Ischemic preconditioning increases the bioavailability of cardiac enkephalins

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Ischemic preconditioning increases the bioavailability of cardiac enkephalins. Am J Physiol Heart Circ Physiol 289: H1652–H1661, 2005; doi:10.1152/ajpheart.01110.2004.—Growing evidence suggests that cardiac enkephalins and their receptors are involved in ischemic preconditioning (IPC). Because there is no evidence for vesicular storage of small bioactive enkephalins in the heart, studies were designed to test the hypothesis that ischemia depletes cardiac enkephalins and that IPC preserves the same enkephalins by accelerating their processing from the larger proenkephalin precursor (PEP) pool. The precursors and two bioactive representatives, Met-enkephalin (ME) and Met-enkephalin-Arg-Phe (MEAP), were separated by size-exclusion chromatography and quantified by radioimmunoassay. Isolated perfused rat hearts were prepared and exposed to global ischemia. After 30 min of global ischemia and 40 min of reflow, the PEP pool was reduced (from 17.99 ± 1.52 to 14.20 ± 2.38 pmol/g wet wt), MEAP increased by 53%, and ME declined by 68%. The sum of the two smaller peptides was unchanged (9.78 ± 0.83 vs. 9.33 ± 2.81). Thus the total enkephalin peptide content was not altered (27.77 ± 1.69 vs. 24.10 ± 4.75). Peptide distribution after ischemia and reflow was also unaltered by pretreatment with peptidase inhibitors. However, when the hearts were preconditioned, the PEP pool remained significantly lower and both of the bioactive peptides, MEAP and ME, were elevated (9.78 ± 0.83 vs. 24.10 ± 4.75), MeAP increased by 53%, and ME declined by 68%. The sum of the two smaller peptides was unchanged (9.78 ± 0.83 vs. 9.33 ± 2.81). Thus the total enkephalin peptide content was not altered (27.77 ± 1.69 vs. 24.10 ± 4.75). Peptide distribution after ischemia and reflow was also unaltered by pretreatment with peptidase inhibitors. However, when the hearts were preconditioned, the PEP pool remained significantly lower and both of the bioactive peptides, MEAP and ME, were elevated (+49% and +86%, respectively). The decline in the PEP pool was prevented by peptidase inhibition and the rise in MEAP was exaggerated. In separate protocols, synthetic enkephalins (ME, MEAP, and Leu-enkephalin) were added to the coronary inflow before 30 min of global ischemia and throughout the subsequent reflow. The added enkephalins (10⁻⁸ M) had no inotropic effect on baseline function but completely prevented the mechanical dysfunction observed in untreated controls during reflow. Thus IPC appears to increase available bioactive enkephalins (MEAP + ME) within the heart by enhancing synthesis of precursors and their subsequent processing from the PEP pool.

opiod peptides; heart; opioid peptide processing

THE ENDGENOUS CARDIAC VIPPEPTIDES and their receptors are considered to be potent modulators of cardiovascular function, inasmuch as they have been implicated in stem cell cardiogenesis (30, 32), cardiac development (27), cardiac aging (3), cardiomyopathy (31, 33), hypertension (38), and hibernation (2). Myocardial infarction increased the abundance of opioid peptide mRNA and the expression of the associated opioid peptides (22). Opioid receptors and their receptors have been demonstrated to protect against myocardial ischemia (8, 18, 21, 26). Opioid receptor blockade prevents the cardioprotective effect of ischemic preconditioning (IPC) (for review see Ref. 14). The cardioprotective effects of opioids are associated with opening of ATP-sensitive K⁺ channels and inhibition of glycogen synthase-β. Thus opioid receptor signaling is an important facet of the myocardial adaptation to stress, although the source of the opioids and the location of their receptors is less well defined.

The concentration of circulating opioid peptides (9) is presumed to be too low to explain their direct action on heart tissues (23, 34). However, bioactive opioid peptides synthesized within the myocardium could produce local concentrations much higher than those achieved in plasma. Moreover, the widespread and rapid enzymatic inactivation of endogenous opioids suggests that their influence is primarily paracrine. Further evidence for the local opioid production derives from the observation that opiate receptor antagonists prevent the beneficial effects of IPC in isolated hearts (13). Despite the abundance of myocardial proenkephalin (PE) mRNA and its translation by cardiac cells (15, 24, 28), the myocardial synthesis and processing of PE during IPC remain largely unknown.

Although isolated cardiomyocytes actively translated and processed PE, the fully processed enkephalins, e.g., methionine-enkephalin (ME), was much lower than that reported for the brain, despite comparable transcript contents in both tissues (11, 28). Because there is no evidence for granular storage of these bioactive peptides, the ventricular myocardium may translate preproenkephalin mRNA continually and then hydrolyze the resulting precursor when needed into an array of intermediate and fully processed enkephalins (1, 37).

ME and ME-arginine-phenylalanine (MEAP) represent >90% of the fully processed bioactive enkephalins in the heart (37). In the absence of vesicular storage, acute changes in ME and MEAP during cellular stress would thus require changes in the synthesis and processing of the larger peptides. The present study provides evidence for a putative scheme of enkephalin peptide processing within the myocardium and tests a three-part hypothesis: 1) that a 30-min period of ischemia and reflow (I/R) reduces posts ischemic ventricular function and depletes large PE precursors (PEP) and small bioactive enkephalins (MEAP and ME), 2) that brief periods of IPC before I/R will preserve ventricular function and increase those same pools of enkephalin, and 3) that preventing enkephalin degradation will substitute for IPC, raise myocardial enkephalin content, and preserve posts ischemic ventricular function.

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METHODS

Isolated rat heart preparation. The animal protocols were approved and conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Hearts for isolated isovolumic perfusions were quickly excised from male Sprague-Dawley rats (350–450 g). The aorta was cannulated, and the coronary arteries were perfused in Langendorff mode at 20 ml/min. Left ventricular systolic pressure (LVP) was recorded from a pressure transducer and fluid-filled catheter attached to a plastic balloon inserted into the left ventricle. The initial volume in the balloon was adjusted to yield a left ventricular end-diastolic pressure (EDP) <6 mmHg. Isolated hearts were perfused with filtered bicarbonate buffer consisting of (in mM) 3.48 KCl, 116.4 NaCl, 26.2 NaHCO3, 1.67 NaH2PO4, 0.69 MgSO4, 1.5 CaCl2, and 11.1 glucose. The perfusate buffer was equilibrated with 95% O2-5% CO2 at 37°C and then supplemented with normal plasma concentrations of 20 amino acids [35].

Experimental groups. Hearts were subjected to 30 min of global ischemia and I/R in the presence or absence of IPC. IPC consisted of three episodes of 2 min of ischemia followed by 5 min of perfusion. Another group of hearts, similarly perfused, were pretreated with a cocktail of peptidase inhibitors (0.1 μmol/ml doses of amastatin, apritin, bestatin, thiorphan, phosphoramidon, and des-Tyr-Leu-enkephalin). Rats received one dose of this cocktail intraperitoneally 24 h before each perfusion and then again 1 h before the heart was collected for isolated perfusion. A third dose was slowly infused into the perfusate during the first 5 min of perfusion of the heart. The experimental protocols are described in detail in Fig. 1. A separate substudy was conducted with hearts prepared as described above, without inhibitor, to evaluate the postsischemic recovery of function after the addition of selected enkephalins to the coronary inflow. ME, MEAP, or leucine-enkephalin (LE) was added to the perfusate (10−8 M) for 20 min before initiation of global ischemia in lieu of the IPC. After 30 min of ischemia, recovery of ventricular function was evaluated during 20 min of reflow with the continued inclusion of the enkephalin. These protocols differed in two other respects: the amino acid complement was omitted, and the hearts were paced at 300 beats/min to reduce functional variation.

Tissue enkephalin extraction. Hearts were blotted, weighed, diced in 2.5 vol of 1 N acetic acid-0.2 N HCl, and boiled for 30 min. After the hearts were cooled, 0.1% β-mercaptoethanol was added, the tissue was homogenized (Polytron, Brinkman), and the supernatant was collected after centrifugation at 25,000 g for 30 min. The pellet was rehomogenized in another 2.5 vol of 1 N acetic acid-0.2 N HCl, and centrifugation was repeated. The supernatants were combined and stored at −80°C. The extracts were thawed, neutralized with 10 N NaOH, and filtered through 0.45-μm syringe filters. Peptides in 2 ml of filtered extract were separated by size-exclusion chromatography on Bio-Gel P-10 columns (1.5 × 10 cm, 15 ml). The samples were eluted with 0.01 M phosphate-buffered saline (PBS), pH 7.0, containing 0.1% gelatin. Fractions corresponding to small (e.g., ME and MEAP), intermediate (e.g., peptide B), and large (e.g., PE) enkephalin sequences were collected [37] and frozen in 0.5-ml aliquots for later radioimmunoassay.

Perfusate enkephalin extraction. Perfusate from the isolated hearts was collected in concentrated acetic acid and HCl, producing final concentrations of 1 and 0.2 N, respectively. β-Mercaptoethanol was added to achieve a 0.1% final concentration. Peptides were extracted on prewetted Sep-Pak C-18 cartridges (Millipore/Waters, Bedford, MA). The cartridges were washed with 10 ml of 0.1% trifluoroacetic acid, the peptides were eluted with 6 ml of 60% acetonitrile in 0.1% trifluoroacetic acid, and the eluent was evaporated under vacuum. The dry extracts were reconstituted in 2 ml of PBS and chromatographically processed as described above.

Radioimmunoassays. Samples were assayed with COOH-terminal-directed antisera specific for ME and MEAP. 125I-labeled ME and MEAP were prepared after oxidation of the iodine with chloramine-T, and the products of the reaction were separated by a combination of size-exclusion and anion-exchange chromatography with Sephadex QAE-A25, as previously described [1, 37].

Enkephalin-hydrolyzing activity. Enkephalin-hydrolyzing activity (EHA) was determined radioimmetrically using 125I-labeled ME as the substrate, and the reaction products were separated by size-exclusion and anion-exchange chromatography. EHA was expressed as (cpm in EA – cpm in EA + inhibitor)/cpm in EA × 100.

Fig. 1. Isolated isovolumic perfused heart experimental protocols. Rats were randomly divided into 8 groups (I–VIII). Isolated heart was perfused, and extracts of the heart were analyzed for enkephalins and enkephalin-hydrolyzing activity (EHA). In group I (time-matched control), the heart was extracted after 106 min of normoxic perfusion. In group III [ischemia-reperfusion (I/R)], after 36 min of control flow, the heart was subjected to total ischemia for 30 min before perfusion was resumed for 40 min, when the heart was extracted for analysis. Group V (IPC + I/R) was treated similarly to group III, except ischemia was preceded by ischemic preconditioning (IPC), i.e., after 15 min of contractile equilibrium, the heart was subjected to 3 episodes of 2 min of ischemia followed by 5 min of perfusion (21 min). Groups II, IV, and VI were perfused as described for groups I, III, and V, respectively, except rats and hearts were pretreated with a cocktail of peptidase inhibitors. Hearts from groups VII (ischemia) and VIII (IPC + ischemia) were extracted immediately after 30 min of global ischemia without prior inhibitor or subsequent reflow.
with multiple comparisons tested post hoc by Tukey’s test. Comparisons tide; LE, Leu-enkephalin; MEAGL, ME-Arg-Gly-Leu.

5% ethanol and quantified by gamma spectrometry.

enzyme reaction mixture consisted of 50 nmol of ME, 5 were performed. Preliminary assays were conducted to determine the in PBS. The homogenates were stored at NH2-terminal signal peptide; peptide B, COOH-terminal pep-

precursor (PEP) pool and selected bioactive products in rat heart. ME, Met-enkephalin; MEAP, ME-Arg-Phe (MEAP); SP, NH2-terminal signal peptide; peptide B, COOH-terminal peptide; LE, Leu-enkephalin; MEAGL, ME-Arg-Gly-Leu.

results

Ventricular contractile function. Contractile parameters did not differ between the groups at baseline before ischemia and IPC interventions (Table 1). In all treatment groups, compared with time-matched controls, I/R resulted in a decrease in LVP, a decrease in the pressure-time derivative (+dP/dt), and an increase in EDP. When IPC preceded reflow, LVP was significantly improved relative to I/R alone (79% of time-matched control vs. 46%). Similarly, IPC blunted the postischemic rise in EDP, but this measure also differed (i.e., was elevated) from that in time-matched controls. Peptidase inhibitors were added to determine whether inhibition of enkephalin degradation might serve as a functional substitute for IPC. Pretreatment with the cocktail of peptidase inhibitors had no significant effect on baseline normoxic contractile function or postischemic recovery of LVP in the I/R group. Peptidase inhibition blunted the IPC-mediated recovery of LVP to 60% of control, despite an increase in enkephalin content compared with the untreated IPC group (see below).

Precursor peptide processing during normoxia. Figure 2 illustrates a putative scheme for the synthesis (step 1), process-

Table 1. Effects of I/R, IPC, and peptidase inhibitor cocktail, on recovery of isovolumic rat heart contractile function

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Baseline (15 min)</th>
<th>Reflow (106 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR, beats/min</td>
<td>LVP, mmHg</td>
</tr>
<tr>
<td>Control</td>
<td>235±16</td>
<td>90.1±11</td>
</tr>
<tr>
<td>Control + inhibitors</td>
<td>214±26</td>
<td>90.5±4.8</td>
</tr>
<tr>
<td>I/R</td>
<td>223±22</td>
<td>90.6±5.5</td>
</tr>
<tr>
<td>I/R + inhibitors</td>
<td>239±30</td>
<td>95.7±11</td>
</tr>
<tr>
<td>IPC + I/R</td>
<td>230±24</td>
<td>86.8±4</td>
</tr>
<tr>
<td>IPC + I/R + inhibitors</td>
<td>211±27</td>
<td>89.9±3</td>
</tr>
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</table>

Values are means ± SD; n = 5 experiments/treatment. Control, time-matched normoxia; I/R, ischemia-reperfusion; IPC + I/R, ischemic preconditioning (IPC) followed by I/R; inhibitors, peptidase inhibitor cocktail pretreatment; HR, heart rate; LVP, left ventricular systolic pressure; EDP, end-diastolic pressure; +dP/dt, peak pressure-time derivative. Main effect of different perfusion protocols on recovery of contractile function parameters, in the presence and absence of peptidase inhibitors, was tested by 2-way ANOVA followed by Tukey’s post hoc test. There was a main effect of perfusion protocol for LVP, EDP, and dP/dt (all P < 0.001). A significant interaction was apparent between perfusion protocol and the presence of peptidase inhibitor cocktail for LVP (P < 0.001) and dP/dt (P < 0.05) only. *P < 0.05 vs. control. †P < 0.05 vs. I/R; ‡P < 0.05 vs. IPC + I/R.

Figure 2. Proposed functional relationships between proenkephalin precursor (PEP) pool and selected bioactive products in rat heart. ME, Met-enkephalin; MEAP, ME-Arg-Phe (MEAP); SP, NH2-terminal signal peptide; peptide B, COOH-terminal peptide; LE, Leu-enkephalin; MEAGL, ME-Arg-Gly-Leu.
ME and MEAP, the principal experimental conditions, I/R and IPC, shows that, regardless of the presence or absence of the conditions, the inhibitors remained in effect. Table 2 also indicates that, despite the homogenization and the substantial dilution required to attain optimal assay conditions, EHA was compared in myocardial homogenates after I/R with and without IPC. To verify the continued efficacy of the inhibitor cocktail during the isolated heart protocols, EHA was quantified by specific radioimmunoassay. Values are means ± SD and correspond to groups I (control, n = 5 experiments) and II (inhibitor cocktail, n = 5 experiments) in Fig. 1. *Significant inhibitor effect (2-tailed unpaired t-test).

**Table 2. Cardiac EHA**

<table>
<thead>
<tr>
<th>Enzyme Activity, μmol/min/g wet wt</th>
<th>Control</th>
<th>Control + inhibitors</th>
<th>I/R</th>
<th>I/R + inhibitors</th>
<th>IPC + I/R</th>
<th>IPC + I/R + inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.01 ± 0.38</td>
<td>1.63 ± 0.63*</td>
<td>3.97 ± 0.46</td>
<td>1.84 ± 0.11*</td>
<td>3.96 ± 0.32</td>
<td>1.72 ± 0.34*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 4–8 experiments/treatment. Enkephalin hydrolyzing activity (EHA) was determined in left ventricular homogenates from hearts with and without peptidase inhibitor pretreatment before cardiac perfusion protocols. EHA was significantly lower after the cocktail (*P < 0.0001), but there were no I/R or IPC + I/R effects, nor was there an interaction with the cocktail (ANOVA).
The third part of the study was designed to test whether inhibiting enkephalin degradation would increase endogenous enkephalin and, thus, represent a strategic substitute for IPC. The animals were treated with peptidase inhibitors in vivo, and the inhibitors were also added to the coronary inflow before I/R. The distribution of myocardial enkephalins was then determined after I/R with and without prior IPC. Figure 5 illustrates the results of adding the inhibitor cocktail in time-matched controls, during I/R, and during IPC + I/R. Peptidase inhibition increased MEAP and decreased ME in time-matched controls to produce a net increase in total enkephalins. Despite the higher net enkephalin content in hearts from time-matched controls, the enkephalin content was much lower after the combination of cocktail and I/R than in time-matched controls and was nearly identical to that in untreated I/R animals. Mechanical function (Table 1) was similarly depressed when cocktail and I/R were combined. In contrast, the combination of IPC and inhibitors restored enkephalins to levels almost matching those of controls treated with inhibitors. Similar to the time-matched controls, much of the restored content was attributed to higher MEAP. In contrast to the inhibitor-free protocols, the higher enkephalin content was associated with a much less robust improvement in mechanical function. The peptidase inhibitors did not alter the PEP pool during I/R. The overall PEP pool declined to a similar degree during I/R whether the inhibitors were present or not.

**ME and MEAP in the myocardial effluent.** ME and MEAP were determined in the coronary effluent to evaluate the role of enkephalin secretion (step 4 in Fig. 2) in altering the myocardial content. In prior studies with the isolated rat heart, constituents of the PEP pool were absent from coronary effluent and ME accounted for the majority of the enkephalin that was released (37). As observed in the earlier studies, there was significantly more ME than MEAP in the coronary effluent in all groups (Table 3). Under control conditions, MEAP recover-
Prior studies have indicated an active turnover of enkephalins within the myocardium (37). When heart extracts were treated with trypsin and carboxypeptidase B, the recovered ME was markedly increased (11), suggesting that there was a pool of large- and intermediate-sized peptides with cryptic ME sequences that were not adequately assessed by standard ME radioimmunoassays. When myocardial extracts were separated by gel filtration and quantified with antisera for specific COOH-terminal sequences, total enkephalin content was >30 times greater than the previously reported ME content (1, 37). Ninety-five percent of the recovered ventricular enkephalins were larger than ME alone. The continuous turnover of newly synthesized cardiac enkephalins was evident from the rapid clearance of pulse-labeled enkephalin from isolated perfused hearts and the steady release of smaller enkephalin into the coronary effluent (37). The present study confirmed prior observations that the majority of the immunoreactive enkephalin in myocardial extracts was associated with high-molecular-weight precursors (Fig. 3). Although unprocessed PE contains four copies of ME and only one copy of MEAP, the representative enkephalins, MEAP and ME, were recovered in nearly equimolar quantities. Because ME was the predominant enkephalin in the venous effluent, the greater mass of ME released might potentially account for the apparent stoichiometric discrepancy in tissue.

The initial contents of various opioid peptides in control hearts shown in Fig. 3 represent a “snapshot” of net balance among synthesis, secretion, and relative activity of a collection of tissue peptidases. These peptidases are responsible for processing the PEP pool to MEAP and ME (step 2 in Fig. 2), for converting MEAP to ME (step 3), and for degrading ME and MEAP (step 5). Thus changes in the distribution of enkephalins resulting from ischemia, reflow, IPC, or introduction of a cocktail of peptidase inhibitors into the coronary perfusate could not be a priori specified. The myocardial MEAP content was very sensitive to peptidase blockade and nearly tripled when the cocktail was applied during control conditions. MEAP must normally have been processed and/or degraded by endogenous peptidase activity. In direct contrast, myocardial ME declined by 50% under the same control conditions. The release of ME and MEAP continued at rates similar to those observed in time-matched controls throughout posts ischemic reflow in the I/R group. However, IPC before I/R decreased the release of ME in the coronary effluent and increased the release of MEAP.

**Exogenous enkephalin restores mechanical function.** A sub-study was conducted to test whether exogenously administered enkephalins could be substituted for the rise in endogenous enkephalin that is presumed to help mediate the effect of preconditioning. Three different cardiac enkephalins, ME, MEAP, and LE, were each evaluated in separate groups of hearts. On the basis of previous pilot dose responses, each enkephalin was added to the coronary inflow at a final concentration that had no effect on contractile activity (i.e., 10−8 M). The enkephalins were administered before initiation of the global ischemia, when the IPC protocol would normally have been conducted, and then continued during 20 min of reflow. There were no differences among groups before treatment (Table 4). I/R reduced left ventricular function during reflow by one-third, and pretreatment with any of the three enkephalins completely restored mechanical function to values indistinguishable from those of the time-matched control. Thus adding enkephalin appears to produce an acute response qualitatively similar to that observed with IPC.

**DISCUSSION**

**Table 3. Quantification of enkephalins released into the coronary effluent by specific radioimmunoassay**

<table>
<thead>
<tr>
<th>Effluent Enkephalin, pmol/h/g wet wt⁻¹</th>
<th>ME</th>
<th>MEAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.49±0.16</td>
<td>0.19±0.05</td>
</tr>
<tr>
<td>Control + inhibitors</td>
<td>0.76±0.04*</td>
<td>0.11±0.01*</td>
</tr>
<tr>
<td>I/R</td>
<td>1.70±0.34</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>I/R + inhibitors</td>
<td>0.82±0.06*</td>
<td>0.11±0.02*</td>
</tr>
<tr>
<td>IPC + I/R</td>
<td>0.92±0.07†</td>
<td>0.28±0.07†</td>
</tr>
<tr>
<td>IPC + I/R + inhibitors</td>
<td>0.30±0.04*†</td>
<td>0.14±0.02*†</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 4–8 experiments/treatment. Methionine-enkephalin (ME) and ME-arginine-phenylalanine (MEAP) were determined in coronary effluent from hearts with and without peptidase inhibitor pretreatment. Main effect of different ischemic treatments in the presence and absence of peptidase inhibitors was tested by 2-way ANOVA followed by Tukey’s post hoc test. *Main effect of added peptidase inhibitor cocktail to reduce peptide secretion regardless of group. †Significant difference between IPC + I/R and both control and I/R.

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conditions. Because MEAP includes an ME sequence, the reciprocal relation between MEAP and ME suggests that some ME was derived from the conversion of MEAP to ME (step 3). Previous work indicated that, when added to myocardial homogenates, ME and MEAP were aggressively degraded by NH$_3$-terminal aminopeptidase activities, with little evidence of interconversion (19). However, when the aminopeptidase activity was blocked, conversion of MEAP to ME was easily demonstrated (20). ME and MEAP may also be processed independently from the PEP pool at different rates, as suggested by the processing arrows in Fig. 2 (step 2). Furthermore, the preferential accumulation of MEAP and decline in ME during peptidase inhibition might also be explained if ME is more aggressively degraded than MEAP by residual myocardial peptidases (19, 20). Thus the relative importance of conversion, degradation, and secretion in determining the relative fates of ME and MEAP remains to be elucidated.

**I/R.** The first part of the hypothesis proposed to test whether the ventricular dysfunction after I/R was associated with depletion of the PEP pool and/or the smaller bioactive peptides. In support of this thesis, the available bioactive peptides, ME and MEAP, declined sharply during ischemia and were collectively restored to near-control values during reflow. Because ME and MEAP are very similar and both were capable of restoring posts ischemic function when administered to isolated hearts, the sum of their contents may be the more critical determinant of opioid-mediated cardioprotection. If cardioprotection requires that collective enkephalins exceed some threshold during reflow, the continued poor mechanical performance during I/R, despite increased MEAP, may reflect the fact that, collectively, MEAP + ME had barely returned to control values.

The PEP pool and the processed enkephalins fell during ischemia, suggesting that synthesis (step 1 in Fig. 2) remained static while processing of precursor (step 2) and degradation of product (step 5) continued. After 30 min of global ischemia, resumption of reperfusion also returned the precursor pool toward control, presumably by reactivating its synthesis (step 1). This presumption is based on the tandem increase in precursors and processed enkephalins (MEAP + ME). The accumulation of the smaller peptides during reflow appears to occur by increasing precursor processing relative to synthesis, as suggested by the incomplete restoration of the PEP pool.

**IPC + I/R.** The second part of the hypothesis proposed to test whether the improved ventricular function after IPC was associated with increased precursor processing and/or an increased supply of ME and MEAP. In contrast to I/R alone, IPC increased MEAP and ME well above the control content at the apparent expense of increased processing from the PEP pool, which declined below the content observed with I/R alone. Thus the improved ventricular function after IPC was, as predicted, associated with a substantive increase in ME + MEAP compared with control or I/R. Compared with I/R, the exaggerated increase in the smaller peptides in the IPC + I/R hearts was accompanied by a less robust recovery in the PEP pool. The accumulation of the smaller peptides after IPC + I/R appears again to occur by a further increase in processing relative to synthesis.

IPC may increase the ME + MEAP content by protecting key processing enzymes from reperfusion injury, perhaps by increasing the cellular content of heat shock proteins, which then serve as protective chaperones.

**Inhibition of peptidase activity.** The third part of the hypothesis proposed to test whether the administered protease inhibitors would inhibit myocardial enkephalin degradation, raise endogenous enkephalins, and restore posts ischemic ventricular function to a level very similar to that expected after IPC. If effective, this strategy might provide a less invasive approach to preconditioning. Because several proteases are likely to be responsible for enkephalin degradation in heart, a cocktail of peptidase inhibitors with a spectrum of specificities was employed. Furthermore, because little is known about the pharmacokinetics of the protease inhibitors in heart, multiple doses were administered to maximize the efficacy. Pretreatment with peptidase inhibitors increased the initial enkephalin content without altering resting function. Despite the higher initial enkephalin content in the peptidase inhibitor-treated group, the combined enkephalin (ME + MEAP) content fell sharply during I/R to values very similar to those observed after I/R alone. Consistent with this observation, ventricular function was similarly not restored in the inhibitor-treated I/R hearts. When protease inhibitor cocktail treatment and IPC + I/R were combined, ME + MEAP content was quantitatively similar to that in untreated IPC + I/R hearts and protease inhibitor-treated time-matched controls. Qualitatively, the mixture was different, in that MEAP predominated when protease inhibitors were present. Ventricular function, although restored in part by protease inhibitor cocktail + IPC, was clearly reduced compared with the cocktail-treated control or IPC without cocktail treatment. The relative failure to improve function, despite the higher enkephalin content, is unexplained. An acute change in content may be required. The extended exposure to protease inhibitors may have provided sufficient time for the development of competing compensatory responses, including the potential downregulation of the participating opioid receptors. Furthermore, the broad specificity of the inhibitors employed
may have reduced the availability of other nonopioid cardio-
protective peptides such as bradykinin. Thus the less-than-
optimal functional result might be improved by the develop-
ment of more specific inhibitors and/or more refined timing in
their administration.

Enkephalin hydrolysis. Cardiac EHA was measured to eval-
uate potential I/R- and IPC-mediated changes in enzyme ac-
tivity and to verify the efficacy of the added peptidase inhibi-
tors. EHA was significant, confirming the myocardial capacity
for the aggressive local degradation of enkephalin (step 5 in
Fig. 2). The near restoration of ME + MEAP during I/R and
the accumulation of ME + MEAP during IPC + I/R are not
likely the result of acute changes in the overall capacity for
enkephalin hydrolysis, because EHA was similar in both pro-
tocols and was not different from that in time-matched controls
(Table 2). The peptidase inhibitors suppressed EHA by >50%;
however, there were no differences when EHA from ischemic
and preconditioned hearts was compared with EHA from cock-
tail-treated controls. The enzyme activity and the effect of
the inhibitors were, however, assessed under optimal substrate
conditions and at a significant dilution of tissue and inhibitor.
Thus the enzyme content was likely unchanged by I/R or
IPC + I/R, although subtle changes in effective enzyme activity
in vivo, secondary to factors such as activation and
available substrate, were not assessed and could have contrib-
uted to differences in the peptide distributions. These data
confirm that myocardial EHA is prodigious in heart (4) and,
thus, represents a significant barrier to effective diffusion of
myocardial enkephalins. This hydrolytic barrier thus reinforces
the paracrine character of myocardial enkephalin and the im-
portance of its local synthesis.

Conversion of MEAP to ME. The idea that myocardial ME
may arise from the hydrolysis of MEAP [conversion (step 3 in
Fig. 2)] is empirically supported by the rise in MEAP and the
reciprocal decline in ME under control conditions when pep-
tidase inhibitors were added. A similar rise in MEAP and
decline in ME were observed after I/R when peptidase activity
may have been impaired by hostile conditions during reflow.
Reduced conversion of MEAP to ME during peptidase inhibit-
ion would not be surprising, however, in the absence of added
inhibitor; a similar reduction in conversion during reflow
suggests again that hostile conditions in the interstitium during
I/R (e.g., pH, oxyradicals, and Ca2+) have modified the puta-
tive converting enzyme activity. Consistent with the thesis that
I/R suppressed the conversion (step 3), IPC + I/R, which
presumably improved interstitial conditions, eliminated the
inverse relation between ME and MEAP. Furthermore, adding
the peptidase cocktail to the IPC + I/R protocol restored the
reciprocal relation by increasing MEAP and decreasing ME.
The relative importance of this conversion is unclear, and some
of the changes might also be attributed to differential changes
in secretion. The greater release of ME than of MEAP (Table
3) in the coronary effluent observed throughout the study
provides some logical support for that alternative. In addition,
the decline in ME secretion after IPC + I/R may have con-
tributed to the robust increase in tissue ME after IPC + I/R.
The relative significance of individual endogenous enkephalins
is also unclear. Although all the endogenous enkephalins are
qualitatively δ-agonists, MEAP may be more potent in some
respects, perhaps in part because of a slower rate of degra-
dation (6, 7, 19). Despite potential differences, however, all three
enkephalins evaluated in the present study were cardioprotec-
tive when added exogenously.

Peptidase inhibitors. Despite the multiple enzyme targets,
comparison of the differential responses to the addition of
peptidase inhibitors provides some insight into enkephalin
processing in the heart. The synthesis of precursor and its
processing to enkephalin were apparently impaired by I/R.
This is based on the observation that the peptide pattern after
I/R was unaltered by the addition of the peptidase cocktail. In
direct contrast, the cocktail produced very similar shifts in the
distribution pattern in time-matched control and IPC + I/R
hearts. Thus it would appear that IPC may have reactivated or
preserved key components of the synthetic and processing
apparatus during reflow. These observations suggest a more
complex interaction between IPC and I/R, which may include
synthesis, differential PEP processing, and conversion of
MEAP to ME (steps 1, 2, and 3, respectively, in Fig. 2). The
net effect of IPC appears to be an increase in the local
availability of bioactive, cardioprotective enkephalins as a
result of an increase in their processing from existing precursor
pools.

Enkephalin secretion. Changes in enkephalin secretion (step
4 in Fig. 2) could contribute to changes in the tissue content.
Because ME secretion during I/R was not different from that
in time-matched controls, an increase in secretion could not
provide a clear explanation for the low myocardial ME content
after I/R. In contrast, however, the clear decline in ME secre-
tion during IPC + I/R might explain in part the dramatic
increase in myocardial ME content observed when I/R was
preceded by IPC. In contrast, the reciprocal increase in MEAP
in the coronary effluent during IPC + I/R may reflect the
parallel rise in MEAP in the tissue available for secretion.
The combined increase in MEAP in tissue and effluent reinforces
the interpretation that IPC increased the generation of MEAP.

Limitations. The inherent limitations associated with tissue
peptide measurements suggested that the broad changes in
constituent peptides were more reliable than the individual
details. Immunoassays of hormones are always relative deter-
minations, and the assay of rapidly degraded peptides in
partially purified tissue extracts should, by their very nature, be
viewed as estimates. Because ME and MEAP have identical
NH2-terminal sequences, the assay for each is directed at the
COOH-terminal epitope, which distinguishes them from each
other. Consequently, the assays are less sensitive to alterations
at the other end (NH2 terminus) of the molecule. Because
NH2-terminal extensions or deletions are generally inactive,
their contribution to the resulting measurements would over-
estimate the biologically active opioid content of the sample.

Significance. Opioid influences are often mediated by reduc-
ing Ca2+ influx and subsequent neurotransmitter release. Car-
dioprotective enkephalins may stabilize the heart by adjusting
neurotransmitter release to ensure an appropriate balance between fuel supply and metabolic demand. Cardiotoxins are energetically inefficient, presumably because they increase cAMP, deplete fuel supplies, and increase intracellular Ca2++.

Reduced energy and excess Ca2++ facilitate myocardial necrosis (25), and chronic sympathetic stimulation facilitates cardiocyte apoptosis (for review see Ref. 10). Protection may also be afforded by limiting Ca2++ overload and the resulting Ca2++-dependent arrhythmias (25, 36). Enkephalin inhibits the chro-

tropic effect of sympathetic stimulation in vivo (29) and reverses the inotropic effect of β-adrenergic stimulation in isolated cardiac myocytes and perfused hearts in vitro (23, 36). The interactions in vitro reduced L-type Ca2++ currents (36) and inhibited cAMP formation (23). Sympatholytic opioid influences in vivo are therefore potentially cardioprotective during metabolic stresses such as ischemia and intense exercise. Ganglionic blockade and chemical sympathetomy increased total myocardial opioids (1, 37), suggesting that the normal processing and release of myocardial opioid peptides depend on continued autonomic input. Continued opioid processing might therefore constitute a form of local feedback that normally moderates or restrains sympathetic stimulation.

Enkephalins may also stabilize the heart by improving vagal transmission (12). As indicated above and elsewhere (12, 16, 22), cardiac enkephalins are elevated during ischemia, and low enkephalin concentrations are vagotonic (12, 16). Thus improved vagal transmission may help protect the heart by reducing myocardial oxygen demand directly and/or indirectly by opposing excitatory sympathetic input.

In summary, myocardial enkephalins were distributed between large precursors and small bioactive products. We have provided evidence for a schematic framework for peptide processing within the heart. The processing was responsive to local myocardial conditions. I/R reduced processing and differentially altered the distribution of bioactive products in favor of MEAP. IPC increased bioactive cardioprotective enkephalins (ME + MEAP), probably by activating, preserving, or restoring the synthesis and processing of existing precursor pools.

REFERENCES


