Evidence that cardioprotection by postconditioning involves preservation of myocardial opioid content and selective opioid receptor activation

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Zatta AJ, Kin H, Yoshishige D, Jiang R, Wang N, Reeves JG, Mykytenko J, Guyton RA, Zhao ZQ, Caffrey JL, Vinten-Johansen J. Evidence that cardioprotection by postconditioning involves preservation of myocardial opioid content and selective opioid receptor activation. Am J Physiol Heart Circ Physiol 294: H1444–H1451, 2008. First published January 18, 2008; doi:10.1152/ajpheart.01279.2006.—Opioids introduced at reperfusion (R) following ischemia (I) reduce infarct size much like postconditioning, suggesting the hypothesis that postconditioning increases cardiac opioids and activates local opioid receptors. Anesthetized male rats subjected to 30 min regional I and 3 h R were postconditioned with three cycles of 10 s R and 10 s reocclusion at onset of R. Naloxone (NL), its peripherally restricted analog naloxone methiodide, δ-opioid receptor (DOR) antagonist naltrindole (NTI), κ-opioid receptor antagonist norbinaltorphimine (NorbNI), and μ-opioid receptor (MOR) antagonist H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP) were administered intra-venously 5 min before R. The area at risk (AAR) was comparable among groups, and postconditioning reduced infarct size from 57 ± 2 to 42 ± 2% (P < 0.05). None of the antagonists alone altered infarct size. All antagonists abrogated postconditioning protection at higher doses. However, blockade of infarct sparing by postconditioning was lost, since tested doses of NL, NTI, NorBNI, and CTAP were lowered. The efficacy of NorBNI declined first at 3.4 μmol/kg, followed sequentially by NTI (1.1), NL (0.37), and CTAP (0.09), suggesting likely MOR and perhaps DOR participation. Representative small, intermediate, and large enkephalins in the AAR were quantified (fmol/mg protein; mean ± SE). I/R reduced proenkephalin (58 ± 9 vs. 33 ± 4; P < 0.05) and sum total of measured enkephalins, including proenkephalin, peptide B, methionine-enkephalin, and methionine-enkephalin-arginine-phenylalanine (139 ± 17 vs. 104 ± 7; P < 0.05) compared with shams. Postconditioning increased total enkephalins (89 ± 8 vs. 135 ± 5; P < 0.05) largely by increasing proenkephalin (33 ± 4 vs. 96 ± 7; P < 0.05). Thus the infarct-sparing effect of postconditioning appeared to involve endogenously activated MORs and possibly DORS, and preservation of enkephalin precursor synthesis in the AAR.

postconditioning; reperfusion injury; opioid receptor activation; myocardial enkephalins

A GROWING BODY OF EVIDENCE supports the concept that postconditioning triggers a cascade of molecular signaling events that induce cardioprotection similar to that of ischemic preconditioning. Indeed, many of the same effectors implicated in preconditioning appear to be involved in postconditioning, producing what may be called a reperfusion injury-tolerant phenotype (11, 16, 38). For example, the G protein-coupled adenosine receptor, which has been strongly implicated in preconditioning, has now been likewise implicated in the reduction in infarct size after postconditioning. Some debate remains, however, regarding which adenosine receptor subtype(s) is responsible for the protection (19, 31, 41). More recently, reactive oxygen species (30), the reperfusion injury salvage kinase pathway (16, 37), protein kinase C (PKC) (30, 31, 44), ATP-sensitive potassium (KATP) channels (25), and glycogen synthase kinase-3β (GSK3β) (27, 36) have all emerged as mediators common to both pre- and postconditioning. Likewise, inhibition of mitochondrial permeability transition pore opening (1,3) is increasingly reported to be a critical end effector in the cardioprotective effects of both processes. Thus a similar array of redundant pathways, triggers, and mediators proposed earlier for preconditioning (6) may also participate in postconditioning. Multiple pathways may be required to realize effective protection, suggesting that postconditioning is likely to be triggered by and dependent on a variety of different, and as yet, unidentified endogenous mediators.

Opioids are well-known endogenous triggers of preconditioning and were recently determined to be effective at reducing infarct size when given at reperfusion through interaction with their cognate G protein-coupled receptors (4, 11–14, 40). Therefore, in light of these reports, the following study tested the hypothesis that the infarct-sparing effects of postconditioning involve endogenous activation of opioid receptors. A series of dose responses were performed with a variety of nonselective and subtype-selective opioid receptor antagonists to determine whether the response might be preferentially mediated by one or more opioid receptor subtypes [μ (MOR)-, δ (DOR)-, and κ (KOR)-opioid receptors]. Previous studies have commonly used opioid antagonists at relatively high doses, thereby making it difficult to assign a specific receptor subtype to its action. The lowest effective dose of each antagonist should enable one to better delineate the most influential of the participating opioid receptors. Moreover, because preconditioning has been shown to increase the bioavailability of cardiac enkephalins (43), the study also tested the hypothesis that postconditioning modulates enkephalin content in the myocardial area at risk during reperfusion. The observations suggest a likely role for MORs and perhaps DORS in the cardioprotective effects of postconditioning. Furthermore, the data also suggest that postconditioning protocols increase or

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preserve cardiac proenkephalin and total enkephalin in the area at risk during the subsequent reperfusion.

**MATERIALS AND METHODS**

Investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publications No. 85-23, revised 1996). The protocol was reviewed and approved by the Institutional Animal Care and Use Committee.

**In vivo rat surgical preparation.** An anesthetized in vivo rat model of myocardial infarction was used for these experiments. The surgical preparation and determination of infarct size have been described previously in detail (44). Following surgical manipulation and stabilization of hemodynamics and blood gases, rats were randomized into experimental groups (Fig. 1).

**Experimental protocol for infarct size studies.** Eighty male Sprague-Dawley rats weighing 280–380 g were subjected to a 30-min left coronary artery (LCA) occlusion (index ischemia) followed by 3 h of reperfusion with or without a postconditioning stimulus (3 cycles of 10 s reperfusion and 10 s reocclusion) initiated at the onset of reperfusion. Opioid antagonists were administered in appropriate groups as a slow 10 s reperfusion and 10 s reocclusion (total intervention time of 1 min) (n = 8); group 3 [naloxone (NL, general opioid receptor antagonist; 7.5 μmol/kg) alone (n = 8)], groups 4–6 [NL + Postcon, postconditioning stimulus initiated in the presence of 0.37 (n = 4), 1.1 (n = 4), or 7.5 μmol/kg (n = 8) NL], group 7 [naloxone methiodide (QNL, peripherally restricted quaternary naloxone derivative; 21 μmol/kg) alone (n = 8)], group 8 [QNL + Postcon (n = 8)], group 9 [naltindole hydrochloride (NTI, DOR selective antagonist; 11 μmol/kg) alone (n = 8)], groups 10–13 [NTI 0.38 (n = 4), 1.1 (n = 4), 3.8 (n = 4), or 11 μmol/kg (n = 8) + Postcon], group 14 [norbornalorphinine dihydrochloride (NorBNI, KOR selective antagonist; 6.8 μmol/kg) alone (n = 8)], groups 15–16 [NorBNI 3.4 (n = 4) or 6.8 μmol/kg (n = 8) + Postcon], group 17 [CTAP (MOR selective antagonist; 0.18 μmol/kg) alone (n = 5)], groups 18–20 [CTAP 0.045 (n = 4), 0.09 (n = 4), or 0.18 μmol/kg (n = 6) + Postcon].

**Tissue enkephalin extraction.** To determine if postconditioning modified the concentration of cardiac enkephalins in area at risk (AAR) myocardium, additional experiments were performed in which cardiac enkephalins were extracted as described by Younes et al. (43). Briefly, a separate series of rats was randomly assigned to one of four groups: 1) sham, subjected to surgical manipulation without LCA occlusion (n = 4); 2) ischemia only, 30 min LCA occlusion with or without reperfusion (n = 4); 3) ischemia-reperfusion (IR), 30 min LCA occlusion and 3 h reperfusion (n = 4); or 4) Postcon (as above) (n = 4). On completion of the experimental protocol, the coronary artery was reoccluded and the AAR was delineated with blue dye. A comparable area of anterior myocardium for sham animals was also demarcated with blue dye by tying off the previously unoccluded sham LCA stitch at the conclusion of the time course. The hearts were rapidly excised, and the AAR was separated from the blue-stained nonischemic zone. The AAR was snap-frozen in liquid nitrogen and stored at 80°C until further processed. The excised myocardium was denatured by boiling in 2.5 ml of 1 N acetic acid-0.2 N HCl for 30 min at 90°C and refrozen in liquid nitrogen and stored at −80°C. Tissue was later thawed, spiked with 2.5 μl of 0.1% β-mercaptoethanol, and homogenized (Polytron; Brinkman). Samples were then centrifuged at 30,000 g for 30 min, and the supernatant was collected. The extracts were neutralized with 10 N NaOH and filtered through 0.45-μm syringe filters. The 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 in 1 N acetic acid-0.2 N HCl with 0.025% gelatin. Fractions corresponding to small [e.g., methionine enkephalin (ME) and methionine-enkephalin-arginine-phenylalanine (MEAP)], intermediate (e.g., peptide B), and large (e.g., proenkephalin) enkephalin-containing sequences were collected and frozen in 0.5-ml aliquots. The acid was evaporated to dryness in a centrifugal evaporator (Savant) and reconstituted in 0.5 ml of PBS with 0.1% gelatin.

**Radioimmunoassay.** Samples were assayed with COOH-terminaldirected antisera specific for ME and MEAP. 125I-labeled ME and 125I-labeled MEAP were prepared after oxidation of the iodine with chloramine T, and the labeled ligands were separated by a combination of size-exclusion and anion-exchange chromatography with Sephadex QAE-A25, as previously described (2, 42). Protein content was determined by the method of Lowry.

**Determination of tissue norepinephrine.** A 10-μl aliquot of the acid extract was neutralized in 0.05 M Tris buffer pH 7.4, and the norepinephrine was extracted with alumina. The norepinephrine was determined by the method of Lowry.
eluted with 0.1 M perchloric acid, separated by HPLC (C-18), and quantified amperometrically.

**Chemicals.** All drugs were purchased from Sigma/RBI (St. Louis, MO). The opioid receptor antagonists were initially dissolved in distilled water and then brought up to volume with 0.9% saline. The highest dose of each antagonist was based on prior studies in which NL (3 mg/kg ≈ 7.5 μmol/kg), QNL (10 mg/kg ≈ 21 μmol/kg), and NTI (5 mg/kg ≈ 11 μmol/kg) were shown to reverse preconditioning-mediated reductions in infarct size (34, 35). QNL is a quaternary derivative of NL that does not easily cross the cerebral nervous system. The selected dose of NorBNI (5 mg/kg ≈ 0.18 μmol/kg) blocked the hypotensive effect of the KOR agonist U-50488H without any observed effect on the cardioprotective effects of preconditioning (33). However, the dose of CTAP (200 μg/kg ≈ 0.18 μmol/kg) was based on its reported inhibitory constant (K_i; see Ref. 21) and its empirical efficacy observed in this study.

**Statistical analysis.** All data are reported as means ± SE. Computerized analyses of variance were conducted (StatSoft, Tulsa, OK), and differences with probabilities of P < 0.05 were considered statistically significant. One-way ANOVA (i.e., AAR, infarct size data, and cardiac enkephalin contents) and multiway ANOVA with repeated measures (cardiodynamics) were used as appropriate. Individual differences were determined post hoc with the Student-Newman-Keul’s test when main effects were detected. Statistical analysis of infarct size was performed over all groups simultaneously; data may be regrouped in Figs. 1–7 for a more logical presentation.

**RESULTS**

**Hemodynamic data.** The systemic hemodynamic data are summarized in Table 1. There were no significant differences observed between control and treatment groups for heart rate, mean arterial pressure, or rate-pressure product at baseline, end of ischemia, and 180 min of reperfusion. Systemic hemodynamic variables remained stable over the course of the experiment (i.e., no significant differences between baseline and the end of reperfusion for any group).

**Infarct-limiting effect of postconditioning involves opioid receptor activation.** The area placed at risk by LCA occlusion, expressed as a percentage of left ventricular mass, was comparable among the 20 groups and ranged from 29 to 41% (Table 2). Regional myocardial ischemia and 3 h reperfusion resulted in an infarct size averaging 57 ± 2% in untreated controls (Fig. 2). Postconditioning significantly reduced infarct size by 15% (42 ± 2 vs. control, P < 0.001) (Fig. 3). Treatment with the highest dose of the general opioid receptor antagonist NL (7.5 μmol/kg) alone at reperfusion did not alter infarct size relative to control (56 ± 1%) (Fig. 2). However, when that same dose of NL preceded the postconditioning protocol, the infarct-sparing effects of postconditioning were blocked, and the resulting infarct (55 ± 3%) was not different from control (Fig. 3). Lowering the dose of NL by 85% to 1.1 μmol/kg did not reduce its ability to block postconditioning. However, NL no longer inhibited postconditioning when the dose was reduced further to 0.37 μmol/kg (5% of the original dose), indicating a maximally effective dose between 1.1 and 0.37 μmol/kg. QNL was administered to test whether the opioid receptor responsible for postconditioning was central or peripheral. Like NL, QNL alone did not alter infarct size relative to control (53 ± 3%). However, the peripherally restricted antagonist did indeed prevent the infarct size reduction observed with postconditioning (53 ± 2% vs. postconditioning, P < 0.01) that was comparable in size to control (Fig. 3). This

**Table 1. Hemodynamic variables during the course of the experiment**

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>25 min Ischemia</th>
<th>180 min Reperfusion</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Heart Rate</td>
<td>MAP</td>
<td>RPP</td>
</tr>
<tr>
<td>Control</td>
<td>327 ± 17</td>
<td>97 ± 11</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>Postcon</td>
<td>334 ± 22</td>
<td>101 ± 9</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>NL</td>
<td>372 ± 18</td>
<td>87 ± 7</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>NL + Postcon</td>
<td>373 ± 15</td>
<td>91 ± 6</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>QNL</td>
<td>350 ± 16</td>
<td>92 ± 3</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>QNL + Postcon</td>
<td>383 ± 14</td>
<td>93 ± 8</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>NTI</td>
<td>373 ± 11</td>
<td>102 ± 8</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>NTI + Postcon</td>
<td>389 ± 11</td>
<td>95 ± 3</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>NorBNI</td>
<td>370 ± 8</td>
<td>91 ± 4</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>NorBNI + Postcon</td>
<td>366 ± 11</td>
<td>94 ± 7</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>CTAP</td>
<td>380 ± 16</td>
<td>93 ± 9</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>CTAP + Postcon</td>
<td>381 ± 14</td>
<td>103 ± 8</td>
<td>39 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE. Values for high antagonist doses shown only. MAP, mean arterial pressure (mmHg); RPP, rate-pressure product/1,000 (mmHg/min); Postcon, postconditioning; NL, naloxone; QNL, naloxone methiodide; NTI, naltrindole; NorBNI, norbinaltorphimine.
observation suggests that the cardioprotective opioid receptors involved are likely located in the periphery.

Three other opioid receptor antagonists were administered as initial evaluations of participation by specific opioid receptor subtypes in the infarct-sparing effects of postconditioning. NTI, NorBNI, and CTAP were employed because of their relative selectivity for DORs, KORs, and MORs, respectively. None of the antagonists alone altered infarct size relative to control (NTI 53 ± 2%; NorBNI 52 ± 4%; CTAP 52 ± 2%) (Fig. 2). All three selective antagonists blocked the infarct-sparing effects of postconditioning at the highest doses (Fig. 4 and 5). The DOR antagonist NTI abrogated the infarct-sparing effect of postconditioning at the highest dose of 11 μmol/kg (infarct size = 56 ± 2%) and maintained this antagonist effect when the dose was reduced by two-thirds to 3.8 μmol/kg (Fig. 4). However, a partial inhibition of postconditioning was observed when the dose was reduced to 1.1 μmol/kg (infarct size = 48 ± 5%) and was no longer effective at blocking postconditioning when the dose of CTAP was further reduced to 3.8 μmol/kg (Fig. 4). In contrast to NorBNI, the maximally effective dose of the MOR antagonist CTAP was between 0.09 and 0.19 μmol/mg, well below those observed for both NorBNI and NTI (Fig. 5). CTAP completely blocked the infarct-sparing effect of postconditioning at 0.18 μmol/kg (infarct size = 54 ± 1%). However, blockade of protection was lost in a stepwise manner when the dose of CTAP was reduced to 0.045 μmol/kg (infarct size = 44 ± 5%) and reduced further to 0.045 μmol/kg (infarct size = 39 ± 4%). Thus the maximally effective dose of the MOR antagonist CTAP was between 0.09 and 0.19 μmol/mg, well below that for NorBNI or NTI. In fact, the effective dose of CTAP was closest to that for NL, which though much less selective than CTAP does have a relative preference for the MOR.

Postconditioning enhances precursor enkephalin content in rat heart. Ischemia without reperfusion reduced total cardiac enkephalins (sum total of measured enkephalins in all fractions; 139 ± 17 vs. 104 ± 7 fmol/mg protein), total precursors [total enkephalin precursors (proenkephalin plus peptide B)]; 112 ± 12 vs. 84 ± 8 fmol/mg protein), and proenkephalin (58 ± 9 vs. 33 ± 4 fmol/mg protein) when compared with
sham heart tissue (Fig. 6, A–C). Ischemia also reduced the tissue norepinephrine content (12 ± 1 vs. 5 ± 1 pmol/mg protein) by >50% (Fig. 6D). A suggestive upward trend in ME was not significant (Fig. 7A). In contrast, ischemia alone reduced the content of MEAP by more than half (23 ± 4 vs. 11 ± 1 fmol/mg protein) (Fig. 7B). Surprisingly, neither the precursors nor the processed enkephalins ME and MEAP were significantly altered after 3 h of reperfusion. Importantly, postconditioning restored the enkephalin precursor content to values observed in sham hearts. PEP in postconditioned hearts was dramatically increased compared with I/R (70 ± 5 vs. 119 ± 5), which was not different from sham hearts (112 ± 12). Much of this increase was attributable to a disproportionate threefold rise in proenkephalin (96 ± 7), well above the content observed in the sham hearts (58 ± 9, Fig. 6C). Furthermore, compared with I/R, postconditioning restored norepinephrine (4 ± 1 vs. 10 ± 1) to values comparable to those observed in sham hearts (12 ± 1, Fig. 6). In contrast, postconditioning did not modify ME levels or MEAP compared with I/R (Fig. 7). Thus, compared with I/R, the increase in total enkephalins (89 ± 8 vs. 132 ± 5) in postconditioned hearts resulted from increased precursor content primarily due to increased proenkephalin.

**DISCUSSION**

The results of the present study demonstrate that the infarct-sparing effects of postconditioning involve the intrinsic activation of opioid receptors. The pharmacological analyses suggest that MORs and perhaps DORs are active participants in the protective effect of postconditioning. The data are less supportive of KOR participation. Postconditioning enhanced cardiac proenkephalin content and thus preserved the pool of total precursor enkephalins. Collectively, these data suggest that the protection induced by postconditioning is functionally associated with opioid receptor activation by local opioids and that maintaining adequate supplies of proenkephalin may be an integral part of the process. If the preservation of protein synthesis extends beyond the opioids, the phenomenon may represent an important general mechanism by which postconditioning exerts protection during reperfusion.

Involvement of specific opioid receptor subtypes in postconditioning. The current antagonist results clearly support the concept that endogenous opioids and their receptors are ac-

![Fig. 6. Effect of ischemia alone (Isch), ischemia-reperfusion (I/R), and Postcon on the content of cardiac enkephalins in the area at risk (AAR) myocardium. A: sum total of measured enkephalins (proenkephalin, peptide B, ME, and MEAP). B: changes in total precursors; PEP. C: changes in proenkephalin. D: changes in norepinephrine. All values are means ± SE; n = 4 in all groups. P < 0.05 vs. sham (*) and vs. Postcon group (†).](image)

![Fig. 7. Effect of Isch, I/R, and Postcon on the content of bioactive enkephalins in the AAR myocardium. Changes in ME (A) and MEAP (B). All values are means ± SE; n = 4 in all groups. P < 0.05 vs. sham (*) and vs. Postcon group (†).](image)
tively involved in the infarct sparing effect of postconditioning. By systematically reducing antagonist doses, the current study attempted to identify which receptor subtype(s) was preferentially involved in the infarct size reduction by postconditioning. Assigning an effect to specific opioid receptor subtypes in vivo is particularly difficult when single and/or high doses of agonist or antagonist are used since selectivity is relative and always dose dependent. The observation that all of the antagonists were effective at the highest dose illustrates this ambiguity, since sufficiently high doses will generally interact with more than the intended target receptor. In addition, the interpretation of antagonist dose responses can also be complicated by differences in bioavailability, volume of distribution, half-life, relative $K_i$, and the local concentrations of the endogenous agonist. Thus even dose responses should be interpreted with some degree of caution.

That said, the MOR antagonist CTAP was fully effective at 0.18 μmol/kg (Fig. 5). NL, which has a reported 10- to 15-fold preference for MOR sites (22, 24) and a similar $K_i$ to CTAP, was the next closest antagonist at six times the CTAP dose. The DOR antagonist NTI and the KOR antagonist NorBNI were both higher yet at 20 and 40 times the molar dose of CTAP, respectively. CTAP has a significant selectivity for MORs relative to DORs and KORs and thus seems unlikely to block DORs or KORs at this low dose when NTI and NorBNI were both ineffective at higher molar equivalents. Despite their relative selectivity, the efficacy of the higher doses of NTI and NorBNI might be explained by nonselective antagonism at the MOR. A likely role for the MOR does not, however, rule out participation by other opioid receptors. Indeed, the DOR antagonist NTI and the KOR antagonist NorBNI both clearly inhibited postconditioning, with NTI being equally effective to NorBNI but at the lower dose (Fig. 4). Thus, if two opioid receptors are involved in postconditioning, the DOR would be the more likely candidate. This is of course consistent with the role of DORs in preconditioning (18, 26, 34), as a reperfusion therapy (see below) and the broad mechanistic overlap between preconditioning and postconditioning.

A growing body of evidence supports the concept that activation of opioid receptors at reperfusion induces cardioprotection (4, 9, 11, 40). Gross et al. (13) demonstrated that morphine or the selective DOR agonist BW-373U86 administered as a pretreatment or 5 min before reperfusion exerted comparable protection against myocardial infarction in rats. Similarly, Forster et al. (9) recently reported that DOR activation with [D-Ala$^2$,D-Leu$^5$]-enkephalin was also successful at reducing infarct size in isolated rabbit hearts when infused 5 min before the onset of reperfusion. Taken together, exogenous (and possibly endogenous) activation of DORs at reperfusion appear to elicit a reperfusion-tolerant phenotype.

The current suggestion that KORs are less influential in postconditioning is consistent with their less well-established role in ischemic preconditioning (33, 39). Nevertheless, administration of KOR agonists both before (7, 28, 29, 32, 39) and after ischemia (11) has been shown to successfully reduce infarct size. Furthermore, delaying the infusion of the selective KOR agonist U-50,488 by 10 s following reperfusion rendered the drug ineffective (11), similar to the loss of infarct sparing when application of the postconditioning stimulus is delayed (20). Collectively, these findings are consistent with the argument that exogenous activation of KORs may be able to mimic postconditioning infarct-sparing effects.

Of interest, morphine has also been determined to be a successful reperfusion mimic (13, 40). Importantly, morphine has a preference for MOR sites (15), and the low dose of morphine employed by Gross and colleagues (13) was unlikely sufficient to activate DORs or KORs. Thus cardioprotection was more likely exerted by activation of MORs. Similarly, Weihrauch et al. (40) determined that an even lower (1/3) dose of morphine reduced infarct size when present at the onset of reperfusion and that morphine enhances isoflurane-induced postconditioning by activating opioid receptors. Although radioligand binding studies have disputed the presence of MORs in the myocardium, more recent data suggest there are functional MORs in rat cardiac myocytes (17), thus participation by MOR in the current study is certainly plausible.

Interestingly, opioids at reperfusion and the postconditioning “stimulus” share a number of common pathways and downstream effectors. Phosphatidylinositol 3-kinase (PI 3-kinase) has been implicated in infarct size reduction by a number of reperfusion therapies, including postconditioning (3, 5, 37, 41), although not without controversy (8). Similarly, initial reports on the cardioprotective effects of opioids at reperfusion unanimously reveal a role for PI 3-kinase (13, 14, 40). Several laboratories have independently demonstrated that postconditioning and opioid-induced protection both modify PI 3-kinase, the target of rapamycin and GSKβ (13, 36). In addition, the infarct-sparing effects of both postconditioning and opioids at reperfusion also involve opening of $K_{ATP}$ channels (4, 25, 30). Moreover, both cardioprotective processes appear to involve a role for PKC (30, 31, 44) consistent with the well-documented involvement of PKC in opioid-mediated protection during ischemia (10, 23, 45).

Enhanced synthesis of enkephalin precursors by postconditioning without altering processed bioactive enkephalin contents. Another significant finding in this study is that postconditioning increased proenkephalin content after ischemia-reperfusion without increasing the bioactive enkephalins ME and MEAP. This observation differs from preconditioned hearts in which postconditioning restored total enkephalins primarily by adding to the pool of processed enkephalin $M + MEAP$ (43). Although the earlier study employed global ischemia in isolated hearts, the effect of ischemia alone was similar to that observed here, i.e., lowered precursor and lowered processed product (43). During ischemia, the processing of existing precursors and the secretion or degradation of the processed products must exceed their synthesis (42, 43). Although both cardioprotective strategies increased total enkephalin content following ischemia, postconditioning appears to increase the precursor and retain less available product, whereas preconditioning appears to retain less precursor and increase the available product. This apparent dichotomy may reflect differences in secretory patterns in vivo and in vitro. Some of these qualitative differences in peptide content between pre- and postconditioned hearts may be due to differences in the model systems and/or $I/R$ protocols used in these two studies. Nonetheless, the observation that postconditioning leads to a restoration of opioid content but does not appear to require an increase above normal endogenous content raises the important possibility that the opioid system is always “on,” as appears for

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the sham data, and that postconditioning prevents I/R from turning these intrinsic cardioprotective mechanisms “down” or “off.”

Like the current study, the restoration of enkephalin in preconditioned hearts was not observed until after reperfusion (43), suggesting that ischemic preconditioning may mediate cardioprotection by providing bioactive peptides during reperfusion. In fact, perfusing the isolated heart with enkephalin also restored postischemic function (43). Thus lower enkephalin production may be responsible for the poor recovery of mechanical function during untreated ischemia and reperfusion (43). Although the presumed beneficial effectors ME and MEAP might be lower in preconditioned hearts, the increased precursor would provide a readily accessible steady-state reservoir of bioactive peptides for continued secretion. Because this snapshot was obtained after 3 h of reperfusion, it may not clearly reflect processing or secretory activity during those initial 60 s or intervening 3 h of reperfusion. The complete restoration of proenkephalin stores, however, clearly attests to the greater viability of intracellular systems in the postconditioned heart. The restoration of norepinephrine content is also indicative that the AAR of this heart is indeed healthier and less injured than the untreated I/R groups.

One of the key consequences of postconditioning may be the protection of protein synthesis in general, not just the processing of enkephalins. The nearly identical pattern for the recovery of norepinephrine content suggests that postconditioning may protect the biosynthetic enzymes for catecholamine synthesis as well. A multiplicity of cardioprotective effects may be invoked to protect essential proteins and their synthesis during the oxidative insult at reperfusion. In this regard, the most vulnerable targets may be those earlier responders (enkephalin, catecholamines) that are quickly consumed by oxidative mechanisms and must then be replaced.

Conclusion

In summary, this study confirmed that postconditioning can significantly reduce reperfusion injury after local coronary occlusion-reperfusion. Opioid antagonists inhibited the infarct sparing effect of postconditioning, suggesting the integral involvement of endogenous opioids in the induction of postconditioning. In particular, the intrinsic activation of MOPRs and possibly DORs by endogenous opioid agonists may be involved in infarct size reduction by postconditioning. Postconditioning may facilitate this opioid effect in part by protecting the enzymes responsible for the synthesis and processing of proenkephalin. Finally, the proposed preservation of protein synthetic mechanisms may be an important avenue for future research, particularly if the protection extends beyond the enkephalins and catecholamines.

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