Fentanyl reduces infarction but not stunning via δ-opioid receptors and protein kinase C in rats

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Langendorff rat hearts were used (i) to examine whether fentanyl reduces stunning, infarction or both, and (ii) to investigate if this protection is mediated by δ-opioid receptors and/or protein kinase C (PKC). In the stunning study, hearts were subjected to global ischaemia (20 min) and reperfusion. This did not produce infarction. Postischaemic mechanical function was measured in hearts treated with or without fentanyl (740 nM). Fentanyl did not affect postischaemic mechanical function. In the infarction study, the left anterior descending coronary artery was occluded for 35 min and infarct size was assessed by triphenyltetrazolium chloride staining. Hearts in the control group exhibited an infarct zone/area at risk (I/R) of 39 (SEM 5)%, whereas the I/R for the fentanyl group was 13 (2)%. When the hearts were treated with a δ-opioid receptor antagonist (naltrindole 1 nM) or a PKC inhibitor (chelerythrine 2 μM), the effect of fentanyl was abolished, with I/R of 37 (1) and 36 (2)% respectively. In our model, we conclude that fentanyl protects against infarction but not against stunning, and that the limitation of ischaemic injury is mediated by both δ-opioid receptors and PKC.

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The last decades have witnessed a large increase in the number of surgical patients with ischaemic heart disease. Anaesthetists very often treat patients with coronary artery disease and make strenuous efforts to minimize perioperative myocardial ischaemia. More than 10 years ago it was reported that halothane and isoflurane protected against myocardial stunning. Additional evidence that inhalational anaesthetics diminish myocardial injury following ischaemia has been reviewed recently by Ross and Foëx.[1] Moreover, morphine has been found to limit infarct size,[2–4] hence there is increasing interest in opioids as cardioprotective agents.

Ischaemic preconditioning is another mechanism by which the myocardium can be protected against ischaemic injury. Ischaemic preconditioning describes the protection against subsequent damage caused by prolonged ischaemia, conferred by brief periods of ischaemia.[5–7] This protective state lasts 1–3 h and has been demonstrated in a wide variety of animal species and in humans.[5 6] Activation of protein kinase C (PKC) seems to play a major role in ischaemic preconditioning.[6 7] PKC reportedly opens K_{ATP} channels, whose central role in ischaemic preconditioning has been generally accepted.[6 7] PKC is stimulated by opioid receptor agonists as well as adenosine A_1, bradykinin and noradrenaline receptor agonists and free radicals.[6 7] All these substances are endogenously released in the myocardium by ischaemia and, therefore, are putative mediators of ischaemic preconditioning.[6 7]

In a previous report, we demonstrated that fentanyl alleviated postischaemic ventricular dysfunction and that this cardioprotective effect was mediated by opioid receptors and K_{ATP} channels.[8] We speculated that the cellular signal conferring the beneficial effect was passed from opioid receptors to K_{ATP} channels through the activation of PKC. Indeed, bradykinin,[9] adenosine,[10 11] endothelin[12] and noradrenaline,[13] which share common signal transduction pathways with opioids, diminish myocardial injury after ischaemia. Of further importance, this reduction in ischaemic damage is blocked by PKC inhibitors[9 10 12 13] or K_{ATP} channel blockers.[11 12] However, our previous study did not directly examine the role of PKC in the protection conferred by fentanyl.

Although fentanyl has a preferential affinity for μ-opioid receptors, it also interacts with δ- and κ-receptors.[14] Of the three major opioid receptors, δ- and κ- but not μ-receptors are expressed in the rat myocardium.[15] κ-Agonists have been reported to be arrhythmogenic and to worsen haemodynamics in ischaemia.[16 17] In contrast, δ-opioid receptors seem to be involved with myocardial protection against ischaemic damage.[3 18–21] This raises our second question,

of whether the attenuation of postischaemic dysfunction by fentanyl resulted from δ-opioid receptor stimulation.

The third question left unsolved was whether fentanyl protects against stunning or infarction, as we assessed left ventricular mechanical function. This can reflect the degree of both types of ischaemic injury, and we used a period of global ischaemia that lasted 30 min, which may result in a mixture of both types of damage.[22]

Therefore, we decided to examine (i) if fentanyl reduces stunning and/or infarction, and (ii) if the protection given by fentanyl is mediated by PKC and/or δ-opioid receptors.

Methods

The study was performed in accordance with the United Kingdom Animal Act (Scientific Procedures) 1986 (Home Office Project Licence number PPL 30/1496).

Preparation of rat hearts

The methods used have been described in detail elsewhere.[8] Adult male Wistar rats (325–400 g) were anaesthetized with intraperitoneal pentobarbitone 60 mg kg^{-1} and heparin 200 IU was administered. The hearts were rapidly excised, arrested in ice-cooled Krebs–Henseleit (KH) buffer, and perfused with KH solution by the Langendorff method. The perfusion pressure was set at 100 cm H_2O and kept constant. A polyvinyl balloon filled with KH solution was inserted through the left atrium into the left ventricle and connected to a pressure transducer. A small thermistor (T200K, Digitron Instruments, Hertford, UK) was placed in the left ventricle and the heart temperature was kept at 37°C during the procedure. All hearts were paced at a fixed rate of 300 bpm. The volume of the balloon was adjusted to achieve an end-diastolic pressure (EDP) between 5 and 10 mm Hg, which was maintained throughout the procedure. In the infarction study, a 3/0 silk suture (W598, Mersilk, Ethicon, Edinburgh, UK) was passed around the main branch of the left anterior descending coronary artery (LAD) to make a snare.

Experimental design

Figure 1 summarizes the procedure.

Stunning study

Groups stn-Con ($n=9$) received no drug. Fentanyl was administered to the perfusate in group stn-Fen ($n=9$) and remained in the perfusate for the rest of the procedure. After the drug incubation period, the aortic flow was stopped and global ischaemia induced. Ventricular pacing was stopped during ischaemia. After 20 min, aortic flow was reintroduced and continued for 120 min. Pacing was reintroduced 8 min into reperfusion and continued until the end of the procedure.

Infarction study

The hearts were allocated to one of six groups depending upon the chemicals added to the perfusate. Group inf-Con was treated with no drug ($n=6$), group inf-Fen with fentanyl ($n=6$), group inf-Nlt with the selective δ-opioid receptor antagonist naltrindole ($n=5$), group inf-Fen+Nlt with fentanyl and naltrindole ($n=5$), group inf-Che with the PKC inhibitor chelerythrine ($n=5$) and group inf-Fen+Che with fentanyl and chelerythrine ($n=5$). Regional ischaemia was induced by tightening the snare around the coronary artery. After an ischaemic period of 35 min, the snare was loosened and the heart was reperfused. Pacing was maintained throughout the procedure.

In both studies, haemodynamic measurements were taken after stabilization (pre-drug), before ischaemia (pre-ischaemia) and at several points during reperfusion. Measurements included developed pressure (DP), EDP, maximum positive and minimum negative derivative of left ventricular pressure ($+dP/dt_{max}$ and $-dP/dt_{min}$ respectively) and coronary flow (CF). Details of haemodynamic data collection have been published elsewhere.[8] At the end of perfusion, infarct size was measured using the triphenyltetrazolium chloride (TTC) staining method (see below). The concentrations of the drugs were fentanyl 740 nM (Janssen-Cilag, Buckinghamshire, UK), naltrindole 1 nM (Research Biochemicals International, MA, USA) and chelerythrine 2 μM (Research Biochemicals International).

Evaluation of infarct area

Stunning study

The heart was frozen and sliced into cross-sectional pieces just over 1 mm thick. The pieces were incubated in 1% TTC (Sigma Chemical Co., Dorset, UK) in 0.1 M phosphate buffer (pH=7.4) at 37°C for 20–30 min. This was used to distinguish unstained (grey) infarct and stained (red) non-infarct zones. The slices were immersed in 10% formalin in saline for 1–2 days. The slices were sandwiched between transparent plates to a thickness of 1 mm. The image of the heart slices were traced on a transparent sheet. Infarct and non-infarct areas were determined by planimetry using SigmaScan (Jandel Corporation, CA, USA).

Infarction study

The LAD occluder was retightened at the end of reperfusion and 0.125% Evans blue in saline was injected into the heart through the aorta for visualization of the non-ischaemic zone. Hearts were then sliced and stained in the same way as in the stunning study. Each non-ischaemic, risk (ischaemic but not infarct) or infarct area was determined by planimetry.

Statistics

All values are expressed as mean (SEM). Differences in haemodynamic indices between groups at various time points were compared using two-way analysis of variance (ANOVA) for treatment and time with repeated measures on a time factor. The comparison of infarct area/area at risk and area at risk was performed by one-way ANOVA combined with Bonferroni's test. Statistical significance was assumed at the $P<0.05$ level.

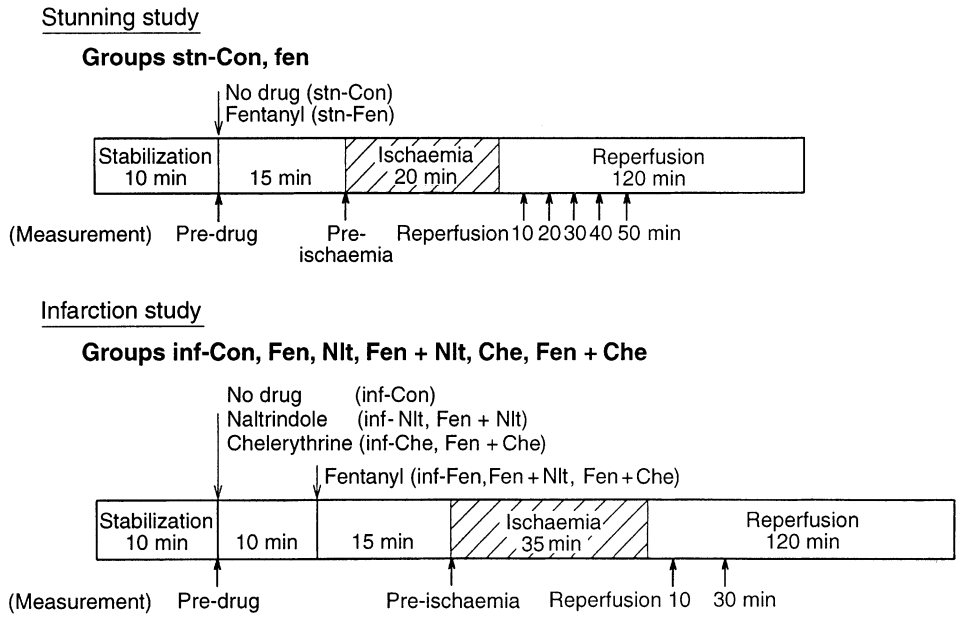

Fig 1 Procedure of the experiments.

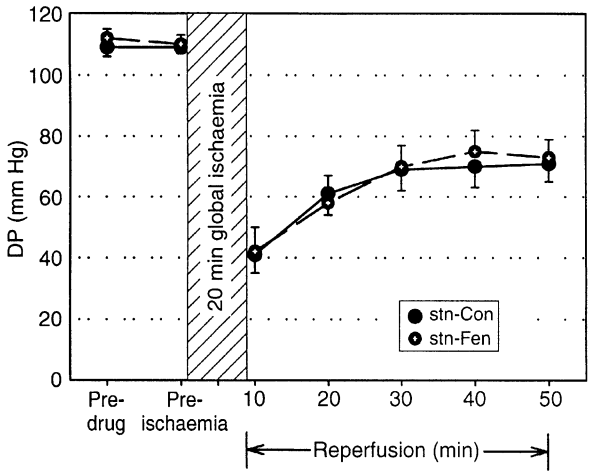

Fig 2 Recovery of developed pressure (DP) in the stunning study. Results are mean (SEM). No significant difference was found between the groups. stn=stunning; Con=control; Fen=fentanyl.

Results

Stunning study

The indices of mechanical function obtained for DP, PSP, EDP, $+dP/dt_{max}$, $-dP/dt_{min}$ and CF are shown in Figure 2 and Table 1. Groups stn-Con and stn-Fen showed similar recovery profiles and there were no significant differences in any index at any time. No unstained (infarct) area was detected by TTC in any hearts.

Infarction study

Table 2 illustrates haemodynamic parameters. No significant difference was found between the groups. Infarct area/area at risk (I/R) was 39 (5)% in the control group, whereas fentanyl significantly reduced I/R to 13 (2)% ($P<0.01$) (Fig. 3). Naltrindole and chelerythrine alone did not affect I/R (39 (2)% in inf-Nlt, 35 (2)% in inf-Che). When fentanyl was given with either naltrindole or chelerythrine, the extent of infarction did not differ from control (I/R was 37 (1)% for group inf-Fen+Nlt and 36 (2)% for group Fen+Che). The area at risk (mm^2) was 212 (12) in group inf-Con, 214 (8) in group Fen and 223 (7) in group Nlt, and 217 (8) in group Fen+Nlt, 207 (8) in group Che and 214 (8) in group Fen+Che (not significant).

Discussion

The present study shows that fentanyl reduced the extent of infarction. A similar effect has been demonstrated for morphine. In response to a LAD occlusion lasting 30 min in isolated rabbit hearts, morphine decreased infarct/area at risk from 32 to 9%.[4] Morphine also reduced necrosis in hearts *in vivo* [2–4] and in isolated ventricular myocytes;[21] this effect was abolished by an opioid receptor antagonist.[2 4 21] The attenuation of infarct area by fentanyl is consistent with these findings.

This cardioprotection produced by fentanyl was eliminated by the selective δ-opioid receptor antagonist naltrindole. Schultz and colleagues showed that a δ_1-opioid receptor agonist limited infarct size,[19] and that ischaemic preconditioning was mediated by δ- but not μ- or κ-receptors.[3 20] In addition, morphine-induced protection was demonstrated to result from δ-receptor activation.[3 21] It should be noted that these studies on opioid subtypes have been conducted in rats, a species in which μ-receptors have not been identified.[15] However, in other species, μ-receptors may be identified in the myocardium, and the activation of μ-receptors could affect the degree of ischaemic injury.

Table 1 Haemodynamic indices before and after ischaemia in the stunning study. Results are mean (SEM). There were no differences between the two groups in any index at any measurement point. DP=developed pressure; EDP=end-diastolic pressure; $+dP/dt_{max}$=maximum positive derivative of left ventricular pressure; $-dP/dt_{min}$=minimum positive derivative of left ventricular pressure; CF=coronary flow; stn=stunning; Con=control; Fen=fentanyl

	Before drug	Before ischaemia	Reperfusion (min)				
			10	20	30	40	50
DP (mm Hg)							
stn-Con	109 (3)	109 (2)	41 (6)	61 (7)	69 (7)	70 (7)	71 (5)
stn-Fen	112 (3)	110 (3)	42 (8)	58 (9)	70 (7)	75 (7)	73 (6)
EDP (mm Hg)							
stn-Con	6 (0)	6 (0)	55 (6)	40 (5)	35 (5)	33 (5)	33 (4)
stn-Fen	5 (0)	5 (0)	55 (7)	40 (5)	34 (5)	32 (4)	32 (4)
$+dP/dt$max (mm Hg s^{-1})							
stn-Con	2782 (77)	2857 (61)	861 (177)	1489 (227)	1778 (238)	1898 (214)	1922 (201)
stn-Fen	2937 (90)	2989 (83)	932 (227)	1500 (242)	1929 (227)	2151 (207)	2176 (176)
$-dP/dt$min (mm Hg s^{-1})							
stn-Con	2203 (126)	2157 (73)	592 (147)	1167 (179)	1342 (180)	1409 (166)	1408 (157)
stn-Fen	2112 (57)	2134 (57)	768 (165)	1148 (175)	1391 (147)	1481 (145)	1463 (125)
CF (ml min^{-1})							
stu-Con	18 (1)	17 (1)	13 (1)	14 (1)	14 (0)	14 (0)	13 (0)
stn-Fen	17 (1)	17 (1)	13 (1)	14 (1)	14 (1)	13 (1)	13 (1)

Table 2 Haemodynamic indices in the infarction study. Results are mean (SEM). No differences were found between groups. DP=developed pressure; EDP=end-diastolic pressure; CF=coronary flow; inf=infarction; Con=control; Fen=fentanyl; Nlt=naltrindole; Che=chelerythrine

	Before drug	Before ischaemia	Ischaemia (min)		Reperfusion (min)	
			5	35	10	30
DP (mm Hg)						
inf-Con	112 (6)	109 (8)	35 (3)	56 (5)	90 (3)	83 (7)
inf-Fen	111 (5)	107 (5)	37 (3)	57 (4)	86 (1)	77 (8)
inf-Nlt	112 (3)	108 (2)	42 (2)	58 (1)	90 (2)	87 (4)
inf-Fen+Nlt	108 (5)	103 (2)	42 (4)	59 (3)	86 (2)	81 (2)
inf-Che	112 (5)	109 (7)	41 (5)	58 (5)	92 (7)	92 (7)
inf-Fen+Che	108 (4)	105 (5)	47 (3)	63 (4)	87 (6)	84 (4)
EDP (mm Hg)						
inf-Con	6 (0)	5 (1)	3 (0)	4 (1)	10 (2)	9 (2)
inf-Fen	5 (0)	5 (1)	4 (0)	3 (0)	7 (1)	7 (1)
inf-Nlt	5 (1)	6 (1)	4 (1)	5 (1)	11 (2)	9 (2)
inf-Fen+Nlt	5 (1)	5 (1)	4 (0)	5 (1)	13 (3)	10 (2)
inf-Che	6 (0)	6 (0)	5 (1)	5 (1)	9 (1)	7 (1)
inf-Fen+Che	6 (1)	5 (0)	4 (0)	4 (0)	8 (1)	6 (1)
CF (ml min^{-1})						
inf-Con	19 (0)	18 (1)	8 (1)	8 (1)	17 (1)	17 (1)
inf-Fen	18 (1)	19 (2)	9 (1)	10 (1)	20 (1)	17 (1)
inf-Nlt	18 (1)	17 (0)	8 (1)	9 (1)	20 (2)	18 (2)
inf-Fen+Nlt	19 (1)	18 (1)	8 (1)	10 (1)	19 (1)	18 (1)
inf-Che	18 (0)	18 (1)	9 (1)	10 (1)	19 (1)	18 (1)
inf-Fen+Che	18 (1)	17 (1)	9 (1)	9 (1)	18 (1)	17 (1)

PKC transfers γ-phosphate from ATP to hydroxyl groups of serine/threonine residues in proteins. In the cardiac myocytes, this phosphorylation controls the function of many cellular effectors, and hence regulates various events, such as contraction and cell growth.[23] Consistent with our hypothesis, the PKC inhibitor chelerythrine eliminated the cardioprotective effect of fentanyl, resulting in increased infarct area. Miki and colleagues also showed in rabbits that chelerythrine abolished the infarct-limiting effect of morphine.[4] Our study also suggests that opioids reduce infarct size through PKC-linked mechanisms. PKC and K_{ATP} channels are signalling effectors shown to be central to ischaemic preconditioning.[6 7] In our previous study, we demonstrated that fentanyl elicits myocardial protection via opioid receptors and K_{ATP} channels. Taking into account previous findings, the present study strongly supports the PKC hypothesis for the protective mechanism of fentanyl; that is, the signal from fentanyl is passed to δ-opioid receptors, PKC and then K_{ATP} channels in the myocardium.

However, the PKC hypothesis remains somewhat controversial. Firstly, the link between δ-opioid receptors and PKC has not been characterized directly in the myocardium. In rat myocytes, Ventura and colleagues demonstrated that δ-receptor stimulation increased the level of inositol 1,4,5-triphosphate.[24] PKC-coupled receptors generally induce phosphoinositide turnover, leading to the production of

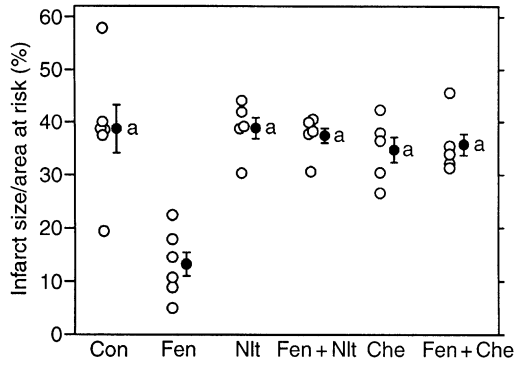

Fig 3 Infarct area/area at risk (I/R) after 35 min regional ischaemia. Open and closed circles represent individual animal I/R and group mean (SEM) respectively. a=$P<0.01$ compared with the group receiving fentanyl alone. Con=control; Fen=fentanyl; Nlt=naltrindole; Che=chelerythrine.

inositol 1,4,5-triphosphate and diacylglycerol; the latter activates PKC directly.[23 25] In glial cells, however, it is widely accepted that δ-opioid receptor agonists activate PKC[26 27], and this could be extrapolated to the myocardium.

Secondly, some authors have taken issue with a number of previous studies which support PKC as a major contributor to preconditioning.[23 28] They argue that what was considered as the effects of PKC agonists or antagonists could have been caused by other kinases. Most of the reports were based on the use of various PKC agonists and antagonists, many of which may not be specific for PKC and may affect other protein kinases. We also used a pharmacological method in this study, and therefore our findings are not free from this criticism. There are currently no reliable standard techniques; thus, the controversy over PKC will not be resolved conclusively until selective PKC inhibitors have been developed.[23 25]

PKC inhibitors have been reported recently to be cardioprotective against ischaemic injury at relatively low doses.[29 30] Chelerythrine 1 μM alleviated ischaemic injury in rats according to Lundmark and colleagues.[30] In contrast, Bugge and colleagues have reported that 2 μM chelerythrine blocked the infarct-reducing effect of ischaemic preconditioning,[31] bradykinin[9] and endothelin[12] without showing protective effects. Our finding is in agreement with the studies by Bugge and colleagues.[9 12 31]

It is generally agreed that K_{ATP} channels, which are primed by PKC, function as pivotal effectors in ischaemic and pharmacological preconditioning.[6 7] There are two kinds of K_{ATP} channels in the myocardium: sarcolemmal and mitochondrial K_{ATP} channels. Recent studies suggest that mitochondrial K_{ATP} mediate cardioprotection.[32] However, the nature of more distal effectors is currently poorly understood, and remains a controversial issue in the PKC hypothesis.

As we used a whole-heart model, the myocardium is not the only fentanyl-sensitive target. We should also take the vascular system in the heart into account. For example, nitric oxide (NO) is released from endothelial cells. Opioids increase the release of NO from the endothelial cells in the vascular system.[33] Hence, fentanyl could produce increased coronary collateral flow to ischaemic areas during ischaemia, reducing infarction. However, we believe this is unlikely, as no significant differences in coronary flow between groups were detected during ischaemia; moreover, collateral vasculature is not well developed in rat hearts.[34] Interestingly, NO is known to stimulate PKC[35] and K_{ATP} channels.[36] Therefore, fentanyl could have activated PKC and/or K_{ATP} channels via NO to reduce ischaemic damage.

This is the first attempt to determine the effect of an opioid on pure myocardial stunning. Our previous study showed that fentanyl did not alter haemodynamic indices in non-ischaemic hearts.[8] Therefore, the present study suggests that fentanyl did not affect the degree of stunning. Our group has reported previously that postischaemic systolic shortening of the left ventricular wall in fentanyl-anaesthetized rabbits was similar to that in isoflurane-anaesthetized animals.[37] This suggested that fentanyl might be protective against ischaemic injury, for there is general consensus that isoflurane attenuates the degree of ischaemic damage.[1] The reason why fentanyl reduced infarction but not stunning remains to be elucidated. A number of cellular events occur after the onset of ischaemia, and they become progressively more severe with time. The sequence is highly variable and as yet no clear border separating cell survival from death exists.[38] However, one hypothesis might be that there is an essential step in the course of developing ischaemic damage which makes the myocardium destined to die, and that fentanyl delays this process. Meldrum and colleagues suggested that different PKC isoforms are linked to different aspects of myocardial protection against ischaemic injury.[39] Fentanyl might have activated the specific PKC isoforms that reduce infarction but not stunning. Another explanation could be that fentanyl reduced infarction by increasing coronary collateral flow. It was not possible for fentanyl to exhibit protection in our stunning model because this involved global rather than regional ischaemia.

Although TTC staining is a reliable method for the quantification of infarct size,[40] it is not sensitive enough to detect necrosis of a single cell. This implies that a very small amount of infarction might have occurred in our stunning model. However, it is highly unlikely that infarction of a small fraction of myocardium affects the haemodynamics of the whole heart. Thus, we consider our model valid in assessing the degree of stunning.

We conclude that fentanyl limits infarction but does not attenuate stunning, and that this protection against infarction is mediated by an δ-opioid receptors and PKC-linked mechanism in our rat model. Further studies are needed to determine whether the contribution of fentanyl in decreasing infarction could be applied to clinical practice. The elucidation of its mechanism could lead to the development of novel interventions to reduce infarction in the future.

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