Surface-modified particles loaded with CaMKII inhibitor protect cardiac cells against mitochondrial injury

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Abstract

An excess of calcium (Ca^{2+}) influx into mitochondria during mitochondrial re-energization is one of the causes of myocardial cell death during ischemic/reperfusion injury. This overload of Ca^{2+} triggers the mitochondrial permeability transition pore (mPTP) opening which leads to programmed cell death. During the ischemic/reperfusion stage, the activated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) enzyme is responsible for Ca^{2+} influx. To reduce CaMKII-related cell death, sub-micron particles composed of poly(lactic-co-glycolic acid) (PLGA), loaded with a CaMKII inhibitor peptide were fabricated. The CaMKII inhibitor peptide-loaded (CIP) particles were coated with a mitochondria targeting moiety, triphenylphosphonium cation (TPP), which allowed the particles to accumulate and release the peptide inside mitochondria to inhibit CaMKII activity. The fluorescently labeled TPP-CIP were taken up by mitochondria and successfully reduced ROS caused by Isoprenaline (ISO) in a differentiated rat cardiomyocyte-like cell line. When cells were treated with TPP-CIP prior ISO exposure, they maintained mitochondrial membrane potential. The TPP-CIP protected cells from ISO-induced ROS production and decreased mitochondrial membrane potential. Thus, TPP-CIP have the potential to be used in protection against ischemia/reperfusion injury.

Keywords

PLGA; CaMKII; mitochondria targeting

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1. Introduction

Myocardial infarction (MI) or heart attack, is a commonly observed symptom of coronary heart disease which is a leading cause of death and disability worldwide[1]. According to the American Heart Association, one American will have a MI approximately every 42 seconds[2]. Typically, reducing acute myocardial ischemic injury and limiting the infarct size using thrombolytic therapy to gain myocardial reperfusion is an effective therapeutic intervention[3]. However, the injury caused during ischemia/reperfusion remains. One of the causes of myocardial cell death during ischemia/reperfusion injury in MI patients is an excess of calcium (Ca^{2+}) influx into mitochondria during mitochondrial membrane potential (Ψ) restoration[4]. This overload of Ca^{2+} leads to mitochondria permeability transition pore (mPTP) opening and increased levels of reactive oxygen species (ROS), which eventually leads to myocardial cell death. The multifunctional calcium/calmodulin (Ca^{2+}/CaM) dependent protein kinase II (CaMKII) enzyme is rapidly activated during the ischemia/reperfusion stage and is responsible for mitochondrial Ca^{2+} influx[5, 6]. Inhibiting CaMKII in the mitochondria reduces cell death from ischemia/reperfusion injury in vivo[5]. A potent CaMKII inhibitor (CaMKIIN) protein is thought to reduce Ca^{2+} flux into mitochondria, thereby preventing mPTP opening[7]. Chang et al. have identified a 21 amino acid sequence (CaMKIIN peptide, Supplementary Figure 1A) derived from CaMKIIN protein at the amino acid sequence 43 – 63, has exhibited potent CaMKII inhibitory activity[8, 9].

To achieve a therapeutic effect, CaMKIIN peptide needs to be able to enter the cells and reach mitochondria. All studies performed so far have focused on modifying the protein/peptide itself to increase cellular uptake or target the mitochondria. Examples include mtCaMKIIN (which contains a mitochondrial localization sequence)[5], palmitoyl-CaMKIIN (which contains a membrane localization motif)[5] and TatCN21 (a membrane permeable CaMKIIN peptide)[10]. Besides delivery of the peptide to cardiomyocyte mitochondria, avoiding physiological degradation of the peptide must be considered. Submicron sized particles made from poly(lactic-co-glycolic acid) (PLGA, structure can be found in Supplementary Figure 1B), an FDA-approved biodegradable polymer, can be loaded with various molecules such as peptides/proteins and protect them from degradation by proteases[11, 12]. The PLGA polymer can be conjugated with various targeting moieties[13, 14] and therefore could be used to increase cellular uptake and deliver the CaMKIIN peptide to mitochondria. The targeting moiety chosen for this current study was the triphenylphosphonium cation (TPP, Supplementary Figure 1D). In 1984, TPP was reported to have an uptake and binding ability to the matrix face and cytosolic face of the mitochondria inner membrane[15]. Since then, TPP has been a popular choice for mitochondria targeting strategies. Moreover, TPP has been reported to promote the rate and amount of cellular internalization of conjugated nanomaterials[16]. There are various TPP-conjugated drug molecules and bioactive compounds such as, vitamin E (MitoVit E)[17], a ubiquinone derivative (MitoQ)[18] and doxorubicin[19]. There are also liposomes formulated with TPP in the lipid layers to target mitochondria[20]. TPP can be conjugated to PLGA-block-poly(ethylene glycol) (PEG) polymer and blended with either non-targeted PLGA-block-PEG-OH or PLGA-COOH to prepare solid PLGA particles using a nanoprecipitation method[21]. These particles can be loaded with lipophilic drugs such as curcumin and 2, 4-

dinitrophenol, and were reported to be taken up into the mitochondria of human cervical cancer (HeLa) cells.[21]

The well characterized cell line, H9c2, derived from rat cardiac tissue[22], was used in this study. This cell line has been used extensively as a model for ischemia/reperfusion injury[22–26] due to its high sensitivity to ischemia/reperfusion injury in terms of cell viability and mitochondrial respiration[24]. Moreover, H9c2 cells have been used in a number of studies involving CaMKII[27–30]. In these studies, the chronic exposure to all-trans retinoic acid was used to induce H9c2 cells to differentiate from cardiomyoblast-like to cardiomyocyte-like cells as has been previously described[31].

Instead of modifying the peptide structure to achieve an increase in both cellular uptake and mitochondrial targeting, in this report, we describe a new drug delivery system for transporting CaMKIIN peptide to mitochondria using functionalized PLGA particles. To the best of our knowledge, this is the first time that a CaMKII inhibitor peptide has been incorporated into a drug delivery system, specifically, into surface-modified PLGA particles. These submicron sized PLGA particles loaded with CaMKIIN peptide were prepared using a modified double emulsion solvent diffusion/evaporation method. The particle surface was conjugated with TPP, using carbodiimide crosslinker chemistry. The ability of the particles to inhibit CaMKII activity was tested using differentiated H9c2 cells that were treated with isoprenaline.

2. Materials and methods

2.1 Fabrication of PLGA Particles

CaMKIIN peptide-loaded particles (CIP) were made using two types of polymer: PLGA (PLGA 50:50 lactic acid: glycolic acid, ester endcap, MW: 24,000 – 38,000 Da; Resomer® RG503, Boehringer Ingelheim Pharma Gmbh & Co., Ridgefield, CT, Supplementary Figure 1B) and PLGA-NH$_2$ (50:50 lactic acid: glycolic acid, diamine endcap, MW: 10,000 – 20,000 Da; PolySciTech Division, Akina, Inc., West Lafayette, IN, Supplementary Figure 1C). The fluorescently-labeled CaMKIIN peptide (H-KRP PKL GQI GRA KRV VIE DDR K(HF488)-NH2) was custom produced (Anaspec, Inc. (EGT group), Fremont, CA) with a purity 90% according to HPLC analysis by the company. CaMKIIN peptide-loaded particles (CIP) were prepared using a double emulsion solvent diffusion/evaporation method with some modifications[32–34]. Briefly, 138 μL containing 625 μg of CaMKIIN peptide in 1% polyvinyl alcohol (PVA, Mowiol® 8–88, Mw ~67,000, Sigma-Aldrich®, St. Louis, MO) was sonicated into a solution of 50 mg of PLGA and 50 mg of PLGA-NH$_2$ dissolved in 150 μL of dimethyl sulfoxide (DMSO) and 2.5 mL of ethyl acetate. Sonication using a Sonic Dismembrator Ultrasonic processor (Fisher Scientific, Pittsburgh, PA) was conducted at 40% amplitude for 45 seconds to create the primary emulsion. To create the secondary emulsion, the primary emulsion was sonicated in 9 mL of 2.5% PVA in 1× PBS containing 1 mL of ethyl acetate at 40% amplitude for 60 seconds. The emulsion was then poured into 2.5% PVA in 1× PBS (51 mL), and stirred in a fume hood for 30 minutes for the particles to solidify. Separation/pelleting of unwanted particles (> 300 nm) was performed by differential centrifugation at 4500 × g for 5 minutes (Eppendorf centrifuge 5804 R, Eppendorf, Hauppauge, NY). The desired particles in the supernatant were then collected by

centrifugation at $10,000 \times g$ for 30 minutes (Thermo Scientific Sorvall™ Legend™ XTR Centrifuge, 230VAC, Cole-Parmer, Vernon Hills, IL) and were washed twice with sterile water, and then frozen and lyophilized using a FreeZone 4.5-L Benchtop Freeze Dry System (Labconco Corporation, Kansas City, MO). This particle preparation method is illustrated in Figure 1A.

Particle surfaces were functionalized with (4-carboxybutyl) triphenylphosphonium bromide (TPP, Sigma-Aldrich) using carbodiimide crosslinker chemistry (ThermoFisher Scientific, Waltham, MA) during the fabrication process. In this reaction, a TPP-derivative containing a carboxylic acid (-COOH) functional group was used along with a mixture of ester end capped PLGA and amine end capped PLGA (PLGA-NH$_2$) in particle fabrication. Thus, the carbodiimide compound was used to activate carboxylic acids for subsequent primary amine conjugation through the formation of amide bonds. While the particles were stirred in the fume hood, 4 mL of (triphenyl phosphate) - (1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide HCl) -(N-hydroxysucciniide) (TPP-EDC-NHS solution) in 0.1 M 2- (N-morpholino) ethanesulfonic acid (MES) buffer solution was added dropwise. Particles were reacted with this solution for 2 hours before being collected, washed and lyophilized. Excess molar ratios of TPP, EDC and NHS were used. For 1 mg PLGA and PLGA-NH$_2$ polymer, 1 mg, 4 mg and 6 mg, of TPP, EDC and NHS were used, respectively.

2.2 Quantification of CaMKIIN peptide loading in CIP

The loading capacity was quantified using a method described by Joshi et al[35]. Since CaMKIIN peptide was fluorescently labeled, the peptide loading in lyophilized particles could be quantified using a SpectraMax® plus 384 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA) (excitation/emission: 500/530). A known amount of lyophilized CIP were degraded using 0.3 N NaOH. After all particles were degraded, the solution was neutralized with 1 N HCl to approximately pH 7 then the fluorescence intensity was measured and compared to the intensity of standard CaMKIIN peptide solutions in $1\times$ PBS. The linear range for the CaMKIIN peptide is between 0.05 – 6.25 μg/mL.

2.3 Morphology, size and zeta potential of particles

The surface morphology of particles was assessed using Scanning Electron Microscopy (SEM: Hitachi S-4800, Hitachi High-Technologies, Ontario, Canada). The lyophilized particles prepared using the method mentioned above were resuspended in deionized water. A drop of this particle suspension (approximately 10 μL) was placed onto a silicon wafer mounted on a SEM stub using double stick carbon tape and air dried. The silicon wafer was then coated with gold-palladium using a sputter coater (K550 sputter coater, Emitech Ltd., Kent, England). The images were collected at 1.5 kV accelerating voltage. The size and zeta potential from each batch of particles were measured using a Zetasizer Nano ZS (Malvern, Worcestershire, UK).

2.4 Confirmation of TPP conjugation on the particle surface using fluorescamine

Fluorescamine interacts with primary amines to yield highly fluorescent derivatives[36]. In this study, fluorescamine was used to quantify the availability of primary amines on the surface of particles prepared using the mixture of PLGA and PLGA-NH$_2$ (as an indirect

measurement of TPP conjugation). Three types of particles were tested, blank (without CaMKIIN peptide) particles made from PLGA and PLGA-NH$_2$ (PLGA:PLGA-NH$_2$, 50:50), TPP conjugated-blank particles made from PLGA and PLGA-NH$_2$ (TPP-PLGA:PLGA-NH$_2$, 50:50) and blank particles made purely from ester-end capped PLGA (PLGA:PLGA-NH$_2$, 100:0).

Lyophilized particles were resuspended in 1 mL PBS. Then, 50 μL of fluorescamine dissolved in acetone (9 mg/ml) was added to the particles. The reaction was allowed to continue for 5 minutes. The particles were then washed three times with PBS. The fluorescence intensity of the particle surface was determined using flow cytometry (LSR II flow cytometer, Becton Dickinson, New Jersey, NJ).

2.5 Cell lines and cell culture

Rat cardiomyoblast-derived cells (H9c2, ATCC® CRL-1446™) were maintained in DMEM medium (Gibco®, Life technologies, Grand Island, NY). All of the media were supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 2 mM Glutamax (Gibco®) and 1% penicillin/streptomycin (Gibco®). Cells were incubated at 37°C and 5% CO$_2$. Subculturing was performed before cells reached 80% confluency in order to prevent the loss of differentiation potential[37].

According to Comelli et al., H9c2 cells can differentiate into cardiomyocyte-like cells using a low percentage (1%) of FBS in combination with retinoic acid[31]. H9c2 cells were seeded at a concentration of 4×10^5 cells/well in 150-mm dishes using DMEM supplemented with 1% FBS, 10 nM all-trans retinoic acid, 2 mM Glutamax (Gibco®) and 1% penicillin/streptomycin (Gibco®). Media was replenished every two days. Differentiated H9c2 cells were used after 7 days of differentiation. Cells were maintained in the differentiation media throughout all experiments.

2.6 Flow cytometry analysis of Mitotracker® Red stained cells

Seven days after the differentiation process, H9c2 cells were gently rinsed with pre-warmed PBS, trypsinized with 0.25% trypsin-EDTA and collected using centrifugation at $230 \times g$ for 5 mins. Cells were then incubated with 200 nM Mitotracker® Red (CMSRos, Molecular Probes, Life technologies, Eugene, OR) for 15 mins in an incubator (37°C, 5% CO$_2$) to stain the mitochondria. Cells were collected by centrifugation and resuspended in pre-warmed PBS without any fixatives. The fluorescent signal from Mitotracker® Red was quantified immediately using flow cytometry (FACScan: Becton Dickinson Immunocytometry Systems, San Jose, CA). Undifferentiated H9c2 cells incubated in DMEM media supplemented with 10% FBS were used as a control group.

2.7 Measurement of intracellular reactive oxygen species (ROS) production by dihydroethidium (DHE) staining

After H9c2 cells were differentiated, the CaMKIIN peptide in either solution or particulate form was added into each 150-mm dish at a concentration of 100 nM, 4 hours prior to adding 125 μM of isoprenaline (ISO). Cells were incubated with particles and ISO for 28 and 24 hours, respectively. The treatment groups (CISol (CaMKIIN peptide in soluble form),

CIP (CaMKIIN peptide loaded particles), TPP-CIP (TPP functionalized CaMKIIN peptide loaded particles) and ISO were maintained in the media until the end of the experiment. After the exposure, media was removed. Cells were gently rinsed with pre-warmed PBS to remove excess particles (see treatment timeline: Figure 1B). Cells were trypsinized with 0.25% trypsin-EDTA and collected by centrifugation at $230 \times g$ for 5 mins. The cells were washed with pre-warmed PBS containing 5 mM sodium pyruvate and incubated at 37°C with dihydroethidium (DHE, 10 μM in DMSO) in PBS containing 5 mM sodium pyruvate. After 40 mins incubation, the cells were analyzed using flow cytometry (FACScan). The relative mean fluorescence intensity (MFI) of 20,000 cells was recorded. All groups were normalized to the untreated control group. Antimycin A or AntA (an electron transport chain blocker, 10 μM in DMSO) was used as a positive control and subsequently increased the DHE oxidation levels by 2.6-fold higher than the control group (data not shown).

2.8 Quantification of particle uptake by differentiated H9c2 cells

The excitation and emission wavelengths of oxidized product from DHE are 535 and 610 nm, respectively[38]. The CaMKIIN peptide was tagged with a fluorophore (HF488, HiLyte Flour™ Dye, Anaspec. Inc,) possessing excitation and emission wavelengths of 500 and 530 nm, respectively. ROS production in H9c2 cells and the fluorescent signal from the CaMKIIN peptide were measured simultaneously using flow cytometry. The relative mean fluorescence intensities (MFI) of 20,000 cells were recorded. All groups were normalized to the untreated control group.

2.9 Measurement of mitochondrial membrane potential by Tetramethylrhodamine, methyl ester, Perchlorate (TMRM) staining

Differentiated H9c2 cells were seeded at 1×10^5 cells per dish one day prior to the experiment. Cells were treated in the same manner as described in section 2.7. After the exposure, the medium was removed. Cells were trypsinized and resuspended in 250 μL of media which contained 25 μM tetramethylrhodamine, methyl ester, perchlorate (TMRM) in Hanks' balanced salt solution (HBSS). Cells were exposed to TMRM containing media in the dark for 30 mins at 37°C. After the incubation was completed, the old media was replaced with DMEM media containing 1% FBS, retinoic acid and sodium pyruvate. Cell images were obtained using an epi-fluorescence, inverted Olympus IX-81.

The fluorescent intensity from H9c2 mitochondria in each group was obtained using the raw intensity data (F1) / background intensity data (F0) which is the area in the image with no cells.

2.10 Statistical Analysis

Data are expressed as mean ± SEM (Scatter dot plot). Statistical significance was determined using an unpaired T-test or One-Way ANOVA with Bonferroni's multiple comparison tests. All statistical tests were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla CA, www.graphpad.com). A p-value less than 0.05 was considered significant.

3. Results and Discussion

3.1 Particle characterization

CIP were fabricated using a water in oil in water double emulsion solvent diffusion/ evaporation method as previously described with some modifications[32, 33]. Ethyl acetate was used as the organic phase with PVA as a stabilizer. This method was developed in order to load water soluble drugs into nanometer-sized particles. The hydrodynamic diameters for CIP (PLGA/PLGA-NH2) and TPP-CIP (TPP-PLGA/PLGA-NH2) were approximately 210.4 ± 3.92 nm (n=5) and 276.5 ± 41.51 nm (n=3), respectively, with a narrow size distribution (Supplementary Figure 2). All particles had a negative overall surface charge. The drug loading of CIP (PLGA/PLGA/NH2) and TPP-CIP (TPP-PLGA/PLGA-NH2) were approximately 0.47 ± 0.04 µg/mg lyophilized particles (n=3) and 0.38 ± 0.06 µg/mg lyophilized particles (n=3), respectively. Entrapment efficiency of CIP was approximately 10.15%. Production yield of NPs (% yield) was approximately 57.16 ± 9.05 (n=5). The surface morphology of the particles was studied using SEM and they were shown to have smooth surfaces, were spherical in shape and were uniform in size (Figure 2). A release study was performed in order to determine the drug release profile from CIP (Supplementary Figure 4). The results indicated that more than 50% of CaMKIIN peptide was released from the particles within the first hour and more than 90% within 13 days.

3.2 TPP was successfully conjugated onto the particle surface

To create mitochondria-targeting particles, TPP was conjugated onto the particle surface using carbodiimide crosslinker chemistry as described in the methods. Two assays were performed to confirm TPP-particle conjugation: 1) zeta potential measurements and 2) detection of residual primary amines with fluorescamine. Since TPP is a lipophilic cation its successful conjugation onto the particle surface results in an increase in zeta potential[39]. As shown in Figure 3, TPP-PLGA:PLGA-NH$_2$, 50:50 particles demonstrated significantly higher zeta potential when compared to unmodified particles (PLGA:PLGA-NH$_2$, 50:50). These results suggest that the TPP group was present on the particle surface of the TPP-PLGA:PLGA-NH$_2$, 50:50 particles. The second method that was used to confirm TPP conjugation involved treating the particles with the amine-reactive dye, fluorescamine. In theory, if the particle surface contains free primary amines, it will react with fluorescamine to yield a highly fluorescent product. However, if the particles surface was occupied with TPP group, there would be lower number of free amines to react with fluorescamine. Thus, TPP-conjugated particles should yield a lower fluorescence intensity compared to unconjugated particles. Schematic images representing the different types of particles made in this experiment are shown in Figure 4A. In Figure 4B, TPP-PLGA:PLGA-NH$_2$, 50:50 particles that were incubated with fluorescamine showed significantly lower fluorescence intensity (p-value < 0.05) compared to PLGA:PLGA-NH$_2$, 50:50 particles that were incubated with fluorescamine. The control group (PLGA particles without primary amines, PLGA:PLGA-NH$_2$, 100:0) showed no significant fluorescence intensity. The results obtained from this experiment were consistent with zeta potential measurements (Figure 3) and suggested that TPP was successfully conjugated onto the surface of the particles.

3.3 Differentiated H9c2 cells have mitochondrial content higher than undifferentiated H9c2 cells

Undifferentiated H9c2 cells are used as an alternative to primary cardiomyocytes as they are recognized as being representative of the cardiomyoblast lineage[22, 40]. These cells have the ability to differentiate into cardiomyocyte-like cells when they are exposed to all-trans retinoic acid in reduced-serum media. After differentiation, H9c2 cells express a more cardiac-like phenotype such as having a greater mitochondrial mass[31], as well as having an increased expression of proteins involved in Ca^{2+} handling and mitochondrial metabolism[41]. Such characteristics are of importance in these studies since the particles being tested were designed to target the mitochondria of cardiomyocytes. Moreover, there have been reports suggesting that differentiated H9c2 cells were more susceptible to cytotoxic substances such as isoprenaline[42] and doxorubicin[37] than undifferentiated cells. Since this study is focused on the protective ability of the particles against isoprenaline-induced toxicity, differentiated cells which are more sensitive to toxic substances, would generate more distinct results than undifferentiated cells.

Flow cytometric analysis of Mitotracker® Red fluorescence was used as a semiquantitative indicator of mitochondrial mass. After 7 days of differentiation in the presence of all-trans retinoic acid and 1% FBS, H9c2 cells had a relative mean fluorescence intensity (MFI) 2.2-fold higher than untreated cells (Figure 5). It has been previously established that treating H9c2 cells with all-trans retinoic acid for 7 days results in an increase in mitochondrial content which is considered one of the markers for the differentiation-associated changes in mitochondrial biogenesis[31]. The aforementioned flow cytometry data was verified using confocal microscopy which showed an increase in mitochondrial mass after Mitotracker® Red staining (Supplementary Figure 5). Taken together, these results confirmed that significant differentiation of H9c2 cells had been achieved.

3.4 TPP-CIP were most efficiently taken up by differentiated H9c2 cells compared to other particle formulations

After differentiated H9c2 cells were incubated with various CaMKIIN peptide formulations and ISO, cells were collected and analyzed for particle uptake using flow cytometry, exploiting the fluorescent signal from the CaMKIIN peptide itself. Cells that were treated with TPP-CIP had the highest fluorescent signal amongst all groups tested which was 14.7-fold higher than the untreated cells (control group, Figure 6A). Cells that were incubated with CIP and CISol had similar levels of fluorescence with 4.1- and 3.5-fold higher than untreated cells, respectively. Thus, using TPP conjugation to the surface of PLGA particles significantly increased cellular uptake by differentiated H9c2 cells.

3.5 TPP-CIP reduced intracellular ROS induced by ISO

In the same set of experiments, differentiated H9c2 cells were also analyzed for intracellular ROS (pro-oxidants such as superoxide) induced by ISO (Figure 6B). Without the CaMKIIN peptide, cells that were treated with 125 μM ISO for 24 hours had 1.6-fold higher fluorescent signal, generated by the oxidized DHE product, than the control group. When cells were pre-treated with CaMKIIN peptide in either soluble form (CISol) or particulate forms (CIP, TPP-CIP) 4 hours prior to treatment with ISO, the intracellular ROS decreased.

Among the different CaMKIIN peptide formulations, TPP-CIP had the lowest fluorescent signal (1.3-fold higher than the control group) and was therefore the most effective at mitigating ROS levels (*p*-value < 0.05 when compared to ISO-treated cells).

3.6 TPP-CIP maintained mitochondrial membrane potential in differentiated H9c2 cells after ISO treatment

Differentiated H9c2 cells were treated with ISO with and without CIP or TPP-CIP pretreatment. Mitochondrial membrane potential was measured using TMRM. Cells treated with ISO alone or cells pretreated with CIP then ISO showed a decrease in membrane potential compared to that of cells pre-treated with TPP-CIP (Figure 7). This suggests that cells pretreated with TPP-CIP had more polarized mitochondria compared to other groups.

In the studies presented here, a CaMKIIN peptide was successfully entrapped inside PLGA particles. These PLGA particles comprised a mixture of PLGA (ester endcapped) with PLGA-NH_2 (amine endcapped) and, in order to impart mitochondrial targeting capabilities to these particles, a test group was conjugated to TPP by linking the carboxylate group on TPP to the amine group on PLGA-NH_2. TPP was conjugated onto the surface of PLGA particles using carbodiimide crosslinker chemistry. Confirmation of successful TPP conjugation to the surface of the particles was achieved through zeta potential measurements of the particles and assessing amine availability on the particle surface.

Using submicron-sized particles is necessary for the purposes of targeting mitochondria. Mitochondria targeting polymeric particles with diameters equal to or smaller than approximately 200 nm have be found in the mitochondria of HeLa cells[21]. Here, the mean hydrodynamic diameter of the particles was approximately 200 nm. The mechanism of how polymeric particles can locate to mitochondria remains unclear. However, it is likely that the TPP group on the particle surface plays an important role in a unique endosomal escape pathway after cellular uptake[43] and facilitates particle transport to the inside of mitochondria with its binding ability to the matrix of the negatively charged mitochondrial inner membrane[44].

Whilst TPP is a desirable agent to include into particulate formulations designed to target mitochondria, excessive TPP accumulation in mitochondria is arguably toxic to cells[45, 46]. Examples of toxic effects include the inhibition of the mitochondrial electron transport chain and inducing mitochondrial proton leakage[45]. In order to minimize possible cytotoxicity of TPP cations, short linker chains of the TPP cation, (4-carboxybutyl) triphenylphosphonium bromide, were used in the studies presented here. Furthermore, the amount of TPP used was minimized by conjugating the TPP group onto the surface of PLGA particles as opposed to every single polymer chain. This was done by adding TPP-EDC-NHS in 0.1 M MES buffer solution into the formulation after the CIP were formed. Since the conjugation was conducted before the particles were washed and lyophilized, the loss in drug loading (CaMKIIN peptide) during formulation was minimized; which is an outstanding feature of this novel method.

TPP-CIP were more readily taken up by cardiomyocyte-like H9c2 cells than non-targeting CIP as indicated in Figure 6A. This result is consistent with previously published

data[16, 39, 46]. Bielski et al. found that conjugating TPP onto poly(amidoamine) (PAMAM) dendrimer nanocarriers increased both cellular internalization and mitochondrial targeting in the human lung adenocarcinoma cell line, A549[16]. Guzman-Villaneuva et al. (2015) reported that TPP-liposomes had higher cellular uptake than liposomes without surface-conjugated TPP[46].

CaMKII is a multifunctional signaling enzyme expressed in heart cells which can be activated by stimulating the β-adrenergic receptor[47]. To study the effect of CaMKII inhibition by TPP-CIP, CaMKII induction is necessary. This can be done by adding a β-adrenergic receptor agonist called isoprenaline (ISO) to differentiated H9c2 cells[48, 49]. It is known that CaMKII activity promotes mitochondrial-triggered cell death due to Ca^{2+} overload and excess ROS[50]. Since ISO treatment increases mitochondrial ROS production, the reduction in intracellular ROS production in cells pretreated with TPP-CIP indicates the ability of these particles to inhibit CaMKII. Cells pretreated with TPP-CIP was the only group which had intracellular ROS (pro-oxidants) levels significantly lower than cells treated with ISO alone, suggesting that TPP-CIP helped to protect cells against ISO induced intracellular ROS generation (Figure 6B).

In addition to intracellular ROS, mitochondrial Ψ of differentiated H9c2 cells was also measured. Ψ is the total transmembrane electrical potential or voltage gradient which is an indicator of the health of cells[51, 52]. TMRM is a fluorescent lipophilic cationic dye which can accumulate within mitochondria at levels that depend on the Ψ value. Since the inner membrane of polarized mitochondria have a higher negative charge than depolarized mitochondria, TMRM can accumulate more in polarized mitochondria[53]. During cellular stress, as when ISO is added, Ψ can be altered by the intracellular ionic charges such as Ca^{2+} [54] which result in Ψ collapse and mitochondria depolarization[53]. From the results shown here, TPP-CIP can protect cardiomyocyte-like cells from ISO-induced mitochondrial damage as seen from the reduction in intracellular ROS (pro-oxidants) (Figure 6B) and the high Ψ (Figure 7).

4. Conclusion

This study reports on the development of a new submicron sized particulate drug delivery system carrying a CaMKIIN peptide designed to protect cells against mitochondrial injury. The cellular uptake and mitochondrial targeting ability was achieved through the surface conjugation of PLGA-based carriers with a mitochondrial targeting molecule, TPP. The conjugation with TPP was confirmed using zeta potential measurements and an assay measuring fluorescamine reactivity. TPP-CIP protected cardiomyocyte-like cells from ISO-induced ROS production and decreased Ψ. TPP-CIP have the potential to be used in protection against ischemia/reperfusion injury in susceptible patients such as patients undergoing heart surgery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

|	
CaMKII	the calcium and calmodulin (Ca^{2+}/CaM) -dependent protein kinase II (CaMKII) enzyme
CaMKIIN	the calcium and calmodulin (Ca^{2+}/CaM) -dependent protein kinase II (CaMKII) inhibitor
CIP	CaMKIIN peptide-loaded particles
CISol	CaMKIIN peptide in soluble form
DHE	dihydroethidium
ISO	isoprenaline
mPTP	the mitochondrial permeability transition pore
PLGA	poly(lactic-co-glycolic acid)
TPP	triphenylphosphonium cation
TPP-CIP	TPP functionalized CaMKIIN peptide loaded particles
Ψ	mitochondrial membrane potential

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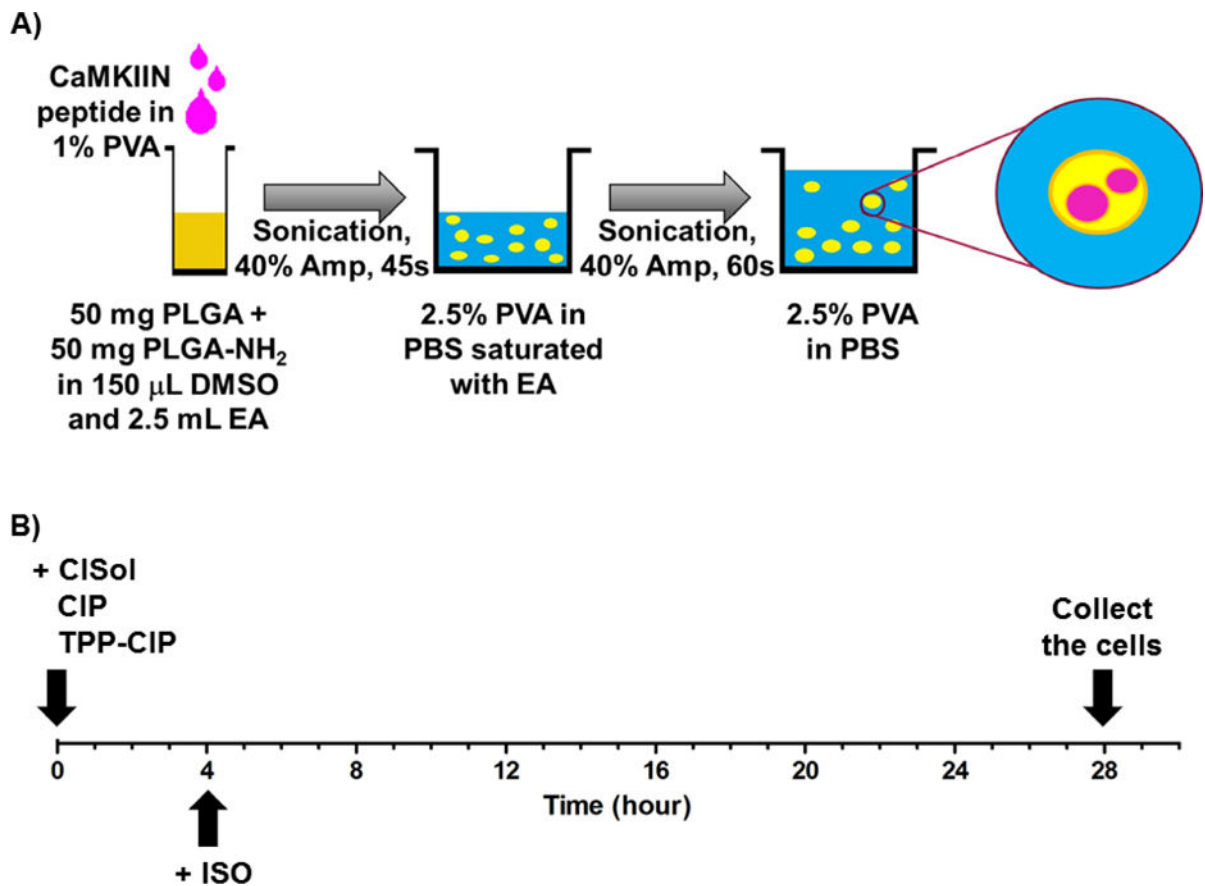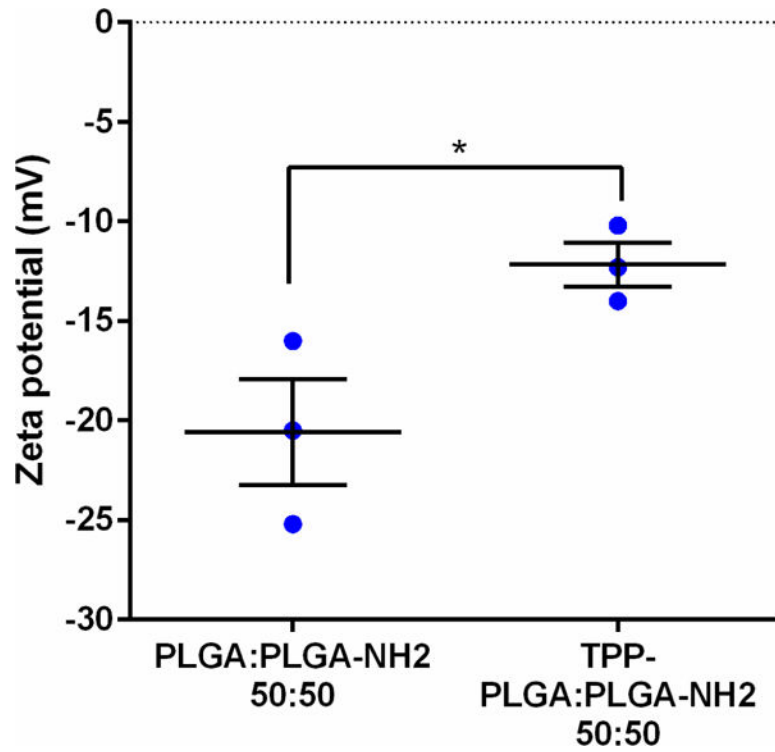
Figure 1.

(A) Fabrication of CaMKIIN-loaded particles (CIP): schematic of the protocol for loading CaMKIIN peptides into PLGA particles (see method for further details). PLGA, poly(lactic-co-glycolic-acid); PLGA-NH$_2$, amine endcapped poly(lactic-co-glycolic-acid); PVA, polyvinyl alcohol; EA, ethyl acetate; Amp, amplitude.

(B) Timeline of in vitro experiments with differentiated H9c2 cells: cells were treated with different formulations of CaMKIIN peptide at t = 0, incubated with ISO at t = 4 and collected at t = 28. CaMKIIN peptide solution (CISol); CaMKIIN loaded particles (CIP); TPP conjugated CaMKIIN loaded particles (TPP-CIP); Isoprenaline (ISO).
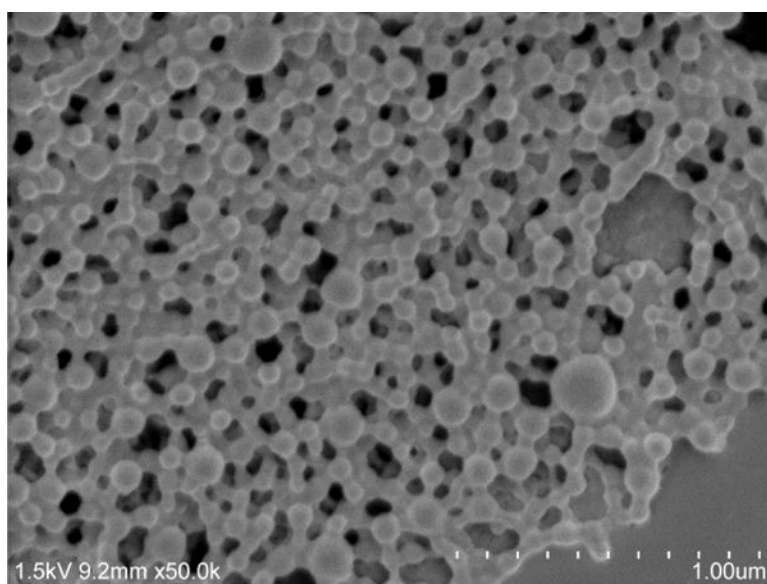
Figure 2. Representative scanning electron micrograph of particles made from PLGA and PLGA-NH$_2$ with TPP functionalization on the particle surface
Scale bars represent 1.00 μm.

Figure 3. Zeta potential of particles before and after TPP conjugation

PLGA: PLGA-NH$_2$, 50:50represents particles prepared using a mixture of PLGA and PLGA-NH$_2$ at the ratio of 50:50. TPP- PLGA: PLGA-NH$_2$, 50:50 represents particles made from mixture of PLGA and PLGA-NH$_2$ at the ratio of 50:50 with TPP conjugation. Data are expressed as scatter plots (n = 3). Unpaired two-tailed t-test was conducted to determine significant differences between PLGA: PLGA-NH$_2$, 50:50 and TPP-PLGA: PLGA-NH$_2$, 50:50.

*$p < 0.05$.
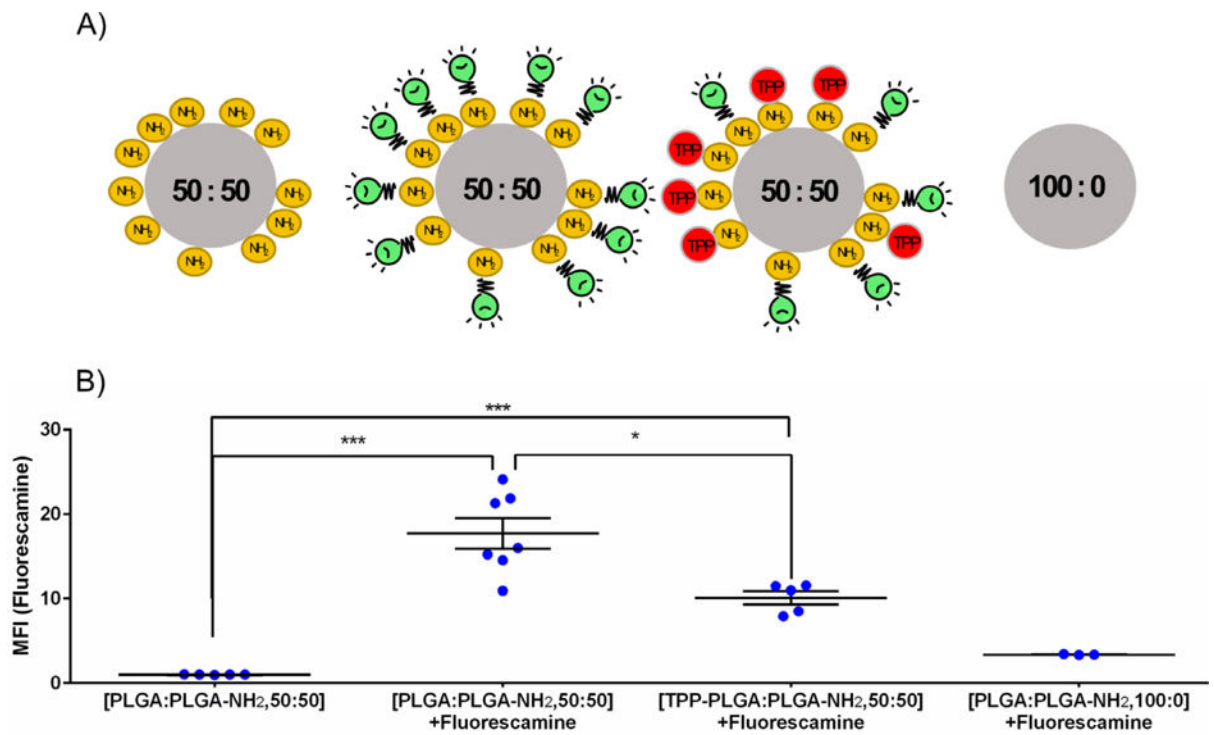
Figure 4. TPP conjugation to PLGA:PLGA-NH$_2$ particles

(A) Schematic of particles with and without TPP conjugation (PLGA:PLGA-NH$_2$ and TPP-PLGA:PLGA-NH$_2$) and incubated with fluorescamine. (B) Mean fluorescence intensity (MFI) obtained from particles treated with/without fluorescamine as indicated. PLGA:PLGA-NH$_2$, 50:50 = particles made from a mixture of PLGA and PLGA-NH$_2$ at the ratio of 50:50; TPP-PLGA:PLGA-NH$_2$, 50:50 = TPP-conjugated particles made from a mixture of PLGA and PLGA-NH$_2$ at the ratio of 50:50; PLGA:PLGA-NH$_2$, 100:0 = particles made purely from PLGA with no amine functionalization. Green light bulbs represent fluorescamine. Data are expressed as scatter plots (n = 3 – 7). One-way analysis of variance with Bonferroni's multiple comparisons test was performed. ***$p < 0.001$, *$p < 0.05$.
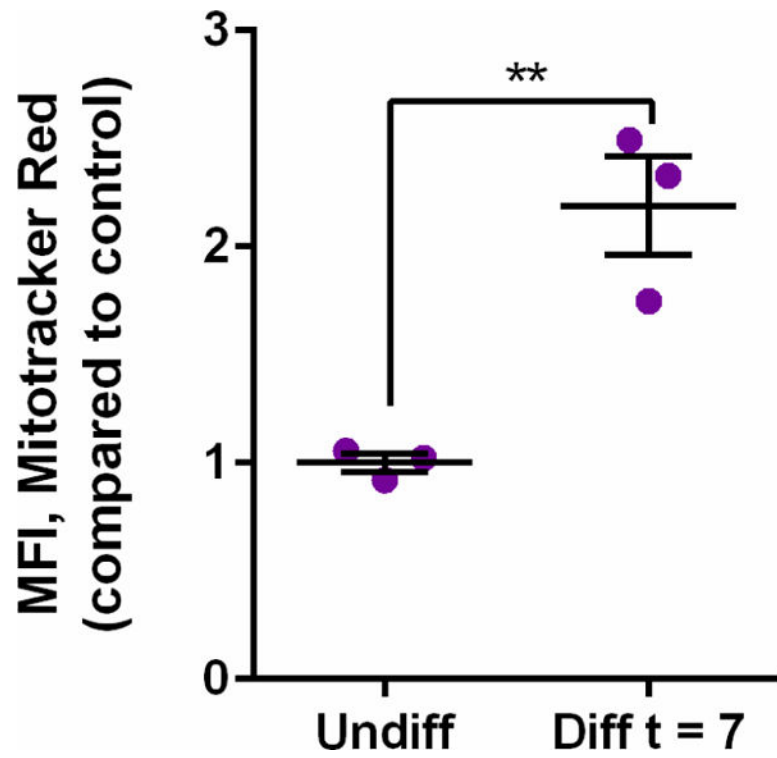
Figure 5. Relative mitochondrial mass as determined using Mitotracker® Red

Relative mean fluorescence intensity (MFI) due to Mitotracker® Red comparing two groups of cells: H9c2 cells that were exposed to the differentiation agent, all-trans-retinoic acid, for 7 days (Diff t = 7) and H9c2 cells not treated with all-trans retinoic acid (Undiff). Data are expressed as scatter plots (n = 3). Unpaired two-tailed t-test was performed. **$p < 0.01$.
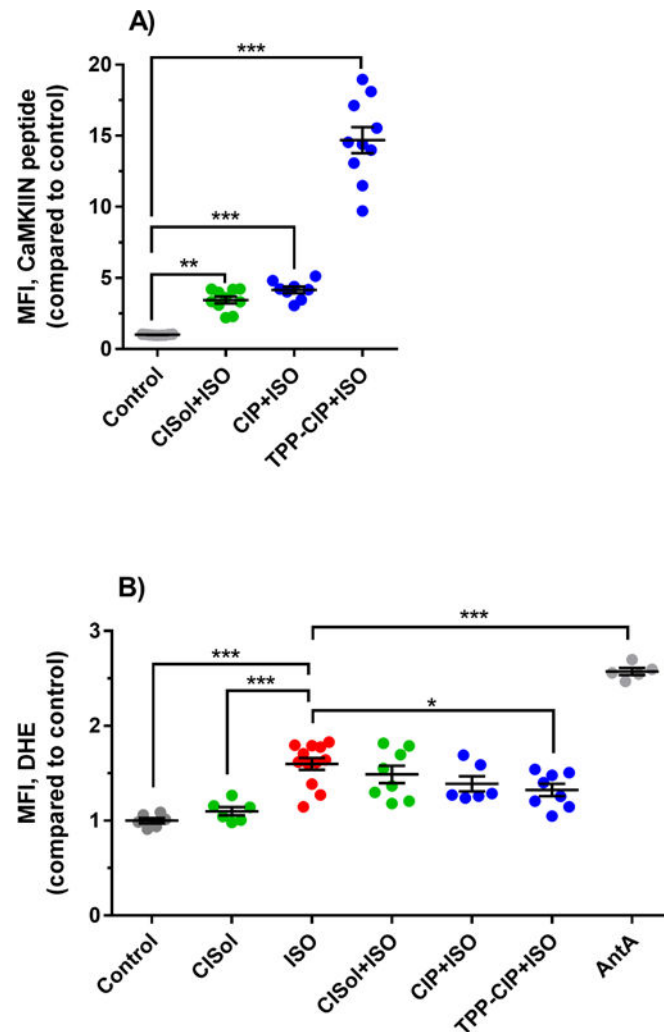
Figure 6. Enhanced uptake of, and reduced pro-oxidants levels due to, TPP-CIP in H9c2 cells
(A) Mean fluorescence intensity (MFI) from fluorescently-tagged CaMKIIN peptide obtained from cells that were incubated with CaMKIIN solution (CISol), CaMKIIN loaded particles (CIP) and TPP-conjugated CaMKIIN loaded particles (TPP-CIP) and treated with isoprenaline (ISO). Data are expressed scatter plots, (n = 8 – 12). One-way analysis of variance with Bonferroni's multiple comparisons test compared to the control was performed. ***$p < 0.001$, **$p < 0.01$. (B) Mean fluorescence intensity (MFI) due to the presence of intracellular pro-oxidants as detected by dihydroethidium oxidation (DHE) obtained from cells that were incubated with CaMKIIN solution (CISol), CaMKIIN loaded particles (CIP) and TPP-conjugated CaMKIIN loaded particles (TPP-CIP) and treated with isoprenaline (ISO). Antimycin A (AntA) was used as a positive control. AntA increased the MFI by 2.6-fold when compared to the control group. Data are expressed as scatter plots, (n = 5 – 12). One-way analysis of variance with Bonferroni's multiple comparisons test compared to the ISO was performed. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.
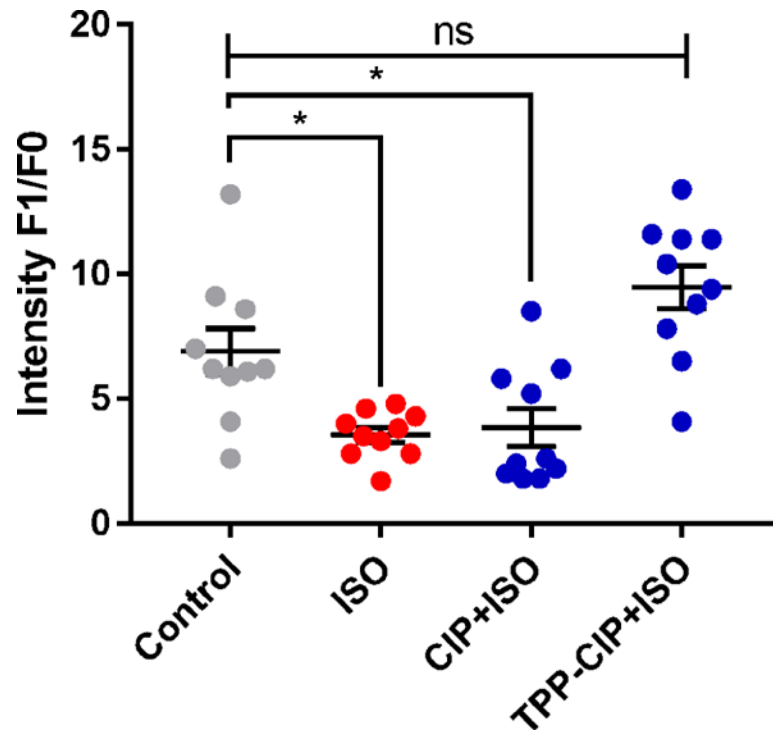
Figure 7. Effect of pretreatment with CIP at maintaining the mitochondria membrane potential in ISO-treated H9c2 cells

TMRM intensity (F1/F0) obtained from epi-fluorescence images of H9c2 cells that were stained with TMRM dye after incubation with either CAMKIIN loaded particles (CIP) or TPP-conjugated CaMKIIN loaded particles (TPP-CIP) then treated with isoprenaline (ISO). F1 represents the raw intensity data. F0 represents the background intensity data which were obtained from the areas of the images that contained no cells. Control group is untreated cells. Data are expressed as scatter plots, (n = 10). One-way analysis of variance with Bonferroni's multiple comparisons test compared to the control group was performed. *$p < 0.05$. ns, not statistically significant.