Enhanced efficiency of superoxide dismutase-induced cardioprotection by retrograde intracoronary administration

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Abstract

Objective: We hypothesized that modification of the infusion route may improve the efficiency of superoxide dismutase (SOD)-induced cardioprotection against reperfusion injury. The routes for SOD delivery previously examined were intravenously, via the left atrium, or by a combination of these, all of which can deliver SOD into the ischemic myocardium only after reperfusion. In contrast, retrograde intracoronary infusion may be able to deliver SOD before reperfusion. We investigated the feasibility and efficiency of the retrograde intracoronary infusion of SOD to attenuate reperfusion injury.

Methods and results: Lewis rats underwent 30-min left coronary artery occlusion followed by reperfusion for 24 h. Just before reperfusion, CuZn–SOD was administered intravenously (15,000 U/kg, V-SOD group) or by retrograde intracoronary infusion (1500 U/kg, R-SOD group) through a catheter inserted into left cardiac vein via left superior vena cava as we have previously reported. This method has been shown to perfuse the whole left ventricular free walls. Controls for each group were injected with phosphate buffer saline only via the same routes (V-PBS and R-PBS group). The R-SOD group demonstrated significantly preserved left ventricular ejection fraction (LVEF; 71.3 ± 1.7% vs. 60.8 ± 2.3%, p = 0.028), reduced infarct size (23.3 ± 2.3% vs. 42.4 ± 3.5%, p < 0.001), and attenuated polymorphonuclear leukocyte (PMNL) infiltration (11.8 ± 0.4 vs. 14.8 ± 0.2 10^3/mm^2, p < 0.001) compared to the V-SOD group. The V-SOD group demonstrated significantly improved reflow (64.3 ± 2.1% vs. 53.4 ± 2.4%, p = 0.017) and attenuated PMNL infiltration (14.8 ± 0.2 vs. 16.8 ± 0.7 10^3/mm^2, p = 0.018) compared to the V-PBS group.

Conclusion: Retrograde intracoronary infusion is a promising, clinically applicable method to enhance the efficacy of SOD-induced myocardial protection against ischemia–reperfusion injury.

Keywords: Reperfusion injury; Free radicals; Cardioprotection; Acute inflammation; Retrograde intracoronary injection

1. Introduction

Although early reperfusion is the only established way to save the ischemic myocardium, it is inevitably associated with reperfusion injury, which remains one of the major issues in clinical settings such as primary angioplasty for acute myocardial infarction [1]. Superoxide dismutase (SOD) has been proposed to be a promising reagent to attenuate reperfusion injury; however, its efficacy has not been fully established in experimental or clinical reports [2–13]. We hypothesized that the efficiency of this form of therapy may be improved by modulating the delivery method. The routes for SOD delivery previously examined were intravenously, via the left atrium or by a combination of these, all of which can deliver SOD into the ischemic myocardium only after reperfusion [4–14]. It is highly unlikely that SOD delivered by these methods efficiently dismutate superoxides, which are generated within seconds after reperfusion [3,15]. For inducing the maximum...
protective effect, SOD has to be delivered into the ischemic myocardium before reperfusion.

Retrograde intracoronary infusion could deliver SOD into the myocardium during ischemia before reperfusion. Using the recently established original model in rat [16,17], we investigated the feasibility and efficiency of retrograde intracoronary infusion of SOD to attenuate reperfusion injury.

2. Methods

2.1. Induction of myocardial ischemia–reperfusion injury

All studies were performed with the approval of the institutional ethics committee and the Home Office, UK. The investigation conforms to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health Publication No. 85-23, 1996). Male Lewis rats (300–350 g, Charles-River, UK) underwent left thoracotomy under anesthesia with sodium pentobarbital (40 mg/kg, intraperitoneal) and mechanical ventilation using a volume-control ventilator (Harvard Apparatus, UK). The left coronary artery (LCA) was completely occluded for 30 min. A 24 gauge catheter (Vialon, UK) was inserted into left cardiac vein through a catheter insertion site, (2) snaring suture to prevent bleeding from the catheter insertion site, (2) snaring suture to prevent the flash back of the reagents into LSVC. LA; left atrium, LV; left ventricle. Fig. 1. Method of retrograde intracoronary infusion. Under left thoracotomy, left coronary artery (LCA) was occluded for 30 min. A 24 gauge catheter was inserted into left cardiac vein (LCV) through left superior vena cava (LSVC). PBS with or without SOD was injected through this catheter under constant pressure (arrow) just before the release of the occlusion of LCA for reperfusion. (1) purse string suture to prevent bleeding from the catheter insertion site, (2) snaring suture to prevent the flash back of the reagents into LSVC. LA; left atrium, LV; left ventricle.

2.2. Administration of SOD

Just before reperfusion, hearts were treated by either retrograde intracoronary or intravenous infusion of SOD after systemic heparinization (100 U/kg, intravenous). Retrograde intracoronary infusion was performed as we have described previously [16,17]. A 24 gauge catheter (Vialon, UK) was inserted into left cardiac vein through a purse string suture placed on the left superior vena cava. The stem of the cardiac vein was snared to prevent flush out of infused reagents by the venous flow into the vena cava (Fig. 1). Through the catheter, 1500 U/kg of CuZn–SOD (Sigma-Aldrich, UK) suspended in 0.5 ml phosphate buffered saline (PBS) or PBS only was injected into the left cardiac vein over a period of 5 s (R-SOD and R-PBS group, respectively). At 25 s after the end of the injection, occlusion of LCA was released for reperfusion. For the V-SOD and V-PBS group, 15,000 U/kg of CuZn–SOD suspended in 1.0 ml PBS and PBS only, respectively, was injected intravenously over a period of 5 s. Then, at 25 s after the end of the injection, occlusion of LCA was released for reperfusion. The dosages of SOD administered were determined according to the previous publications [4–13]. The rationale for the SOD dosage is further discussed in the Discussion. The suture used for LCA occlusion was left in situ. The chest was closed and the rats returned to their cages after extubation. All these procedures were randomly assigned and carried out in a blind manner.

2.3. Assessment of cardiac function and structure

At 24 h after the reperfusion, the rats were again anesthetized with intraperitoneal injection of 30 mg/kg sodium pentobarbital for echocardiography (n = 16 for each group) with the Sequoia 512 system and 15-MHz probe (Acuson). Both 2-dimensional (2-D) and M-mode images were taken at the mid-papillary muscle level. LV diastolic (LVDd) and systolic (LVDs) dimension was measured with M-mode. Left ventricular ejection fraction (LVEF) was measured by 2-D tracing using the formula; LVEF (%) = [(LV diastolic area − LV systolic area)/LV diastolic area] × 100, as previously described [18]. During the measurement, we carefully regulated the factors, which may affect the afterload, such as body temperature and anesthesia, as these may affect the cardiac function. After the echocardiography examination, the rats were subsequently used for further assessments: measurement of infarct size (n = 6), evaluation of regional myocardial blood flow (n = 5) and histological analysis (n = 5). All these assessments were randomly assigned and carried out in a blind manner.

2.4. Measurements of infarct size

The heart was exposed through the previous incision under mechanical ventilation. Immediately after re-occlusion of the LCA with the suture left in situ, 1.5 ml of 2% Evans Blue dye (Sigma-Aldrich, UK) was injected into the left femoral vein to estimate the area perfused by the occluded LCA. The heart was then removed and the LV was cut into 5 segments parallel to the apex–base axis, which were incubated with 4% triphenyltetrazolium chloride (TTC, Sigma-Aldrich, UK). Area at risk of infarction (negative for Evans Blue) and infarct area (negative for TTC) was assessed with NIH Image program [19]. Infarct...
Cardiac function before and after ischemia–reperfusion

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<th>V-PBS</th>
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<tr>
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<td>444±8</td>
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<td>LVDd (mm)</td>
<td>5.9±0.2</td>
<td>6.0±0.3</td>
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<td>LVDs (mm)</td>
<td>4.4±0.2</td>
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<td>LVEF (%)</td>
<td>54.7±2.9</td>
<td>52.4±2.3</td>
<td>60.8±2.3</td>
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Data were measured at 24 h after reperfusion with echocardiography. LVDd and Ds were assessed with M-mode and LVEF with 2-D tracing. Data are presented as mean±SEM. *p<0.05 vs. V-PBS, R-PBS and V-SOD.

size was calculated as a percentage of the infarct area to the area at risk of infarction.

2.5. Evaluation of regional myocardial blood flow

The heart was exposed and 6×10^5 coloured microspheres (15 μm diameter, Dye-Trak, Triton Technologies) suspended in 0.5 ml of saline containing 0.02% Tween 80 were injected into the LV cavity [20]. The heart was then excised, the atria and the right ventricular free wall were removed. In this assessment, we could not stain the samples by Evans blue and TTC, as these dyes mask the detection of the blue microspheres. The area at risk of infarction was therefore determined by referring to the typical samples for the infarct size measurement and separated from the rest of the ventricular wall. Each sample was digested with overnight incubation in 1 mol/1 KOH at 37 °C. The number of microspheres contained in each sample was counted with a counting chamber (improved Naubauer type). Regional myocardial blood flow (RMBF) was defined as the microsphere number divided by the weight of the sample. The degree of reflow in the ischemic area was calculated as a percentage of RMBF in the area at risk of infarction to that in the area without risk of infarction.

2.6. Histological analysis for acute inflammation

Histological analysis was performed on separate hearts. The heart was collected, cut into 4 sections and frozen in OCT compound (BD Biosciences, UK). The embedded samples were cut into 10-μm cryosections and stained with haematoxylin and eosin. The number of polymorphonuclear leukocytes (PMNLs) per high-power field was calculated as an indicator of the degree of acute myocardial inflammation [21]. Nine different fields of the area at risk of infarction in each section were investigated.

2.7. Statistical analysis

All values are expressed as means±SEM. Statistical comparison of the data was performed using 1-way ANOVA followed by Bonferroni test for individual significant difference. A value of p<0.05 was considered statistically significant.

3. Results

3.1. Preserved cardiac function by retrograde intracoronary SOD infusion

Cardiac function and dimension were measured with echocardiography. Baseline values (intact normal animals; n=7) of heart rate, LVDd, LVDs and LVEF were 385±17 bpm, 6.6±0.2 mm, 4.0±0.2 mm and 77.5±1.6%, respectively. After 30-min ischemia and 24-h reperfusion, all the groups demonstrated significantly (p<0.05) decreased LVEF compared to the baseline data. Importantly, the R-SOD group demonstrated a significantly higher LVEF compared with any other groups (p<0.05, Table 1). LVEF in the V-SOD group tends to be higher than that in the V-PBS group (p=0.111). There was no difference in LVEF between the R-PBS and V-PBS groups. Heart rate in every group tends to be higher than the baseline presumably due to surgical stress. Neither LV diastolic nor systolic dimensions showed significant differences among any groups.

3.2. Reduced infarct size by retrograde intracoronary SOD infusion

The area at risk of infarction and infarct area after ischemia–reperfusion injury were determined by Evans Blue and TTC staining. The area at risk of infarction was similar among all groups (50.7±2.6% in the R-SOD, 50.1±1.9% in the V-SOD, 55.3±1.7% in the R-PBS and 50.6±2.2% in the V-PBS group). Infarct size was calculated as a percentage of the infarct area to the area at risk of infarction. The R-SOD group (23.3±2.3%) demonstrated the smallest infarct size among all groups including the V-SOD group (42.4±3.5%) (Fig. 2). In contrast, the difference of infarct size between the V-SOD and V-PBS (47.4±3.4%, p=0.330) groups was not significant. Infarct size in the R-PBS group (48.9±2.8%) was similar to the V-PBS group.

![Fig. 2. Infarct size. The infarct area and the area at risk of infarction were determined with Evans Blue and TTC staining. The infarct area was calculated as the percentage to the area at risk of infarction. Infarct size in the R-SOD group was the smallest among all other groups. The V-SOD group did not show significant difference compared with the V-PBS groups. *p<0.01 vs. V-SOD, R-PBS and V-PBS. n=6 in each group.](https://academic.oup.com/cardiovascres/article-abstract/69/2/459/283772)
3.3. Improved reflow by retrograde intracoronary SOD infusion

The degree of reflow in the area at risk of infarction after ischemia–reperfusion was evaluated with coloured microspheres. At 24 h after reperfusion, the degree of reflow in the V-SOD (64.3 ± 2.1%) and R-SOD (69.8 ± 2.3%) groups was significantly larger than that of the R-PBS and the V-PBS groups. \( *p < 0.01 \) vs. R-PBS and V-PBS. \( ^{1}p < 0.05 \) vs. R-PBS and V-PBS. \( n=5 \) in each group.

The difference between R-SOD and V-SOD groups was not statistically significant (\( p=0.556 \)).

Fig. 3. Degree of reflow. RMBF was measured with coloured microspheres at 24 h after the reperfusion. Degree of reflow was defined as RMBF in the area at risk of infarction divided by that in the area without risk of infarction. The degree of reflow both in the R-SOD and the V-SOD group was significantly larger than that of the R-PBS and the V-PBS groups. \( *p < 0.01 \) vs. R-PBS and V-PBS. \( ^{1}p < 0.05 \) vs. R-PBS and V-PBS. \( n=5 \) in each group.

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The degree of reflow in the area at risk of infarction after ischemia–reperfusion was evaluated with coloured microspheres. At 24 h after reperfusion, the degree of reflow in the V-SOD (64.3 ± 2.1%) and R-SOD (69.8 ± 2.3%) groups was significantly larger than that in the R-PBS (54.4 ± 2.4%, \( p=0.017 \) vs. R-SOD) and R-PBS (54.4 ± 2.4%, \( p=0.001 \) vs. V-SOD and R-SOD) groups (Fig. 3). The difference between R-SOD and V-SOD groups was not statistically significant (\( p=0.556 \)).

Fig. 4. Histological findings. Acute inflammatory response was evaluated with haematoxylin and eosin staining at 24 h after reperfusion. Representative picture in each group was shown. PMNLs was infiltrating into the gap of disrupted myocardium. It can be seen that number of PMNLs is the smallest in the R-SOD group (D) compared with the V-SOD (C), the R-PBS (B) and the V-PBS (A). Bar=100 μm.

Fig. 5. Number of PMNL infiltrating into myocardium. The number of PMNL infiltrating into the area at risk of infarction per high-power field was calculated as an indicator of acute inflammatory response at 24 h after reperfusion. The R-SOD group demonstrated the smallest number of PMNLs among all other groups. The V-SOD group showed significant reduction compared with the V-PBS groups. \( *p < 0.01 \) vs. V-SOD, R-PBS and V-PBS. \( ^{1}p < 0.05 \) vs. R-PBS and V-PBS. \( n=5 \) in each group.

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3.4. Reduced inflammation by retrograde intracoronary SOD infusion

Acute inflammatory response after ischemia–reperfusion was evaluated by haematoxylin and eosin staining (Fig. 4). The myocardium lost its regular structure and cardiomyocytes were enlarged in the area at risk of infarction with PMNLs infiltration into the gap of disrupted myocardium in all samples analysed. The R-SOD group demonstrated significant reduction in the number of PMNL infiltrating into the area at risk of infarction compared with the V-SOD group (11.8±0.4 vs. 14.8±0.2 10^3/mm^2, p<0.001), (Fig. 5). PMNL infiltration in the V-SOD group was significantly reduced compared with the V-PBS group (16.8±0.7 10^3/mm^2, p=0.018). There was no significant difference in the number of PMNL between the R-PBS (18.0±0.6 10^3/mm^2) and the V-PBS group.

4. Discussion

Using an original experimental model in rat, we have demonstrated that SOD-induced cardioprotection is enhanced by retrograde intracoronary administration compared to intravenous systemic administration. While intravenous infusion of SOD improved the Reflow and attenuated acute inflammation following ischemia–reperfusion injury compared to intravenous PBS infusion, retrograde intracoronary infusion furthermore preserved LVEF and reduced infarct size, with further attenuated acute inflammatory response in the area at risk of infarction, compared to intravenous SOD infusion. Retrograde intracoronary PBS infusion resulted in similar findings to that of intravenous PBS infusion in terms of cardiac function and acute inflammation, suggesting that mechanical injury in the myocardium caused by this method is negligible.

The high level of free radicals generated during myocardial ischemia–reperfusion causes cell necrosis as well as apoptosis, impede RMBF and trigger inflammatory cascades associated with induction of pro-inflammatory cytokines [2,3]. Based on many previous reports, SOD has been expected to be a useful reagent to attenuate such myocardial ischemia–reperfusion injury by scavenging free radicals [2–11,14]. Experiments using transgenic mice [22] or in vivo gene transfection [14] consistently demonstrated that SOD overexpression improves myocardial tolerance against the reperfusion injury. However, on the other hand, when recombinant CuZn–SOD is administered after reperfusion, the cardioprotective effect is inconsistent [4–13]. The reasons for such inconsistency may include the insufficient amount of SOD used as well as the inappropriate delivery method. The total amount of SOD used in previous studies was in the range of 15,000–130,000 U/kg with 15,000 U/kg being the most common dosage [4–13]. This was infused either intravenously, via the left atrium or by a combination of these. In the present study, we used 15,000 U/kg CuZn–SOD for the V-SOD group, while one-tenth of this amount (1500 U/kg) was used for the R-SOD group, as blood flow perfusing the heart is considered to be 5–10% of the systemic blood flow. As a result, intravenous infusion of SOD demonstrated only a mild level of cardioprotection. This is understandable considering that the previous reports using the same amount of SOD demonstrated the inconsistent results [5,6,8,9]. It may be possible to improve the cardioprotective effect by administrating a higher dosage of SOD intravenously. However, in contrast, retrograde intracoronary infusion of one-tenth (proportionally the same) dosage of SOD showed markedly enhanced cardioprotection, highlighting the superior efficiency of this route.

Retrograde intracoronary infusion has been reported to be useful in administrating cardioplegic solution, recombinant peptide and vectors for gene therapy into the myocardium [23–25]. We have demonstrated that skeletal myoblasts are disseminated homogenously into the ischemic/infarcted myocardium when transplanted through this route in rat [17]. In the present study, we have shown that retrograde intracoronary infusion of SOD provides enhanced protection against reperfusion injury compared to intravenous injection. This may be due to unique abilities of retrograde intracoronary infusion to efficiently deliver SOD into the myocardium. First, this route can effectively supply SOD to the right place (post-capillary venules, where inflammatory response takes place). In addition, retrograde intracoronary infusion can perfuse the ischemic myocardium before reperfusion, as we have described previously [16,17]. This method also allows for certain incubation time (25–30 s in our study) after injection, which will encourage the delivered SOD to permeate into the myocardial tissues and cells. This ability to provide SOD at the right place, at the right time, with a high efficiency, is considered to be of great value. Free radical generation is reported to start during the first 10 s of reperfusion and peak at 10 min [15,26]. Elevating the myocardial SOD level before reperfusion by this treatment will effectively scavenge the free radicals initially generated immediately after reperfusion, resulting in enhanced reduction of subsequent and overall reperfusion injury.

Bokestegers et al. [27,28] have recently reported the safety and efficacy of retrograde intracoronary infusion of arterial blood in attenuating myocardial ischemia during percutaneous transluminal coronary angioplasty in human. This suggests the possibility of clinical application of the proposed strategy in the present paper, namely pretreatment of the ischemic myocardium by retrograde intracoronary infusion of SOD for myocardial protection against reperfusion injury. This treatment is feasible by using a balloon catheter in clinical settings of percutaneous coronary intervention (PCI) for treating acute myocardial infarction. Disadvantageously, it requires additional procedures (catheter insertion into the coronary sinus) to the usual PCI, which will delay the recanalization by several minutes.
Nevertheless, we consider that the treatment will induce greater benefits which far outweigh the loss caused by the "several-minute delay" of recanalization, improving the overall outcome of the PCI therapy. This concept, however, has to be confirmed by further laboratory research in a relevant experimental model using large animals. Investigation of the efficiency of retrograde intracoronary SOD infusion at later time points of reperfusion, which is more easily applicable to patients, would be also worthwhile.

It has been reported that combined administration of SOD and catalase effectively reduces myocardial reperfusion injury [4]. It is therefore considered that co-administration of catalase may further enhance the cardioprotective effect of retrograde intracoronary infusion of SOD. Other reagents such as Na+/H+ exchanger inhibitors, calcium antagonists, renin–angiotensin system antagonists, adenosine and nitric oxide donors have been shown to provide cardioprotection during primary angioplasty for acute myocardial infarction [29]. Although efficiency of these reagents has been demonstrated using models of antegrade intracoronary or intravenous infusion, retrograde intracoronary infusion may enhance the efficiency by the same mechanisms as the present study using SOD. In addition, antegrade intracoronary and intravenous administration of anti-P-selectin and anti-intercellular adhesion molecule (ICAM)-1 antibodies are also known to attenuate myocardial ischemia–reperfusion injury [30]. As these molecules theoretically work at postcapillary venules, retrograde intracoronary infusion, which has direct access to this part, may be the ideal injection route for these antibodies.

In conclusion, this study has shown that retrograde intracoronary infusion is a promising clinically applicable method to enhance the efficacy of SOD-induced myocardial protection against ischemia–reperfusion injury.

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References


