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Original Research

Effects of calcitonin gene-related peptide and BIBN4096BS on myocardial ischemia in anesthetized rats

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KEY WORDS calcitonin gene-related peptide; BIBN4096BS; myocardial infarction; reperfusion injury

ABSTRACT

AIM: The cardioprotective effect of calcitonin gene-related peptide (CGRP) was investigated in an ischemia rat model. **METHODS**: Ischemia-reperfusion injury was provoked by 60 min left main coronary artery occlusion followed by 60 min of reperfusion in anesthetized rats. The transverse slices of ventricles were stained by 2,3,5-triphenyltetrazolium chloride to determine the infarct area. Plasma creatine phosphokinase levels were determined by means of a creatine phosphokinase (CPK) kit. A radioimmunoassay was used to determine plasma CGRP levels. **RESULTS**: Intravenous infusion of CGRP (1 nmol·kg^{-1}·h^{-1}) 10 min before occlusion until the end of reperfusion reduced infarct size by 89 % ± 5 %. The reduction in infarct size was accompanied by a decrease in circulating levels of creatine phosphokinase. Infusion of the same dose of CGRP commencing from the start of reperfusion until its end induced a 40 % ± 3 % reduction of the infarct size. The cardioprotective effects of CGRP were blocked by the novel CGRP antagonist BIBN4096BS (20 nmol·kg^{-1}·h^{-1}). Although cardiac ischemia resulted in an almost 50 % increase in plasma CGRP levels in blood sampled from right cardiac ventricle, intravenous infusion of the CGRP antagonist BIBN4096BS before occlusion until the end of reperfusion had no statistically significant effect on the infarct size. **CONCLUSION**: The present study demonstrates that CGRP is a potent myocardial protective substance.

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide that is predominantly synthesized and stored in sensory neurons. It can be released from both the central and peripheral axons of these neurons[1]. CGRP-containing nerve fibers have been identified throughout the cardiovascular system, in association with blood vessels, in particular the coronary arteries, and around the sinoatrial and atrioventricular nodes[2,3]. CGRP is a potent vasodilator peptide and it exerts positive chronotropic and inotropic effects in rats and humans[4-7]. It has been shown to exert extremely potent vasodilator activity in isolated bovine, porcine, and human coronary arteries[8-10]. In addition, CGRP appears to be a more potent vasodilator in the small-diameter coronaries when compared with the large-diameter coronaries[11]. In patients with acute myocardial infarction, an almost two-fold increase of plasma CGRP level was observed within 24 h after hospital admission. This rise may result from the reflex release of CGRP in response to the reduction in myocardial perfusion[12,13]. CGRP has been tested in patients with chronic stable angina caused by coronary artery disease. It was shown to dilate coronary arteries at the site of atheromatous stenoses and to delay the onset of myocardial ischemia during exercise testing[14].

We previously reported on the first small molecule selective CGRP antagonist: BIBN4096BS, [R-(R, (R', S'))-*N*-[2-[[3-amino-1-[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl)-, 1-piperidinecarboxamide] which possesses high affinity and selectivity for human CGRP-receptors[15]. For the putative CGRP-1 (eg, car-

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diac tissue) and CGRP-2 receptors in rat tissue the affinity (pK_b) of BIBN 4096BS amounts to 8.5 and 7.1, respectively[16]. In the present study, we investigated the effects CGRP and BIBN4096BS on myocardial ischemia provoked by left main coronary artery occlusion followed by reperfusion in anaesthetised rats.

MATERIALS AND METHODS

Male Wistar rats (Chbb: Thom), weighing 350 – 380 g were used in this study. All experiments were performed according to institutional guidelines.

Measurement of myocardial infact size One day fasted rats were anaesthetised with sodium pentobarbitone: induction with 60 mg·kg^{-1} ip and then maintainence with an infusion of 30 mg·kg^{-1}·h^{-1} sc in the abdominal skin through a 23 G needle using a solution of 10 g·L^{-1}. The trachea was cannulated and the animals were artificially ventilated (80 strokes·min^{-1}) with room air supplemented by oxygen. The body temperature was maintained at 37 ℃ with a heating pad. The right *femoral artery and left jugular vein were cannulated for* the continuous measurement of arterial blood pressure and intravenous infusion of test agents (or vehicle: saline), respectively. Heart rate was derived from the blood pressure signal.

A left sided thoracotomy was performed at the level of the fifth intercostal space. A 5 – 0 silk suture was placed around the left anterior descending coronary artery approximately 1 – 2 mm from its origin. Four pieces of number 16 sewing cotton were ligated along with the coronary artery to facilitate reperfusion. The coronary artery was occluded for a period of 60 min followed by 60 min of reperfusion. Reperfusion was instituted by removing the ligature. Blood pressure and heart rate were measured continuously throughout the experiment. At the end of the reperfusion period, the heart was removed. Both atria and the roots of the great vessels were removed. The entire ventricle was cut from the apex to base into four transverse slices and incubated in 2,3,5-triphenyltetrazolium chloride (10 mg·mL^{-1} in phosphate buffer) for a period of 10 min at 37 ℃ to visualize the infarct area[17,18]. Each section was scanned by a colour image scanner (Hewlett Packard ScanJet IIcx, Germany) and infarct size was determined using the Photoshop 4.0 program.

Drug treatment: Test agents (saline, CGRP (1 nmol·kg^{-1}·h^{-1}), BIBN4096BS (20 nmol·kg^{-1}·h^{-1}), and CGRP (1 nmol·kg^{-1}·h^{-1}) + BIBN4096BS (20 nmol·kg^{-1}·h^{-1}) were infused into the jugular vein at a rate of 3.3 mL·kg^{-1}·h^{-1}. In the first series of experiments (protocol Ⅰ), the infusion of each compound commenced 10 min before occlusion and was maintained until the end of reperfusion. In the second series of experiments (protocol Ⅱ), the infusion of each compound commenced from the start of reperfusion and was maintained until the end of reperfusion. The doses were selected on the basis of prior testing: infusion of CGRP (1 nmol·kg^{-1}·h^{-1}, for 2 h), induced a 25 % decrease in blood pressure from 10 min after starting of infusion until the end of infusion in anesthetized rats. When CGRP (1 nmol·kg^{-1}·h^{-1}) and BIBN4096BS (20 nmol·kg^{-1}·h^{-1}) were infused together, BIBN4096BS reduced the effect of CGRP on blood pressure by 50 %.

Measurement of creatine phosphokinase activity In protocol Ⅰ groups, femoral artery blood samples were removed before infusion of the test agent, 50 min after occlusion, and 50 min after reperfusion. The blood samples were promptly centrifuged at 14 000 × *g*, 4 ℃, for 15 min, and the plasma thus obtained was stored at −80 ℃ until assay. Creatine phosphokinase (CPK) levels were determined by means of a CPK kit.

Measurement of CGRP levels Animals were divided into three groups. In group Ⅰ, blood samples were taken from the right ventricle of the heart after anaesthesia. In groups Ⅱ and Ⅲ, animals were subjected to 60 min coronary artery occlusion followed by 60 min of reperfusion, and the infusions of saline and CGRP (1 nmol·kg^{-1}·h^{-1}) were commenced 10 min before occlusion and were maintained until the end of reperfusion, respectively. Femoral artery blood samples were removed before occlusion and 50 min after reperfusion from group Ⅱ animals. Blood samples from the right ventricle of the heart were removed 50 min after reperfusion from both group Ⅱ and group Ⅲ animals. The blood samples were promptly centrifuged at 14 000 × *g*, 4 ℃, for 15 min, and the plasma was stored at −80 ℃ until assay.

Peptide extraction: Plasma samples were mixed with a double volume of 0.1 % trifluoroacetic acid (TFA) (v/v) and centrifuged at 14 000 × *g*, 4 ℃, for 15 min. The supernatants were pooled and applied on preprimed C18 Sep-Pak cartridges (Waters Corporation, Massachusetts, USA). The Sep-Pak cartridges were washed with 20 mL 0.1 % TFA at a flow rate of 3 mL·min^{-1}, and then eluted with 3 mL of 60 % acetonitril (v/v) containing 0.1 % TFA (v/v) at a flow rate of 2 mL·min^{-1}. The eluates were freeze dried, and stored at −80 ℃ until radioim-

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munoassay was performed. Recovery was determined by addition of ^{125}I-rCGRP to plasma prior to extraction, and was (75.4 ± 1.1) % (n = 8).

Radioimmunoassay: A competitive radioimmunoassay was used to specifically measure rat (r)CGRP concentrations in plasma extracts. The procedure was applied according to the instructions of the rCGRP RIA kit. Plasma extracts were assayed in duplicate using an antibody raised against rCGRP. The label (^{125}I-rCGRP) was added after the samples had been incubated with the antibody (rabbit anti-rCGRP) for 1 d. Subsequent to another 1 d incubation, the antibody bound ^{125}I-rCGRP was separated using goat anti-rabbit antibody, and the radioactivity counted on a gamma counter (Canberra-Packard GmbH, Germany). Rat CGRP was used as standard.

Statistical analysis Myocardial infarct size was expressed as a percentage of the surface of the transection of the ventricles. All results are expressed as $x \pm s_x$, $n = 5 - 6$. Comparisons were carried out by means of analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ were considered to be significant.

Drugs used The following drugs and analytical tools were used: h-αCGRP was purchased from Polypeptide, Wolfenbüttel, Germany; the rCGRP RIA kit was purchased from DRG Instruments GmbH, Marburg, Germany; the CPK kit was purchased from Sigma, Steinheim, Germany; BIBN4096BS was synthesized by Boehringer Ingelheim Pharma KG, Biberach an der Riss, Germany. BIBN4096BS was dissolved in a small volume (20 μL) HCl 1 mol/L, further diluted with saline, and then adjusted to pH 6.5 ~ 7.0 by NaOH 1 mol/L. Solutions were diluted to final concentrations with saline.

RESULTS

Hemodynamic changes during coronary artery occlusion and reperfusion in control and in drug treated rats As shown in Tab 1, occlusion of the main left coronary artery resulted in a reduction in mean blood pressure and heart rate, and remained unchanged throughout the experiment. Intravenous infusion of CGRP (1 nmol·kg^{-1}·h^{-1}) before occlusion resulted in a significant decrease in mean blood pressure (mmHg), from 110 ± 8 to 82 ± 7. Infusion of BIBN4096BS (20 nmol·kg^{-1}·h^{-1}) and CGRP (1 nmol·kg^{-1}·h^{-1}) + BIBN4096BS (20 nmol·kg^{-1}·h^{-1}) did not induce significant changes in mean blood pressure. However, there was no significant difference between treated groups and control group after coronary artery occlusion and reperfusion.

Immunoreactive CGRP levels We observed a small but significant increase in plasma CGRP level in the blood sampled from the right ventricle of the heart 50 min after reperfusion in group Ⅱ, compared to the basal level of control animals (Fig 1). The levels were (133 ± 14) ng·L^{-1} in ischemic hearts compared to (84 ± 12) ng·L^{-1} in the controls ($P < 0.05$). We found no increase in the systemic blood (data not shown). In CGRP treated animals (Ⅲ), the CGRP level was (798 ± 103) ng·L^{-1} in the blood sampled from the right ventricle of the heart 50 min after reperfusion.

Tab 1. Hemodynamics in control and drug treatment groups. $n = 6$. $x \pm s_x$. $^bP < 0.05$ *vs* **the control group.**

	Baseline	After 5 min drug treatment	Occlusion 30 min	Repertusion 1 h	Repertusion 2 h
MBP (mmHg)					
Control	117 ± 9	118 ± 9	65 ± 5	69 ± 5	67 ± 5
CGRP (Pre-Isch)	110 ± 8	82 ± 7^b	67 ± 5	69 ± 5	70 ± 5
BIBN4096 (Pre-Isch)	116 ± 8	117 ± 9	67 ± 6	62 ± 4	68 ± 4
CGRP + BIBN4096 (Pre-Isch)	122 ± 9	110 ± 8	64 ± 4	64 ± 5	63 ± 5
CGRP (Pre-Rep)	112 ± 8	112 ± 8	68 ± 6	67 ± 4	70 ± 5
BIBN4096 (Pre-Rep)	111 ± 8	112 ± 8	68 ± 5	65 ± 4	68 ± 5
HR (bpm)					
Control	427 ± 22	426 ± 22	312 ± 18	319 ± 20	311 ± 18
CGRP (Pre-Isch)	418 ± 23	436 ± 24	355 ± 17	352 ± 19	350 ± 17
BIBN4096 (Pre-Isch)	413 ± 24	416 ± 25	313 ± 20	318 ± 22	320 ± 20
CGRP + BIBN4096 (Pre-Isch)	425 ± 26	429 ± 24	328 ± 22	307 ± 18	312 ± 18
CGRP (Pre-Rep)	422 ± 20	422 ± 21	341 ± 23	338 ± 20	344 ± 19
BIBN4096 (Pre-Rep)	417 ± 21	418 ± 23	321 ± 19	310 ± 20	311 ± 19

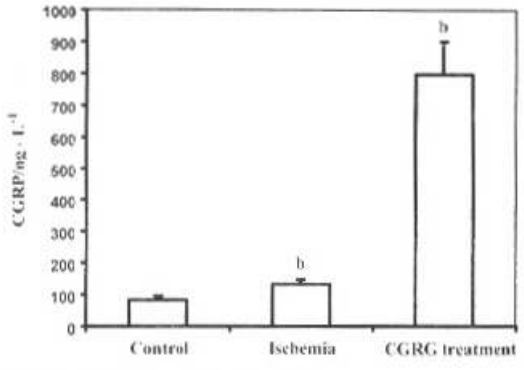

Fig 1. CGRP levels in the blood samples taken from the right ventricle of the rat heart. The influence of ischemia-reperfusion and CGRP treatment of the animals is shown. $n = 6$. $x \pm s_x$. $^bP < 0.05$ *vs* **saline control.**

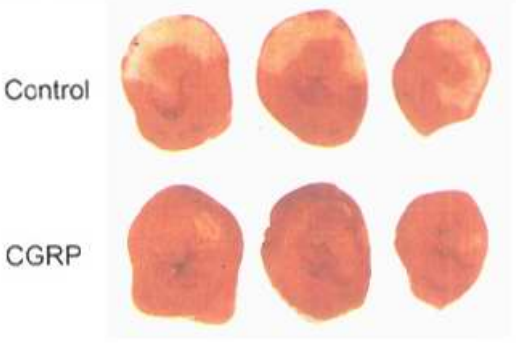

Fig 2. Scanning graphs of the infarct area (surface of each slice).

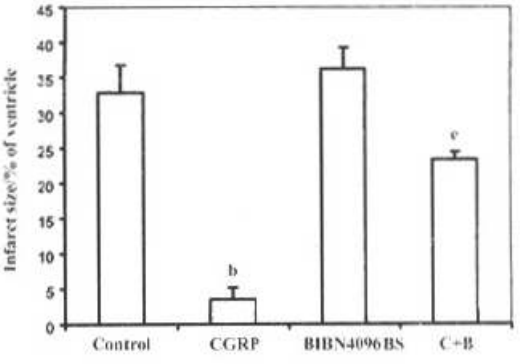

Fig 3. The effects of CGRP (1 nmol · kg⁻¹ · h⁻¹), BIBN4096BS (20 nmol·kg⁻¹·h⁻¹), and CGRP (1 nmol·kg⁻¹·h⁻¹) + BIBN4096BS (20 nmol · kg⁻¹ · h⁻¹) on infarct size, expressed as a percentage of the surface of both ventricles. $n = 6$. $x \pm s_x$. $^bP < 0.05$ *vs* **with saline control.** $^cP < 0.05$ *vs* **the group receiving CGRP alone.**

The release of creatine phosphokinase Tab 2 illustrates the difference in CPK activity in each group. Plasma creatine phosphokinase levels were significantly elevated 50 min after occlusion of the coronary artery. This increase was further enhanced 50 min after the start of reperfusion. Treatment with CGRP (1 nmol · kg^{-1} · h^{-1}) reduced the increase during the reperfusion period ($P < 0.05$). Treatment with BIBN4096BS (20 nmol · kg^{-1} · h^{-1}) did not influence plasma creatine phosphokinase activity compared to saline treatment controls. However, BIBN4096BS significantly attenuated the effect of CGRP on CPK activity ($P < 0.05$).

Myocardial infarct size Occlusion of the left anterior descending coronary artery for a period of 60 min followed by 60 min of reperfusion resulted in substantial injury to the myocardium. In a group of control rats occlusion and reperfusion produced an infarct amounting to (33 ± 4) % of the area of ventricles (Fig 2).

Fig 3 shows the infarct size expressed as a percentage of both ventricles after treatment with saline and test agents, respectively. The infusion was started 10 min before ligation of the coronary artery and continued until

Tab 2. The effects of CGRP (1 nmol·kg^{-1}·h^{-1}), BIBN4096BS (20 nmol·kg^{-1}·h^{-1}), and CGRP (1 nmol·kg^{-1}·h^{-1}) + BIBN4096BS (20 nmol·kg^{-1}·h^{-1}) on creatine phosphokinase levels (U·mL^{-1}) in the blood of anaesthetized rats. The left descending coronary artery was occluded for a 60-min period followed by 60 min of reperfusion and femoral artery blood samples were taken 5 min before compound infusions, 50 min after occlusion, and 50 min after reperfusion, respectively. $n = 5-6$. $x \pm s_x$. $^bP < 0.05$ *vs* **saline control group.** $^cP < 0.05$ *vs* **the CGRP treatment group.** $^hP < 0.05$ *vs* **the base level (before occlusion).**

	Saline	CGRP	BIBN4096BS	CGRP + BIBN4096BS
Before occlusion	5.8 ± 1.3	5.1 ± 1.7	7.7 ± 2.0	7.1 ± 0.7
50 min after occluson	11.5 ± 1.6^b	13.1 ± 2.1^b	20 ± 4^b	12.9 ± 1.0^c
50 min after reperfusion	149 ± 40	50 ± 5^b	124 ± 25	108 ± 15^c

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the end of reperfusion. Treatment with CGRP (1 nmol·kg^{-1}·h^{-1}) reduced the infarct size by 89 % ± 5 %. The reduction in infarct size by CGRP was counteracted by BIBN4096BS. However, treatment with BIBN4096BS alone (20 nmol·kg^{-1}·h^{-1}) had no statistically significant effect on myocardial infarct size.

Fig 4 shows the infarct size after treatment with saline, CGRP (1 nmol·kg^{-1}·h^{-1}), and BIBN4096BS (20 nmol·kg^{-1}·h^{-1}), respectively, when the compounds were infused at the start of the reperfusion and maintained until the end of reperfusion. CGRP reduced the infarct size by (40 ± 3) %. BIBN4096BS showed no influence on the infarct size compared to control group.

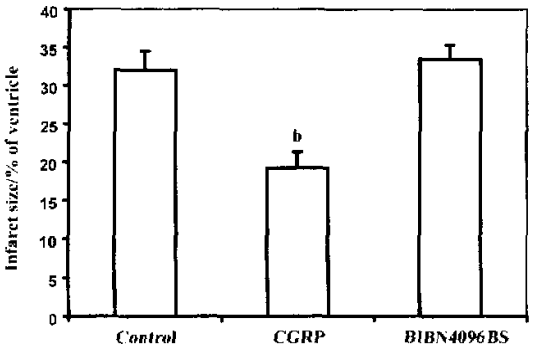

Fig 4. The effects of infusion of CGRP (1 nmol·kg^{-1}·h^{-1}) and BIBN4096BS (20 nmol·kg^{-1}·h^{-1}) from the start of reperfusion and maintained until the end of reperfusion on infarct size, expressed as a percentage of the surface of both ventricles. $n = 6$. $x \pm s_x$. $^bP < 0.05$ *vs* with saline control.

DISCUSSION

Early reperfusion of an occluded coronary artery is a well known and effective means of reducing ischemia-induced myocardial damage[19-22]. However, reperfusion has been shown to cause significant cardiac injury by itself[23,24]. In the present study, occlusion of the left anterior descending coronary artery for a period of 60 min followed by 60 min of reperfusion resulted in substantial injury to the myocardium. In a group of control rats, occlusion and reperfusion produced an infarct of (33 ± 4) % of the ventricle. In accordance with literature[25,26], a progressive rise in the CPK release during reperfusion, resulting from myocardial reperfusion injury, was observed.

In order to investigate the cardioprotective effect of CGRP in anaesthetized rats subjected to coronary artery occlusion followed by reperfusion, two different experimental protocols were used. When the infusion was started 10 min before ligation of the coronary artery until the end of reperfusion, CGRP (1 nmol·kg^{-1}·h^{-1}) reduced the infarct size by (89 ± 5) % compared to controls. The same dose of CGRP reduced the infarct size by (40 ± 3) % while the infusion commenced from the start of reperfusion until the end of reperfusion. CGRP did not alter CPK levels during ischemia but there was a marked decrease in CPK release during reperfusion. These findings indicate that post-ischemia tissue damage induced by reperfusion can be markedly attenuated by CGRP. CGRP appears to maximize the myocardial salvage achieved by reperfusion and may hence preserve ventricular function. In both protocols, infusion of BIBN4096BS (20 nmol·kg^{-1}·h^{-1}), a novel CGRP antagonist, did not signigicantly influence myocardial infarct size and CPK activity by itself. However, BIBN4096BS (20 nmol·kg^{-1}·h^{-1}) significantly antagonized the effects of CGRP on cardioprotection as well as on CPK activity.

In patients with congestive heart failure or myocardial infarction, an almost two fold increase of endogenous levels of CGRP level has been observed[12,13]. It seems likely that circulating CGRP acts primarily as a vasodilator and it may thus play a role in the regulation of systemic blood pressure and regional organ blood flow. Since CGRP is stored in the peripheral terminal of sensory nerves, it can be speculated that when the tissue is subjected to ischemia or during the excitation of sensory nerves, CGRP might be released by an axon reflex mechanism, thus increasing local blood flow. CGRP-immunoreactive nerves are abundantly present around the coronary artery, and CGRP exerts extremely potent coronary vasodilator activity. Therefore, the regulation of coronary blood flow by endogenous CGRP can be imagined. We observed an almost 50 % increase in plasma CGRP levels in blood from the right ventricle of the hearts 50 min after reperfusion compared to the basal level. However, no increase was observed in systemic blood samples. BIBN4096BS did not significantly increase in infarct size which is somewhat in contrast to a study by Kallner *et al*[27], showing that capsaicin pretreatment augmented myocardial infarction. This could be explained by the fact that receptor antagonism is a more specific way to block CGRP mediated effects than depleting neuropeptides from sensory nerves through capsaicin treatment. Alternatively, it can be imagined that

the release of endogenous CGRP in the experimental setting used is insufficient to produce effective cardioprotection. Only high plasma levels CGRP may cause cardioprotection. In the rats that were treated with CGRP (1 nmol·kg^{-1}·h^{-1}), the CGRP levels were about 10 fold higher than the basal level of the control group when blood was sampled from the right ventricle.

Although it has recently been shown that CGRP might be involved in ischemia preconditioning[28], the exact mechanism and role for CGRP release under pathophysiological condition is not yet fully understood. It has been proposed that CGRP is not released during the acute phase of myocardial infarction or anginal pain in human but could be considered as an inflammatory reaction rather than a response to ischemia[29].

Nevertheless, the present investigation demonstrates a cardioprotective effect of CGRP in anaesthetized rats which were subjected to coronary artery occlusion followed by reperfusion. Accordingly, these results support the hypothesis that CGRP is a very potent myocardial protective substance. However, the endogenous release of CGRP under experimental ischemia conditions used in this study was not sufficient to produce effective cardioprotection.

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降钙素基因相关肽和 BIBN4096BS 对麻醉大鼠心肌缺血的作用

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关键词 降钙素基因相关肽; BIBN4096BS; 心肌梗死; 再灌注损伤

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