Morphine preconditioning attenuates neutrophil activation in rat models of myocardial infarction

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Abstract

Previous results from our laboratory have suggested that morphine can attenuate neutrophil activation in patients with acute myocardial infarction. To elucidate if morphine preconditioning (PC) has the same effects via activation of neutrophil endopeptidase 24.11 (NEP), we measured serum levels of intercellular adhesion molecule-1 (ICAM-1), gp100 and NEP in adult Wistar rats subjected to ten different protocols (n = 10 for each) at baseline, immediately after and 2 h after morphine PC. All groups were subjected to 30 min of occlusion and 2 h of reperfusion. Similarly, morphine-induced PC was elicited by 3-min drug infusions (100 µg/kg) interspersed with 5-min drug-free periods before the prolonged 30-min occlusion. Infarct size (IS), as a percentage of the area at risk (AAR), was determined by triphenyltetrazolium staining. Pretreatment with morphine increased NEP activities (9.86 ± 1.98 vs. 5.12 ± 1.10 nmol/mg protein in control group; p < 0.001). Naloxone (µ-opioid receptor antagonist) (4.82 ± 1.02 nmol/mg protein) and phosphoramidon (NEP inhibitor) (4.66 ± 1.00 nmol/mg protein) inhibited morphine-activated NEP, whereas glibenclamide (ATP-sensitive potassium channel antagonist) and chelerythrine (protein kinase C inhibitor) had no effects. The ICAM-1 and gp100 of the third sampling were lowest for those with morphine PC (280 ± 30 ng/ml and 2.2 ± 0.7 µg/ml; p < 0.001), but naloxone (372 ± 38 ng/ml and 3.8 ± 0.9 µg/ml) and phosphoramidon (382 ± 40 ng/ml and 4.2 ± 1.1 µg/ml) abolished the above phenomenon. IS/AAR were definitely lowest for those with morphine PC (24 ± 7%; p < 0.05). Morphine preconditioning increases NEP activities to attenuate shedding of gp100(MEL14) and to ICAM-1 and, thus, provides myocardial protection.

Keywords: Adhesion molecules; Myocardial infarction; Opioid receptors; Naloxone

1. Introduction

Activation of neutrophils and endothelium appears to be a component of atherosclerosis [1,2] and of ischemia–reperfusion myocardial injury [3,4]. Recent experiments demonstrated massive leukocyte extravasation at sites of myocardial ischemia and reperfusion [5–7]. Leukocytes are considered to potentiate ischemic myocyte damage by microvascular obstruction and generation of cytotoxic metabolites [6,8]. Inhibition of neutrophil adhesion was shown to reduce myocardial infarct size [5,9,10].

Intercellular adherence is necessary for leukocyte accumulation at sites of inflammation. Intercellular adhesion molecule-1 (ICAM-1) is located primarily at surfaces of activated endothelial cells and is needed for optimal migration of neutrophils out of the blood vessels and for adhesion of neutrophils to myocytes [11]. Recent studies demonstrated expression of ICAM-1 on human atherosclerotic plaques [12]. On the other hand, gp100(MEL14) and L-selectin (human analogue of murine gp100(MEL14)), which are also members of the integrin family, mediate lymphocyte and neutrophil adhesion to specialized endothelium [13–15]. Treatment of monoclonal antibody against ICAM-1 [5,16] and L-selectin [9,10] resulted in a significant reduction in infarct size in animal experiments. These studies indicated that adhesion molecules might play a role in the development of ischemic myocyte damage. Soluble

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isoforms of these adhesion molecules shed from the surfaces of activated cells can now be quantified in peripheral blood [17]. Increased serum concentrations have been observed in a variety of diseases, including acute coronary syndrome [16–18].

Neutral endopeptidase 24.11 (NEP, 'enkephalinase'), a kind of zinc metalloprotease, hydrolyzes a variety of naturally occurring peptides, including the endogenous opioid pentapeptides met- and leu-enkephalin [19–21]. Met-enkephalin has been shown to induce morphologic changes, and directed migration and aggregation of neutrophils [22–24]. Morphine can activate NEP and, in turn, increased the amount of met-enkephalin required to trigger changes in the shape and size of these cells by several orders of magnitude [24]. Our recent study demonstrated that intravenous morphine can attenuate neutrophil activation in patients with acute myocardial infarction [25]. Recent reports have revealed that morphine mimicked ischemic preconditioning (PC) via a glibenclamide-dependent pathway [26]. Whether or not neutrophil activation was also affected deserves further investigation. The aim of this study was to measure the serum levels of ICAM-1, and neutrophil NEP activities in rats during morphine PC, and to evaluate the effect of intravenous morphine on these parameters.

2. Methods

2.1. Animal preparation

Male Wistar rats, weighing 350–450 g, fed a standard diet, were included in this study. After anesthesia with intraperitoneal pentobarbital (50 mg/kg body weight), all of the animals underwent tracheotomy and endotracheal intubation. Mechanical ventilation support was given by a Harvard Apparatus Rodent Respirator (Boston, MA, USA). The rats were ventilated with room air at 60 to 70 breaths per minute. The right carotid artery was cannulated to measure mean blood pressure (MBP) and heart rate via a Coul PE-50 pressure transducer, which was connected to a Grass polygraph. The right jugular vein was cannulated to infuse saline or drugs. A left thoracotomy was performed to expose the heart at the fifth intercostal space. After removing the pericardium, a 6-0 prolene ligation, along with a snare occluder, was placed around the left coronary artery, close to the place of origin. Regional ischemia was achieved by pulling the snare and securing the threads with a mosquito hemostat. Ischemia was confirmed by a substantial fall in left ventricular developed pressure.

2.2. Study groups and experimental protocols

The present study consisted of rats that were randomly assigned to one of ten experiment groups (ten animals for each group). All hearts experienced an initial 30 min stabilization period. Group I (the control group) was subjected to 30 min of occlusion and 2 h of reperfusion. In group II (MOR PC group), to determine if µ-opioid receptor stimulation mimicked preconditioning as described in a previous report [26], morphine was given as three 5-min infusions interspersed with 5-min drug-free intervals (100 µg/kg per infusion; total dose, 300 µg/kg). In group III (NL+MOR PC group), naloxone (3 mg/kg i.v.), a nonselective opioid receptor antagonist, was administered 10 min before morphine-induced PC. Group IV (GLI+MOR PC group) consisted of rats that were administered glibenclamide (0.3 mg/kg i.v.) 30 min before morphine PC, to test the interactions of ATP-sensitive potassium channels and the µ-opioid receptor in myocardial protection and neutrophil activation. In group V (CLR+MOR PC group), to test the interaction between protein kinase C and the µ-opioid receptor, chelerythrine (2 mg/kg) was given 30 min before morphine-induced PC. In group VI (PPR+MOR PC group), phosphoramidon (2.5 mg/kg i.v.), a NEP antagonist, was administered 10 min before morphine-induced PC. In addition, another four groups were also enrolled as comparative groups. Group VII (MOR group) comprised animals treated with three cycles of morphine without occlusion–reperfusion. Group VIII (NL group) consisted of animals treated with naloxone alone, without occlusion–reperfusion. Group IX (NL+control group) was subjected to naloxone alone, with subsequent occlusion–reperfusion. Finally, animals in group X were treated as group II except that an additional three cycles of morphine were administered after occlusion and reperfusion.

2.3. Determination of infarct size

After the end of the experiment, the silk snare was tightened and patent blue dye was injected to stain the normally perfused region of the heart. This procedure allowed for visualization of the normal, non-ischemic region and the area at risk (AAR). The heart was then weighed and frozen overnight before being cut into 2-mm thick slices, thawed, and stained by incubation for 20 min in 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4). Thereafter, the slices were immersed in 10% formalin, to enhance the contrast of the stain. The infarct area (TTC negative) and AAR were determined by planimetry. The volumes of the left ventricle, the infarct volume and the volume of AAR were then calculated by multiplying each area by the slice thickness and summing the products. The infarct size (IS) was expressed as a percentage of the AAR.

2.4. Measurements of ICAM-1 and gp100<sup>MEL•14</sup>

Venous blood was sampled from an inferior vena cava (at The Department of Emergency Medicine) at baseline
(before any drug administration), immediately after PC and 2 h after PC (but after the second morphine PC in group X). Blood was allowed to clot at 4°C for 30 min and was centrifuged at 1000 rpm for 10 min. Serum was frozen at −80°C until it was used. Serum ICAM-1 and gp100 were measured by enzyme-linked immunosorbent assay (ELISA) with specific kits for rat adhesion molecules. The ICAM-1 ELISA kit was purchased from T Cell Diagnostics (Cambridge, MA, USA) and the gp100 kit was purchased British Bio-Technology Products (Abingdon, UK). The assay was performed in duplicate for each sample. The specificities of the ICAM-1 ELISA kit and the gp100 kit were 98 and 99%, respectively. The sensitivities of detection of serum ICAM-1 and gp100 were 0.35 ng/ml and 0.05 μg/ml, respectively.

2.5. Preparation of neutrophil and enzyme assays

Heparinized rat blood was obtained via the vena cava. In certain experiments, peripheral blood was separated by Ficoll–Hypaque (Pharmacia, Piscataway, NJ, USA) density gradient centrifugation and peripheral blood granulocytes were isolated from Ficoll pellets by dextran sedimentation and were resuspended in the media RPMI 1640 (GIBCO, Gaithersburg, MD, USA) containing <0.03 U endotoxin/ml, without additions for further analysis. Sterile plastic pipettes, test tubes and flasks were used in all experiments to avoid endotoxin contamination.

Purified neutrophils in RPMI without additives were analyzed for NEP enzymatic activity with an established fluorometric assay using glutaryl–ala–ala–phe–4-methoxy–2-naphthylamine (Enzyme System Products, Livermore, CA, USA) as a substrate [19]. Cleavage of this substrate by NEP yields phe–4-methoxy–2-naphthylamine, which is converted to the fluorescent product, 4-methoxy–2-naphthylamine, in the presence of aminopeptidase. Protein was determined by a modified Lowry method [27].

2.6. Histochemical evaluation of neutrophil adhesion

The myocardium was stained for 10 h by a histochemical reaction for myeloperoxidase [28]. Neutrophils thus appear as yellow–brown cells. Neutrophils adhering to the walls of blood vessels were counted at a magnification of 400× in regions that each measured 6×106 μm² in area, located both in TTC-positive and TTC-negative areas.

Neutrophils were judged to be inside venules by their position within blood vessels and by their shape. Shape was used as a criterion because it has been found that most neutrophils that adhere to the endothelium of blood vessels have a round or tear-drop shape, whereas neutrophils outside blood vessels have an irregular, amoeboid shape, as found in previous investigations of other tissues. The numbers of neutrophils that migrated within and through the endothelium into the myocardium were accepted as the amount of neutrophil adhesion and were counted quantitatively, excluding neutrophils within vessels. The amount of neutrophil adhesion was expressed as the number of neutrophils per square millimeter of myocardium.

2.7. Drugs

Glibenclamide (Sigma) was dissolved in a 1:1:1:2 cocktail mixture of polyethylene glycol, 95% ethanol, 0.1 N sodium hydroxide and 0.09% saline. Chelerythrine (Sigma) was dissolved in specific buffer immediately before use. TTC (Sigma) was dissolved in 100 mmol/l phosphate buffer (pH 7.4). Naloxone (Research Biochemicals), phosphoramidon (Sigma) and morphine were dissolved in saline.

2.8. Statistics

All values are expressed as mean±SD. Categorical variables were analyzed with the Fisher’s Exact Test. Continuous variables were analyzed by a paired Student’s t-test or by analysis of variance with Scheffe comparison, where appropriate. A value of p<0.05 was considered to be statistically significant.

3. Results

3.1. Infarct size

Fig. 1 depicts the IS/AAR in all groups. There were no significant differences in left ventricular and AAR weights among groups (data not shown). Morphine PC (group II) decreased the IS/AAR ratio significantly (24±7 vs. 58±8% for control group or group I, p<0.01). In addition, group X (MOR-2, 24±6%) also had a significant cardio-protective effect (p<0.01 vs. control group). IS/AAR for groups I (control, 58±8%), III (NL+MOR PC, 62±10%)

![Fig. 1. Infarct size (IS)/area at risk (AAR) ratios among animals treated with ten different protocols (see text) (*) p<0.01 vs. control group (group I); † p<0.05 vs. morphine PC group (group II).](https://academic.oup.com/cardiovascres/article-abstract/40/3/557/275515/fig1_c)
and VI (PRP+MOR PC, 49+9%) were significantly higher than those for the other three groups (p<0.001). These results indicate that nonspecific μ-opioid antagonist (naloxone) and NEP inhibitor (phosphoramidon) could block the effects of morphine PC. However, groups IV (GLI+MOR PC group, 37±11%) and V (CLR+MOR PC, 38±10%) also had higher IS/AAR ratios than group II (MOR PC, 24±7%) (p<0.05). The doses of naloxone, glibenclamide and chelerythrine used in the present study did not change the IS when either agent was administered to non-PC hearts (data not shown).

3.2. Hematologic parameters

The comparison of hematological values for the ten groups is shown in Table 1. The baseline neutrophil counts were similar among the ten groups. However, the final neutrophil counts were significantly lower in groups VII and VIII than in other groups (p<0.01). There were no significant differences in hemoglobin and platelet counts among the groups.

3.3. Serum levels of adhesion molecules

Table 2 depicts serum levels of adhesion molecules and NEP activities for each group during the experiments. The baseline ICAM-1 and gp100\textsuperscript{MEL,14} levels were similar for all groups. In the control group (group I), the serum levels of ICAM-1 increased progressively after coronary ligation and were increased significantly at the third sampling (380±40 ng/ml vs. 268±32 ng/ml at baseline, p<0.01). Morphine PC (group II) blunted the increase in ICAM-1 (280±30 ng/ml at first sampling vs. 278±34 ng/ml at baseline; p>NS). However, naloxone (group III, 372±38 vs. 270±42 ng/ml; p<0.01) and phosphoramidon (group VI, 390±44 vs. 270±34 ng/ml; p<0.01) inhibited the protective effects of morphine PC. Overall, the serum levels of ICAM-1 were slightly lower in groups II (MOR PC), IV (GLI+MOR PC), V (CLR+MOR PC), VII (MOR), VIII (NL) and X (MOR-2) than in other groups at the second sampling, although this did not reach the level of significance. The ICAM-1 levels at the third sampling were, however, significantly higher in the above six groups (groups II, IV, V, VII, VIII and X) than in other groups.
(p<0.001 by ANOVA; or p<0.05 for each of these six groups vs. control group), whereas the level of group X was lowest (258±32 ng/ml) (Table 2). The results shown for group X may indicate an accumulative effect of morphine.

Similar findings can be found in the measurements of gp100MEL14. The gp100MEL14 levels were also comparable among the ten groups at baseline and were slightly lower in groups II (MOR PC), IV (GLI+MOR PC), V (CLR+MOR PC), VII (MOR), VIII (NL) and X (MOR-2) at the second sampling. In control group (group I), the serum levels of gp100MEL14 were elevated progressively after coronary ligation and reached significantly increased levels at the third sampling (4.0±1.0 vs. 2.3±0.9 μg/ml at baseline; p<0.01). Morphine PC (group II) inhibited the increase in gp100MEL14 (2.2±0.7 μg/ml at the third sampling vs. 2.4±0.8 μg/ml at baseline; p=NS), whereas the protective effects were further blocked by naloxone (group III, 3.8±0.9 vs. 2.4±0.8 μg/ml; p<0.01) and phosphoramidon (group VI, 4.4±1.2 vs. 2.5±1.0 μg/ml; p<0.01). Overall, the gp100MEL14 levels at the third sampling were significantly lower in groups II (MOR PC), IV (GLI+MOR PC), V (CLR+MOR PC), VII (MOR), VIII (NL) and X (MOR-2) than in other groups (p<0.001 by ANOVA, or p<0.05 for each of the six groups vs. control group), whereas the level in group X was the lowest (2.0±0.7 μg/ml) (Table 2).

These data indicate that morphine PC attenuates the shedding of ICAM-1 and gp100MEL14 via an opioid receptor-specific pathway. In addition, the inhibitor of NEP can block morphine effects on the above circulating adhesion molecules.

3.4. Measurement of enzyme activities

There was no significant difference in baseline NEP activities among the ten groups. However, the NEP activities at the second blood sampling were significantly higher in groups II (MOR PC, 9.86±1.98 nmol/mg protein), IV (GLI+MOR PC, 9.76±1.88 nmol/mg protein), V (CLR+MOR PC, 9.70±1.74 nmol/mg protein) and X (MOR-2, 9.80±1.90 nmol/mg protein) than in the other groups (p<0.001). These data demonstrated that morphine PC activated NEP, whereas glibenclamide (ATP-sensitive potassium channel blocker) and chelerythrine (protein kinase C inhibitor) had no definite effects on the above phenomenon. The NEP activities remained remarkably high in these four groups (Table 2). In addition, both naloxone (group III) and phosphoramidon (group VI) inhibited morphine-related NEP activation.

3.5. Assay variation

The intra-assay coefficients of variation for serum ICAM-1 and gp100MEL14 were 4.8 and 5.4%, respectively. The inter-assay coefficients of variation were 6.0 and 7.4%, respectively. The intra-assay and inter-assay coefficients of variation for NEP were 5.6 and 8.4%, respectively.

3.6. Histochemical evaluation of neutrophil adhesion

Table 3 depicts the number of adherent neutrophils in myocardial vessels, shown in the histogram. Neutrophil adherence was significantly predominant in groups I (110±18 cells/mm³), III (108±15 cells/mm³), VI (116±20 cells/mm³) and IX (106±18 cells/mm³) (p<0.001) in TTC-positive areas. Similar results were also found in TTC-negative areas. The numbers of adherent neutrophils were also slightly higher in the TTC-negative areas for groups IV and V, but the results were not significant.

4. Discussion

Clinically, intravenous administration of morphine remains an essential step in the initial treatment of acute myocardial infarction [29]. It is supposed that morphine provides alleviation of pain and anxiety, attenuation of sympathetic activation and a decrease in myocardial oxygen consumption. Recent animal studies also revealed that pretreatment with morphine may mimic ‘preconditioning’ and decrease infarct size via a glibenclamide-sensitive mechanism [26]. In this report, we demonstrated that morphine increased neutrophil NEP activities and, thus, attenuated shedding of ICAM-1 and gp100MEL14. These data suggest that morphine-related inhibition of neutro-

<table>
<thead>
<tr>
<th>Group</th>
<th>TTC-positive area (cells/mm³)</th>
<th>TTC-negative area (cells/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (control)</td>
<td>110±18</td>
<td>136±30</td>
</tr>
<tr>
<td>II (MOR PC)</td>
<td>64±14</td>
<td>84±22</td>
</tr>
<tr>
<td>III (NL+MOR PC)</td>
<td>108±15</td>
<td>150±28</td>
</tr>
<tr>
<td>IV (GLI+MOR PC)</td>
<td>90±15</td>
<td>130±28</td>
</tr>
<tr>
<td>V (CLR+MOR PC)</td>
<td>116±20</td>
<td>134±26</td>
</tr>
<tr>
<td>VI (PPR+MOR PC)</td>
<td>54±12</td>
<td>154±30</td>
</tr>
<tr>
<td>VII (MOR)</td>
<td>60±12</td>
<td>Nil</td>
</tr>
<tr>
<td>VIII (NL)</td>
<td>60±12</td>
<td>160±30</td>
</tr>
<tr>
<td>IX (NL+)</td>
<td>62±12</td>
<td>78±20</td>
</tr>
<tr>
<td>X (MOR-2)</td>
<td>62±12</td>
<td>78±20</td>
</tr>
</tbody>
</table>

CLL, chelerythrine; GLI, glibenclamide; MOR, morphine; NL, naloxone; PC, preconditioning; PPR, phosphoramidon; TTC, triphenyltetrazolium chloride.

For experimental protocol for each group, see text.

1. p<0.001 by ANOVA among nine groups; 2. p<0.001 by ANOVA among seven groups.

3. p<0.05 for all neutrophil numbers in TTC-negative area vs. TTC-positive area.

4. p<0.001 vs. control group.
phine cannot be excluded. In general, blockade of adhesion interactions between leukocytes and the endothelium, and between leukocytes and the vascular compartment of platelets. All of the selectins can rapidly arrest freely flowing leukocytes and mediate rolling of the cells along the endothelium of blood vessels [30,31]. Activated neutrophils undergo coordinated changes in the expression of the adhesion molecule gp100MEL14 (or the human analogue L-selectin) in early phase [32]. The shedding of gp100MEL14 into peripheral blood is also proportional to the degree of neutrophil activation [16]. In other words, our observation that serum levels of gp100MEL14 decreased after morphine treatment suggested that neutrophil adhesion was inhibited.

On the other hand, ICAM-1 is known to be a major ligand on endothelial cells for adhesion of activated leukocytes and subsequent passage of leukocytes into the myocardium [16,31,32]. Both serum ICAM-1 and gp100MEL14 levels were elevated in patients with acute myocardial infarction, suggesting that both rolling and postrolling events [18] play an important role in ischemic myocardial injury. Because intravenous administration of morphine can suppress the elevation of ICAM-1 and gp100MEL14, the mechanism should involve early and late phases of leukocyte–endothelium activation.

This study demonstrates that morphine PC firstly activates NEP within 30 min and, subsequently, attenuates neutrophil–endothelium activation during myocardial ischemia. A NEP antagonist, such as phosphoramidon, can almost completely inhibit the shedding of ICAM-1 and gp100MEL14. It is reasonable that morphine-induced attenuation of the neutrophil–endothelium reaction is based upon activation of NEP. The neuropeptide met-enkephalin functions as an inflammatory mediator [22–24] and cell surface CD10/NEP regulates met-enkephalin-associated inflammatory responses by controlling the local concentration of the peptide [24]. Shipp et al. [24] revealed that inhibition of NEP enzymatic activity reduces the amount of met-enkephalin required to trigger changes in the shape and size of neutrophils. The mechanism, however, remains to be elucidated. Several lines of evidences, as described above, suggest that morphine and other endogenous opioids be linked to neutrophil–endothelium activation via the common acute lymphoblastic leukemia antigen (CALLA) (CD10)/NEP pathway. The mechanism by which the opiate receptors in the neutrophils operate on NEP remains to be investigated. In addition, because opioid receptors are widely distributed, especially in the central nervous system [33], the systemic effect of morphine cannot be excluded. In general, blockade of adhesion, or lack of adhesion, results in transient neutrophilia. The phenomenon did occur in our study. The systemic effects of morphine, which induce release from marrow stores, may be tested in the future.

In this study, morphine PC activates NEP and pretreatment with naloxone, a μ-opioid receptor antagonist, will abolish this phenomenon. On the other hand, pretreatment with chelerythrine (a protein kinase C inhibitor) or glibenclamide (an ATP-sensitive potassium channel antagonist) did not alter the action of morphine PC on neutrophil–endothelium activation, although some degree of reduction in infarct size did exist. This means that neither protein kinase C nor an ATP-sensitive potassium channel is involved in the effects of morphine. The μ-opioid receptor-specific pathway may therefore be responsible. The modest reduction in infarct size in the chelerythrine and glibenclamide-treated groups provides evidence that multiple signaling pathways are involved in myocardial PC.

In conclusion, morphine increases NEP activities to attenuate shedding of L-selectin and to ICAM-1 via a μ-opioid-receptor-specific pathway. These data support the theory that morphine PC provides an additional cardioprotective effect that was previously unrecognized.

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