Role of endogenous opioids in ischemic preconditioning but not in short-term hibernation in pigs

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Schulz, Rainer, Petra Gres, and Gerd Heusch. Role of endogenous opioids in ischemic preconditioning but not in short-term hibernation in pigs. Am J Physiol Heart Circ Physiol 280: H2175–H2181, 2001.—Endogenous opioids are involved in ischemic preconditioning (IP) in several species. Whether or not opioids are important for IP and short-term myocardial hibernation (STMH) in pigs is currently unknown. In 34 enflurane-anesthetized pigs, the left anterior descending coronary artery was flow constantly perfused. Subendocardial blood flow (Endo), infarct size (IS; percent area at risk), and the free energy change of ATP hydrolysis (ΔG) were determined. After 90-min severe ischemia and 15-min reperfusion reduced IS to 9.9 ± 6.1% (means ± SE) (n = 8; Endo: 0.047 ± 0.009 ml·min⁻¹·g⁻¹). IP by 10-min ischemia and 15-min reperfusion reduced IS to 9.9 ± 3.8% (P < 0.05, n = 8; Endo: 0.044 ± 0.009 ml·min⁻¹·g⁻¹). After naloxone (1 mg·kg iv followed by 2 μg·kg⁻¹·min⁻¹), IS averaged 25.8 ± 7.0% (n = 6; Endo: 0.039 ± 0.008 ml·min⁻¹·g⁻¹) without and 24.7 ± 4.7% (n = 6; Endo: 0.044 ± 0.006 ml·min⁻¹·g⁻¹) with IP. At 5-min moderate ischemia in the presence of naloxone, Endo decreased from 0.90 ± 0.07 to 0.28 ± 0.03 ml·min⁻¹·g⁻¹ and ΔG decreased from −58.6 ± 1.0 to −52.6 ± 0.4 kJ/mol. Prolongation of ischemia to 90 min did not alter Endo, but ΔG recovered toward control values (57.7 ± 1.1 kJ/mol), and the myocardium remained viable. These responses are identical to those of nonnaloxone-treated pigs. Endogenous opioids are involved in IP but not in STMH in pigs.

infarction; myocardial ischemia; free energy change

p-ENDORPHINE PLASMA CONCENTRATIONS are increased in patients during acute myocardial ischemia, such as during percutaneous transluminal coronary angioplasty (PTCA) (15, 30) and after myocardial infarction (36). In these experiments, there is good evidence that opioids attenuate the consequences of ischemia-reperfusion. Blockade of opioid receptors with naloxone abolishes 1) the ischemia-induced decrease in mean arterial pressure (16) and 2) the morphine-induced decreases in heart rate and arterial pressure and the ischemia-induced arrhythmias in anesthetized pigs (5).

Ischemic preconditioning is the earliest stress response that occurs during episodes of brief ischemia and reperfusion, and it can render the myocardium more tolerant to subsequent lethal ischemic injury (39). Ischemic preconditioning occurs in two different phases: first, an early immediate effect [classic ischemic preconditioning (IP)] and, second, a late effect (late phase of preconditioning) (3, 20, 57). Naloxone abolishes the infarct size reduction achieved by IP in rats (17, 45, 46) and rabbits (13, 34), and it prevents the attenuation of electrocardiogram changes during repeated PTCA in humans (52). Conversely, activation of opioid receptors reduces infarct size during subsequent ischemia to the same extent as IP in rats (27, 42, 44); the important opioid receptor for such cardioprotection in rats appears to be the δ-opioid receptor (1, 43). The activation of δ-opioid receptors also induces the late phase of preconditioning in rats (17). The signal cascade after activation of opioid receptors involves activation of pertussis toxin-sensitive G proteins in rats (44), protein kinase C in rabbits (34), and ATP-dependent potassium channels in rats (27, 42, 44) and isolated cardiomyocytes from chick embryos (31). Whether or not endogenous opioids are also involved in infarct size reduction by IP in pigs is unclear at present, because certain species differences in the mechanisms of ischemic preconditioning exist (12).

During more moderate ischemia, the myocardium does not inevitably undergo necrosis but can adapt to reduced blood flow through a regulatory reduction in contractile function (22, 38). Therefore, loss of contractile function in patients with coronary artery disease frequently does not indicate infarction but a downregulated state of myocardial “hibernation” (38). A close relationship between reduced myocardial blood flow and contractile function, i.e., perfusion-contraction matching, is a key feature of “short-term myocardial hibernation” (STMH) in the experimental setting (40). Further characteristics of myocardial hibernation are recovery of energy metabolism during ongoing ischemia, persistence of an inotropic reserve, recovery of contractile function on reperfusion, and, almost by definition, absence of necrosis (22). No mechanism underlying myocardial hibernation, other than reduced calcium responsiveness (24), has so far been identified.
Whereas the term hibernation was initially borrowed from zoology as a paradigm to characterize the endogenous cardiac protection during ischemia, recent studies indicate that the serum of truly hibernating mammals does indeed contain an opioid-like protein (25) that acts to preserve myocardial ultrastructure (8, 27) and improve functional recovery from ischemia (7, 8, 27) when given to nonhibernating animals. However, whether or not the development of successful STMH during ischemia is also triggered by endogenous opioids is unclear at present. Pigs were used for the study of involvement of endogenous opioids in both IP and STMH because their coronary anatomy (55), extent of collateral flow (56), and time course of infarct development most closely resemble those observed in humans (41).

We therefore tested in an established pig model of IP and STMH whether or not blockade of opioid receptors with naloxone 1 abolishes infarct size reduction by IP and 2 interferes with the development of STMH.

MATERIALS AND METHODS

Experimental Preparation

Thirty-four Göttinger minipigs (20–40 kg) of either sex were initially sedated using ketamine hydrochloride (1 g im) and then anesthetized with thiopental (Trapanal; 500 mg iv). Through a midline cervical incision, the trachea was intubated for connection to a respirator (Dräger; Lübeck, Germany). Anesthesia was then maintained using enflurane (1–1.5%) with an oxygen/nitrous oxide mixture (40:60%). Arterial blood gases were monitored frequently in the initial stages of the preparation until stable and then periodically throughout the study (Radiometer; Copenhagen, Denmark). Rectal temperature was monitored, and body temperature was kept between 37 and 38°C using heating pads. Pigs were instrumented for the measurement of left ventricular pressure and wall thickness (23, 47). After heparinization, the left anterior descending (LAD) coronary artery and vein were cannulated, and the artery was perfused by an extracorporeal circuit including an occlusive roller pump. Heart rate was held constant by left atrial pacing.

Regional Myocardial Function, Blood Flow, and Metabolism

Regional systolic wall thickening (sonomicrometry) was calculated. Regional myocardial blood flow was measured with microspheres, and myocardial oxygen and lactate consumptions were calculated (23). Transmural biopsies were taken to determine the free energy change of ATP hydrolysis (32).

Morphology

Six transverse myocardial slices from each heart were incubated in triphenyltetrazolium chloride (TTC) solution to identify necrotic tissue (47).

Experimental Protocols: Ischemic Preconditioning

Group 1 (n = 8). After control measurements of systemic hemodynamics, regional myocardial function, blood flow, and metabolism were performed, the myocardium was subjected to one cycle of 10-min preconditioning ischemia, with a 90% reduction in regional myocardial function, and 15-min reperfusion. During reperfusion, coronary arterial pressure was maintained at the level measured before ischemia by continuously adapting coronary inflow with the roller pump. After reperfusion, coronary inflow was once again reduced to the same level as during the preconditioning ischemia. Thereafter, the protocol of group 2 was identical to that of group 1.

Group 3 (n = 6). After control measurements of systemic hemodynamics, regional myocardial function, blood flow, and metabolism were performed, naloxone was given as a bolus of 1 mg/kg iv, followed by a continuous intravenous infusion of 2 μg·kg⁻¹·min⁻¹ until the end of the 90-min ischemic period. This dose of naloxone has been previously shown to completely block the morphine-induced decreases in heart rate and blood pressure in anesthetized pigs (5). Thirty minutes after the bolus injection of naloxone was given, all measurements were repeated before coronary inflow was reduced.

Group 4 (n = 6). The protocol of group 4 was identical to that of group 2 except that the naloxone administration was started 30 min before the first ischemia. Once again, naloxone was given as a bolus of 1 mg/kg iv, followed by a continuous intravenous infusion of 2 μg·kg⁻¹·min⁻¹ until the end of the 90-min ischemic period.

Data Analysis and Statistics

Data are reported as mean values ± SE. Statistical analysis for groups 1–4 comprised two-way ANOVA for repeated measures and Fisher’s least significant difference tests when significant overall effects were detected. Data in group 5 were analyzed by one-way ANOVA and Fisher’s least significant difference tests. A P value < 0.05 was accepted as indicating a significant difference in mean values.

In groups 1–4, linear regression analyses between subendocardial blood flow at 5-min ischemia in the left ventricular area at risk and infarct size (expressed as a percentage of the area at risk) were performed. Regression lines were compared by analysis of covariance.

RESULTS

Data on systemic hemodynamics, regional myocardial function, blood flow, and metabolism in groups 1–5 are summarized in Tables 1 and 2. Heart rate was held constant by left atrial pacing. Regional myocardial function of the posterior control wall remained stable throughout the experimental protocol in each group.

Ischemic Preconditioning

Systemic hemodynamics, regional myocardial function, blood flow, and metabolism were not different
among groups 1–4 at baseline. Naloxone did not alter any of the measured parameters at baseline.

Systemic hemodynamics, regional myocardial function, blood flow, and metabolism were not different among groups 1–4 during ischemia except for a tendency toward a better-preserved left ventricular peak pressure and maximum first derivative of left ventricular pressure during ischemia in groups 1 and 3 (Table 1).
Table 2. Systemic hemodynamics, regional myocardial function, blood flow, and metabolism in pigs during short-term hibernation with naloxone

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Naloxone</th>
<th>5-Min Ischemia</th>
<th>85-Min Ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>104 ± 5</td>
<td>102 ± 5</td>
<td>102 ± 5</td>
<td>102 ± 1</td>
</tr>
<tr>
<td>LVPP</td>
<td>94 ± 4</td>
<td>92 ± 5</td>
<td>88 ± 4</td>
<td>82 ± 6</td>
</tr>
<tr>
<td>dPdI/dt max</td>
<td>1.258 ± 0.5</td>
<td>1.192 ± 0.5</td>
<td>1.061 ± 0.47</td>
<td>1.022 ± 0.73</td>
</tr>
<tr>
<td>CAP</td>
<td>111 ± 3</td>
<td>118 ± 6</td>
<td>42 ± 2*</td>
<td>39 ± 2*</td>
</tr>
<tr>
<td>CBF</td>
<td>31.7 ± 2.0</td>
<td>33.3 ± 2.2</td>
<td>15.8 ± 1.0*</td>
<td>15.8 ± 1.0*</td>
</tr>
<tr>
<td>AWT ref</td>
<td>12.69 ± 1.19</td>
<td>12.84 ± 1.24</td>
<td>12.21 ± 1.17</td>
<td>11.68 ± 1.25</td>
</tr>
<tr>
<td>AWT</td>
<td>38.6 ± 3.3</td>
<td>37.6 ± 7.4</td>
<td>23.2 ± 3.6*</td>
<td>20.6 ± 3.7*</td>
</tr>
<tr>
<td>TMF</td>
<td>0.79 ± 0.08</td>
<td>0.84 ± 0.10</td>
<td>0.35 ± 0.04*</td>
<td>0.37 ± 0.04*</td>
</tr>
<tr>
<td>Endo</td>
<td>0.86 ± 0.07</td>
<td>0.90 ± 0.07</td>
<td>0.28 ± 0.03*</td>
<td>0.29 ± 0.06*</td>
</tr>
<tr>
<td>MVCo2</td>
<td>64.9 ± 6.9</td>
<td>67.3 ± 5.4</td>
<td>37.0 ± 3.2*</td>
<td>35.1 ± 3.7*</td>
</tr>
<tr>
<td>MVlac</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>-0.4 ± 0.2*</td>
<td>-0.1 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. baseline.

Infarct Size

The area at risk was comparable between groups 1 and 4, averaging 43.6 ± 3.1, 48.7 ± 3.4, 47.3 ± 2.5, and 42.0 ± 3.5%, respectively (Fig. 1). After 90-min severe myocardial ischemia and 120-min reperfusion, infarct size averaged 28.3 ± 5.4% (group 1) (Fig. 1). IP by one cycle of 10-min ischemia and 15-min reperfusion reduced infarct size to 9.9 ± 3.8% (P < 0.05, group 2). The relationship between infarct size and subendocardial blood flow in group 2 was significantly shifted downward compared with the relationship obtained in group 1 (Fig. 2).

In the presence of naloxone after 90-min sustained ischemia and 120-min reperfusion (group 3), 25.8 ± 7.0% of the area at risk was infarcted, and infarct size was unchanged (24.7 ± 4.7%) with IP (group 4) (Fig. 1). Also, the relationships between infarct size and subendocardial blood flow in groups 3 and 4 were superimposable (Fig. 3).

Short-Term Myocardial Hibernation

Naloxone did not alter any of the measured parameters at baseline. Left ventricular pressure slightly decreased with the initiation of ischemia and decreased further when ischemia was prolonged to 90 min.

With the reduction in coronary inflow, mean coronary arterial pressure, anterior systolic wall thickening, transmural and subendocardial blood flows, myocardial oxygen consumption, and the free energy change of ATP hydrolysis in the anterior wall were decreased (Table 2 and Fig. 4). Myocardial lactate consumption was reversed to net lactate production. Prolongation of ischemia to 85 min did not result in a further change (Table 2) except that the free energy change of ATP hydrolysis recovered toward control values (Fig. 4). Myocardial necrosis (TTC staining) was
absent in all animals after 90-min moderate ischemia and 120-min reperfusion.

This is exactly the same pattern of responses as seen in previous studies (23, 32) on STMH with the use of the same animal model in the absence of naloxone.

**DISCUSSION**

The major findings of the present study are that blockade of opioid receptors with naloxone completely abolished the infarct size reduction achieved by IP and that the same dose of naloxone did not interfere with the development of STMH.

**Critique of Methods**

The strengths and limitations of the experimental model have been discussed in detail elsewhere (23, 47). Pigs were used for the study of involvement of endogenous opioids in both IP and STMH because their coronary anatomy (55), extent of collateral flow (56), and time course of infarct development most closely resemble those observed in humans (41).

Naloxone was given at a dose of 1 mg/kg iv, followed by a continuous infusion of 2 μg·kg⁻¹·min⁻¹, resulting in a total dose of ~1.2 mg/kg. This dose of naloxone did not alter baseline systemic hemodynamics or regional myocardial function. Similarly, naloxone at concentrations of up to 10 mg/kg had no effect on systemic hemodynamics in squirrel monkeys (11). This dose of naloxone, however, effectively blocks opioid receptors, as previously shown by blockade of the morphine-induced decreases in heart rate and blood pressure in anesthetized pigs (5) and evidenced by loss of the infarct size reduction by IP in the present study. Naloxone had no direct unspecific effect on the myocardium, because infarct size in untreated (group 1) and naloxone-treated (group 3) pigs was similar after 90-min ischemia and 120-min reperfusion.

**Ischemic Preconditioning**

In rats and rabbits, naloxone has been previously shown to block the infarct size reduction achieved by IP (13, 34, 45, 46). In naloxone-treated patients, S-T segment changes and cardiac pain severity during a second PTCA balloon inflation were similar to those observed during the first inflation, whereas they were significantly attenuated in patients given placebo (52). This effect on S-T segment changes has been regarded as evidence for IP in the human heart; however, experiments in rabbits revealed that electrocardiogram changes associated with IP protocols are, rather, epiphenomena and do not reflect the protection of IP (6).

Our data confirm, for the first time in pigs, those previous findings of a protective effect of endogenous opioids during severe myocardial ischemia. The signal cascade of IP in pigs involves several endogenous triggers such as adenosine (49) and bradykinin (47), subsequent activation of protein kinase C and a protein tyrosine kinase (54), and activation of ATP-dependent potassium channels (48). More recently, the activation of p38 and extracellular signal-regulated kinases (ERK)/mitogen-activated protein kinases (MAPK) has also been demonstrated during IP in pigs (2, 4). The present study adds one more endogenous trigger to those involved in the signal cascade of IP in pigs, i.e., opioids. Infusion of opioid receptor agonists reduced infarct size to the same extent as IP in pigs, i.e., opioids. Infusion of opioid receptor agonists reduced infarct size to the same extent as IP in rats (27, 42). The important opioid receptor for such cardioprotection was identified as the δ-opioid receptor (1, 43), whereas activation of the κ-opioid receptor appears to be even detrimental (1) with the possibility of putting the heart into an “antipreconditioning” state (14). Blockade of opioid receptors with naloxone in the present and other studies worsened the outcome of ischemia and reperfusion, indicating that endogenous opioids act primarily on δ-opioid receptors rather than κ-opioid receptors.

The interaction of triggers of IP is rather complex (19). Bradykinin appears to be an important trigger with a weaker/more short-lasting IP stimulus, whereas adenosine gains more importance with stronger/longer stimuli (19, 47). In pigs, infarct size reduction achieved by 3-min preconditioning ischemia and 15-min reperfusion is completely abolished by blockade of bradykinin receptors, whereas the infarct size reduction after 10-min preconditioning ischemia and 15-min reperfusion is largely attenuated by destruction of adenosine with adenosine deaminase (47, 49). As blockade of opioid receptors (present paper) also almost completely abolishes infarct size reduction by IP in swine, both adenosine and opioids appear to trigger IP in an interactive fashion. Whether both signals are oriented in parallel or back-to-back remains to be established. The latter explanation has recently been suggested from a study (26) in isolated rat hearts in which the fentanyl-induced increase in contractile function after ischemia-reperfusion was abolished by pretreatment with an adenosine receptor antagonist.
The mechanisms involved in infarct size reduction by opioid receptor activation in other species involve activation of pertussis toxin-sensitive G proteins in rats (44), protein kinase C in rabbits (34), and ATP-dependent potassium channels in rats (27, 42) and isolated cardiomyocytes from chick embryos (31). The further signal cascade leading finally to cardioprotection has not yet been established. In vitro data in transfected COS-7 cells (African green monkey kidney cells) suggested that opioid receptor activation, especially that of the δ- and κ-subtype, is associated with increased phosphorylation of ERK but not p38 MAPK (21). While ERK are activated during ischemia-reperfusion in rabbits (37) and pigs (4) in vivo, their importance for the infarct size reduction by IP remains controversial (29, 51). The signal cascade of opioid receptor activation-induced protection in pigs is entirely unknown; however, in a recent study (51), blockade of ERK abolished IP in pigs.

**Short-Term Myocardial Hibernation**

We used an established model of STMH in which regional low-flow ischemia was induced by controlled hypoperfusion of the LAD (23, 32). Myocardial blood flow was reduced by ~50% such that regional contractile function decreased to ~50% of baseline. Under these conditions, the initially impaired metabolism recovered over time, and necrosis was absent even when ischemia was extended to 90 min. The hemodynamic and metabolic data in the animals used for the present study in the presence of naloxone are comparable to previously published data in the absence of naloxone (23, 32).

Opioids appear to be involved in true mammalian hibernation. Plasma from a hibernating bear caused ground squirrels, normally active during the summer, to hibernate. This induction of summer hibernation is effectively blocked by naloxone (25). Similarly, infusion of a δ-opioid receptor agonist induces hibernation in ground squirrels (25). The hibernation-inducing trigger or hibernation-related factor has been identified as an opioid-like 88-kDa protein (25), which may be either a precursor or a potent releaser of endogenous opioids (10, 35). Whereas blockade of opioid receptors with naloxone thus abolishes the induction of true mammalian hibernation, it had no effect on the development of STMH in vivo in the present study.

Therefore, endogenous opioids have a role similar to that of endogenous adenosine and ATP-dependent potassium channels in that they are involved in the endogenous cardioprotection provided by IP in pigs (48, 49) but not in the development of STMH (50). The present data once more support the notion that IP and STMH, although both are cardioprotective phenomena initiated during ischemia, are mechanistically different.

**Clinical Implications**

Given the experimental data, one can assume that patients receiving opioid receptor agonists during cardiac surgery involving ischemia-reperfusion (33, 53) will potentially benefit apart from and in addition to pain relief. Conversely, patients receiving opioid receptor antagonists for detoxification after a narcotic overdose or as part of the treatment of drug abuse (9, 18, 28) are potentially at increased risk.

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