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Cardioprotection by Mild Hypothermia During Ischemia Involves Preservation of ERK Activity

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Abstract

Cooling the ischemic heart by just a few degrees protects it from infarction without affecting its mechanical function, but the mechanism of this protection is unknown. We investigated whether signal transduction pathways might be involved in the anti-infarct effect of mild hypothermia (35°C). Isolated rabbit hearts underwent 30 min of coronary artery occlusion/2 h of reperfusion. They were either maintained at 38.5°C or cooled to 35°C just before and only during ischemia. Infarct size was measured. The effects of the protein kinase C (PKC) inhibitor chelerythrine, the nitric oxide synthase (NOS) inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME), the phosphatidylinositol 3-kinase (PI3K) antagonist wortmannin, or either of the mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitors PD98059 and U0126 on cooling's protection were examined. Myocardial ATP assays were performed and the level of phosphorylation of extracellular signal-regulated kinase (ERK) and MEK was examined by western blotting. To investigate an effect of cooling on protein phosphatase (PPase), a PPase inhibitor cantharidin was tested in the infarct model and the effect of mild hypothermia on PP2A activity *in vitro* was measured. Infarct size was 34.4 ± 2.2% of the ischemic zone in normothermic (38.5°C) hearts, but only 15.6 ± 8.7% in hearts cooled to 35°C during ischemia. Mechanical function was unaffected. Neither chelerythrine, L-NAME, nor wortmannin had any effect, but both PD98059 and U0126 completely eliminated protection. Ischemia rather than reperfusion was the critical time when ERK had to be active to realize protection. Phosphorylation of ERK and MEK fell during normothermic ischemia, but during hypothermic ischemia phosphorylation of ERK remained high while that of MEK was increased. Cooling only slightly delayed the rate at which ATP fell during ischemia, and ERK inhibition did not affect that attenuation suggesting ATP preservation was unrelated to protection. Cantharidin, like cooling, also protected during ischemia but not at reperfusion, and its protection was dependent on ERK phosphorylation. However, mild hypothermia had a negligible effect on PP2A activity in an *in vitro* assay. Hence mild hypothermia

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Disclosures

None

preserves ERK and MEK activity during ischemia which somehow protects the heart. While a PPase inhibitor mimicked cooling's protection, a direct effect of cooling on PP2A could not be demonstrated.

Keywords

hypothermia; cardioprotection; ATP; ERK; ischemia/reperfusion; protein phosphatase

Introduction

It is well documented that mild hypothermia (core temperature no lower than 32°C) is highly protective against myocardial infarction when introduced before or during the ischemic period [5, 10, 13, 14, 24, 27, 29, 41]. Deep hypothermia has been used for decades to protect the heart during surgery, but in that case the arrested heart is cooled to a temperature low enough to protect it by reducing metabolism and conserving energy stores in the myocardium. Mild hypothermia has received the attention of cardiologists because in that temperature range the heart continues to function normally and no external cardiovascular support is needed. Mild hypothermia was shown to protect the beating heart from infarction as early as 1983 when the coronary sinus of dogs was retroperfused with cold blood during regional ischemia [12]. Chien et al. [4] showed that small changes in the heart's temperature had a profound effect on infarct size in open-chest rabbits and that was later confirmed by Duncker et al. [7] in a porcine model. Because mild hypothermia has been shown to modestly delay the fall in high energy phosphates during ischemia [23, 37], it has been assumed that ATP preservation was the mechanism of that protection. But the theory has never been critically tested.

Chick cardiomyocytes cooled to 25°C during the last 10 min of 70 min of simulated ischemia and the first hour of reperfusion are very protected [35]. That protection could be attenuated by either an inhibitor of nitric oxide synthase (NOS), N^ω-nitro-L-arginine methyl ester (L-NAME), or a selective inhibitor of PKC-ε indicating that cellular signal transduction pathways were involved in this protection. But because those quiescent avian myocytes were cooled to a temperature far below the range of mild hypothermia, this model may not be an accurate representation of mild hypothermia in an intact and beating mammalian heart. Accordingly in the present study we explored the possibility that those or some other signal transduction pathways might also be responsible for the protection seen in rabbit hearts experiencing mild hypothermia.

Methods

All protocols were approved by the Institutional Animal Care and Use Committee and conformed to published guidelines [30]. Rabbit hearts were isolated and mounted on a Langendorff apparatus, and perfused with Krebs buffer. Regional ischemia was produced by ligating a prominent epicardial coronary artery for 30 min followed by 2 h of reperfusion. The experimental protocols are presented in Fig. 1. Blocking agents were added to the buffer and administered to hearts starting either 5 min prior to ischemia or at the beginning of reperfusion and continued in the buffer until either the onset of reperfusion or after 20 min of reflow. After the experiment the coronary artery was reoccluded and the risk zone delineated with fluorescent microspheres injected into the aortic root. The heart was then frozen and sliced into transverse sections, and infarct size was measured by triphenyltetrazolium chloride staining. Hypothermia was induced by quickly switching the source of the water supplied to the in-line heat exchanger's water jacket from a water bath set to 38.5°C (normothermic) to one set to 35°C (cool). This allowed the heart's temperature

to be changed in less than 5 minutes. The temperature was continually monitored during experiments by putting a thermocouple into the Krebs's solution within which the hearts were immersed. Blocking agents for one of 4 signal transduction elements known to be required for protection in ischemic preconditioning were added to the perfusion buffer: 2.8 μ M chelerythrine (PKC inhibitor) [19], 10 μ M PD98059 (ERK inhibitor) [1], 0.5 μ M U0126 (ERK inhibitor) [6], 100 nM wortmannin [phosphatidylinositol (PI) 3-kinase inhibitor] [34], or 200 μ M L-NAME [43]. Cantharidin, a protein phosphatase inhibitor, was tested in normothermic hearts at 100 nM [22]. It was administered either from 5 min prior to coronary occlusion until the end of ischemia or for 1 h starting just prior to reperfusion.

ATP assay

Left ventricular biopsies from the central ischemic territory were fast frozen and ATP was measured by a luciferase assay. Two approaches were used. In the first, drill biopsies were obtained at baseline from a beating heart before the onset of ischemia and then every 10 min during 30 min of regional ischemia. In the second, a baseline biopsy was obtained and then 2 strips of myocardium weighing ~2 gm each were cut from the left ventricle. Total ischemia was simulated by placing the strips into containers of Krebs buffer. One container was maintained at 38.5°C, while the other was cooled to 35°C. Thirty min later the strips were assayed for ATP.

PP2A assay

PP2A activity was measured by using the fluorogenic substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) as previously described [31]. Briefly, the dephosphorylation reactions (200 μ l final volume) were carried out in a 96-well plate with a reaction buffer containing 30 mM HEPES pH 7.0, 1 mM DTT, 1 mM sodium ascorbate, 0.1 mg/ml BSA, 0.1 mM MnCl_2 and 100 μ M DiFMUP. A plate with wells containing buffer with or without enzyme was pre-incubated in the plate reader at 35°C for 10 min prior to starting the reaction by addition of substrate. The formation of the fluorescent product, 6,8-difluoro-4-methylumbelliferone, was measured at 10-sec intervals in a SpectraMax M5 plate reader (Molecular Devices) with an excitation wavelength of 358 nm and an emission wavelength of 450 nm. For the first 5 min of measurements, the plate reader's incubator temperature was set to 35°C. After 5 min the incubator temperature was changed to 39°C and data were collected for another 10 min. A linear least-squares fit to data points in the first 5 minutes was taken to represent the activity at 35°C and a linear fit to data collected in the last five minutes was taken to represent the activity at 39°C.

Western blotting

In isolated rabbit hearts undergoing global ischemia at either 38.5°C or 35°C, one myocardial sample was obtained to measure baseline levels of phospho-ERK1/2 and phospho-MEK1/2 after 40 min of stabilization at 38.5°C. Then samples were obtained after 10', 20', and 30' of global ischemia and 5' of reperfusion. Samples were homogenized and subjected to SDS-PAGE. The lanes were loaded with equal amounts of protein and this was checked by Ponceau S staining. After blocking with milk the membranes were incubated with anti-phospho-Thr202/Tyr204-ERK (Cell Signaling Technology, Danvers, MA), anti-phospho-Ser217/221-MEK1/2 (Cell Signaling Technology), p44/42 MAPK mouse mAb (Cell Signaling Technology), MEK 1 antibody (Cell Signaling Technology) or actin antibody (Sigma-Aldrich) overnight while shaking at 4°C. After incubation with appropriate anti-mouse or anti-rabbit horseradish peroxidase-coupled secondary antibody (Cell Signaling Technology), bands were visualized with ImmobilonTM according to manufacturer's instructions.

Statistics

Data are expressed as mean $\pm$ SEM. Infarct sizes among groups were compared by one-way analysis of variance (ANOVA) with Student-Newman-Keuls post hoc testing. ATP data in *in vitro* hearts were compared by one-way and two-way ANOVA. ATP data in myocardial strips were compared with Student's paired t-tests. ERK1/2 and MEK1/2 phosphorylation levels were first standardized to the level of total ERK1/2 and MEK1, respectively, and then were normalized to the baseline pERK1/2 and pMEK1/2. We found that there were large differences in baseline ERK and MEK phosphorylation among the hearts. The cause of this variability is unknown but it clearly introduces noise in our measurements. Normalizing the data to the baseline sample in each heart greatly reduced the noise but did not eliminate it. Differences at the various time points were compared by ANOVA with Student-Newman-Keuls post hoc testing. $P < 0.05$ was considered significant.

Results

Fig. 2 and Table 1 present infarct size data. Although animal and heart sizes were different in a few groups, importantly risk zone sizes did not differ among groups. Cooling hearts to 35°C during ischemia resulted in dramatic protection. We looked for evidence of signal transduction in the protected cooled rabbit hearts by using selective blockers of known cardioprotective signaling molecules. When wortmannin, L-NAME, or chelerythrine was present during both ischemia and early reperfusion there was no attenuation of cooling's profound infarct-sparing effect. To eliminate the possibility that we might have preconditioned the hearts with cooling prior to ischemia we also tested whether 5' of cooling followed by normothermic ischemia affected infarction (35°C 5' Pre Isch group). It did not. Neither did cooling 5 min before and during the first 10 min of ischemia (35°C 5' Pre + 10' Isch group).

Next we tested if ERK might be involved in the protection. PD, a MEK inhibitor [1], did block protection when it was present during both ischemia and reperfusion (full) (Fig. 3). To reduce the possibility of a non-specific effect of PD we also tested a second MEK blocker with a different molecular structure, U0126 [6]. Again protection was completely blocked. We next investigated the timing of ERK's effect. When PD was administered only during reperfusion, protection was not blocked. However, PD given only during ischemia did abort protection. We already had observed that neither PD nor U0126 administered as a 20-min infusion at reperfusion had any effect on infarction in the control normothermic heart subjected to ischemia/reperfusion [44]. Because we had not previously tested the effect of either PD or U0126 infused for the entire duration of ischemia as well as the first 20 min of reperfusion, we added additional groups in which PD or U0126 was given to hearts undergoing ischemia at 38.5°C at the "full" dose and schedule. Fig. 3 reveals that neither PD nor U0126 had any effect on infarction in normothermic hearts.

Cold is known to reduce metabolism in biological systems. Therefore, an obvious hypothesis is that the protection of mild hypothermia is related to ATP preservation during ischemia. To examine this, we obtained serial biopsies from the ischemic left ventricle which were fast frozen and analyzed for ATP content. Four biopsies were collected from each heart. Three groups were studied: normothermic, cool and cool + PD. ATP was significantly preserved in all cooled hearts after 30 min of regional ischemia compared to normothermic hearts ($p < 0.001$) (Fig. 4). It should be noted, however, that PD had no independent effect on cooling's preservation of ATP. Since PD blocks cooling's infarct-sparing effect (Fig. 3), equivalent preservation of myocardial ATP in the cool + PD and the cool groups suggests that ATP preservation during ischemia cannot be the protective mechanism of mild hypothermia.

One-way ANOVA of the data for the 30-min samples of Fig. 4 indicated that ATP was significantly higher in the two cooled groups than the normothermic group and that there were no differences among the 3 groups at baseline. However, a more appropriate analysis would be a two-way ANOVA. When that was done, a time but not a group effect was demonstrated. To further test the ATP hypothesis we redesigned the experiment to make it more sensitive. Several biopsies serving as baseline samples were obtained while the heart was perfused with oxygenated buffer containing PD. Then the heart was immediately excised and two transmural strips weighing ~ 2 gm each were cut from the anterior wall of the left ventricle. One was placed in 38.5°C Krebs buffer and the other in 35°C Krebs buffer. After 30 min a sample from each of the strips was frozen in liquid nitrogen and all samples were analyzed for ATP. The results are shown in Fig. 5. One-way ANOVA showed that cooling alone preserved ATP despite the presence of PD in both the warm and cold ischemic tissues. Thus, blocking ERK did not affect preservation of ATP in cooled myocardium despite its successful abrogation of protection.

We measured levels of ERK and MEK phosphorylation in normothermic and cooled ischemic hearts. In normothermic hearts pMEK1/2 and pERK1/2 fell markedly during ischemia (Fig. 6). However, in cooled hearts there was a dramatic increase of pMEK1/2 after 10 min of ischemia there was also a modest but insignificant increase of pERK. MEK and ERK phosphorylation decreased as ischemia continued in cooled hearts but at the end of ischemia ERK phosphorylation was still near the preischemic level while MEK phosphorylation was nearly twice the preischemic value. Upon reperfusion pERK and pMEK increased above the preischemic value in cooled hearts but remained low in warm hearts.

We hypothesized that cooling might have inhibited a protein phosphatase involved in deactivation of ERK or MEK. We therefore tested whether a protein phosphatase inhibitor would be protective. The protein phosphatase inhibitor cantharidin administered during only ischemia was protective (Fig. 7), although not quite as much as seen with hypothermia. As with cooling the protection could be blocked by U0126. Also, like cooling, cantharidin was not protective when started at reperfusion. We biopsied several cantharidin-treated hearts, but did not see any sign of preservation of ERK or MEK phosphorylation (data not shown). Since inhibition of PP2A, a prominent phosphatase in the heart, has previously been implicated in cardioprotection [8, 42], we investigated whether mild hypothermia could inhibit a protein phosphatase *in vitro*. The effect of mild hypothermia on an *in vitro* assay of PP2A is demonstrated in Fig. 8. A 4°C drop in temperature had a negligible effect on the rate of dephosphorylation of the test substrate by the isolated catalytic subunit of PP2A (PP2Ac). Of course, in the intact heart hypothermia could still affect regulatory subunits or even post-translational modifications of the enzyme that might influence overall enzyme activity. But these data do argue against a direct effect of cooling on protein phosphatases.

Discussion

Our data suggest that mild cooling protects by preserving the activity of ERK in the heart. ERK has been proposed to be a key player in ischemic preconditioning in which its activation in the first minutes of reperfusion is required to prevent reperfusion injury [16, 39]. The end-effector for preconditioning appears to be the mitochondrial permeability transition pore. This pore forms when ischemically injured myocardium is reperfused, and preconditioning blocks pore formation [18, 21]. When formed, these pores uncouple the mitochondria, and thus stop ATP production. They also cause mitochondria to swell and burst releasing cytochrome C into the cytosol which can trigger apoptosis [11]. Preconditioning's and postconditioning's protection is dependent (at least in rat and rabbit hearts) on activation of ERK in the first minutes of reperfusion [16, 20, 39]. Blocking ERK

during only the ischemic period does not affect preconditioning's protection [25]. Unlike the case with preconditioning, ischemia rather than reperfusion is the critical time for cooling-induced protection [41]. And, as shown in this study, this protection is again dependent on ERK but in this case during the ischemic phase. It should be noted that both ERK inhibitors that we tested actually inhibit MEK1/2, the upstream kinases that activate ERK1/2. Moreover the changes in phosphorylation of MEK1/2's activations site was much more dramatic than that seen for ERK1/2. It is tempting to ask whether MEK rather than ERK might be responsible for the protection. However, to our knowledge ERK is MEK's only target. It should also be noted that we did not actually verify that MEK was inhibited by the blockers in the cooled hearts. But because both inhibitors blocked cooling's protection and because MEK phosphorylation was greatly increased in cooled hearts, it seems very likely that MEK activation was involved in cooling's protection.

ERK1/2 is a member of the RISK family of protective kinases [17] and RISK activation can protect rabbit and rat hearts from infarction after ischemia/reperfusion, although RISK activation does not seem to be involved in myocardial protection in pigs [38]. That would suggest that there are major differences among species. Mild hypothermia protects pig hearts [7] and it would be important to see if cooling's protection is ERK-dependent in a large animal species closer to human [15]. If species differences do affect cooling's mechanism, then it is possible that the human heart may respond differently from rabbits.

It is not known how ERK prevents injury during ischemia. It is possible that ERK somehow lessens the injury during ischemia so that transition pores are less likely to form at reperfusion. The mechanism whereby ERK and MEK phosphorylation is affected by cooling is not known either. One attractive possibility is that cooling may have slowed the activity of the phosphatase responsible for removing the two phosphate groups from ERK's and/or MEK's activation site. Inhibition of phosphatases has previously been shown to be cardioprotective [2] and the selective PP2A inhibitor fostriecin was found to protect against infarction when present during the ischemic period [42].

Unfortunately fostriecin is no longer available in sufficient quantities to do infarct size studies. We therefore tested a much less selective PPase inhibitor, cantharidin. Five μM cantharidin during ischemia in rat hearts depressed post-ischemic function and did not limit infarct size [8]. While 1 μM in our preliminary experiments was also ineffective (data not shown), a 10-fold lower dose (100 nM) had an anti-infarct effect (Fig. 6). Cantharidin's IC_{50} varies widely among the many PPases in cells [40], and 100 nM fortuitously seems to provide favorable differential inhibition. As in cooling, ischemia rather than reperfusion was the critical time for cantharidin's protection and its protection could be blocked by an ERK inhibitor which at least suggests a similar mechanism. We did not see any sign of preservation of ERK or MEK phosphorylation in cantharidin-treated hearts. That failure may have been related to the reduced level of protection it caused, but we cannot exclude the possibility that ERK phosphorylation has nothing to do with cantharidin's protection and U0126 just happened to block its protection by a non-specific action.

Because of our previous studies with fostriecin, we hypothesized that PP2A might be cooling's target. However, our *in vitro* assay showed that human PP2Ac is not very temperature-dependent making it unlikely to be a direct target for mild hypothermia. There are many PPases and the one that is responsible for MEK's dephosphorylation may be more temperature-sensitive than PP2Ac. On the whole, however, we found little evidence to support phosphatase inhibition as the mechanism of MEK's activation by cooling.

It is obvious that pMEK somehow gets activated in the cooled hearts. It is possible that cooling stimulates some temperature-sensitive receptors or modulates the release of some

receptor agonists during ischemia which subsequently activate pMEK1/2. Studies report that affinity of adenosine A₁ receptors can be modulated by temperature [3], and it has also been shown that temperature may regulate nucleoside transporter activity [28]. Clearly further investigation is warranted.

In the present study levels of pERK and pMEK were also evaluated after 5 min of reperfusion. While there were significant differences for pERK1 and pERK2 in normothermic and cooled hearts, the difference for pMEK during reperfusion did not achieve significance. Since PD administered during reperfusion did not block the cardioprotection of cooling, we assume that these differences during reperfusion were not contributing to the protection.

Blocking either PI3-kinase, NOS or PKC would also have been expected to abrogate cooling's protection had cooling somehow invoked the preconditioning mechanism. Surprisingly none of the three signaling elements identified by Shao et al. [35] as being required for protection in their hypothermic chick myocyte model appeared to be involved in mild hypothermia's protection in our intact rabbit hearts. There are significant differences in the two models other than species. Shao et al. [35] studied isolated myocytes in which ischemia was simulated. They also used a much lower temperature (25°C) and applied it for only the final minutes of ischemia and first minutes of reoxygenation. In that model simulated reperfusion rather than simulated ischemia was the critical time for cooling's protection. Shao et al. [36] recently extended their observations to isolated murine cardiomyocytes cooled to 32°C which is in the mild hypothermia range. Protection was again seen, but cooling was begun 20 min before the end of the 90-min period of simulated ischemia and lasted for 1 h following reoxygenation. Therefore one cannot exclude the possibility that it was cooling in the period of simulated ischemia that was responsible for the protection. Of note, Akt phosphorylation was increased at the end of simulated ischemia in the cooled cardiomyocytes, and protection was abrogated by blockade of either Akt or NOS. Unfortunately ERK was not studied. Finally, our negative data with these inhibitors must be interpreted with caution, however, because we did not actually verify that the targets were inhibited by the administered antagonists. It is possible that the reduced temperature interfered with their ability to block.

Deep hypothermia has been used to protect the heart during transplantation and open-heart surgery for years. Cooling the arrested heart to a low temperature slows metabolism and clearly preserves ATP. With mild hypothermia metabolic slowing is much less pronounced although the protective effect is quite profound. Jones et al. [23] found that the ATP content of canine myocardium after total ischemia was inversely dependent on the temperature over the entire range of mild hypothermia and this relationship even extended into hyperthermia. Simkhovich et al. [9] measured the rate of ATP depletion in their model of regional ischemia and found that cooling the heart to 32°C caused a 43% increase in ATP content at the end of ischemia. They concluded that the increased preservation of ATP was the mechanism of mild hypothermia's protection as well. However, our investigations of the effect of cooling on myocardial ATP level suggest that preservation of ATP is only an epiphenomenon unrelated to the mechanism of mild cooling's protection since inhibiting ERK prevented mild cooling's anti-infarct effect but did not interfere with cooling's preservation of high energy phosphates.

Clinical significance

Several studies reveal that the degree of protection achieved with mild hypothermia is proportional to the magnitude of reduction of the normothermic ischemic time [24, 29]. The sooner the subject is cooled prior to reperfusion, the smaller the infarct will be. There has

been an interest in trying to use whole body hypothermia to protect the hearts of patients with acute myocardial infarction. However, an insurmountable problem has been finding a reasonably non-invasive method to cool the patients early enough and quickly enough to significantly reduce the normothermic ischemic time prior to recanalization. But to date all methods for inducing whole body hypothermia have been either too slow (e.g., surface cooling with ice packs [26]) or are so invasive (e.g., intravascular thermodes [32, 33]) that there has been reluctance to use them because their implementation would either delay reperfusion therapy or add to the patient's risk. Our data raise the possibility that activation of this pathway with a pharmacologic agent could confer the same protection. If such a drug were identified, then it could even be given by EMS personnel while the patient is being transported to the hospital to minimize the unprotected ischemic time.

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Fig. 1.

Experimental protocols. Abbreviations: chel = chelerythrine ischem = ischemia L-NAME = N^{ω} -nitro-L-arginine methyl ester PD = PD98059 reper = reperfusion Wort = wortmannin

Fig. 2.

Infarct size as a percentage of risk zone in isolated rabbit hearts. Open circles represent individual animal data points, while closed circles are group means $\pm$ SEM. The perfusate was either maintained at 38.5°C (Control) or cooled to 35°C. All hearts were subjected to 30 min of regional ischemia and 2 h of reperfusion. In some hearts cooling was confined to a 5-min period immediately prior to regional ischemia (Pre-isch), whereas in other hearts cooling was extended 10 min into the ischemic period (5' Pre + 10' Isch). In all other hearts subjected to cooling, hypothermia was initiated and within 5 min when the temperature of the immersed heart reached 35°C the coronary artery was occluded. Hypothermia was extended to the end of the 30-min period of ischemia. Some hearts were treated continuously with either wortmannin (Wort), N^ω-nitro-L-arginine methyl ester (L-NAME), or chelerythrine (Chel) to block phosphatidylinositol 3-kinase, nitric oxide synthase, or protein kinase C, resp., from 5 min before ischemia through 20 min of reperfusion (full). Cooling to 35°C significantly reduced infarct size and this protective effect could not be blocked by either Wort, L-NAME, or Chel. Cooling during only the pre-ischemic phase or during the latter and the initial 10 min of ischemia was not protective. * $p < 0.001$

Fig. 3.

Infarct size as a percentage of risk zone in isolated rabbit hearts. Open circles represent individual animal data points, while closed circles are group means $\pm$ SEM. The perfusate was either maintained at 38.5°C (Control) or cooled to 35°C. Either PD98059 (PD) or U0126, ERK antagonists, was added to the perfusate continuously during the entire period of ischemia and the first 20 min of reperfusion (full), or PD was infused during only the phase of regional ischemia (isch) or the initial 20 min of reperfusion (reper). Cooling's protection was blocked when PD or U0126 was added to the perfusate for combined ischemia and reperfusion or when PD was restricted to the ischemic phase. Neither PD nor U0126 had any effect in the normothermic heart. * $p < 0.001$

Fig. 4.

Tissue ATP levels in biopsies of rabbit left ventricular myocardium obtained before coronary occlusion (baseline) and again 10, 20, and 30 min after the onset of ischemia in each heart. Group averages $\pm$ SEM are presented for normothermic hearts (filled circles), hearts cooled to 35°C (open circles), and cooled hearts also treated with PD98059 (PD) (triangles). Ischemia resulted in a dramatic decline in myocardial ATP. The fall was comparable in each group for the initial 20 min of regional ischemia. Thereafter the decline was significantly greater in the normothermic hearts than in either of the groups of cooled hearts. There was no difference between Cool and PD + Cool. * $p < 0.05$

Fig. 5.

Tissue ATP levels in myocardial strips cut from isolated rabbit hearts perfused with PD98059. Two strips were obtained from each heart. One was placed in a beaker of Krebs buffer maintained at 38.5°C, while the other was placed in buffer cooled to 35°C. Thus the myocardium previously loaded with PD98059 was ischemic for 30 min and similarly treated in the two beakers except for temperature. After 30 min of ischemia samples of each muscle strip were prepared for ATP assay. ATP was significantly higher in tissue maintained at 35°C. Hence the presence of PD98059 in the tissue did not appear to affect the ATP level.

* $p < 0.02$

Fig. 6.

Western blots of pERK1/2 and pMEK1/2 during ischemia and reperfusion in globally ischemic rabbit hearts. (A) Representative blots of biopsies. Abbreviations: B=prior to ischemia (baseline); 10'I, 20'I, 30'I = after 10', 20', 30' of ischemia; 5'R = after 5' of reperfusion. (B), (C) and (D) are the statistical (composite) data for pERK1, pERK2 and pMEK1/2 respectively. n = 6 for each group. *p<0.05

Fig. 7.

Infarct size as a percentage of risk zone in isolated rabbit hearts. Open circles represent individual animal data points, while closed circles are group means $\pm$ SEM. In all groups the perfusate was maintained at 38.5°C except in the one group cooled to 35°C. When cantharidin was infused from just prior to ischemia until the onset of reperfusion, infarct size was significantly decreased, while co-administration of U0126 blocked the protection. If the cantharidin infusion were instead begun just before reperfusion (Reper), it no longer protected. * $p < 0.01$ ** $p < 0.001$

Fig. 8.

The results of an *in vitro* activity assay for bovine PP2A. Decreasing the temperature of the assay reaction by 4°C had a negligible effect on the rate of dephosphorylation of the substrate (n=8). Baseline fluorescence was subtracted from each data point before plotting.

Table 1

Infarct size data

Group	BW (kg)	HW (g)	Risk Zone (g)	Ischemic Zone (g)	RZ/IZ (%)
Control-38.5°C	2.1 ± 0.1	7.0 ± 0.1	1.24 ± 0.10	0.43 ± 0.04	34.4 ± 2.2
35°C	2.4 ± 0.0	7.8 ± 0.0	1.37 ± 0.07	0.13 ± 0.04 [*]	9.3 ± 2.4 [*]
35°C + Wort (Full)	2.6 ± 0.1 [‡]	8.0 ± 0.1 [‡]	1.41 ± 0.10	0.18 ± 0.04 [*]	12.5 ± 1.8 [*]
35°C + L-NAME (Full)	2.2 ± 0.1	7.5 ± 0.2	1.20 ± 0.05	0.10 ± 0.03 [*]	8.6 ± 2.7 [*]
35°C + Chel (Full)	1.9 ± 0.0	7.1 ± 0.1	1.18 ± 0.05	0.15 ± 0.03 [*]	12.8 ± 2.4 [*]
35°C Pre-isch	1.9 ± 0.0	6.8 ± 0.1	1.07 ± 0.05	0.34 ± 0.05	31.8 ± 3.8
35°C 5' Pre + 10' Isch	2.1 ± 0.1	7.2 ± 0.3	1.36 ± 0.12	0.38 ± 0.07	27.0 ± 3.1
35°C + PD (Full)	2.4 ± 0.1	7.9 ± 0.2 [‡]	1.44 ± 0.09	0.44 ± 0.06	30.6 ± 4.2
35°C + PD (Reper)	2.0 ± 0.2	7.0 ± 0.3	1.34 ± 0.06	0.22 ± 0.05 [‡]	16.2 ± 3.1 [*]
35°C + U0126 (Full)	1.7 ± 0.0 [‡]	6.9 ± 0.1	1.37 ± 0.12	0.51 ± 0.08	36.4 ± 2.8
35°C + PD (Isch)	1.9 ± 0.0	6.6 ± 0.2	1.21 ± 0.16	0.43 ± 0.09	34.3 ± 3.2
38.5°C + PD (Full)	2.6 ± 0.1 [‡]	7.8 ± 0.3	1.38 ± 0.10	0.45 ± 0.05	32.4 ± 1.6
38.5°C + U0126 (Full)	2.3 ± 0.0	7.6 ± 0.4	1.40 ± 0.07	0.52 ± 0.04	37.6 ± 3.4
Cantharidin Ischemia	2.4 ± 0.1	8.3 ± 0.2 [*]	1.24 ± 0.09	0.29 ± 0.05	22.6 ± 2.3 [‡]
Cantharidin Ischemia + U0126	2.0 ± 0.0	7.2 ± 0.2	1.14 ± 0.16	0.42 ± 0.05	37.4 ± 1.6
Cantharidin Reperfusion	2.0 ± 0.0	7.3 ± 0.1	1.21 ± 0.09	0.49 ± 0.05	40.7 ± 2.1

Mean ± SEM

Abbreviations: BW = body weight Chel = chelerythrine HW = heart weight Isch = ischemia IZ = ischemic zone L-NAME = N^ω-nitro-L-arginine methyl ester PD = PD98059 Reper = reperfusion RZ = risk zone Wort = wortmannin

Statistical significance of difference between experimental group and Control:

[‡] p<0.05

[‡] p<0.01

^{*} p<0.001