminescence (ECL) kit (Amersham). PKC was quantified densitometrically using suitable autoradiographs.

Statistical analysis Values are presented as means ± s.e.mean. Risk and infarct sizes were compared among groups by one-way analysis of variance (ANOVA) combined with Bonferroni's *post hoc* test for multiple comparisons. The differences in blood pressure and heart rate over the time course among the groups were assessed by two-way repeated measures ANOVA. Student's *t*-test was used to assess the differences in glycogen and lactate concentrations between pairs of groups. Time-dependent changes in the subcellular distribution of PKC were analysed by one-way ANOVA, and differences between groups at each time point were assessed by Student's *t*-test. Values of $P < 0.05$ were considered significant.

Results

Infarct size study

Among the 40 rabbits used for the infarct-size study, one died in each of the chelerythrine + MOR-14 and chelerythrine + saline groups because of ventricular fibrillation. The remaining 38 rabbits were used for the data analysis. No significant differences in blood pressure or heart rate were seen among the four groups at any time point during ischaemia or after reperfusion (Table 1). Similarly, the areas at risk were not significantly different among the four groups (Figure 1A). MOR-14 significantly reduced infarct size expressed as a per cent of the area at risk as compared with saline (17 ± 2% *vs* 46 ± 5%), which is consistent with our earlier findings (Figure 1B). The infarct size-reducing effect of MOR-14 was completely abolished when chelerythrine was administered prior to MOR-14. Infarct size in the chelerythrine + MOR-14 group was 39 ± 4%, significantly larger than in the MOR-14 group and not different from the saline group. Infarct size in the chelerythrine + saline group (43 ± 4%) was similar to that in the saline group.

Myocardial glycogen and lactate

Myocardial glycogen content (mg g wet wt^{-1}) in the ischaemic region was significantly preserved in the MOR-14 group than in the saline group at 30 min of ischaemia

Table 1 Haemodynamics in each group

	20 min before occlusion	*10 min before occlusion*	*Occlusion 0 min*	*Occlusion 10 min*	*Occlusion 30 min*	*Reperfusion 20 min*
Systolic blood pressure (mmHg)						
MOR-14 group		121 ± 5	120 ± 4	110 ± 4	109 ± 4	99 ± 4
Saline group		120 ± 5	120 ± 5	108 ± 4	109 ± 5	102 ± 4
Chelerythrine + MOR-14 group	124 ± 5	127 ± 7	124 ± 6	115 ± 5	112 ± 5	105 ± 4
Chelerythrine + saline group	124 ± 6	129 ± 6	127 ± 6	117 ± 5	115 ± 5	105 ± 4
Diastolic blood pressure (mmHg)						
MOR-14 group		91 ± 2	89 ± 3	80 ± 3	81 ± 3	71 ± 4
Saline group		89 ± 2	89 ± 2	76 ± 3	77 ± 1	72 ± 2
Chelerythrine + MOR-14 group	89 ± 3	94 ± 5	93 ± 4	81 ± 3	80 ± 2	73 ± 2
Chelerythrine + saline group	90 ± 3	95 ± 4	95 ± 4	83 ± 4	82 ± 4	75 ± 4
Heart rate (beats per minute)						
MOR-14 group		266 ± 7	259 ± 7	250 ± 7	245 ± 7	232 ± 6
Saline group		245 ± 5	243 ± 6	239 ± 8	234 ± 8	210 ± 8
Chelerythrine + MOR-14 group	279 ± 10	272 ± 6	259 ± 8	253 ± 7	248 ± 6	230 ± 6
Chelerythrine + saline group	275 ± 8	270 ± 6	262 ± 7	256 ± 6	251 ± 6	235 ± 7

There was no significant difference in the haemodynamic parameters among the groups.

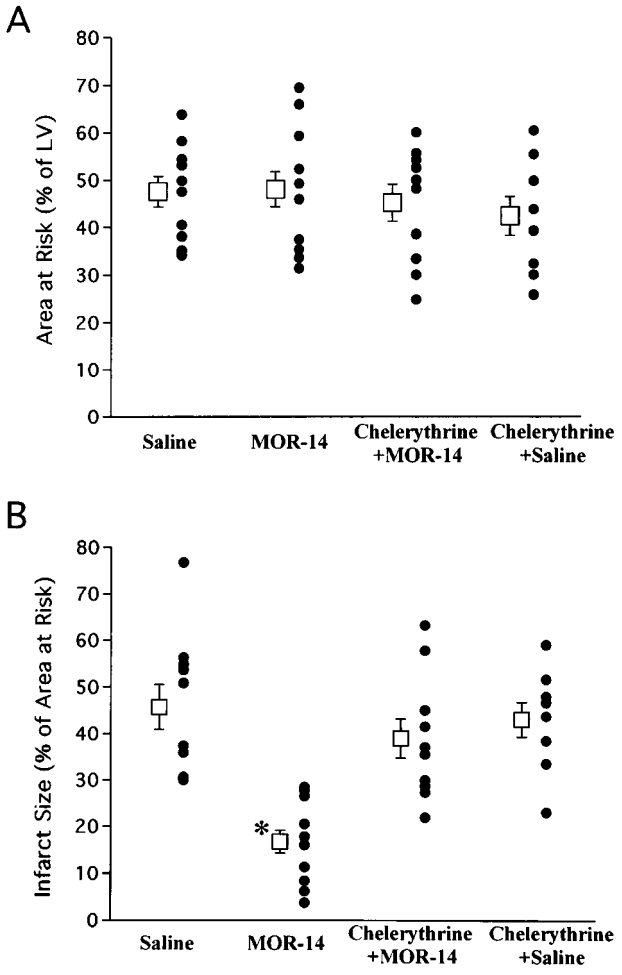

Figure 1 Comparison of areas at risk as percentages of the left ventricle (LV) (A) and of infarct areas as percentages of the area at risk (B). $*P<0.05$ *vs* saline group. Bars represent means ± s.e.mean.

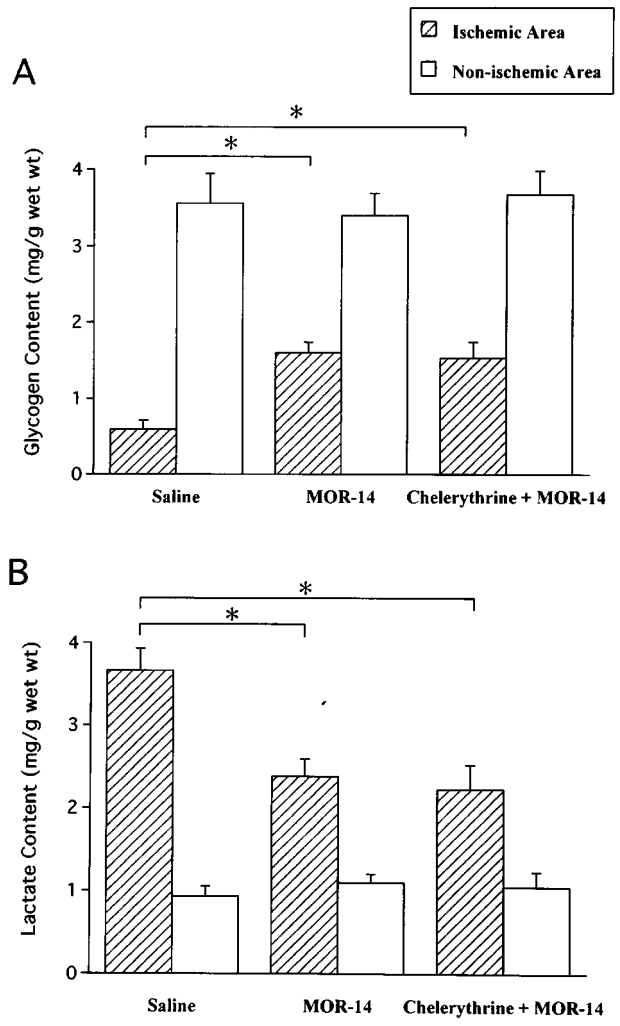

Figure 2 Myocardial glycogen (A) and lactate (B) concentrations in ischaemic and non-ischaemic areas at 30 min of ischaemia. $*P<0.05$ for each comparison. Bars represent means ± s.e.mean.

(1.6 ± 0.1; $n=11$ *vs* 0.6 ± 0.1; $n=11$) (Figure 2A). Addition of chelery-thrine had no effect on the capacity of MOR-14 to inhibit glycogenolysis. In the chelerythrine + MOR-14 group, glycogen content at 30 min of ischaemia (1.5 ± 0.2; $n=8$) was not different from the MOR-14 group and was significantly higher than the saline group. Not surprisingly, chelerythrine did not affect MOR-14-induced attenuation of lactate accumulation. Lactate content (mg g wet wt^{-1}) in the ischaemic region in the chelerythrine + MOR-14 group was 2.2 ± 0.3, which was similar to that in the MOR-14 group (2.4 ± 0.2) and significantly less than that in the saline group (3.7 ± 0.3) (Figure 2B). The glycogen and lactate content of non-ischaemic regions were not different among the three groups (glycogen: 3.4 ± 0.3, 3.6 ± 0.3, and 3.7 ± 0.3; lactate: 1.1 ± 0.1, 0.9 ± 0.1, and 1.1 ± 0.2 in the MOR-14, saline, and chelerythrine + MOR-14 groups, respectively) (Figure 2A and B).

Subcellular translocation of PKC isoforms

In the Langendorff-perfused rabbit hearts, left ventricular developed pressure, positive dP/dt, and coronary flow at baseline were 76 ± 2 mmHg, 3112 ± 132 mmHg s^{-1}, and 51 ± 4 ml min^{-1}, respectively. The infusion of MOR-14 for 10 min had no effects on these parameters.

The representative immunoblots in Figure 3 illustrate the time-dependent changes in the levels of PKC-ε in the particulate (A) and cytosolic (B) fractions of heart homogenates. PKC-ε was detected as a single band with molecular masses of 98 kDa. The quantitative densitometric analyses illustrated in Figure 4 show that MOR-14 did not affect the subcellular distribution of PKC-ε during periods of stable perfusion prior to ischaemia. Ischaemia caused translocation of PKC-ε from the cytosolic to the particulate fraction. The PKC content of the particulate fraction peaked at 20 min of ischaemia, after which it declined somewhat (Figure 4A). Concomitantly, there was a continuous, time-dependent decline in the PKC-ε content of the cytosolic fraction (Figure 4B).

In the MOR-14 treated group, the amount of PKC-ε in the membrane fraction at 20 and 30 min of ischaemia had increased to 356 ± 61% and 193 ± 17% of baseline, respectively, which were significantly greater than those in the saline group (229 ± 45% and 84 ± 14%, respectively) (Figure 4A). In

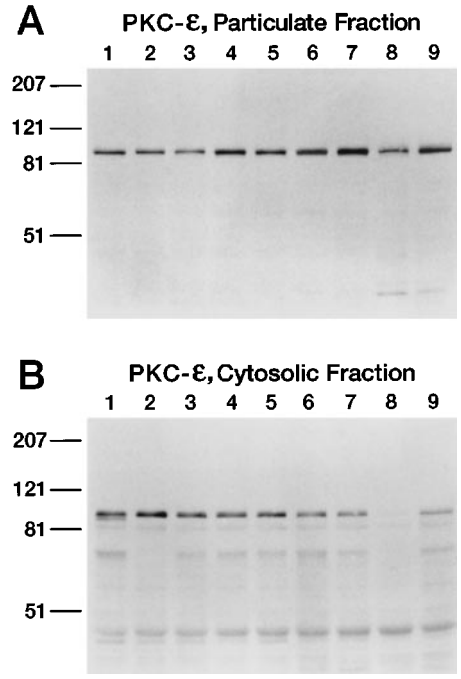

Figure 3 Representative Western blots showing PKC-ε in the particulate (A) and cytosolic (B) fractions of ventricular homogenates. Lane 1 corresponds to the sample obtained at baseline (before infusion of saline or MOR-14). Lanes 2, 4, 6 and 8 respectively correspond to the samples obtained from the saline-treated hearts at 0, 10, 20 and 30 min of ischaemia. Lanes 3, 5, 7 and 9 respectively correspond to the samples obtained from MOR-14-treated hearts at 0, 10, 20 and 30 min of ischaemia. PKC-ε appeared as a 98 kDa band in both the particulate and cytosolic fractions.

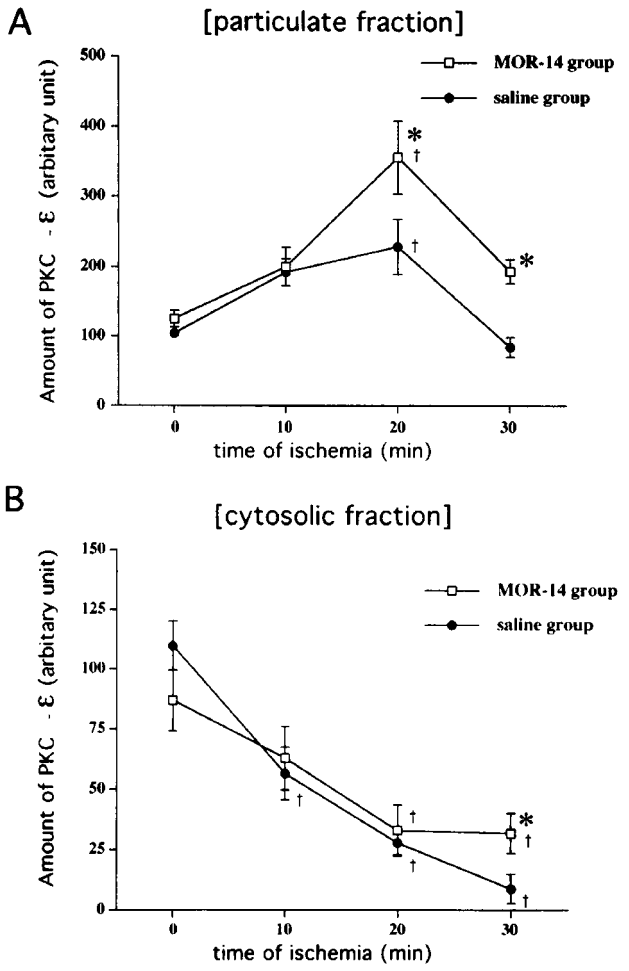

Figure 4 Quantitation of PKC-ε in the prticulate (A) and cytosolic (B) fractions in saline- and MOR-14-treated hearts. PKC-ε levels were expressed in arbitrary units representing the ratio of the content in each fraction (%) to the content of hearts after stabilizing perfusion. $*P<0.05$ compared with the value in the saline group at each time point. $\dagger P<0.05$ *vs* the value at the onset of ischaemia (0 min) in each fraction in each group. Bars represent means ± s.e.mean of eight samples.

addition, preischaemic treatment with MOR-14 also preserved the PKC-ε content in the cytosolic fraction at 30 min of ischaemia as compared with control (32 ± 8% *vs* 10 ± 7% of baseline; $P<0.05$) (Figure 4B).

Global ischaemia in isolated perfused rabbit hearts elicited a time-dependent decline in myocardial glycogen content (mg g wet wt^{-1}) in both the absence (saline) and presence of MOR-14 (Figure 5A). And there was a concomitant rise in myocardial lactate content (Figure 5B). At the onset and at 10 min of ischaemia, there were no differences in glycogen or lactate content between the MOR-14 and saline groups. However, glycogen content was significantly preserved and lactate accumulation was significantly attenuated at 20 and 30 min of ischaemia in hearts pretreated with MOR-14.

Discussion

The relation between infarct size, inhibition of glycogenolysis, and PKC-ε activation

In the present study, the infarct size-reducing effect of MOR-14, an α-1,6-glucosidase inhibitor, was blocked by chelerythrine. On the other hand, chelerythrine had no effect on MOR-14-induced attenuation of glycogen breakdown and lactate accumulation. Cytotoxicity of chelerythrine cannot explain the abolition of infarct reduction, because the inhibitor had no discernible effect on infarct size in the saline-treated hearts. It has been reported that inhibiting anaerobic glycolysis by removing substrate may trigger activation of PKC, thereby reducing myocardial ischaemic damage (Armstrong *et al*., 1994; Goto *et al*., 1995). Specifically, Armstrong *et al*. (1994) showed that the protection of isolated rabbit cardiomyocytes from subsequent ischaemia afforded by glucose-free preincubation was blocked by calphostin C, a PKC inhibitor. And more recently, Goto *et al*. (1995) demonstrated that transient perfusion with glucose-free buffer mimicked ischaemic preconditioning in isolated perfused rabbit hearts and that this protection was blocked by polymyxin B, another PKC inhibitor. Thus, we considered that PKC acting downstream of the MOR-14-induced inhibition of glycogen utilization contributes markedly to the capacity of MOR-14 to reduce infarct size.

PKC-ε was found to be more abundantly distributed in the particulate fraction of MOR-14-treated hearts than control hearts at 20 and 30 min of global ischaemia, but not at 10 min of ischaemia. Analogously, pretreatment with MOR-14 significantly attenuated glycogenolysis and lactate produc-

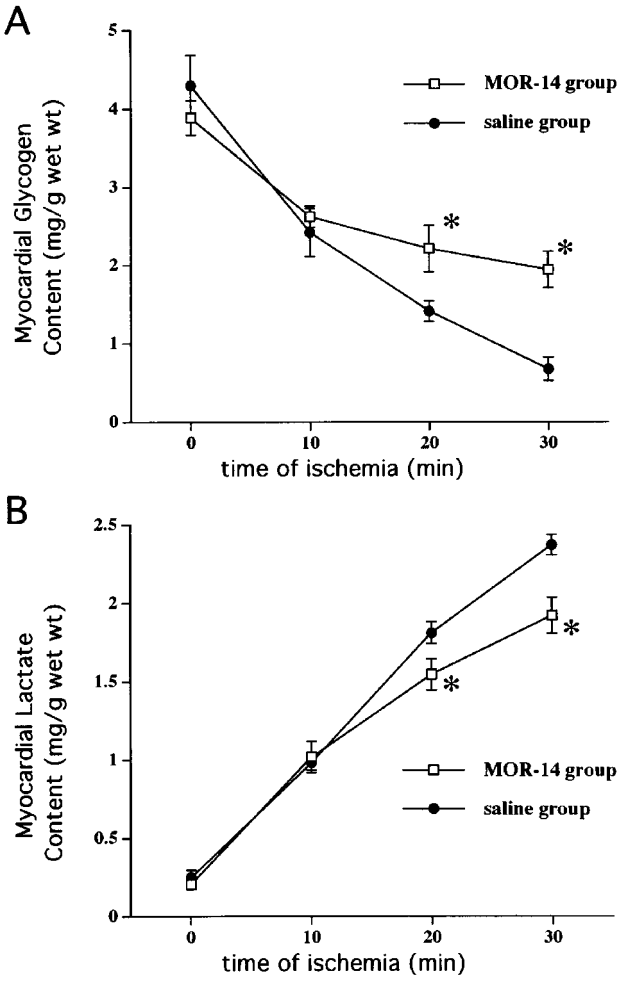

Figure 5 Time course of the decline in myocardial glycogen (A) and concomitant lactate accumulation (B) during global ischaemia in isolated Langendorff-perfused rabbit hearts. $*P<0.05$ *vs* the values in the saline group at each time point. Bars represent means ± s.e.mean.

tion at 20 and 30 min of ischemia, but not at 10 min. The good temporal agreement between the metabolic effects of MOR-14 and the subcellular distribution of PKC suggests that metabolic inhibition by MOR-14 is associated with a redistribution of some PKC isoforms. The glycogenolysis that persisted during early period of ischaemia may have been mediated by intact glycogen phosphorylase. However, this enzyme cannot cleave the four glucosyl residues located distal to a glucose having an α-1,6-linked glucosyl unit, which means that glycogen would be expected to decrease slowly in hearts whose α-1,6-glucosidase was blocked.

In contrast to ischemic preconditioned hearts, in which some PKC isoforms are translocated before the onset of sustained ischaemia (Mitchell *et al*., 1995; Ping *et al*., 1997; Kitakaze *et al*., 1996), MOR-14 did not induce redistribution of PKC-ε until at least 10 min of ischaemia. One might therefore hypothesize that MOR-14-induced alterations in the distribution of PKC would occur too late to be cardioprotective. However, an earlier study from Miura *et al*. (1989) showed that myocardial infarct size does not increase linearly over the time in ischaemic rabbit hearts. Instead, infarct size remains small early in the period of ischaemia and then progressively increases after 20 min. As a result, infarcts evoked in rabbits by 30 min of ischaemia were more than twice as large as those evoked after 20 min. This non-linear time course is consistent with idea that the augmented PKC-ε content in the particulate fraction, observed at 20 min of ischaemia, may contribute to MOR-14-induced cardioprotection. Further, since PKC-ε levels in the particulate fraction peaked at 20 min of ischaemia, it seems likely that the cardioprotective action of PKC-ε was maximally exerted at about that time point. It follows, therefore, that our observation that chelerythrine abolished MOR-14-induced reductions in infarct size strongly suggests that translocation of PKC was a key mediator of the cardioprotection seen in MOR-14-treated hearts.

Mechanisms underlying MOR-14-induced subcellular redistribution of PKC

There are two possible explanations for the mechanisms by which metabolic inhibition by MOR-14 may induce subcellular redistribution of PKC. The simplest explanation would be that MOR-14 facilitates ischaemia-induced translocation of PKC-ε from the cytosolic to the particulate fraction. The mechanism for this facilitated translocation could be increased ATP catabolism resulting from glycolytic inhibition (Goto *et al*., 1995), which would increase adenosine levels, stimulate adenosine A_1 receptors, and activate PKC. However, the hypothesis of facilitated translocation does not provide a good explanation for the present finding that, compared with the saline group, the MOR-14-treated group had significantly higher PKC-ε levels in both the cytosolic and particulate fractions at 30 min of ischaemia.

We could assume that MOR-14 might inhibit degradation of PKC-ε in both the particulate and cytosolic fractions. PKC levels in the particulate fraction decreased during the period between 20 and 30 min of ischaemia, which is in good agreement with the earlier report from Yoshida *et al*. (1996). Those investigators also showed that degradation of PKC by proteolytic enzymes could account for its disappearance. It is possible that the significant increases in the membrane-associated PKC-ε content seen in the MOR-14-treated hearts was due to suppressed degradation, rather than enhanced translocation from the cytosol. And the same mechanism could account for the preservation of PKC-ε in the cytosolic fraction at 30 min of ischaemia. Suppressed degradation of PKC is also consistent with cardioprotective effect because inhibitors against PKC proteolysis reduce myocardial damage as assessed by creatine kinase release (Yoshida *et al*., 1995; Urthaler *et al*., 1997).

Calpain is an important proteolytic enzyme contributing to the degradation of PKC, and is activated by Ca^{2+} influx during ischaemia (Yoshida *et al*., 1993). Increased anaerobic glycolysis leading to intracellular acidification should activate Na^+/H^+ exchange, which coupled to increased Na^+/Ca^{2+} exchange, could elevate intracellular Ca^{2+}. Thus, we speculated that by reducing anaerobic glycolysis and the production of protons, preischaemic treatment with MOR-14 might attenuate intracellular accumulation of Ca^{2+} and in turn the activity of calpain. Further studies such as the measurement of calpain activity would be warranted to clarify the detailed mechanisms of PKC-dependent cardioprotective effect of MOR-14.

Limitation of the study

According to the recent study (Ping *et al*., 1997), ten PKC isoforms (α, $\beta1/\beta2$, γ, δ, ε, ζ, η, ι, λ, and μ) have been detected in the rabbit heart, although there is some controversy among investigators (Armstrong *et al*., 1996; Rouet-Benzineb *et al*., 1996). We focused on PKC-ε because in rabbit hearts, it is the predominant isoform of PKC (Ping *et al*., 1997), and is mainly responsible for the protection afforded by ischaemic preconditioning (Ping *et al*., 1999; Liu *et al*., 1999). Although further studies to assess the involvement of other PKC isoforms are warranted, our data strongly suggest that at least one PKC isoform is an effector mediating MOR-14-induced cardioprotection.

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References

ARAI, M., MINATOGUCHI, S., TAKEMURA, G., UNO, Y., KARIYA, T., TAKATSU, H., FUJIWARA, T., HIGASHIOKA, M., YOSHIKUNI, Y. & FUJIWARA, H. (1998). *N*-methyl-1-deoxynojirimycin (MOR-14), an α-1,6-glucosidase inhibitor, markedly reduced infarct size in rabbit hearts. *Circulation,* **97,** 1290 – 1297.

ARMSTRONG, S., DOWNEY, J.M. & GANOTE, C.E. (1994). Preconditioning of isolated rabbit cardiomyocytes: induction by metabolic stress and blockade by the adenosine antagonist SPT and calphostin C, a protein kinase C inhibitor. *Cardiovasc. Res.,* **28,** 72 – 77.

ARMSTRONG, S. & GANOTE, C.E. (1994). Preconditioning of isolated rabbit cardiomyocytes: effect of glycolytic blockade, phorbol esters, and ischaemia. *Cardiovasc. Res.,* **28,** 1700 – 1706.

ARMSTRONG, S.C., HOOVER, D.B., DELACEY, M.H. & GANOTE, C.E. (1996). Translocation of PKC, protein phosphatase inhibition and preconditioning of rabbit cardiomyocytes. *J. Mol. Cell. Cardiol.,* **28,** 1479 – 1492.

ASAOKA, Y., NAKAMURA, S.I., YOSHIDA, K. & NISHIZUKA, Y. (1992). Protein kinase C, calcium and phospholipid degradation. *Trends. Biochem. Sci.,* **17,** 414 – 418.

ASIMAKIS, G.K., INNERS-McBRIDE, K., MEDELLIN, G. & CONTI, V.R. (1992). Ischemic preconditioning attenuates acidosis and preischemic dysfunction in isolated rat heart. *Am. J. Physiol.,* **263,** H887 – H894.

AWAN, M.M., MAKAULA, S., FORRESTI, S. & OPIE, L.H. (1999). Metaboilic preconditioning: substrates and signaling. (abstract). *Circulation,* **100,** 1 – 716.

BERGMYER, H.U. (1974). *Methods of Enzymatic Analysis*. New York, NY: Academic Press Inc; **4,** 1446 – 1451.

FISHBEIN, M., MEERBAUM, S., RIT, J., LANDO, V., KANMATSUSE, K., MERCIER, J., CORDAY, E. & GANZ, W. (1981). Early phase of acute myocardial infarct size quantification: validation of triphenyl-tetrazolium chloride tissue enzyme staining technique. *Am. Heart J.,* **101,** 593 – 600.

GOLDBERG, M., ZHANG, H.L. & STEINBERG, S.F. (1997). Hypoxia alters the subcellular distribution of protein kinase C isoforms in neonatal rat ventricular myocytes. *J. Clin. Invest.,* **99,** 55 – 61.

GOTO, M., TSUCHIDA, A., LIU, Y., COHEN, M.V. & DOWNEY, J.M. (1995). Transient inhibition of glucose uptake mimics ischemic preconditioning by salvaging ischemic myocardium in the rabbit heart. *J. Mol. Cell. Cardiol.,* **27,** 1883 – 1894.

HU, K. & NATTEL, S. (1995). Mechanisms of ischemic preconditioning in rat hearts. Involvement of α1B-adrenoceptors, pertussis toxin-sensitive G proteins, and protein kinase C. *Circulation,* **92,** 2259 – 2265.

JENNINGS, R.B., MURRY, C.E. & REIMER, K.A. (1991). Energy metabolism in preconditioned and control myocardium: effect of total ischemia. *J. Mol. Cell. Cardiol.,* **23,** 1449 – 1458.

KEPPLER, W. & DECKER, K. (1984). *Methods of Enzymatic Analysis*. New York, NY: Academic Press Inc; **6,** 11 – 18.

KITAKAZE, M., FUNAYA, H., MINAMINO, T., NODE, K., SATO, H., UEDA, Y., OKUYAMA, Y., KUZUYA, T., HORI, M. & YOSHIDA, K. (1997). Role of protein kinase C-alpha in activation of ecto-5′-nucleotidase in the preconditioned canine myocardium. *Biochem. Biophys. Res. Commun.,* **239,** 171 – 175.

KITAKAZE, M., NODE, K., MINAMINO, T., KOMAMURA, K., FUNAYA, H., SHINOZAKI, Y., CHUJO, M., MORI, H., INOUE, M., HORI, M. & KAMADA, T. (1996). Role of activation of protein kinase C in the infarct size-limiting effect of ischemic preconditioning through activation of ecto-5′-nucleotidase. *Circulation,* **93,** 781 – 791.

LI, Y. & KLONER, R.A. (1995). Does protein kinase C play a role in ischemic preconditioning in rat hearts? *Am. J. Physiol.,* **268,** H426-H431.

LIU, G.S., THORNTON, J., VAN WINKLE, D.M., STANLEY, A.W., OLSSON, R.A. & DOWNEY, J.M. (1991). Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. *Circulation,* **84,** 350 – 356.

LIU, G.S., COHEN, M.V., MOCHLY-ROSEN, D. & DOWNEY, J.M. (1999). Protein kinase C-epsilon is responsible for the protection of preconditioning in rabbit cardiomyocytes. *J. Mol. Cell. Cardiol.,* **31,** 1937 – 48.

LIU, Y., TSUCHIDA, A., COHEN, M.V. & DOWNEY, J.M. (1995). Pretreatment with angiotensin II activates protein kinase C and limits myocardial infarction in isolated rabbit hearts. *J. Mol. Cell. Cardiol.,* **27,** 883 – 892.

MINATOGUCHI, S., ARAI, M., UNO, Y., KARIYA, T., NISHIDA, Y., HASHIMOTO, K., KAWASAKI, M., TAKEMURA, G., FUJIWARA, T. & FUJIWARA, H. (1999). A novel anti-diabetic drug, miglitol, markedly reduced myocardial infarct size in rabbits. *Br. J. Pharmacol.,* **128,** 1667 – 1672.

MITCHELL, M.B., MENG, X., AO, L., BROWN, J.M., HARKEN, A.H. & BANERJEE, A. (1995). Preconditioning of isolated rat heart is mediated by protein kinase C. *Circ. Res.,* **76,** 73 – 81.

MIURA, T., DOWNEY, L.M., OOIWA, H., OGAWA, S., ADACHI, T., NOTO, T., SHIZUKUDA, Y. & IIMURA, O. (1989). Progression of myocardial infarction in a collateral flow deficient species. *Jpn. Heart J.,* **30,** 695 – 708.

NISHIZUKA, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature,* **334,** 661 – 665.

PING, P., TAKANO, H., TANG, X.L., QIU, Y., LI, R.C., BANERJEE, S., DAWN, B., BALAFONOVA, Z. & BOLLI, R. (1999). Isoform-selective activation of prorein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. *Circ. Res.,* **84,** 587 – 604.

PING, P., ZHANG, J., QIU, Y., TANG, X.L., MANCHIKALAPUDI, S., CAO, X. & BOLLI, R. (1997). Ischemic preconditioning induces selective translocation of protein kinase C isoforms ε and η in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ. Res.,* **81,** 404 – 414.

QIU, Y., PING, P., TANG, X.L., MANCHIKALAPUDI, S., RIZVI, A., ZHANG, J., TAKANO, H., WU, W.J., TESCHNER, S. & BOLLI, R. (1998). Direct evidence that protein kinase C plays an essential role in the development of late preconditioning against myocardial stunning in conscious rabbits and that epsilon is the isoform involved. *J. Clin. Invest.,* **101,** 2182 – 2198.

ROUET-BENZINEB, P., MOHAMMADI, K., PERENNEC, J., POYARD, M., BOUANANI, N.E.H. & CROZATIER, B. (1996). Protein kinase C isoform expression in normal and failing rabbit hearts. *Circ. Res.,* **79,** 153–161.

TSUCHIDA, A., LIU, Y., LIU, G.S., COHEN, M.V. & DOWNEY, J.M. (1994). α1-Adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C. *Circ. Res.,* **75,** 576–585.

URTHALER, F., WOLKOWICZ, P.E., DIGERNESS, S.B., HARRIS, K.D. & WALKER, A.A. (1997). MDL-28170, a membrane-permeant calpain inhibitor, attenuates stunning and PKCε proteolysis in reperfused ferret hearts. *Cardiovasc. Res.,* **35,** 60–67.

WALL, T.M., SHEEHY, R. & HARTMAN, J.C. (1994). Role of bradykinin in myocardial preconditioning. *J. Pharmacol. Exp. Ther.,* **270,** 681–689.

YOSHIDA, K., HIRATA, T., AKITA, Y., MIZUKAMI, Y., YAMAGUCHI, K., SORIMACHI, Y., ISHIHARA, T. & KAWASHIMA, S. (1996). Translocation of protein kinase C-α, β and ε isoforms in ischemic rat heart. *Biochim. Biophys. Acta.,* **1317,** 36–44.

YOSHIDA, K., SORIMACHI, Y., FUJIWARA, M. & HIRONAKA, K. (1995). Calpain is implicated in rat myocardial injury after ischemia or reperfusion. *Jpn. Circ. J.,* **59,** 40–48.

YOSHIDA, K., YAMASAKI, Y. & KAWASHIMA, S. (1993). Calpain activity alters in rat myocardial subfractions after ischemia or reperfusion. *Biochim. Biophys. Acta.,* **1182,** 215–230.

YTREHUS, K., LIU, Y. & DOWNEY, J.M. (1994). Preconditioning protects ischemic rabbit heart by protein kinase C activation. *Am. J. Physiol.,* **266,** H1145–H1152.

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