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SPECIAL REPORT

Involvement of protein kinase C in the delayed cytoprotection following sublethal ischaemia in rabbit myocardium

G.F. Baxter, [1]F.M. Goma & [2]D.M. Yellon

The Hatter Institute for Cardiovascular Studies, Division of Cardiology, University College London Hospital and Medical School, Grafton Way, London WC1E 6DB

Rabbit hearts were preconditioned with four 5 min coronary artery occlusions 24 h before 30 min coronary occlusion with 120 min reperfusion. Preconditioning significantly reduced the percentage of myocardium infarcting within the risk zone from 49.1 ± 4.3% to 31.8 ± 3.5% ($P<0.05$). When the protein kinase C (PKC) inhibitor, chelerythrine, was administered just before preconditioning, the delayed protection against infarction 24 h later was abolished. We conclude that the delayed cytoprotective response associated with ischaemic preconditioning of myocardium is likely to involve the early activation of one or more PKC subtypes.

Keywords: Ischaemic preconditioning; second window of protection; myocardial protection; ischaemia; infarction; protein kinase C (PKC); chelerythrine

Introduction Recently, a delayed phase of resistance to ischaemia in myocardium has been described that develops many hours after preconditioning with transient ischaemia. This 'second window of protection' (Yellon & Baxter, 1995) is associated with infarct size reduction in the rabbit (Marber *et al.*, 1993; Baxter *et al.*, 1994) and the dog (Kuzuya *et al.*, 1993), and with anti-arrhythmic effects in the dog (Vegh *et al.*, 1994). Alterations in the transcriptional regulation of protective proteins, as part of the adaptive response to sublethal ischaemic stress, may be involved in this late protection (Hoshida *et al.*, 1993; Marber *et al.*, 1993). Adenosine A_1 receptor activation during ischaemic preconditioning may be an important trigger for the delayed protection in the rabbit (Baxter *et al.*, 1994) but the intracellular signalling cascade is unknown. Since the A_1 receptor is known to link to protein kinase C (PKC) and since PKC can regulate gene transcription (Hug & Sarre, 1993), we tested the hypothesis that PKC activation during preconditioning is involved in the development of the delayed protection.

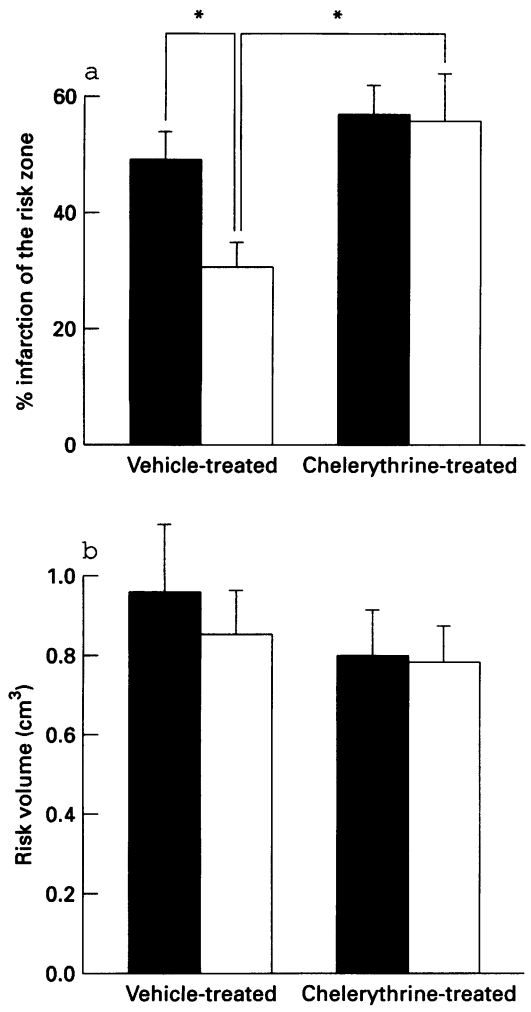
Methods The two-stage experimental protocol has been described in detail (Baxter *et al.*, 1994). Under anaesthesia ('Hypnorm' and diazepam), male New Zealand White rabbits (2.0-3.0 kg) underwent a midline sternotomy. Preconditioning was effected by four 5 min occlusions of an anterolateral branch of the circumflex coronary artery, each separated by 10 min reperfusion. Sham-operated animals served as controls. During these procedures, animals received either chelerythrine chloride 5 mg kg^{-1} (Calbiochem, Nottingham, UK) or vehicle (water with 7% v/v ethanol, total volume 6 ml), administered by slow i.v. injection over 5 min, beginning 8-10 min before the first coronary occlusion or sham preconditioning period. Four experimental groups were prepared: preconditioned + vehicle ($n=7$); preconditioned + chelerythrine ($n=6$); sham + vehicle ($n=8$); sham + chelerythrine ($n=5$). Twenty four hours later the animals were re-anaesthetized with pentobarbitone sodium and the coronary artery was occluded for 30 min with 120 min reperfusion. The myocardial risk volume (R) was determined *ex vivo* by infusion of zinc cadmium sulphide microspheres (Duke Scientific, Palo Alto, CA, U.S.A.) and the infarcted zone (I) was determined with triphenyltetrazolium chloride staining (Sigma, Poole, UK). The ratio I/R was calculated. Data were analysed with ANOVA followed by Fisher's least significant difference test and $P<0.05$ was considered as significant.

Results The principal endpoint of protection in this study was I/R (Figure 1a). I/R was reduced from 49.1 ± 4.3% in sham + vehicle to 31.8 ± 3.5% in preconditioned + vehicle ($P<0.05$), a reduction in infarct size constituting the second window of protection. Chelerythrine during the sham operation had no significant effect on infarct size 24 h later (I/R 56.9 ± 3.6% v 49.1 ± 4.3% in sham + vehicle). However, chelerythrine during preconditioning abolished the protection 24 h later (preconditioned + chelerythrine 55.4 ± 8.4% v preconditioned + vehicle 31.8 ± 3.5%; $P<0.05$). Since the rabbit is a species deficient in preformed collateral vessels, the baseline predictors of infarct size in this model are R and systemic haemodynamic status during the infarction procedure. R was similar in all the experimental groups at around 0.8-1.0 cm^3 (Figure 1b). Arterial pressure and heart rate did not differ among the experimental groups at any time point (Table 1).

Discussion This study extends our earlier observation of adenosine A_1 receptor involvement in the delayed protection (Baxter *et al.*, 1994) and suggests that a PKC signalling pathway may link adenosine receptor activation during preconditioning to the observed cytoprotection many hours later. These data provide the first evidence that PKC activation is a pivotal step in the development of the delayed protection after ischaemic preconditioning *in vivo*. Chelerythrine has been reported to be a very potent inhibitor of PKC (IC_{50} approximately 0.7 μM), showing marked selectivity for this kinase rather than other protein kinases (Herbert *et al.*, 1990). Little is known about other actions of chelerythrine. For example, inhibition of hepatic alanine aminotransferase and Na-K ATPase have been reported to occur in the micromolar range. Thus, we cannot exclude the possibility that non-kinase related activity of the compound may have been involved in our observation.

Many oncogenes and transcription factors are known to be activated by various PKC subtypes (Hug & Sarre, 1993). We have hypothesized that alterations in the regulation of heat stress proteins and/or anti-oxidant enzyme genes in the preconditioned myocardium may play a role in the mediation

[1] On leave from the Department of Physiology, University of Zambia Medical School, Lusaka
[2] Author for correspondence.



of this protection (Yellon & Baxter, 1995) while Vegh *et al.* (1994) have suggested that upregulation of the inducible nitric oxide synthase and/or cyclo-oxygenase may be involved. The nuclear translocation of activated PKC subtypes could be an important step in the transcription or modulation of these putative cytoprotective proteins. Evidence to support a direct link between adenosine receptor activation and subsequent enhancement of endogenous anti-oxidant activity has come recently from cell culture studies by Maggirwar *et al.* (1994). In addition, Yamashita *et al.* (1994) showed that hypoxic preconditioning of rat isolated cardiomyocytes resulted in enhanced tolerance to a more prolonged hypoxic period 24 h later. This delayed protection was associated with elevation of Mn-SOD activity in response to hypoxic preconditioning and was abolished by incubation with staurosporine, a relatively non-selective protein kinase inhibitor.

In summary our results provide a further demonstration of an infarct limiting effect 24 h after ischaemic preconditioning in rabbit myocardium and show that this delayed protection can be abolished by PKC inhibition with chelerythrine during the preconditioning stimulus. Thus, it seems that the second window of protection against infarction in the rabbit is likely to involve the early activation of chelerythrine-sensitive PKC subtype(s).

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Figure 1 (a) Percentage infarction of the risk zone (I/R) in rabbit hearts subjected to 30 min left coronary occlusion and 120 min reperfusion. Twenty fours hours previously, rabbits were treated with either chelerythrine or vehicle and were preconditioned (open columns) or were sham-operated (solid columns). In vehicle-treated rabbits, there was a marked reduction in infarct size in the group preconditioned 24 h earlier. When chelerythrine was given at the same time as preconditioning, the subsequent protection against infarction was abolished. (b) Volume of myocardium at risk during coronary occlusion was similar in all the experimental groups. The values given are mean ± s.e.mean for 5–8 animals. $*P<0.05$ (ANOVA).

Table 1 Summary of systemic haemodynamic parameters during the infarction protocol

	Baseline	*29 min ischaemia*	*60 min reperfusion*	*120 min reperfusion*
Sham + vehicle, $n=8$				
HR (min^{-1})	261 ± 12	239 ± 13	228 ± 11	241 ± 12
MAP (mmHg)	69 ± 3	55 ± 1	57 ± 3	57 ± 2
Preconditioned + vehicle, $n=7$				
HR (min^{-1})	236 + 9	234 + 16	230 ± 16	225 ± 17
MAP (mmHg)	60 + 3	46 ± 4	50 ± 4	48 ± 4
Sham + chelerythrine, $n=5$				
HR (min^{-1})	256 ± 19	250 ± 18	240 ± 21	215 ± 20
MAP (mmHg)	66 ± 5	54 ± 6	49 ± 6	56 ± 3
Preconditioned + chelerythrine, $n=6$				
HR (min^{-1})	248 ± 21	248 ± 20	252 ± 21	253 ± 22
MAP (mmHg)	68 ± 2	59 ± 2	55 ± 3	60 ± 4

Heart rate (HR) and mean arterial pressure (MAP) were recorded continuously from a cannula placed in the right carotid artery. Data are expressed as mean ± s.e.mean.

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