The delivery of superoxide dismutase encapsulated in polyketal microparticles to rat myocardium and protection from myocardial ischemia-reperfusion injury

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Abstract

Oxidative stress is increased in the myocardium following infarction and plays a significant role in death of cardiac myocytes, leading to cardiac dysfunction. Levels of the endogenous antioxidant Cu/Zn-superoxide dismutase (SOD1) decrease following myocardial infarction. While SOD1 gene therapy studies show promise, trials with SOD1 protein have had little success due to poor pharmacokinetics and thus new delivery vehicles are needed. In this work, polyketal particles, a recently developed delivery vehicle, were used to make SOD1-encapsulated-microparticles (PKSOD). Our studies with cultured macrophages demonstrated that PKSOD treatment scavenges both intracellular and extracellular superoxide, suggesting efficient delivery of SOD1 protein to the inside of cells. In a rat model of ischemia/reperfusion (IR) injury, injection of PKSOD, and not free SOD1 or empty particles was able to scavenge IR-induced excess superoxide 3 days following infarction. In addition, only PKSOD treatment significantly reduced myocyte apoptosis. Further, PKSOD treatment was able to improve cardiac function as measured by acute changes in fractional shortening from baseline echocardiography, suggesting that sustained delivery of SOD1 is critical during the early phase of cardiac repair. These data demonstrate that delivery of SOD1 with polyketals is superior to free SOD1 protein therapy and may have potential clinical implications.

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1. Introduction

Ischemic heart diseases, leading to myocardial infarction and heart failure, are a leading cause of global morbidity and mortality [1]. Loss of myocytes through necrosis and programmed cell death (apoptosis) following insults such as ischemia/reperfusion (IR) is mainly regional [2], suggesting the potential benefit of a localized therapy in preventing the development of cardiac dysfunction. However, because the disease is progressive in nature, localized therapy must also be sustained to deliver a consistent amount of drug over relevant times. Oxidative stress has been proposed as the unifying mechanism behind various risk factors of heart diseases [3] and it is implicated in many pathological disease states of the heart including hypertrophy [4], IR injury [5] and myocardial stunning [6]. Thus, a therapy that could address the high oxidative radicals over a sustained period of time will have great potential in prevention of cardiac dysfunction.

Direct measurements and indirect inferences have implicated the role of excess superoxide levels in the pathogenesis of infarct development, reperfusion injury and eventual myocardial dysfunction [7,8]. Additionally, mRNA expression levels of superoxide dismutase (SOD), an endogenous superoxide scavenger, decrease significantly after myocardial infarction thereby potentially exacerbating superoxide levels [9]. Antioxidant therapy with SOD including transgenic overexpression and gene therapy studies improve cardiac function following infarction, but the clinical relevance of these studies is still unclear as questions still remain regarding the safety and efficacy of gene therapy [10,11].

Despite the large role of oxidative stress in cardiac dysfunction, there is a strong lack of consistency in the efficacy of Cu/Zn SOD (SOD1) protein therapy and many large animal trials have failed to show a significant benefit [12,13]. Possible factors contributing to these discrepancies are the unfavorable pharmacokinetics and the rapid protein half life of SOD1. Half life of circulating wild type bovine SOD1 in rat blood is about six minutes [14] and depending on the modifications made to the protein, its half life can increase to about six hours [14–16]. Thus, there is significant interest in developing better SOD delivery systems, and many modifications have been made to the SOD protein to improve its pharmacokinetics.
and delivery including addition of cell penetrating molecules and targeting sequences [17]. Although, these modified proteins have better efficacies than the native SOD protein, intracellular delivery of the drug remains a challenge. In addition, high doses of these modified SODs are used in most studies [17] which by itself can exacerbate the cardiac pathologies [18].

The goal of the present study was to demonstrate that a direct myocardial injection of SOD1-encapsulated within polyketel microparticles can reduce both extracellular and intracellular superoxide levels, generate a sustained level of SOD1 within the myocardium and improve the cardiac function following IR injury. Here poly(cyclohexane-1,4-diyl acetone dimethylene ketal) (PCADK) – a recently developed polymer – was used as a delivery vehicle for SOD1 protein.

2. Materials and methods

2.1. PKSOD and empty PCADK (PK) particle preparation

PKSOD particles were made by a double emulsion process as described by Lee et al. [19] with slight modifications. In our method, a protein to polymer ratio of 0.05 was taken and larger particles were created by stirring the water in oil emulsion at 6000 rpm for one minute. PK particles were made in a similar manner without the addition of SOD1 protein. PCADK microparticles encapsulating a p38 inhibitor (SB239063) (PK-p38i) was made as described previously [20]. Fluorescein isothiocyanate (FITC) conjugated SOD (FSOD) was made by stirring SOD1 (5 mg/ml pH 9.0 carbonate buffer) with FITC (1 mg/ml DMSO) overnight at 4°C. The conjugated protein was purified by dialysis (8 k molecular weight cutoff) and lyophilized and fluorescent FSOD loaded PK particles (PKFSOD) were made similarly as described above.

2.2. Macrophage culture

RAW264.7 macrophages were maintained in DMEM (Fisher) supplemented with 10% fetal bovine serum (Hyclone), l-glutamine, and penicillin/streptomycin (Invitrogen). For experiments involving PMA stimulation, cells were seeded on a 12 well plate (2 million cells/well) and quiesced overnight in serum free DMEM. The media was then aspirated and replaced with treatment media (Kreb’s Hepes buffer (KHB)) containing SOD1 (50 U), PKSOD or PK, and incubated for 5 h at 37 °C. Cells were then washed with ice cold KHB buffer followed by the addition of media with or without 10 μM PMA for 20 min at 37 °C.

2.3. Intracellular and extracellular superoxide measurement

Superoxide was detected using dihydroethidium (DHE) and a high-performance liquid chromatography (HPLC) based assay [21]. In the cell culture experiments, macrophages were stimulated with 10 μM PMA along with 20 μM DHE and incubated at 37 °C for 20 min protected from light. For extracellular superoxide measurement, 100 μl of reaction buffer was removed and suspended in 300 μl methanol. For intracellular superoxide measurement, the cells were mechanically homogenized and suspended in 300 μl methanol. In order to separate ethidium, oxyethidium and unreacted DHE, the samples were loaded on a C18 column for reverse-phase HPLC analysis using an acetonitrile gradient [21].

2.4. Animals

A randomized and blinded study was conducted using adult Sprague-Dawley rats (obtained from Charles River) weighing 250 g. Rats were divided into two time points (3 and 21 days) containing five groups (n = 7 to 10 per group) each. While one group was subjected to sham surgery, the other four groups received IR surgery (30 min coronary artery ligation followed by reperfusion), with or without the injection of 100 μl of saline containing either 80U SOD1, 10 mg/ml of PK or 10 mg/ml PKSOD with or without 5 mg/ml SB239063 (p38 inhibitor), into the perimeter of cyanotic ischemic zone (3 locations) through a 30-gauge needle immediately after reperfusion. The animals were sacrificed after the specific time point and the hearts were melted in OCT compound and snap frozen in liquid nitrogen.

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and all animal studies were approved by Emory University Institutional Animal Care and Use Committee.

2.5. Superoxide detection following IR

In situ superoxide production was detected using DHE as described previously with slight modifications [22]. Briefly, 10 μM DHE was topically applied over unfixed frozen 20 μm heart sections and incubated at 37 °C in a light protected CO2 incubator for 5 min. After 2 min of DAPI staining, the slides were mounted with anti-fading medium (Vectashield® HardSet™; Vector laboratories) and analyzed with Axioscope fluorescence microscope with identical camera acquisition settings [8]. DHE fluorescence intensity was quantified using Imagej software (NIH).

Fig. 1. SOD can be encapsulated within Poly(cyclohexane-1,4-diyl acetone dimethylene ketone) to create micron sized particles. (A) Histogram of the microparticles analyzed with Imagej software demonstrating similar size distribution. (B) SEM image of empty polymer (PK) and PKSOD (Scale bar: 20 μm). (C) SOD1 protein encapsulated in PKSOD as measured by micro-BCA method.
2.6. Apoptosis detection

Roche Diagnostics TUNEL (terminal transferase-mediated dUTP-fluorescein nick end labeling) assay kit was used as per the manufacturer’s protocol to determine the number of apoptotic cells in the tissue sample. The slides were imaged using Axioscope fluorescence microscope and the number of TUNEL positive cells among the myocytes was reported as a percentage.

2.7. Echocardiography

Anesthetized rats were subjected to echocardiography prior to and after 3 or 21 days of IR surgery. Short axis values of left ventricular end systolic (ES) and end diastolic (ED) dimension were obtained using Acuson Sequoia 512 echocardiography workstation with 14 MHz transducer. An average of 2 consecutive cardiac cycles was used for each measurement and were made three times in an investigator-blinded manner. Changes in fractional shortening (calculated as (ED–ES)/ED) were determined after normalizing each rat to its own baseline value.

2.8. Statistics

All statistical analyses were performed using Graphpad Prism software as described in the figure legends. P values of less than 0.05 were considered significant.

3. Results

3.1. SOD encapsulation within PCADK microparticles

A double emulsion method was used to encapsulate SOD within the PCADK particles (PKSOD). Particle analysis with ImageJ software revealed that the size ranged from about 2 µm to 30 µm with a mean particle size of 11.4 ± 4.9 µm (Fig. 1A bottom panel). Representative SEM images are shown in Fig. 1B. Empty particles (PK) were similar in size and morphology to PKSOD particles (10.8 ± 5.9 µm, Fig. 1A top panel). Micro-BCA protein analysis of the encapsulated protein revealed an encapsulation efficiency of 50% (expressed as the ratio of actual SOD1 loading to the theoretical maximum), corresponding to almost 100 U of SOD1 per mg of polymer (Fig. 1C).

3.2. In vitro superoxide scavenging

The ability of PKSOD particles to scavenge superoxide radicals was investigated in macrophage cell culture (RAW 264.7) using quantitative DHE-HPLC. PMA stimulation resulted in a 1.6-fold increase in extracellular superoxide production as compared to control cells (p < 0.01). This increase was significantly inhibited by application of exogenous SOD1 (50 U/ml) (p < 0.001; Fig. 2A). Cells pretreated with PK particles also showed a significant PMA-induced increase in superoxide levels (p < 0.01 vs vehicle treated control), whereas cells treated with PKSOD for 5 h prior to PMA stimulation had no significant increase in superoxide levels compared to control cells. This inhibition was evident at both 0.25 mg and 0.125 mg of polymer per million cells (Fig. 2A).

Analysis of intracellular superoxide levels revealed that PMA stimulation significantly increased superoxide levels 3.9-fold as compared to control cells (p < 0.01). In contrast to the results observed in extracellular superoxide measurement, treatment with exogenous SOD1 was not able to reduce PMA-induced intracellular superoxide production (Fig. 2B, p < 0.001 vs control). Similarly, PK pretreatment also had no significant effect on intracellular superoxide levels (p > 0.01 vs vehicle treated control; Fig. 2B). Interestingly, PKSOD pretreatment was able to dose-dependently reduce the PMA-induced intracellular superoxide levels (Fig. 2B), suggesting efficient delivery of SOD1 to the intracellular space.

3.3. PKSOD retention in the myocardium

In order to determine the ability of PKSOD to remain in the heart after IR, either fluorescent SOD1 (FSOD) or PKFSOD were injected intramyocardially (n = 3) and analyzed by confocal microscopy three days following IR surgery (Fig. 3). Three-dimensional reconstruction of images showed bright fluorescence from PKFSOD particles scattered in the left ventricular infarct zone of the myocardium (Fig. 3A) with no fluorescence in the non-infarcted tissue (data not shown). Additionally, data obtained in our laboratory demonstrates retention of the particles for up to 10 days in native myocardium [20]. This fluorescence was totally absent in FSOD injected hearts (Fig. 3B).

3.4. In vivo superoxide scavenging

In preliminary studies, we found a significant increase in superoxide production at 3 days following IR that was completely inhibited by PKSOD treatment as measured by cytochrome C reduction...
A p38 inhibitor (PK-p38i) that, in prior published studies had no we investigated whether dual delivery with particles containing rats were not significantly improved compared with IR alone. Thus, seen in PKSOD treated animals as compared to PK or free SOD1, the a similar trend was observed with cardiac functional improvement the chronic cardiac function as measured by absolute change in frac- tional shortening from baseline to 21 days. While there was no effect of free SOD1 or empty PK, treatment with PKSOD significantly reduced it to basal levels (Fig. 4). Examination of the infarct core demonstrated no significant increase in DHE fluorescence with IR (data not shown).

3.5. Myocyte apoptosis following IR

In order to measure apoptosis, rats subjected to different treatments were sacrificed after three days and TUNEL staining was performed on 5 μm frozen sections and the percentage of TUNEL positive myocytes were determined by manual counting in a blinded manner. IR injury significantly increased the percentage of TUNEL positive myocytes within the left ventricle infarct zone greater than 4-fold as compared to sham operated animals. There was no significant decrease in apoptosis with either PK or free SOD1 treatment; however, PKSOD treatment significantly reduced the TUNEL positive myocyte count (Fig. 5).

3.6. Cardiac function following IR

To determine the effect of sustained SOD delivery on cardiac function, echocardiography data was collected prior to surgery to compare basal function, and then again after indicated time points (3 and 21 days post-surgery) as described in methods. As shown in Fig. 6A, IR significantly reduced cardiac function as measured in absolute change in fractional shortening from baseline to 3 days post-injury. While there was no effect of free SOD1 or empty PK, treatment with PKSOD significantly improved function to sham levels. Similarly, IR injury significantly impaired chronic cardiac function as measured by absolute change in fractional shortening from baseline to 21 days. Although a similar trend was observed with cardiac functional improvement seen in PKSOD treated animals as compared to PK or free SOD1, the rats were not significantly improved compared with IR alone. Thus, we investigated whether dual delivery with particles containing a p38 inhibitor (PK-p38i) that, in prior published studies had no acute effect but improved chronic cardiac function due to inhibition of fibrosis, could improve function in these rats. Interestingly, dual treatment with PKSOD and PK-p38i significantly improved cardiac function compared to IR alone, suggesting the need for multiple therapeutics to combat the different phases of the disease.

4. Discussion

The overproduction of superoxide plays a central role in the progression of ischemia/reperfusion (IR) injury and causes a rapid loss of cardiomyocytes and decreased cardiac function. In spite of convincing studies on the role of superoxide in causing cardiac pathologies and the ability of SOD1 to reverse this, conflicting results exist on the efficacy of SOD treatment likely due to its poor stability. Our primary findings indicate that PCADK microparticles can be used for sustained and intracellular delivery of SOD1 protein which improves cardiac function during the acute phase of reperfusion injury.

In this report, PCADK – a polyketal based biodegradable polymer – was used as a carrier to deliver SOD1 to scavenge both intra- and extra- cellular superoxide. Unlike polyester based biomaterials, these polymers do not have acidic degradation products and their potential to encapsulate SOD has been reported previously. Importantly, PCADK causes no significant inflammatory response and we have recently demonstrated its ability to treat inflammatory diseases like cardiac dysfunction with small molecule anti-inflammatory drugs making it an ideal carrier for in vivo applications.

Particle size analysis revealed that PKSOD microparticles in the 2 to 30 μm size range can be made using a double emulsion method by employing low speeds during the homogenization step. This size distribution allows the particles to be retained by a single injection in vascular tissues such as the myocardium, while still having particles small enough to be taken up by macrophages. Published data demonstrates that SOD1 protein maintains its activity following encapsulation and our data show that we can successfully encapsulate SOD1 into larger particles more suitable for cardiac delivery.

Superoxide generation occurs both inside and outside of cells during IR and we therefore measured the effects of PKSOD on both intracellular and extracellular superoxide production. Our results were not surprising for free SOD1, as our data demonstrate its ability...
to only scavenge extracellular superoxide, as large proteins do not cross the cell membrane. In stark contrast, our PKSOD particles were able to significantly reduce superoxide levels intra- and extracellular, suggesting the particles were taken up by the macrophages and the contents released intact within. Our unpublished data suggests that active processes are involved in uptake of the particles as inhibitors of phagocytosis and endocytosis partially inhibit uptake (data not shown). This finding suggests potential advantages of PKSOD over other SOD delivery vehicles, as intracellular superoxide buildup cannot be adequately addressed by a simple parenteral administration of SOD1 as it requires modifications to increase cell permeability, such as coupling it to polyethylene glycol (PEG). Not only does this entail more synthetic steps, but PEG itself may induce an oxidative response [26]. Scavenging excess superoxide levels within macrophages is known to reduce production of inflammatory cytokines such as tumor necrosis factor alpha [27] which causes damage to native cardiomyocytes [28]. Other cell types such as endothelial cells and cardiac stem cells also internalize these particles (data not shown) and a reduction of superoxide build up in different cell types in the myocardium may have additional beneficial effects.

The heart is a highly vascularized organ and as such most small molecules and proteins are rapidly cleared [29]. Therefore, many therapies usually involve daily or multiple injections of drugs and proteins over extended time periods [30,31]. Although gene therapy could provide a solution for this problem [32], targeting specific tissues and cell types is very difficult through this method. Further, prolonged overexpression of drugs such as SOD1 could

Fig. 4. PKSOD scavenges superoxide in the border zone following ischemia-reperfusion. Pictures shown are representative images of dihydroethidium (DHE) fluorescence in the myocardium, imaged with identical camera settings. Individual images were quantified for red fluorescence intensity using ImageJ software in a blinded manner and represented as mean ± SEM (n ≥ 5 per group). Only PKSOD significantly reduced DHE fluorescence 3 days following IR, with no effect of empty PK or free SOD1. *p < 0.05 ANOVA, followed by Dunnett’s multiple comparison test.
exacerbate cardiac pathologies [18]. Intracellular delivery of SOD1 using cell penetrating peptides may protect the myocardium against ischemic insult [33] however through this method the protein was delivered systemically, increasing the chances for nonspecific effects and protein loss. Thus given the fact that intramyocardial injections are considered safe [34], a one-time localized administration of drugs that could sustain antioxidant levels in the heart during disease progression could offer advantage over existing treatment methods. To determine whether our micron sized particles were retained in the heart, we initially created fluorescent dye (rubrene) loaded particles and injected them directly into healthy myocardium. Confocal microscopy showed the particles to be scattered in the myocardium three days following surgery and even over a week later [20]. Additionally, our prior studies demonstrated that the half life of these particles was quite stable in neutral environments with no inflammatory response, thus we expect the particles to be quite stable for several weeks in the myocardium [19,20]. We then tested the retention of PKSOD particles in ischemic myocardium by injecting fluorescent PKFSOD or FSOD intramyocardially following IR. The confocal images clearly show the local retention of PKFSOD in the myocardium three days after IR surgery.

To determine the effect of prolonged SOD1 retention with polyketal, we injected the PKSOD particles in a randomized and blinded study to the myocardium of IR-injured rats, injecting treatments immediately after reperfusion. Our data show that 3 days following IR, excess superoxide is generated in the border zones of the ischemic left ventricle. This increase in superoxide is not affected by administration of free SOD1 protein, but completely inhibited by intramyocardial injection of PKSOD several days after injection. This finding is striking not only due to the prolonged effect of PKSOD administration, but also because of the dose given (single injection of 1 mg per rat, corresponding to 80 U of SOD1). This dose is several orders of magnitude lower than previous published reports demonstrating infusion of over 1,000 U/kg prior to infarction followed by continuous infusion of over 1,000 U/kg thereafter [35]. We speculate that our treatment was more effective due to the localized nature of the superoxide generation (border zones only) and the fact that our microparticles are retained within the injection site. These conclusions are supported by our imaging data demonstrating that the native protein is not retained in the myocardium following injection. Thus, large doses of free SOD1 would be needed to maintain pharmacologically relevant doses in the affected area.

Superoxide generation is known to cause cardiomyocyte apoptosis [36], which is generally held to be responsible for progressive loss of cardiomyocytes after IR in spite of wide variations in the percentage of apoptotic myocytes (0.05 to 35%) [37,38]. The majority of cardiomyocyte apoptosis occurs within the first 72 h of reperfusion, thus we hypothesized that the sustained reduction of superoxide by PKSOD administration would be beneficial in reducing early cardiomyocyte apoptosis. Interestingly only PKSOD treatment significantly reduced cardiomyocyte apoptosis, suggesting that prolonged superoxide scavenging plays an important role in the survival of endogenous cardiomyocytes. This is in contrast to some studies showing free SOD1 infusion reduces apoptosis and infarct size, however it should be noted that the levels used to correspond to our PKSOD treatment (80 U) were much lower than levels used in those studies. Finally, we examined the effect of prolonged superoxide scavenging by PKSOD on cardiac function measured by...
echochardiography. Change in fractional shortening from baseline echocardiograms demonstrated a significant improvement in function due to PKSOD treatment three days following treatment, with function similar to sham operated animals. In contrast, no functional improvements were observed with free SOD1 or PK treatment suggesting a critical need for sustained therapy. Our apoptosis data show a low percentage of apoptotic cells (about 0.5%) after IR and a reduction of that percentage to about 0.1% by PKSOD treatment was able to show a significant functional recovery. Whereas inhibiting initial apoptosis is critical in maintaining normal function and homeostasis, we speculate that the sustained inhibition of superoxide levels may play important role outside of cell survival, such as improving vasculogenesis [39], expression of contractile proteins [40] and recruitment of regenerative cells [39]. Current studies are underway to examine whether prolonged SOD1 presence induces migration of regenerative cells that may aid in functional recovery.

During the chronic phase (21 days following IR), no significant improvement in cardiac function was observed with PKSOD. Though there was a trend toward improved function with PKSOD treatment (30% better than IR alone) as compared to PK and free SOD1, this result did not achieve statistical significance. It is entirely possible that the PKSOD was completely consumed in the early phase and a supplement dose would be needed later. It may also be possible that increased superoxide alone does not determine the fate of orchestrated set of events happening in myocardial disease progression. Thus a single antioxidant alone may not be sufficient to rescue function in the chronic phase of disease development. Supporting this, our recent study using polyketals to deliver small molecule inhibitors demonstrated no acute effect of p38 pathway inhibition, though sustained delivery had a striking effect on chronic remodeling. To address this interesting possibility, we delivered both PKSOD and PK-p38, following reperfusion and measured function at baseline and 21 days following injury. The combined treatment significantly improved cardiac function as compared to IR alone, suggesting the potential need for multiple therapies to treat this progressive disease. Current studies are underway to understand the various mechanistic processes in different phases of myocardial infarction and making modifications to the polyketals to allow for delivery of multiple factors.

5. Conclusion

In summary our work demonstrates that PCADK microparticles can be used as a carrier for sustained and intracellular delivery of SOD1. SOD encapsulation within PCADK improves the duration of superoxide scavenging following ischemia/reperfusion injury. Prior SOD1 protein therapy for myocardial infarction was inconclusive, involving repeated administration of large quantities of the enzyme. Unlike other modifications made to SOD1 to improve stability, encapsulation within PCADK also allows for intracellular delivery, an important factor as intracellular superoxide production may account for a significant proportion of superoxide generated following injury. This sustained scavenging of superoxide led to increased cardiomyocyte survival following injury and ultimately preserved cardiac function. These results may have broad clinical implications in treatment of cardiac dysfunction and other diseases where sustained oxidative stress contributes to pathology.

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Conflict of Interest

The authors report no conflict of interest.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2009.10.045.

Appendix

Figure with essential colour and discrimination. Certain figures in this article, in particular Figs.3 and 4 are difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2009.10.045.

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