Long-term infusion of Met<sup>5</sup>-enkephalin fails to protect murine hearts against ischemia-reperfusion injury

Koh Kuzume, Kazuyu Kuzume, Zhiping Cao, Lijuan Liu, and Donna M. Van Winkle

1Research and 2Anesthesiology Services, Veterans Affairs Medical Center, and 3Department of Anesthesiology, Oregon Health and Sciences University, Portland, Oregon

Submitted 12 March 2004; accepted in final form 13 November 2004

Kuzume, Koh, Kazuyu Kuzume, Zhiping Cao, Lijuan Liu, and Donna M. Van Winkle. Long-term infusion of Met<sup>5</sup>-enkephalin fails to protect murine hearts against ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 288: H1717–H1723, 2005. First published November 18, 2004; doi:10.1152/ajpheart.00257.2004.—Recently, we reported that exogenous administration of Met<sup>5</sup>-enkephalin (ME) for 24 h reduces infarct size after ischemia-reperfusion in rabbits. In the present study, we tested whether ME-induced cardioprotection is exhibited in murine hearts and whether chronic infusion of this peptide can render hearts tolerant to ischemia. Barbital-anesthetized open-chest mice (C57BL/6J) were subjected to regional myocardial ischemia-reperfusion (45 min of occlusion and 20 min of reperfusion). Mice received saline vehicle or ME for 24 h or 2 wk before undergoing regional myocardial ischemia-reperfusion or for 24 h followed by a 24-h delay before regional myocardial ischemia-reperfusion. Infarct size was measured with propidium iodide and is expressed as a percentage of the area at risk. Infarcts were smaller after infusion of ME for 24 h than with vehicle control: 49.2 ± 9.0% vs. 22.2 ± 3.2% (P < 0.01). In contrast, administration of ME for 2 wk failed to elicit cardioprotection: 36.5 ± 9.1% and 41.4 ± 8.2% for control and ME, respectively (P = not significant). When a 24-h delay was imposed between the end of drug treatment and the onset of the ischemic insult, cardioprotection was lost: 38.5 ± 6.1% and 42.8 ± 6.6% for control and ME, respectively (P = not significant). Chronic sustained exogenous infusion of the endogenously produced opioid peptide ME is associated with loss of the cardioprotection that is observed with 24 h of infusion. Furthermore, in this in vivo murine model, ME failed to induce delayed tolerance to myocardial ischemia-reperfusion.

infarction; ischemic preconditioning; myocardial; peptides; opioid

METHODS

Animals were allowed access to food and water ad libitum until induction of anesthesia. All procedures were approved by the local Institutional Animal Care and Use Committee, and all animals received humane treatment in compliance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Research, National Research Council, National Academy Press, 1996).

Surgical Preparation

Male C57BL/6 mice (24.0–31.2 g body wt) were anesthetized with thiobutabarbitosal (90 μg/g ip; Inactin, Research Biochemicals International, Natick, MA), and anesthesia was maintained with one intraperitoneal supplement if needed. After induction of anesthesia, the mice were placed in a supine position, a tracheostomy was performed, and the mice were mechanically ventilated at 120 breaths/min with a tidal volume of ~0.5 ml. Core body temperature was measured via a rectal temperature probe and maintained at ~37°C with a miniature heating blanket. The ECG was monitored continuously throughout the surgery and experimental protocol. A 1.4-Fr transducer-tipped catheter (model SPR-671, Millar Instruments, Houston, TX) was inserted through the right carotid artery to the aortic arch to record blood pressure. The right jugular vein was cannulated with a PE-10 catheter for intravenous access. With the aid of a dissecting microscope, a left thoracotomy was performed in the fourth intercostal space. A small opening was made in the pericardium, and a 7-0 silk suture on a curved taper needle was passed under the proximal left anterior descending coronary artery, and both ends of the suture were passed through a segment of PE-10 tubing to form a snare.

Induction of ischemia. Mice were subjected to 45 min of regional myocardial ischemia followed by 20 min of reperfusion. Ischemia was confirmed by cyanosis, regional akinesis, and ECG changes. At the end of the ischemic period, the snare was released, and reperfusion was confirmed by hyperemia.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: D. M. Van Winkle, Anesthesiology Service, P3ANES, DVA Medical Center, 3710 SW US Veterans Hospital Rd., Portland, OR 97239-2999 (E-mail: Donna.Vanwinkle@med.va.gov).
Measurement of Risk and Infarct Sizes

At 5 min of reperfusion, propidium iodide (PI, 10 mg/kg; Sigma Chemical, St. Louis, MO) was administered via a jugular catheter (8, 11, 18, 19). After 15 min, the coronary snare was retightened, and ZnCd particles (Duke Scientific, Palo Alto, CA) were infused intraarterially to delineate the ischemic zone. The heart was then excised and weighed. PI is membrane impermeant and, thus, is excluded from viable cells; however, in nonviable cells with membrane failure (an early event after irreversible injury), this red-fluorescent dye binds to DNA by intercalating between bases. Because PI does not depend on washout of enzymes and cofactors (as does the triphenyltetrazolium chloride staining method of infarct assessment), sustained reperfusion after myocardial ischemia is not necessary to demonstrate infarction. The ZnCd particles fluoresce bright green under ultraviolet light and delineate the area at risk as a negative image. Hearts were sliced perpendicularly to the long axis of the heart into 1-mm-thick slices with the aid of a mouse brain matrix (Vibratome, St. Louis, MO). Slices were then placed between two glass slides that were separated by 0.67 mm with four cover-glass shims. Heart slices and a calibration square (5 cm\(^2\), calibration of 5 mm\(^2\), square (5 cm\(^2\)) were placed between two glass slides that were separated by 0.67 mm with four cover-glass shims. Heart slices and a calibration square (5 cm\(^2\), calculated and translated to cubic millimeters using the calibration model 58, UVP, Upland, CA) using a digital camera (model DC290, Wacom Technology, Vancouver, WA), and the risk zone was quantified by subtracting the green-fluorescing area (ZnCd) from the total biventricular area. The pixel number of this area was calculated and translated to cubic millimeters using the calibration square (5 × 5 mm). Red-fluorescing areas (infarcted tissue) were delineated in the same way. The resultant areas were multiplied by slice thickness (0.67 mm) to calculate volume. All risk and infarct measurements were performed by one person in a blinded fashion.

Experimental Protocols

ME and saline vehicle were delivered by subcutaneously implanted osmotic minipumps (Alzet, Cupertino, CA). Three separate experimental series were conducted.

Protocol 1. To determine whether exogenous delivery of ME reduces infarct size in mice [as was previously reported for rabbits (10)], animals received a 24-h infusion of ME (0.125 mg·kg\(^{-1}\)·h\(^{-1}\), 1 μl/h) immediately followed by regional myocardial ischemia-reperfusion. The infusion pump was removed just after the onset of ischemia to ensure cessation of ME delivery. Experimental groups were designated SAL24 (saline vehicle) and ME24 (24 h of ME).

Table 1. Heart rate

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>Ischemia 30 min</th>
<th>Reperfusion 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sal24</td>
<td>5</td>
<td>379±25</td>
<td>371±23</td>
<td>383±23</td>
</tr>
<tr>
<td>ME24</td>
<td>5</td>
<td>357±23</td>
<td>355±10</td>
<td>369±10</td>
</tr>
<tr>
<td>Sal2</td>
<td>7</td>
<td>336±10</td>
<td>358±12</td>
<td>367±12</td>
</tr>
<tr>
<td>ME2</td>
<td>7</td>
<td>348±16</td>
<td>368±17</td>
<td>376±13</td>
</tr>
<tr>
<td>Sal24/24</td>
<td>9</td>
<td>340±16</td>
<td>346±12</td>
<td>368±14</td>
</tr>
<tr>
<td>ME24/24</td>
<td>13</td>
<td>344±14</td>
<td>374±13</td>
<td>386±15</td>
</tr>
</tbody>
</table>

Values are means ± SE in beats/min. Sal24, 24-h infusion of saline; ME24, 24-h infusion of Met\(^{\beta}\) enkephalin (ME); Sal2, 2-wk infusion of saline; ME2, 2-wk infusion of ME; Sal24/24, 24-h washout after saline infusion; ME24/24, 24-h washout after ME infusion.

Protocol 2. To determine whether chronic extension of the ME infusion is associated with continued cardioprotection, animals were given ME for 2 wk (0.125 mg·kg\(^{-1}\)·h\(^{-1}\), 0.5 μl/h) and immediately subjected to regional myocardial ischemia-reperfusion. These groups were designated Sal2 and ME2.

Protocol 3. To determine whether ME can elicit delayed cardioprotection, a 24-h period was interposed between the end of a 24-h ME infusion (0.125 mg·kg\(^{-1}\)·h\(^{-1}\), 1 μl/h) and the onset of myocardial ischemia. The experimental groups were designated Sal24/24 and ME24/24.

Assessment of Myocardial Opioid Receptors

To determine whether the loss of protection with sustained ME infusion is a result of decreased opioid receptor levels, δ- and κ-opioid receptor proteins were measured by Western blot. Animals received saline vehicle or ME for 24 h or 2 wk (Sal24/24, ME24/24, SAL2-WB, and ME2-WB, respectively). Under pentobarbital sodium anesthesia (90 mg/kg ip), hearts were excised and immediately placed in liquid nitrogen. Samples were kept at -80°C until analysis. Left ventricular (LV) tissue was lysed in SDS sample buffer (Bio-Rad) using a Polytron. Lysates were boiled for 5 min, centrifuged for 5 min at 12,000 g, and stored at -80°C. LV proteins (6 μg/lane) in SDS sample buffer were separated on 4–20% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN 3, Bio-Rad) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for 5 min at room temperature and incubated overnight at 4°C with primary antibodies (1:4,000 dilution in 5% dry milk). The antigens were detected by Western blot technology (ECL-plus Western blotting detection kit, Amersham) with peroxidase-linked anti-rabbit (1:1,000 dilution in 5% dry milk; Cell Signaling Technology, Beverly, MA). The intensity of the immoblot bands was assessed with the Kodak 1D Image Analysis System (EDAS 290). Data are expressed as percent change of control values.

CAMP Assay

To determine whether sustained ME treatment results in desensitization of opioid receptors, we examined production of cAMP in the absence and presence of the adenylate cyclase activator forskolin in saline- and ME-treated hearts. Stimulation of opioid receptors has been previously reported to decrease cAMP levels in the heart (14). Mice were anesthetized with pentobarbital sodium (50 mg/kg ip), and the hearts were rapidly excised and mounted on a nonrecirculating Langendorff apparatus. Hearts were perfused at 37°C for 15 min with oxygenated Krebs-Henseleit buffer (in mM: 118.5 NaCl, 24.8 NaHCO\(_3\), 10.0 glucose, 4.7 KCl, 2.0 CaCl\(_2\), 1.2 KH\(_2\)PO\(_4\), and 1.2 MgSO\(_4\), pH 7.4) with or without 1.0 μM forskolin. After perfusion,
Data analysis was performed with a personal computer statistical software package (Prism 4.0, GraphPad Software). The primary dependent variable analyzed to assess presence or absence of cardioprotection was infarct size. Because the amount of LV myocardium that progresses to infarction depends on the size of the risk zone, infarct size was normalized as a percentage of the risk zone. Infarct size (expressed as a percentage of the area at risk) and the size of the area at risk were analyzed between groups using the Mann-Whitney test.

**Table 3. Myocardial weights and risk volumes**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Wt, g</th>
<th>Heart Wt, mg</th>
<th>Risk, mm³</th>
<th>Risk/Heart, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protocol 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sal24</td>
<td>5</td>
<td>27.0 ± 1.3</td>
<td>117 ± 6</td>
<td>38.0 ± 5.1</td>
<td>32.9 ± 4.7</td>
</tr>
<tr>
<td>ME24</td>
<td>5</td>
<td>25.5 ± 0.7</td>
<td>120 ± 7</td>
<td>38.7 ± 3.2</td>
<td>32.2 ± 2.2</td>
</tr>
<tr>
<td><strong>Protocol 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sal2</td>
<td>7</td>
<td>28.5 ± 0.9</td>
<td>139 ± 8</td>
<td>41.9 ± 3.7</td>
<td>30.5 ± 2.8</td>
</tr>
<tr>
<td>ME2</td>
<td>7</td>
<td>28.3 ± 0.5</td>
<td>143 ± 2</td>
<td>44.5 ± 3.4</td>
<td>31.0 ± 2.2</td>
</tr>
<tr>
<td><strong>Protocol 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sal24/24</td>
<td>9</td>
<td>26.1 ± 0.4</td>
<td>122 ± 3</td>
<td>36.3 ± 1.2</td>
<td>30.0 ± 1.4</td>
</tr>
<tr>
<td>ME24/24</td>
<td>13</td>
<td>27.2 ± 0.4</td>
<td>129 ± 4</td>
<td>40.2 ± 2.1</td>
<td>31.2 ± 1.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. See Table 1 footnote for explanation of experimental groups.

Data Analysis

Hemodynamic data are shown in Tables 1 and 2. Two-way ANOVA showed no interaction between the treatment and the time course in heart rate and mean arterial pressure in any protocol.

Infarct Size

Body weight, biventricular heart weight, area at risk (mm³), and risk size as a percentage of biventricular heart weight are shown in Table 3. There were no significant differences in these parameters between the groups in each protocol.

**Protocol 1.** ME infusion for 24 h decreased infarct size 55%: 49.2 ± 9.0% and 22.2 ± 3.2% for SAL24 and ME24, respectively (P < 0.01). When risk volume (mm³) was assessed as a covariate of infarct volume, infarct size was significantly smaller in the ME24 than in the SAL24 group (P < 0.05 by ANCOVA, analysis of covariance.

**Results**

**Hemodynamic Data**

Additional, linear regression was performed for pooled area at risk and infarct data of each series, and then the vertical difference of the individual data points from the common regression line (residuals) was calculated. The means of these differences were compared by unpaired t-test. Hemodynamic valuables in the time course between groups were evaluated by two-way ANOVA. Each time point within a group was analyzed by one-way ANOVA with Dunnett’s test, in which each time point is compared with the baseline value. Opioid receptor levels were normalized as a percentage of results from saline-treated animals and compared using the Mann-Whitney test. Values are means ± SE, and statistical significance is assumed for P < 0.05.
ANCOVA). Residuals from SAL24 hearts fell mainly above the pooled regression line, and those from ME24 hearts fell below the line; t-test of these residuals showed a significant difference (5.6 ± 4.0 and −5.6 ± 1.7 for SAL24 and ME24, respectively, P < 0.05 by unpaired Student’s t-test), indicating that infarct size was significantly smaller in the ME24 than in the SAL24 group (Fig. 1).

Protocol 2. When the infusion of ME was sustained for 2 wk, infarct limitation was lost: 41.9 ± 3.7% and 44.5 ± 3.4% for SAL2 and ME2, respectively [P = not significant (NS)].
Infarct size analyses using ANCOVA and t-test of the residuals from the pooled regression line (−0.3 ± 3.9 and 0.3 ± 3.9 for SAL2 and ME2, respectively, \( P = \text{NS} \)) also failed to demonstrate significant cardioprotection (Fig. 2).

Protocol 3. Introduction of a 24-h delay between cessation of the 24-h ME infusion and the onset of myocardial ischemia also resulted in loss of cardioprotection: 38.5 ± 6.1% and 42.8 ± 6.6% for SAL24/24 and ME24/24, respectively (\( P = \text{NS} \)). Similarly, infarct volume analyzed with risk as a covariate was not significantly different between groups (\( P = \text{NS} \) by ANCOVA), nor were the residuals from the pooled regression line: −0.5 ± 2.3 and 0.4 ± 2.7 for SAL24/24 and ME24/24, respectively (\( P = \text{NS} \); Fig. 3).

Western Blot of Cardiac Opioid Receptors

There was a trend for \( \delta \)-opioid receptor levels to be lower after 2 wk than after 24 h of ME treatment, but this did not achieve statistical significance: 1.10 ± 0.15 and 0.89 ± 0.03 for 24 h and 2 wk, respectively (\( P = 0.26 \) by t-test with Welch’s correction for unequal variances). There were no differences in levels of \( \kappa \)-opioid receptors by treatment duration: 1.05 ± 0.05 and 0.99 ± 0.06 for 24 h and 2 wk, respectively (\( P = \text{NS} \) by t-test; Fig. 4).

cAMP Production

Basal cAMP levels were reduced ~60% in hearts treated with ME for 24 h compared with saline-treated hearts (1.53 ± 0.13 vs. 0.64 ± 0.07 pmol/mg, \( P < 0.01 \)) but were unchanged in hearts treated for 2 wk with ME (1.44 ± 0.05 vs. 1.18 ± 0.11 pmol/mg, \( P = \text{NS} \)). Forskolin augmented cAMP production in hearts treated for 24 h and 2 wk with saline (from 1.53 ± 0.13 to 2.41 ± 0.20 pmol/mg, \( P < 0.01 \), and from 1.44 ± 0.05 to 2.62 ± 0.22 pmol/mg, \( P < 0.001 \), respectively; Fig. 5). In hearts treated with ME for 2 wk, administration of forskolin elicited an increase in cAMP production similar to that in saline-treated hearts: 2.43 ± 0.17 vs. 2.62 ± 0.22 pmol/mg, respectively (\( P = \text{NS} \)). However, in hearts treated with ME for 24 h, administration of forskolin did not result in augmented cAMP levels: 1.71 ± 0.09 and 2.41 ± 0.20 pmol/mg for ME + forskolin and saline + forskolin, respectively (\( P < 0.05 \)).

DISCUSSION

The principal findings of this study are as follows: 1) chronic exogenous infusion of ME for 2 wk fails to reduce infarct size, and 2) ME does not elicit delayed cardioprotection after a 24-h infusion of peptide.

In 1995, Shultz et al. (15) demonstrated that infarct limitation due to IP is blocked by the nonselective opioid antagonist naloxone in rats; subsequently, we showed that this effect of naloxone is stereoselective (i.e., opioid receptor mediated) and occurs locally in the heart (3). These observations suggest that the cardiac endogenous opioid system plays a crucial role in the infarct limitation of IP. PP with opioid agonists has also been demonstrated: the nonselective opioid agonist morphine mimics the cardioprotection of IP (12), and the narcotic anesthetic fentanyl has also been shown to limit infarct size (9). Studies using synthetic selective opioid receptor agonists or antagonists have identified the \( \delta \)-opioid receptor as the primary opioid receptor subtype responsible for infarct limitation (6). However, \( \kappa \)-opioid receptor stimulation has also been reported to be cardioprotective, through infarct limitation and a reduction of malignant arrhythmias (20).
Studies of PP with endogenous opioid peptides are few, probably because it is difficult to achieve a cardioprotective concentration of these peptides at cardiomyocytes because of their short half-lives. We previously reported that ME, Leu\textsuperscript{2}-enkephalin, and Met\textsuperscript{2}-enkephalin-Arg\textsuperscript{6}-Phe\textsuperscript{7} protect isolated adult rabbit cardiomyocytes against simulated ischemia (16), as does dynorphin B (2). In vivo, we found that 24 h of ME infusion immediately before the onset of ischemia-reperfusion (i.e., there was no drug-free period before coronary ligation) limits infarct size in rabbits. The present study corroborates the rabbit data in an in vivo murine model, demonstrating robust infarct limitation with 24 h of ME infusion. The dose of ME was 0.125 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}, which was the same as in the previous rabbit study; similarly, the extent of infarct limitation was comparable (55% in mice and 60% in rabbits).

Temporal extension of this opioid-induced protective state would be very desirable for clinical medicine; however, in the present study, extension of the infusion period to 2 wk was associated with loss of protection. This is reminiscent of a previous report by Hashimi et al. (7) and Tsuchida et al. (17), who demonstrated that chronic (72 h) adenosine receptor agonist infusion immediately preceding myocardial ischemia-reperfusion resulted in tachyphylaxis and loss of cardioprotection. GPCRs typically desensitize in three general ways: transduction efficacy is reduced because of alterations that limit interaction with G proteins (receptor inactivation); they are internalized, thus eliminating access to their ligand (receptor sequestration); and they undergo lysosomal degradation subsequent to internalization (receptor downregulation) (1). The tachyphylaxis observed in the present study is likely due to receptor desensitization (inactivation or sequestration), rather than receptor downregulation, because absolute receptor levels were unchanged by chronic ME treatment (Western blot of opioid receptor protein would be expected to include an internalized receptor protein that has not yet undergone degradation). The cAMP production data at 2 wk, showing preserved forskolin-induced cAMP production and loss of ME-mediated inhibition of forskolin-induced cAMP production, suggest that opioid receptor coupling to adenylate cyclase is impaired, through receptor inactivation or internalization.

In our previous study in which ME was administered for 24 h to rabbits, there was no drug-free interval before the onset of myocardial ischemia-reperfusion (10). Therefore, it was difficult to determine whether the 24-h infusion of ME was eliciting acute or delayed cardioprotection. Delayed opioid-induced protection has been reported for a number of opioid agonists such as TAN-67, BW-373U86, and SNC-121, but in each of these instances, the drug was given as a bolus or a short infusion (6). Dana et al. (4) showed that administration of an A\textsubscript{3} adenosine receptor agonist for 48 h followed by ischemia-reperfusion 48 h later results in preserved cardioprotection. However, when we interposed a 24-h drug-free interval between the end of the 24-h ME administration and the onset of ischemia-reperfusion in mice, cardioprotection was lost. The reason for the inability of 24 h of ME infusion to elicit delayed protection is not known. It is conceivable that this duration of infusion results in receptor desensitization that does not become manifest immediately. It is unlikely that the loss of ME-induced cardioprotection after the subsequent 24-h drug-free interval is due to receptor degradation, because 2 wk of treatment of ME was not associated with appreciable receptor loss. Alternatively, it is possible that ME does not elicit the production of reactive oxygen species, which are known to trigger delayed preconditioning. BW-373U86 and SNC-121 have been reported to elicit delayed preconditioning and do so via reactive oxygen species production; this protection is not sensitive to blockade of opioid receptors (6).

In summary, we found that exogenous infusion of the endogenously produced opioid pentapeptide ME for 24 h reduces ischemia-reperfusion injury in mice in vivo when given immediately, but not 24 h, before ischemia. In addition, ME administration for 2 wk was associated with loss of the cardioprotective effect.

GRANTS

This work was supported by a Department of Veterans Affairs Medical Research Service Merit Review Grant to D. M. Van Winkle.

REFERENCES

7. Hashimi MW, Thornton JD, Downey JM, and Cohen MV. Loss of myocardial protection from ischemic preconditioning following chronic exposure to Rp(−)-N\textsubscript{2}-(2-phenylisopropyl)adenosine is related to defect at the adenosine A\textsubscript{3} receptor. Mol Cell Biochem 186: 19–25, 1998.


20. Wong TM, Lee AYS, and Tai KK. Effects of drugs interacting with opioid receptors during normal perfusion or ischemia and reperfusion in the isolated rat heart—an attempt to identify cardiac opioid receptor subtype(s) involved in arrhythmogenesis. *J Mol Cell Cardiol* 22: 1167–1175, 1990.