The δ-opioid receptor agonist DADLE at reperfusion protects the heart through activation of pro-survival kinases via EGF receptor transactivation

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Förster K, Kuno A, Solenkova N, Felix SB, Krieg T. The δ-opioid receptor agonist DADLE at reperfusion protects the heart through activation of pro-survival kinases via EGF receptor transactivation. Am J Physiol Heart Circ Physiol 293: H1604–H1608, 2007. First published June 1, 2007; doi:10.1152/ajpheart.00418.2007.—The specific δ-opioid receptor agonist [d-Ala²,d-Leu⁵]enkephalin (DADLE) protects against infarction in the heart when given before ischemia. In rabbit, this protection leads to phosphorylation of the pro-survival kinases Akt and extracellular signal-regulated kinase (ERK) and is dependent on transactivation of the epidermal growth factor receptor (EGFR). DADLE reportedly protects rat hearts at reperfusion. We therefore tested whether DADLE at reperfusion could protect isolated rabbit hearts subjected to 30 min of regional ischemia and 120 min of reperfusion and whether this protection is dependent on Akt, ERK, and EGFR. DADLE (40 nM) was infused for 1 h starting 5 min before reperfusion and reduced infarct size from 31.0 ± 2.3% in the control group to 14.6 ± 1.6% (P = 0.01). This protection was abolished by cotreatment of the metalloproteinase inhibitor (MPI) and the EGFR inhibitor AG1478. In contrast, 20 nM DADLE, although known to be protective before ischemia, failed to protect. Western blotting revealed that DADLE’s protection was correlated to increase in phosphorylation of the kinases Akt and ERK1 and -2 in reperfused hearts (2.5 ± 0.5, 1.6 ± 0.2, and 2.3 ± 0.7-fold of baseline levels, P < 0.05 vs. control). The DADLE-dependent increases in Akt and ERK1/2 phosphorylation were abolished by either MPI or AG1478, confirming a signaling through the EGFR pathway. Additionally, DADLE treatment increased phosphorylation of EGFR (1.4 ± 0.2-fold, P = 0.03 vs. control). Thus the δ-opioid agonist DADLE protects rabbit hearts at reperfusion through activation of the pro-survival kinases Akt and ERK and is dependent on the transactivation of the EGFR.

It is well established that activation of opioid receptors before ischemia can protect the heart against infarction, and indeed opioid receptor activation is partially responsible for the protection seen with ischemic preconditioning (8). Opioid receptors constitute a family of G protein-coupled receptors (GPCR) that mediate their protective properties via kinase signaling, including protein kinase C (PKC), phosphatidylinositol 3-kinase (PI 3-kinase), Akt, and extracellular signal-related kinase (ERK). Various reports have demonstrated that some G₂-coupled GPCRs propagate their signals through epidermal growth factor receptor (EGF) transactivation (4). Cross-communication between GPCRs and EGFR is still not fully understood, but in some cases receptor agonist binding leads to metalloproteinase-dependent cleavage of heparin-binding EGF-like growth factor (HB-EGF) from membrane-associated pro-HB-EGF, and HB-EGF then presumably interacts directly with the ectodomain of the EGFR to activate the intracellular signal in a Src-dependent manner (16). We recently presented evidence that this pathway is used in the heart during pharmacological preconditioning with ACh (14). That evidence included the observation that ACh-induced cardioprotection could be aborted by blocking metalloproteinases, a pivotal step in the EGFR transactivation pathway, and that the EGFR antagonist AG1478 could block ACh-induced phosphorylation of Akt. A similar coupling was described by Cao et al. (2) for opioid signaling in response to the endogenous opioid peptide Met²-enkephalin (ME). Protection afforded by ME again occurred via EGFR transactivation and was dependent on Src, PI 3-kinase/Akt, ERK, and PKC. Furthermore, we recently observed that pretreatment with the highly selective δ-opioid agonist [d-Ala²,d-Leu⁵]enkephalin acetate (DADLE) decreased infarct size in isolated rabbit hearts and was again dependent on EGFR transactivation (3). That report also revealed that not all protective receptors use EGFR transactivation in their signaling. Bradykinin’s protection couldn’t be blocked with the metalloproteinase inhibitor (MPI), indicating that bradykinin’s pathway did not involve the EGFR, but, like protection from the muscarinic receptor, was still dependent on PI 3-kinase, Src kinase, and ERK. We (22) and others (1) have found bradykinin to have a profound anti-infarct effect when given just before reperfusion after a lethal ischemic insult. Yet ACh, which stimulates an identical pathway downstream from EGFR, was not protective when given at reperfusion (22). This would suggest that success of a drug given at reperfusion might depend on whether or not it coupled through EGFR. Gross et al. (9) recently reported that the nonselective opioid receptor agonist morphine and the selective δ-opioid receptor agonist BW-373U86 given right at reperfusion in an in vivo rat heart model could reduce infarct size, and this protection was also dependent on PI 3-kinase/Akt and the Akt downstream target GSK-3β. We, therefore, asked whether the δ-agonist DADLE would also be able to protect the ischemic rabbit heart if started at reperfusion. Moreover, we tested whether DADLE could phosphorylate the protective kinases Akt and ERK and whether these steps were dependent on EGFR transactivation.

METHODS

All experiments were performed in accordance with The Guide for the Care and Use of Laboratory Animals (National Institutes of Health). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
isolated heart model. Male New Zealand White rabbits were used as previously described (5). Briefly, animals were anesthetized with intravenous pentobarbital sodium (35 mg/kg). After a tracheotomy, animals were ventilated with 100% oxygen. The chest was opened via a left thoracotomy, and the heart was exposed by opening the pericardium. A 2-0 silk suture was passed around a major branch of the left coronary artery. The heart was rapidly removed and mounted on a Langendorff apparatus. Perfusion was started immediately using a modified Krebs-Henseleit bicarbonate buffer containing (in mM): 118.5 NaCl, 24.8 NaHCO3, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, and 10 glucose.

A fluid-filled latex balloon was inserted in the left ventricle and inflated to record the heart rate and pressure throughout the experiment. After equilibration, hearts underwent a 30-min period of regional ischemia by occluding the encircled artery followed by 2 h of reperfusion. Infarct size was measured in seven groups of hearts. Control hearts received no treatment, whereas one group received DADLE (40 nM) for 1 h beginning 5 min before reperfusion. The next two groups received DADLE as mentioned above and, additionally, the metalloproteinase inhibitor MPI (0.8 μM) or the EGFR inhibitor AG1478 (1 μM), starting 5 min before the DADLE treatment. MPI and AG1478 were tested alone to rule out any unspecific effects of the inhibitors. The final group received DADLE in a lower concentration of 20 nM since we observed previously that 20 nM for 5 min was sufficient to precondition the rabbit heart before ischemia (13).

Infarct size measurement. At the end of reperfusion, the snared artery was reoccluded, and 2–9 μm green fluorescent microspheres were infused to delineate the risk zone. Hearts were frozen and cut into 2-mm transverse slices, and infarction was measured with triphenyltetrazolium chloride staining. Areas of risk zone and infarction were determined by planimetry of each slice, and volumes were calculated by multiplying area by slice thickness. Volumes were summed for each heart. Infarction is presented as a percentage of the risk zone.

Biochemical studies. The biochemical studies were performed as described previously in detail (5). Briefly, rabbit hearts were retrogradely perfused as described above. A protocol with 30 min of global ischemia followed by 5 min of reperfusion was applied. Serial transmural biopsies were taken with a motorized biopsy tool as indicated in Fig. 1. The first biopsy was taken before ischemia and served as the baseline. At the end of reperfusion, a second sample was collected. The following groups were studied. Hearts of the first group were untreated and served as controls. In the second group, DADLE (40 nM) was administered at reperfusion as described above. The next groups were treated with DADLE as above but in the presence of either MPI III (3 μM), the EGFR blocker AG1478 (1 μM), or the δ-opioid receptor antagonist naltrindole (10 nM). Finally, DADLE was used at the concentration of 20 nM, and naltrindole was tested alone.

Biopsies were snap-frozen, pulverized in liquid nitrogen, and transferred to tubes containing ice-cold lysis buffer to which the protease inhibitor phenylmethylsulfonyl fluoride (1 mM) was added. Samples were centrifuged at 13,000 g for 15 min at 4°C, and the protein content of the supernatant was analyzed by BCA protein assay (Pierce Biotechnology, Rockford, IL). Dithiothreitol-containing Laemmli sample buffer was added to the remaining supernatant. Equal amounts of each sample (50 μg for Akt and ERK, and 100 μg for EGFR) were electrophoresed on a 10% polyacrylamide gel (6% for the EGFR measurements) followed by transfer to nitrocellulose membranes. Afterward, membranes were blocked in 5% milk and treated with the primary antibodies directed against the phosphorylated form of Akt (1:1,000), ERK1/2 (1:2,000), and EGFR (1:400). A horseradish peroxidase-conjugated secondary antibody (1:5,000) was used to detect immunoreactive proteins by enhanced chemiluminescence.
31.0 ± 2.3% in the control group, *P = 0.01). Cotreatment with the metalloproteinase inhibitor MPI and the EGFR inhibitor AG1478 abolished this protection (31.0 ± 3.8% for MPI and 35.3 ± 3.6% for AG-1478, both *P < 0.05 vs. DADLE). MPI and AG1478 alone had no effect (31.1 ± 3.7 and 28.3 ± 1.7%). Although protective when given before ischemia, DADLE at a lower concentration of 20 nM failed to protect when given before reperfusion (33.9 ± 4.6%, *P = 0.003 vs. 40 nM DADLE).

Biochemical studies. Phosphorylation of Akt (Ser473) in the myocardium reperfused for 5 min was expressed as a ratio of that seen before ischemia. In untreated hearts, phosphorylation was slightly decreased at reperfusion (0.7 ± 0.2-fold), whereas treatment of the hearts with DADLE at reperfusion led to a significant increase in phosphorylation (2.5 ± 0.5-fold, *P < 0.001 vs. control). This increase was totally abolished by cotreatment with the metalloproteinase inhibitor MPI (1.1 ± 0.2-fold), the EGFR blocker AG1478 (0.8 ± 0.2-fold), and the δ-opioid receptor antagonist naltrindole (0.8 ± 0.08-fold).

DADLE at the lower concentration and naltrindole alone had no effect (0.8 ± 0.2-fold for 20 nM DADLE, and 0.7 ± 0.2-fold for naltrindole alone, not significant). Figure 3 shows the data of at least four independent experiments for each group.

Similar results were obtained by probing the samples with a phosphospecific antibody against ERK1 (Fig. 4A) and ERK2 (Fig. 4B). Administration of DADLE (40 nM) at reperfusion resulted in an increase in the phosphorylation of both ERK isoforms when compared with control hearts (1.6 ± 0.2-fold for ERK1, and 2.3 ± 0.7-fold for ERK2, both *P < 0.01 vs. control). Cotreatment with MPI blocked this DADLE-induced phosphorylation of both kinases. The same was true for AG1478. Unlike the case with Akt, ERK phosphorylation remained increased in the lower DADLE dose of 20 nM, although it did not reach significance with five experiments.

Furthermore, treatment with DADLE (40 nM) led to a significant increase in EGFR phosphorylation compared with untreated controls (1.4 ± 0.2- vs. 0.8 ± 0.1-fold, *P = 0.03, Fig. 5). Therefore, these data indicate that DADLE is signaling through the EGFR transactivation pathway.

**DISCUSSION**

The outcome of the present investigation supports the concept that the δ-opioid receptor agonist DADLE protects when given at the onset of reperfusion in isolated rabbit hearts. DADLE’s cardioprotection as well as its activation of the prosurvival kinases Akt and ERK1/2 could be blocked by either an matrix metalloproteinase (MMP) inhibitor or an EGFR inhibitor, suggesting a mechanism involving transactivation of the EGFR. A higher dose of DADLE was required to protect when given at reperfusion as opposed to treatment before ischemia.

Preconditioning with short ischemic periods or pharmacological triggers is a powerful tool to reduce infarct size using the heart’s own endogenous mechanisms. A similar procedure of transient ischemia-reperfusion cycles at the beginning of reperfusion, called postconditioning, is equally protective and has a higher clinical relevance, since patients with myocardial infarction can potentially be treated following the ischemic event (20). When compared with preconditioning, many signaling elements of postconditioning have been found to be identical, like redox signaling through mitochondrial ATP-sensitive potassium channels (15) and downstream activation of PI 3-kinase, Akt, ERK1/2, and NOS (11). Here, we could show for the first time that the specific δ-opioid receptor agonist DADLE given at reperfusion protects the heart through a metalloproteinase-dependent transactivation of the EGFR, which is the same protective signaling cascade it uses to trigger preconditioning (2).

There is evidence that opioid receptors have cross talk with EGFR via G protein subunits, and EGFRs are well known to signal through PI 3-kinase, Akt, and mitogen-activated protein kinases. One signaling mechanism for the transactivation of EGFRs involves metalloproteinases that contain a disintegrin and a metalloproteinase domain (ADAM). Although the protective properties of opioid receptors are well established, there is still debate whether their mode of action requires the above-described utilization of the EGFR. In HEK cells, Schulz et al. (18) found that ERK activation after opioid receptor stimula-

![Fig. 2. Infarct size expressed as a percentage of the risk zone. DADLE (40 nM) given for 1 h starting 5 min before reperfusion reduced infarct size, and this protection could be blocked by both the metalloproteinase inhibitor MPI and the EGFR receptor blocker AG1478. The blockers alone and DADLE at 20 nM had no effect. Individual data points: * group averages with SE. *P < 0.05 vs. control (*) and vs. 40 mM DADLE (#).](http://ajpheart.physiology.org/)

![Fig. 3. Mean levels of phosphorylated Akt normalized for Akt at baseline.](http://ajpheart.physiology.org/)
tion was dependent on the activation of MMPs and the transactivation of the EGFR, whereas Kramer et al. (12) had previously suggested an EGFR-independent activation. Cao et al. (2) demonstrated the ability of the opioid receptor agonist ME to protect isolated rat myocytes via MMPs and EGFR. There are also conflicting data in whole heart models. Fryer et al. (7) reported in an in vivo rat model that the opioid agonists DADLE and TAN-67, when given before ischemia as preconditioning agents, could not be blocked by either the Src inhibitor PP2 or Lavendustin, a tyrosine kinase inhibitor that has selectivity for the EGFR. On the other hand, in isolated rabbit hearts, DADLE's protection before ischemia could be abolished with inhibitors of MMPs or EGFR (3, 13). In the present report, we show for the first time that DADLE's protection at reperfusion could also be abolished with an MMP inhibitor as well as AG1478, a highly selective EGFR blocker. We also saw a phosphorylation of the EGFR as a result of DADLE treatment, further supporting a transactivation of this receptor system. How opioid receptors control metalloproteinases and subsequently the transactivation of the EGFR is still poorly understood. The literature provides examples of GPCRs causing an MMP-dependent cleavage of HB-EGF, which in turn leads to EGFR dimerization and, hence, receptor tyrosine kinase autophosphorylation (16, 17, 21). None of these signaling elements was investigated in the present study.

It is well established that virtually all preconditioning mimetics protect by stimulating downstream targets, including PI 3-kinase, Akt, and ERK early in the reperfusion period (11). In the present study, we observed an opioid receptor-dependent activation of Akt and ERK that was correlated with protection against infarction. This is in contrast to a report of Fryer et al. (6) who observed in situ rat hearts only ERK1 activation after δ-opioid stimulation with TAN-67, whereas ERK2 remained unchanged (6).

Although DADLE in the lower dose of 20 nM was protective in our hands when given before ischemia, it failed to protect when given at reperfusion in the present study. A higher dose of 40 nM, however, did prove to be protective. Because we observed that the highly selective δ-opioid receptor antagonist naltrindole could block DADLE’s ability to increase Akt phosphorylation, and others showed δ-opioid receptor selectivity at even higher concentrations (19), we assume that DADLE is still selective at 40 nM. Yet, a distinction between the two isoforms δ1 and δ2 cannot be made. This difference between the two DADLE concentrations was mirrored in the total lack of Akt phosphorylation and the blunted ERK phosphorylation seen in hearts treated with the lower dose. Correlation of Akt phosphorylation and protection was also seen in a recent report from Yang’s (22) group, where ACh, which is highly protective as a preconditioning agent, also caused a robust increase in Akt phosphorylation. However, ACh at reperfusion did not protect the hearts, and that correlated with a lack of effect on Akt phosphorylation.

Our results are in agreement with a study done by Gross et al. (9) in which the nonselective opioid receptor agonist morphine and the δ-selective opioid receptor agonist BW-373786 given at reperfusion were able to decrease infarct size in an in vivo rat model. The same group also showed kinase signaling, including Akt phosphorylation, after opioid treat-

Fig. 4. A and B: mean levels of phosphorylated extracellular signal-regulated kinase (ERK) 1 (A) and ERK2 (B) normalized for those seen at baseline. The same myocardial samples used in Fig. 3 were assayed for ERK1 and ERK2, drugs were infused, and biopsies were taken as depicted in Fig. 1. The DADLE group showed a significant increase of both ERK1 and ERK2 phosphorylation (*P < 0.01), and both could be blocked by the inhibitors MPI and AG1478, suggesting a role for EGF receptor transactivation. ERK phosphorylation was increased in the lower DADLE dose (20 nM) but did not reach significance (ns).

Fig. 5. Hearts (n = 4) were treated with DADLE (40 nM) at reperfusion accordingly. Mean levels of phosphorylated EGF receptor (EGFR) normalized for values seen at baseline showed a significant increase after DADLE treatment. *P = 0.03 vs. control.
ment at reperfusion with morphine and the δ-opioid agonist fentanyl isothiocynate (10).

In conclusion, we found compelling evidence that the δ-opioid receptor agonist DADLE given at reperfusion protects against infarction by activating the prosurvival kinases Akt, ERK1, and ERK2 through a metalloproteinase-dependent activation of the EGFR. DADLE’s protection at reperfusion requires higher concentration compared with that for treatment before ischemia and that correlated with the kinase phosphorylation.

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GRANTS

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