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Modulation of α7nAchR for Cardioprotection against Isoprenaline-Induced Myocardial Injury

(Modulasi α7nAchR untuk Kardioperlindung terhadap Kecederaan Miokardium Aruhan Isoprenalin)

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ABSTRACT

The α7nAchR gene is expressed in immune cells, including macrophages, and is linked to cardiovascular diseases. The cardioprotective effects of α7nAchR activation against inflammation in isoprenaline-induced myocardial injury are unclear. This study aims to assess the cardioprotective effects of α7nAChR activation on isoprenaline-induced myocardial injury rats. We hypothesized that α7nAChR activation provides cardioprotection against the myocardial injury via the cholinergic anti-inflammatory pathway. Isoprenaline hydrochloride (85 mg/kg) was injected subcutaneously in rats to induce myocardial injury and activated the α7nAchR through daily transcutaneous tragus stimulation for 14 days at 20 Hz, 0.2 ms, and 2 mA. Examination on Langendorff isolated heart perfusion technique showed improvement in cardiac functions. The electrical stimulation affected collagen deposition, circulating troponin T, and circulating inflammatory marker TNFα. The effects were abolished with pharmacological inhibition of α7nAChR. Further, the role of α7nAChR in ischemia-induced inflammation was studied in RAW264.7 macrophages. Inflammation was activated in RAW264.7 macrophages that were treated with glucose-free media, flushed with 95% N_2, and 5% CO_2 for 1 h, and reoxygenated in a normoxic incubator for 24 h. α7nAchR stimulation in the activated macrophages reduced pro-inflammatory markers (NO, TNFα) but did not affect anti-inflammatory (IL10, TGFβ) expression levels. The reduction in pro-inflammatory factors was linked to the modulation of the transcriptional regulator NFκB, but not STAT3. Our findings suggest that activation of the cholinergic anti-inflammatory pathway may protect against isoprenaline-induced cardiac injury by improving cardiac performance and regulating inflammatory macrophage activity potentially via the NFκB/TNFα pathway.

Keywords: Inflammation; isoprenaline; myocardial injury; transcutaneous tragus stimulation; α7nAchR

ABSTRAK

Gen α7nAchR diekspresikan dalam sel imun, termasuk makrofaj dan dikaitkan dengan penyakit kardiovaskular. Kesan kardiopelindung pengaktifan α7nAchR terhadap kecederaan miokardium aruhan isoprenalin adalah tidak diketahui. Kajian ini bertujuan untuk menilai kesan kardiopelindung pengaktifan α7nAChR pada tikus yang mengalami kecederaan miokardium aruhan isoprenalina. Kami membuat hipotesis bahawa pengaktifan α7nAChR menyediakan perlindungan terhadap kecederaan miokardium yang disebabkan oleh isoprenalin melalui laluan anti-radang kolinergik. Isoprenalin hidroklorida (85 mg/kg) telah disuntik secara subkutaneus pada tikus untuk menginduksi kecederaan miokardium dan mengaktifkan α7nAchR melalui rangsangan tragus transkutaneus harian selama 14 hari pada 20 Hz, 0.2 ms dan 2 mA. Pemeriksaan menggunakan teknik perfusi jantung terasing Langendorff menunjukkan peningkatan dalam fungsi jantung. Rangsangan elektrik mempengaruhi pemendapan kolagen, troponin T dalam peredaran dan penanda keradangan TNFα dalam sistem peredaran. Kesan tersebut dihapuskan dengan perencatan farmakologi α7nAChR. Selain itu, peranan α7nAChR dalam keradangan aruhan iskemia dikaji pada makrofaj RAW264.7. Makrofaj RAW264.7 telah mengalami pengaktifan melalui rawatan di dalam medium bebas glukosa, pengaliran dengan 95% N_2, 5% CO_2 selama 1 jam dan dioksigenkan semula dalam inkubator selama 24 jam. Rangsangan α7nAChR dalam makrofaj yang telah diaktifkan

mengurangkan penanda pro-radang (NO, TNFα) tetapi tidak mempengaeruhili tahap pengekspresan anti-radang (IL10, TGFβ). Pengurangan faktor pro-radang dikaitkan dengan modulasi pengawal selia transkripsi NFκB, tetapi bukan STAT3. Penemuan kami mencadangkan bahawa pengaktifan laluan anti-radang kolinergik mungkin melindungi daripada kecederaan jantung aruhan isoprenalina dengan meningkatkan prestasi jantung dan mengawal aktiviti makrofaj keradangan, berpotensi melalui laluan tapak NFκB/TNFα.

Kata kunci: Isoprenalin; kecederaan miokardium; keradangan; rangsangan tragus transkutaneus; α7nAchR

INTRODUCTION

Cardiovascular diseases (CVD) remain a health threat to the global population. The current global incidence rate of CVD in individuals younger than 60 years has been determined as 3.8%, and in individuals aged 60 years and above, the rate has reached 9.5% (Salari et al. 2023). The anticipated rise in the worldwide elderly population afflicted with CVD, which is projected to reach a rate of 1,845 per 100,000 individuals by 2030, elicits apprehension due to the potential for a significant financial strain on the healthcare infrastructure (Khan et al. 2020).

Myocardial injury (MI) can be an immediate manifestation of underlying CVD, with the mismatch between oxygen demand and supply acting as a precursor to the inflammatory response in MI. During acute hypoxia, a pro-inflammatory response is initially activated to remove necrotic cell debris, followed by an anti-inflammatory response that initiates tissue healing and scar formation to maintain the structural integrity of the heart (Chung et al. 2020). A balanced transition between the pro-inflammatory and anti-inflammatory phases is required for proper cardiac tissue repair. Any discrepancy in the dynamic interplay between these phases leads to fibrosis formation, ventricular wall thickening, and ultimately, a cardiac de-compensatory state (Chung et al. 2020). Hence, management of myocardial inflammation following cardiac insult is critical to limit cardiac vascular remodelling (Shook, Singh & Singh 2023).

Isoprenaline (ISO) is a β-adrenergic receptor agonist with the ability to increase chronotropism and inotropism in the heart muscle. ISO treatment also causes peripheral blood vessel dilation. Thus, the combined effects of ISO treatment result in unmet oxygen demand by the myocardium, making it a suitable model for experimental MI, such as myocardial ischemia and takotsubo cardiomyopathy (Ali et al. 2020; Forte et al. 2021). For example, ISO has been shown to induce infarct-like lesions in rats when administered at high doses (85–160 mg/kg) (Forte et al. 2021; Wang et al. 2022; Yovas et al. 2022). Indeed, ISO-induced MI causes metabolic and morphological changes in the heart tissue of experimental animals, with features like those seen in humans (Forte et al. 2021; Rroku et al. 2022). Furthermore, the relationship between cardiac inflammation and fibrogenesis in ISO-model MI has been well documented (Forte et al. 2021; Zhang et al. 2020); ISO-associated cardiac injury activates CD4+ helper T cells that induce B cells to generate anti-heart auto-antibodies, a hallmark sign of autoimmune response in MI (Forte et al. 2021).

Electrical stimulation of the auricular branch of the vagus nerve in the tragus has been proposed as an effective approach for treating failing hearts (Chung et al. 2020). Specifically, applying transcutaneous tragus stimulation (TTS) in individuals diagnosed with heart failure has been shown to improve the occurrence of ventricular arrhythmia (Stavrakis et al. 2017; Yu et al. 2017). Furthermore, tragal stimulation improved the interstitial cardiac fibrosis score, ventricular tachycardia, arrhythmia, infarct size, and left ventricular ejection fraction equivalent to invasive vagus nerve stimulation (VNS) in animals with ischemic MI induced by coronary artery ligation (Chen et al. 2022; Dasari et al. 2021; Kulkarni et al. 2021). However, the cardioprotective potential of TTS in ISO-induced MI models has not yet been tested.

The α7-nicotinic acetylcholine receptor (α7nAchR), a five subunit of ligand gated ion channel is one of the target receptor from the vagus nerve stimulation to induce cholinergic anti-inflammatory pathway. Acetylcholine mediates neural transmission in the ganglion synapses of parasympathetic neuron in the postganglionic vagal efferent neuron to resolve inflammatory response (Chung et al. 2020). Stimulating α7nAChR markedly reduced the level of inflammatory cytokine and modulates macrophages infiltration potentially via the intracellular signaling pathway, janus kinase2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway (Arunrungvichian et al. 2024). The activation of cholinergic anti-inflammatory pathway eventually lead to negative modulation of NF-κB resulting in reduction of pro-inflammatory cytokines (Arunrungvichian et al. 2024). Pre-treatment with α-bungarotoxin blocked the cardioprotective effects of α7nAchR in mice with myocardial injury (Kong et al. 2018).

The primary aim of this study was to examine the cardioprotective effects of α7nAChR activation on ISO-induced MI using TTS. We used the Langendorff isolated heart preparation to assess left ventricle function and fibrosis, examining circulating markers for cardiac injury and inflammation. We further investigated the anti-inflammatory mechanism from α7nAChR activation on macrophages exposed to hypoxia conditions, as mismatches in oxygen demand and supply have been linked to ISO-induced cardiac injury (Radhakrishnan et al. 2023). This study could elucidate TTS-induced α7nAChR activation as an adjunct therapy for MI resulting from decreased oxygen accessibility.

MATERIALS AND METHODS

IN VIVO INVESTIGATION OF THE CARDIOPROTECTIVE EFFECTS OF α7nAchR ACTIVATION IN ISO-INDUCED MYOCARDIAL INJURY

Experimental animals

Male Sprague-Dawley rats (age: 6 weeks; weight: 150-200 g) were purchased from the Universiti Kebangsaan Malaysia (UKM) Laboratory Animal Resource Unit (LARU). Male rats are preferred to eliminate the biological sex variations in cardiac physiology that have been reported in female rodents (Blenck et al. 2016). All rats were acclimatized for 3 days and housed with free access to pellets and water in the UKM animal laboratory under standard laboratory conditions (Capdevila et al. 2007). The Animal Ethics Committee of UKM (UKMAEC) approved all experiments described herewith, which were conducted according to the guidelines and regulations based on the Malaysian Code of Practice for the Care and Use of Animals for Scientific Purposes (2015) (Reference: FF/2018/MOHD KAISAN/28-NOV./964-NOV.-2018-AUG.-2021). All experiments also followed the three R's (Replace, Reduce, Refine) principles, and the animals were treated with care before being humanely euthanized at the end of the experiment.

Experimental design

The rats were randomly divided into five groups. Rats in the control group (n=6) were administered normal saline (0.9%) subcutaneously for two consecutive days. Similarly, ISO hydrochloride (85 mg/kg; Sigma-Aldrich, USA) was administered subcutaneously for two days to induce MI (n=6). MI + Sham (n=6) received electrical stimulation at the scapha since this region lacks vagal innervation (Mahadi et al. 2019). MI + TTS (n=6) received electrical stimulation at the tragus since this region contained auricular branch of the vagus nerve. The role of α7nAChR in MI was further studied in MI + TTS + α-bungarotoxin (αB) (n=6). αB is an α7nAChR inhibitor (ThermoFisher, USA) and administered intraperitoneally at a dose of 1 µg/kg for 1 h before the daily TTS until day 14.

Anaesthesia protocol

The ketamine-xylazine-zoletil (KTX) cocktail was purchased from the Universiti Kebangsaan Malaysia Laboratory Animal Resource Unit. KTX stock solution was prepared by mixing zoletil-50 (a mixture of 125 mg of tiletamine and 125 mg of zolazepam) with 2.5 mL of ketamine (250 mg/mL) and 12.5 mL xylazine (250 mg/mL). The KTX cocktail was made by diluting 1 mL of the KTX stock solution with 4 mL of normal saline (ratio 1:4) and stored in 4 °C. Rats from all experimental groups, including the control group, were subjected to daily intraperitoneal anaesthesia using a dosage of 0.15 mL/100 g of body weight. The response to noxious stimuli, such as the loss of the forelimb, hindlimb, eyelid, and tail pinch, was used to determine anaesthetic depth (Tsukamoto et al. 2018). A subsequent dose of 0.1 mL was injected after 30 min to maintain the depth of anaesthesia.

Transcutaneous tragus stimulation

After confirming the loss of pedal reflex via toe pinch, a set of electrodes was attached to the rats' tragus, and electrical stimulation at 20 Hz, 0.2 ms, and 2 mA was applied daily for 14 consecutive days (ADInstruments, Australia), an hour per day (Mahadi et al. 2019). As our previous study found that the autonomic control of the heart is affected by the time of day, the stimulation time was kept constant throughout the experiment at 10 am-11 am (Mahadi et al. 2019). Sham received stimulation at the scapha (sham region) at the same intensity as MI followed by TTS.

Isolated Langendorff heart perfusion

The Langendorff technique was performed to evaluate heart function *ex vivo* as previously described (ADInstruments, Australia) (Aziz et al. 2021). To prevent blood clot formation, the rats were first administered 500 IU of heparin. The rats were then sedated using a KTX cocktail. The rats were placed in a supine position after confirming the loss of pedal reflex via a toe pinch, and a transabdominal incision was made to expose the thoracic cavity. To stop the heartbeat, the heart was isolated and placed in a cold Krebs-Henseleit buffer. The heart was then connected to the cannula and perfused with warm Krebs-Henseleit buffer (37 °C, 95% oxygen, 5% carbon dioxide). The fat tissue from the heart was removed first, followed by the left atrial appendage. Then, a latex balloon was inserted through the mitral valve into the left ventricle to measure changes in left ventricular pressure, that is, left ventricular developed pressure (LVDP), maximum rate of left ventricular pressure increase ($+dp/dt_{max}$), maximum rate of left ventricular pressure decrease ($-dp/dt_{max}$), and heart rate in an *ex vivo* setting.

Picrosirius red staining

Picrosirius red staining was performed in accordance with an established protocol, with minor modifications to achieve the best coloration (Ali et al. 2019). The left ventricle was isolated and fixed for 24 h in 10% formalin. The left ventricle was subjected to dehydration, cleaning, and impregnation. The left ventricle was embedded in paraffin wax and sectioned at 4 µm after tissue processing to create a tissue ribbon. The tissue ribbon was then transferred onto a slide and air-dried overnight. The slides were deparaffinized, rehydrated, dehydrated, and cleaned prior to staining with Picosirius Red. The slides were mounted with DPX solution and air-dried overnight. Under a light microscope, 10 sets of images were captured for each slide at 100x magnification. To determine the

percentage of collagen deposition, 10 measurements were taken at random, and the mean percentage of collagen deposition was analyzed using Sirius Red Macro in ImageJ version 7.0 (Bethesda, Maryland, USA). The histological assessment was carried out by a second assessor who was not aware of the animal treatments.

Serum biochemical analysis

1.0 mL of blood was drawn from the tail vein, collected in a vacutainer, and stored at room temperature for an hour on D0 and D15. The blood was then centrifuged at 2000 × g for 10 min at 20 °C to obtain the serum. Serum from D0 was used to quantify the concentration of circulating troponin T (TnT) for the validation of MI. Meanwhile, serum from D15 was used to determine the concentrations of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), cardiac TnT, and matrix metallopeptidase-9 (MMP9) by enzyme-linked immunosorbent assay (ELISA), (Elabscience, China).

IN VITRO ELUCIDATION OF ANTI-INFLAMMATORY RESPONSES FOLLOWING α7nAchR ACTIVATION IN ISCHEMIC MACROPHAGES

Experimental design

RAW264.7 cells were separated into four groups. The control group consisted of RAW264.7 cells (n=3) incubated in a normoxic chamber with normal DMEM solution. The oxygen-glucose derived/reoxygenation (OGD/R) group (n=3) included RAW264.7 cells that were cultured in glucose-free DMEM and placed in a hypoxic chamber (Stemcell Technologies; Cat. No. 27310) before being transferred for reoxygenation. The OGD/R + PNU282987 (n=3) treatment group used α7nAchR agonist. The negative control is OGD/R + PNU282987 + αB (n=3), which inhibits nAchR subtypes irreversibly.

Oxygen glucose deprivation and reoxygenation

The hypoxia chamber was kept in a 37 °C incubator for 1 h before hypoxia induction. With the outlet valve open, the hypoxic gas (95% N2, 5% CO2) was flushed for 4 min at 20 L/min. The valve was later clamped, and the chamber was maintained at 37 °C within the incubator. The gas was flushed again under the same conditions for one hour. To reoxygenate hypoxic cultures, cells were placed in a regular normoxic incubator (95% air, 5% CO_2) and incubated for 24 h.

Treatments

In preparation for agonist (PNU282987) and antagonist-agonist treatments, RAW264.7 cells were cultured in 6-well plates with a seeding density of 5×10^5 cells per well, 24 h before to the experiment. Once the cell confluency reached 65% to 80%, the cells were initially exposed to oxygen-glucose deprivation and reperfusion (OGDR). During the reoxygenation phase, the cells were subjected to either a 10 nM agonist treatment or a 200 nM antagonist treatment, which was administered 5 h before the addition of a 10 nM agonist.

NO measurement

The presence of NO in the supernatant of RAW264.7 cells was determined using the Griess assay. The BV2 supernatant (50 µL) was transferred in triplicate to a 96-well plate and supplemented with 50 µL of Griess reagent. The Griess reagent is composed of a solution containing 1% sulfanilamide and 0.1% NED, which are dissolved in 2.5% phosphoric acid (Sigma-Aldrich St. Louis, MO, USA). The mixture was subjected to incubation at ambient temperature for 10 min, following which it was transferred to a microplate reader for analysis, with absorbance measurements taken at 530 nm. The concentration of nitrite was determined using a formula derived from a standard curve constructed by serially diluting sodium nitrite ($NaNO_2$) in a two-fold manner. The concentrations used for the dilutions were 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, and 0 µM. To eliminate the background reading, the absorbance values of all wells were normalized by subtracting the absorbance of the 0µM $NaNO_2$ solution. The outcomes are presented in the form of NO_2- concentration expressed in micromolar (µM) units.

RT-PCR

Total RNA was isolated from RAW264.7 cells using TRIzol Reagent (Invitrogen, US) according to the manufacturer's instructions. A Nanodrop 2000 spectrophotometer (ThermoFisher, USA) was used to measure the extracted RNA. RNA (900 ng) was then converted to cDNA as directed. The reverse transcription reaction was performed using a SensiFAST™ SYBR® No-ROX Kit by the manufacturer's instructions (Bioline, UK). In a final reaction volume of 10 µL, the PCR contained 5 µL SYBR Green PCR mix, 0.4 µM each primer, and 4.2 µL (50 ng) cDNA template and water (ThermoFisher, Japan). Using a CFX connect real-time PCR system (BIO-RAD, US),

the cycling conditions were 95 °C for 2 min; 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) and the corresponding primers (Table 1) were normalized to GAPDH using the 2Ct method and presented as relative expression. As an internal control, GAPDH was used.

STATISTICAL ANALYSIS

All statistical tests were carried out using the R Statistical Package Jamovi (Sydney, Australia). All data are presented as mean ± SEM. ANOVA was used to examine differences between multiple groups. The statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey's post hoc test.

RESULTS

IN VIVO INVESTIGATION OF CARDIOPROTECTION ELICITED BY α7nAchR ACTIVATION

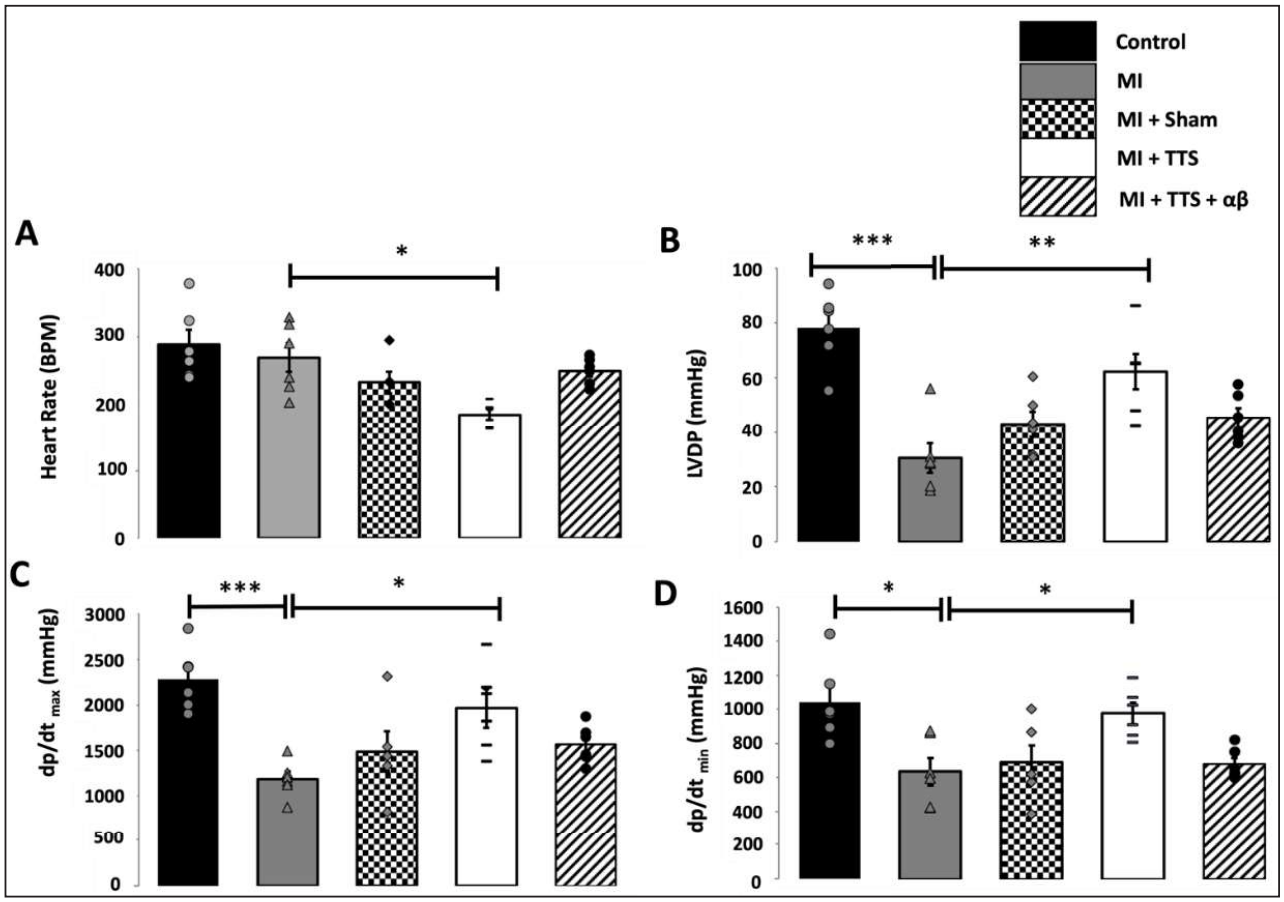
Effects on left ventricular function

In general, tragal stimulation improved myocardial contractility parameters, LVDP, and $+dp/dt_{max}$. TTS also influenced myocardial diastolic capability, including $-dp/dt_{max}$ and average heart rate (HR). As shown in Figure 1(A), the average HR did not differ significantly between the control (288 ± 54 bpm), MI (269 ± 52.5 bpm), sham (232 ± 39.1 bpm), and αB groups (249 ± 19.5 bpm). However, the average HR in the TTS group was significantly lower (184 ± 17.9 bpm; $p < .05$) than that in untreated MI group. The LVDP measurements (Figure 1(B)) showed that animals with MI significantly had lower LVDP (30.6 ± 13.4 mmHg) than the control group (78.3 ± 13.5 mmHg; $p < .001$). Compared with animals with MI, LVDP showed a significant increase in the TTS-treated animals (62.1 ± 15.6 mmHg; $p < .01$). LVDP in the sham (42.8 ± 11.1 mmHg) and αB (45.1 ± 8.6 mmHg) groups showed non-significant changes, almost identical to MI alone.

MI alone had a significantly lower $+dp/dt_{max}$ (1186 ± 201 mmHg/s) than the control group (2287 ± 342 mmHg/s; $p < .001$; Figure 1(C)). In contrast, TTS-treated animals showed a significant increase in $+dp/dt_{max}$ (1963 ± 468 mmHg/s; $p < .05$) when compared to those with untreated MI. Sham (1488 ± 539 mmHg/s) and αB (1568 ± 209 mmHg/s) treatment did not affect $+dp/dt_{max}$, as the recorded value was nearly equivalent to that of MI alone. Additionally, MI alone had a significantly lower dp/dt_{min} (632 ± 201 mmHg/s) than the control group (1042 ± 228 mmHg/s; $p < .05$; Figure 1(D)). Compared to the MI group, TTS-treated animals showed a significant increase in dp/

TABLE 1. Primer sequences

Gene	Primer	Sequence (5′–3′)
TNFα	Forward	GGTGCCTATGTCTCAGCCTCTT
	Reverse	GCCATAGAACTGATGAGAGGGAG
IL-6	Forward	TGTATGAACAACGATGATGCACTTG
	Reverse	AGGTAGCTATGGTACTCCAGAAGAC
IL-10	Forward	CGGGAAGACAATAACTGCACCC
	Reverse	CGGTTAGCAGTATGTTGTCCAGC
TGF-ß	Forward	TGATACGCCTGAGTGGCTGTCT
	Reverse	CACAAGAGCAGTGAGCGCTGAA
GAPDH	Forward	AACTTTGGCATTGTGGAAGG
	Reverse	ACACATTGGGGGTAGGAACA
NF-kB p65	Forward	TCCTGTTCGAGTCTCCATGCAG
	Reverse	GGTCTCATAGGTCCTTTTGCGC
STAT3	Forward	AGGAGTCTAACAACGGCAGCCT
	Reverse	GTGGTACACCTCAGTCTCGAAG



*denotes significant difference p < .05. ** denotes significant difference p < .01. *** denotes a significant difference p < .001. MI: myocardial injury; TTS: transcutaneous tragus stimulation

FIGURE 1. Cardioprotective effects of TTS measured by Langendorff isolated heart preparation (n = 6 for every group). (A) HR was significantly reduced in the TTS-treated group, and (B-D) Cardiovascular parameters, including LVDP, +dp/dtmin, and -dp/dtmax, significantly deteriorated in animals with MI. TTS significantly reversed cardiac dysfunction. Cardioprotection was abolished in the sham control and αβ groups. Values are presented as mean SEM. The statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey's post hoc test

dt_{min} (975 ± 145 mmHg/s; $p < .05$), whereas this effect was absent in the sham (688 ± 246 mmHg/s) and αB (676 ± 89 mmHg/s) groups.

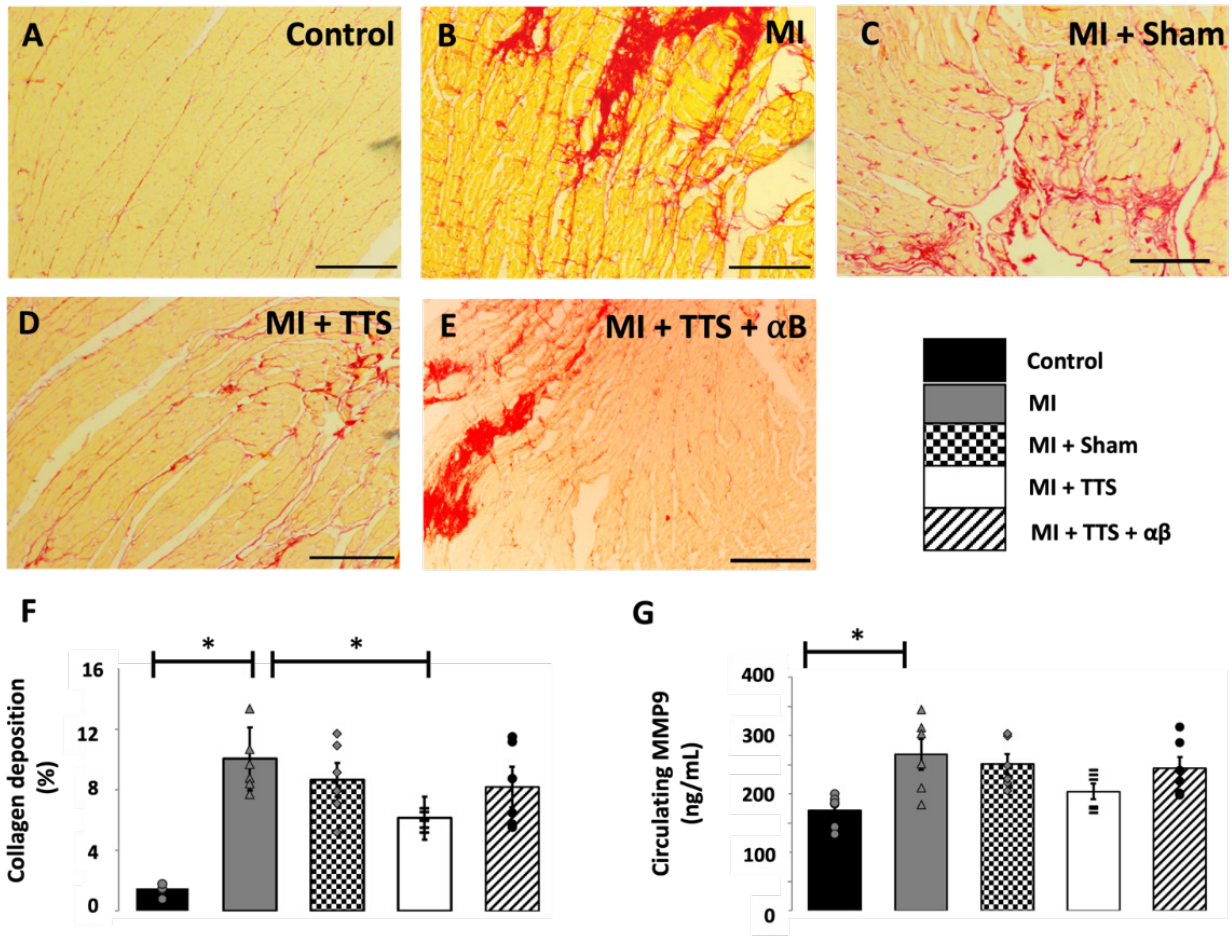
Effects on collagen deposition in the left ventricle

Picrosirius red staining was used to examine the effect of TTS on collagen deposition (Figure 2(A)-2(E)). In terms of collagen deposition (%) (Figure 2(F)), MI had a significant increase (P<.01) in collagen deposition in the left ventricular region (10.1 ± 4.7%) when compared to the control group (1.5 ± 0.4%). Cardiomyocytes in the TTS-treated group (6.1 ± 3.2%) had a significantly lower percentage (P <.05) of collagen deposition than the MI group (10.1 ± 4.7%). This effect of TTS was eliminated in sham (8.7 ± 2.7%) and αB (8.2 ± 3.0%), as evidenced by an increase in the percentage of collagen deposition in both groups. For the circulating levels of MMP9 (Figure 2G), MI (268.2 ± 63.6 ng/mL) were significantly higher than the control (172.1 ± 28.1 ng/mL; P < .05). The levels of circulating MMP9 in TTS (204.3 ± 32.71 ng/mL) Sham (251.3 ± 42.11 ng/mL) and αB (244.3 ± 47.6 ng/mL) did not change significantly.

IN VIVO EXAMINATION OF CIRCULATING MARKERS FOLLOWING α7nAchR ACTIVATION

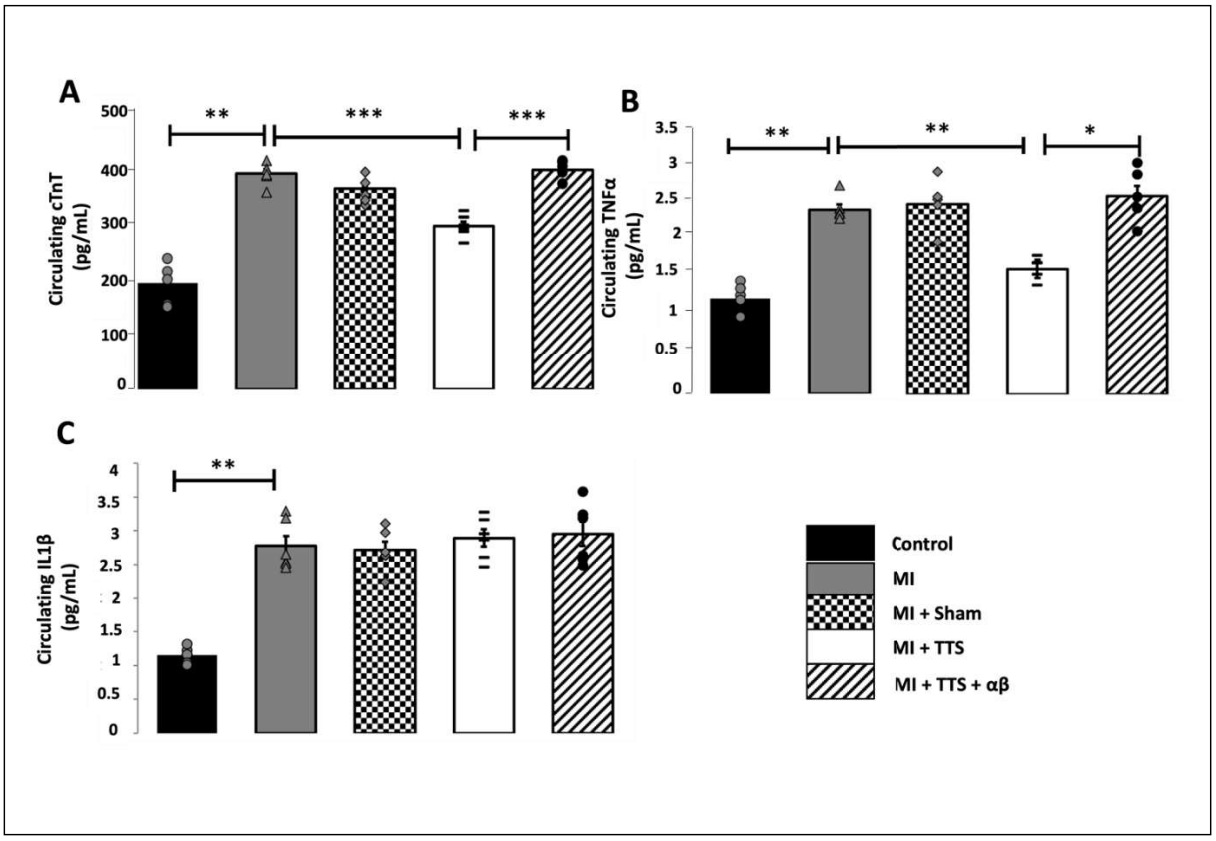
The animals with MI exhibited significantly elevated levels of cTnT (Figure 3(A)) compared with the healthy groups (192.8 ± 13.9 pg/mL vs. 388.3 ± 7.7 pg/mL; $p < .01$). We found no statistically significant differences in the circulating cTnT levels between the sham stimulation group (361.5 ± 9.9 pg/mL) and the MI group. However, the experimental group that underwent TTS exhibited a significant reduction in circulating levels of cTnT (295.2 ± 8.2 pg/mL; $p < .001$) compared to the MI group. The group administered with αB exhibited a statistically significant increase in circulating cTnT levels (394.9 ± 6.4 pg/mL; $p < .001$) compared to the group subjected to TTS treatment.

We also assessed the concentrations of TNFα in the animals' circulation (Figure 3(B)). These levels were found to be significantly higher in the MI group (2.47 ± 0.07 pg/mL; $p < .001$) compared to the control group (1.28 ± 0.07), while the sham control group exhibited TNFα concentrations (average = 2.54 ± 0.12 pg/mL) comparable to those observed in the MI group. The circulating levels of TNFα were found to be significantly lower (1.68 ± 0.06 pg/mL) after tragus stimulation compared to the levels in the MI group. However, administration of αB to MI rats



*denotes significant difference when compared with MI group P < .05. **denotes significant difference when compared with MI group P<.01. ***denotes significant difference when compared with MI group P<.001. MI: myocardial injury, TTS: transcutaneous tragus stimulation

FIGURE 2. TTS effects on fibrogenesis in rats with MI. (A-E) Representative results of picrosirius red stained fibrotic on myocardial tissue (n = 6 for every group). Magnification 100x; scale bar 200 (E) MI significantly increased collagen deposition. TTS reduced collagen deposition in myocardial tissue, and (F) The fibrosis biomarker MMP9 was significantly higher in the MI group. The TTS-treated rats showed no significant reduction in circulating MMP9. Values are represented as mean SEM. The statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey's post hoc test



*denotes significant difference p < .05. **denotes significant difference p < .01. ***denotes a significant difference p < .001. MI: myocardial injury; TTS: transcutaneous tragus stimulation; αβ: α-bungarotoxin; cTnT: cardiac troponin T; TNFα: tumour necrosis factor-alpha; IL-1β: interleukin one beta

FIGURE 3. Circulating biomarker analysis (n = 6 for every group) measured by ELISA. (A) Elevated circulating cardiac injury marker cTnT in MI was reverted by TTS therapy and exacerbated by αB injection, (B) The circulating pro-inflammatory marker TNFα was not affected in MI and TTS-treated animals, and (C) IL-1β levels were elevated in MI and were unaffected by TTS therapy. Values are presented as mean SEM. The statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey's post hoc test

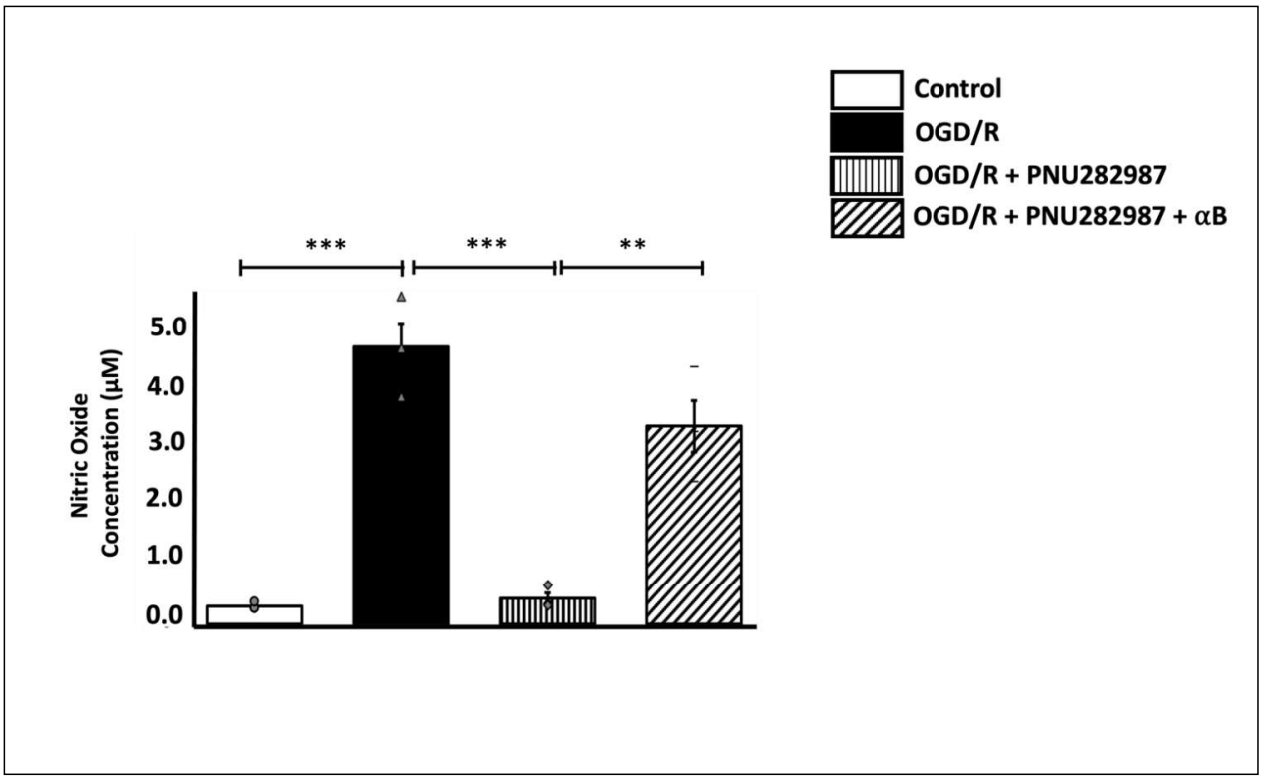
before TTS attenuated its beneficial effect and resulted in a significant increase in the levels of circulating TNFα (2.64 ± 0.13 pm/mL; $p < .05$). Finally, the circulating levels of IL-1β (Figure 3C) in MI-induced animals were significantly higher than in the controls (1.2 ± 0.1 ng/mL vs. 2.8 ± 0.4 ng/mL; $p < .05$). The levels of circulating IL-1β in the TTS (2.9 ± 0.3 ng/mL), sham (2.7 ± 0.3 ng/mL) and αB (2.9 ± 0.4 ng/mL) groups did not change significantly when compared to MI.

IN VITRO INVESTIGATION OF α7nAchR ACTIVATION IN RAW264.7 MACROPHAGES INDUCED BY OXYGEN-DEFICIENT AND REOXYGENATION

To validate the anti-inflammatory observed in ISO-induced MI *in vivo*, we investigated the effects of α7nAchR activation in RAW264.7 macrophages conditioned to OGDR.

NO concentration

The production of NO by macrophages indicates an activation to modulate immune responses. A Griess assay (Figure 4) showed that the NO concentration was negligible in the control group (0.3 ± 0.03 μM) but was significantly increased in the OGD/R group (4.9 ± 0.4 μM; $p < .001$). However, PNU282987 treatment significantly curbed the increase in NO concentration induced by OGD/R (0.5 ± 0.1 μM; $p < .001$). Compared to the PNU282987 treatment, pretreatment with αB on RAW264.7 cells resulted in a significant increase in the NO concentration in the culture media following OGD/R (3.5 ± 0.5 μM; $p < .01$).



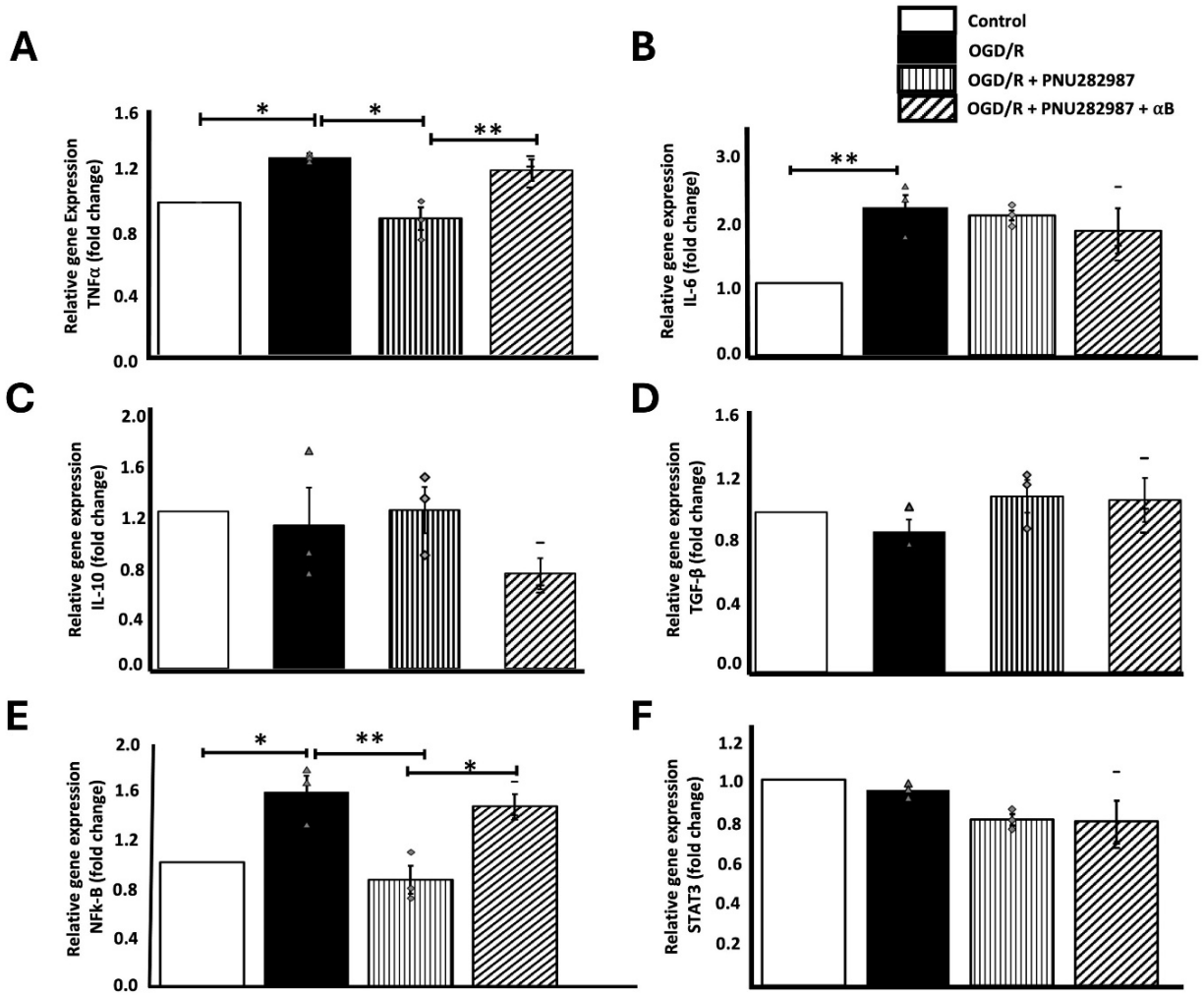
*denotes significant difference $p < .05$. **denotes significant difference $p < .01$.
***denotes a significant difference $p < .001$. NO: Nitric oxide

FIGURE 4. NO concentration according to the Griess Assay (n = 3). The levels of NO exhibited a substantial increase after the deprivation of oxygen and glucose and subsequent reoxygenation. Administration of PNU286287 reduced NO released by RAW264.7 macrophages. The inclusion of an α7nAchR antagonist resulted in the restoration of NO levels similar to those observed in the oxygen-glucose deprivation/reoxygenation group alone. Values are presented as mean ± SEM. The statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey's post hoc test

Inflammatory gene expression from the cholinergic anti-inflammatory pathway in vitro

TNFα is a key mediator produced by activated macrophages. Following OGD/R, TNFα expression (Figure 5(A)) was significantly increased in RAW264.7 (1.3 ± 0.03-fold change; $p < .05$). Gene expression was significantly reduced following PNU286287 treatment after OGD/R (0.9 ± 0.1-fold change; $p < .05$). The TNFα gene expression was significantly increased in the treatment group that received both αB and PNU286287 compared with the group that only received PNU286287 (1.2 ± 0.1-fold change; $p < .05$). Other than TNFα, IL-6 is a key inflammatory mediators released by activated macrophages. Following OGD/R, IL-6 expression (Figure 5(B)) was significantly increased in RAW264.7 (2.1 ± 0.1-fold change; $p < .01$). The gene expression levels observed after treatment with PNU286287 (1.9 ± 0.1-fold change) and pre-treatment with αB (1.9 ± 0.2-fold change) did not show any statistically significant differences compared with the OGDR group alone.

NFκB is a key transcriptional factor for the pro-inflammatory factors released by activated macrophages. NFκB gene expression (Figure 5(E)) was significantly higher in the OGDR group (1.6 ± 0.1-fold change; $p < .05$). PNU286287 agonists resulted in a significant decrease in NFκB gene expression (0.9 ± 0.1-fold change; $p < .01$). When compared with the agonist group, the αB pre-treatment resulted in a significant increase in NFκB gene expression (1.5 ± 0.1-fold change; $p < .05$). The anti-inflammatory factor gene expression levels in RAW264.7 macrophages conditioned with OGDR were also investigated. However, the expression levels of IL-10 (Figure 5(C)), TGF-β (Figure 5(D)), and STAT3 (Figure 5(F)) were not significantly altered by treatment with PNU286287 or αB compared to the OGD/R group alone.



*denotes significant difference $p < .05$. **denotes significant difference $p < .01$. ***denotes a significant difference $p < .001$. αB: α-bungarotoxin; IL-6: interleukin 6; IL-10: interleukin 10; OGD/R: oxygen-glucose deprivation/reoxygenation; TGF-β: transforming growth factor beta; TNF-α: tumour necrosis factor alpha

FIGURE 5. Expression of inflammatory genes in RAW264.7 cells (n = 3). (A) OGD/R significantly increased the TNFα gene expression inhibited by PNU286287 and reverted by αB, (B) IL-6 was significantly increased by OGD/R but not affected by PNU286287 and αB, (C-D) No significant effects were observed on IL-10 and TGF-β across all groups, (D) OGD/R significantly increased the NFκB gene expression inhibited by PNU286287 and reverted by the αB, and (E) No significant effects were observed on STAT3 across all groups. Values are presented as mean ± SEM

DISCUSSION

Activation of α7nAchR in rats with ISO-induced MI was accomplished by administering TTS at the tragus daily for 2 weeks at 20 Hz, 0.2 ms, and 2 mA. We investigated the cardioprotective effects of TTS using Langendorff isolated heart preparations and found that the TTS improved left ventricular contractility and relaxation functions. Picrosirius red histology of the left ventricle showed a significant decrease in collagen deposition in the TTS-treated group. Additionally, TTS reduced the levels of circulating markers for cardiac injury and inflammation, as measured by cTnT and TNFα, respectively. However, the presence of αB (nAchR antagonist) abolished these effects, indicating that the α7nAchR potentially plays a critical role in mediating the cardioprotective effects by TTS in ISO-induced MI.

Furthermore, we investigated the role of α7nAchR in RAW264.7 macrophages in response to hypoxia,

simulating the imbalance of oxygen demand and supply as well as a hypoxic state in ISO-induced MI *in vivo* (Siddiqui et al. 2016). The activation of the α7nAchR with an agonist (PNU282987) inhibited the expression of pro-inflammatory markers, such as NO, TNFα, and NFκB genes. In contrast, α7nAchR agonists did not affect the expression of anti-inflammatory markers IL-10, TGF-β, or STAT3. Moreover, α7nAchR agonists in hypoxia-induced macrophages did not significantly affect IL-6 gene expression, contradicting previous findings (Tan et al. 2022).

α7nAchR STIMULATION MITIGATED CARDIAC INJURY AND VENTRICULAR REMODELLING IN RATS WITH ISOPRENALINE-INDUCED MYOCARDIAL INJURY

Cardiac troponin, which is the gold standard for diagnosing acute MI (Crapnell et al. 2022), is a regulatory protein complex that can be found in the cytoplasm of cardiomyocytes and facilitates actin–myosin interactions during cardiac contraction. Upon the onset of injury, cellular membrane rupture causes spillage of intracellular contents, such as troponin, into the extracellular space and bloodstream, subsequently leading to an increase in circulating troponin concentration (Crapnell et al. 2022). While we discovered a significant increase in troponin spillage in rats with MI, our findings suggest that 2 weeks of TTS treatment reversed the spillage of cardiac injury markers. However, circulating cTnT levels were significantly higher in the αβ-treated group, indicating worsened cardiac injury and highlighting the importance of the α7nAchR in repairing MI.

Cardiac remodelling refers to changes in ventricular morphology, structure, and function that occur due to damage repair in the injured myocardium. Previously, we showed that cardiac remodelling associated with ISO-induced MI may occur as early as 7 days in animal models (Ali et al. 2019) and decompensates into cardiac dysfunction within 4 weeks after injury (Si et al. 2017). ISO can auto-oxidize into toxic compounds and react with oxygen to form reactive oxygen species (ROS), a main determinant for cardiac injury and remodelling (Ali et al. 2019). In this study, we found that activating the α7nAchR in rats with ISO-induced injury resulted in a significant reduction in collagen deposition in the ventricle, which is an important component of cardiac remodelling. Collagen could be made of collagen-1 (Col-1), collagen-3 (Col-3), and fibronectin, which are stimulated to compensate for cardiomyocyte loss (Ali et al. 2019). The prolonged fibrotic process will eventually lead to stiffening of the heart and will disrupt cardiac function. Our findings are consistent with previously reported results that pyridostigmine (cholinergic stimulation) improved oxidative stress and prevented adverse cardiac remodelling in MI rats induced by coronary ligation (Bezerra et al. 2017). Nonetheless, our findings on the circulating marker for cardiac remodelling, MMP9, showed no effects after α7nAchR activation. The effects of TTS on cardiac adverse remodelling via ROS, Col-1, Col-3, fibronectin and MMP9 are unknown and should be investigated further.

α7nAchR STIMULATION PROVIDES CARDIOPROTECTION AGAINST ISO-INDUCED MYOCARDIAL INJURY BY MODULATING INFLAMMATORY MACROPHAGE ACTIVITY

Inflammation is one of the key factors in determining the severity of cardiac injury and remodelling (Chung et al. 2020). Our *in vivo* study showed that the improvement in cardiac functions following α7nAchR was associated with a significant inhibition of circulating TNFα. To better understand the role of α7nAchR in modulating the cardioprotection, we mimic the cardiogenic conditions in ISO-induced MI *in vivo* with hypoxia-reperfusion conditions *in vitro* by focusing on macrophages activity. The phenotypic plasticity of macrophages is contingent upon the specific stimuli encountered, and the resulting immune response mediated by macrophages will dictate the extent of injury, such as in the case of MI (Chung et al. 2020).

Our current study indicates that activation of the α7nAchR in macrophages exposed to hypoxia conditions regulates the expression of pro-inflammatory factors. The NFκB, a protein complex, acts as a transcription factor, and during cellular quiescence, it typically remains inactive or sequestered within the cytoplasm. After activation, NFκB undergoes translocation into the nucleus, where it interacts with DNA sequences, facilitating or impeding the transcription of DNA (i.e., cytokines and chemokines). Activation and translocation of NFκB have been observed in pro-inflammatory macrophages and are commonly associated with cellular damage (Downton et al. 2023). According to the current findings, the pro-inflammatory mediators NO_2, TNFα, IL-6, and NFκB were expressed at significantly higher levels in RAW264.6 macrophages after OGD/R treatment. This observed effect replicated the pathological characteristics commonly observed in cases of MI (Kiss et al. 2017; Rroku et al. 2022; Wu et al. 2017). However, administration of PNU264287 led to a significant decrease in NFκB and pro-inflammatory factors, indicating its pivotal role as a central mediator of α7nAchR stimulation.

While we found that α7nAchR stimulation affects the gene expression of pro-inflammatory factors NFκB and TNFα, there was no difference in anti-inflammatory factors or STAT3 gene expression across groups. The role of the JAK2/STAT3 signalling pathway in mediating downstream pathways from α7nAchR activation in ischemic injury has yet to be determined (Niu et al. 2023). An earlier model proposed by de Jonge et al. (2005) described the mechanism by which α7nAchR agonists induce the recruitment of Jak2 for the phosphorylation of STAT3 (p-STAT3). Upon phosphorylation, p-STAT3 molecules undergo dimerization and subsequently translocate into

the nucleus, where they function as negative regulators of the pro-inflammatory response. Another model proposed by Peña et al. (2010) suggests that anti-inflammatory responses are mediated by the unphosphorylated STAT3 (uSTAT3), not p-STAT3. uSTAT3 forms a complex with NF**κ**B and decreases inflammatory factors (Peña et al. 2010; Peng-Fei et al. 2021). Hence, the anti-inflammatory effects of α7nAchR stimulation in hypoxia conditions via uSTAT3 and p-STAT3 regulation should be investigated further.

Our *in vitro* study demonstrated a statistically significant elevation in IL-6 levels in macrophages induced by hypoxia. Nevertheless, the administration of an agonist on the α7nAchR did not yield any discernible effects on the expression of the IL-6 gene, indicating that IL-6 may not be a crucial determinant in the anti-inflammatory pathway. This finding contradicts those reported by Tan et al. (2022), who demonstrated that α7nAchR activations resulted in a substantial reduction in the expression of the IL-6 gene and protein compared to other cytokines, such as TNFα and IL1β. The regulation of IL-6 by α7nAchR appears to be contingent on various experimental factors, including the nature of the stimuli employed and the overall inflammatory milieu (Hirano 2021). Furthermore, IL-6 exhibits pleiotropic characteristics because it regulates diverse functions of immune cells, such as promoting their movement from the bloodstream to injured tissue and initiating the NFκB pathway to induce a localized inflammatory reaction (Akabane, Murakami & Murakami 2023). Therefore, the intricate association between IL-6 and α7nAchR exhibits a high level of complexity and can potentially be influenced in a non-canonical fashion by other inflammatory pathways. Further clarification is necessary to address the inconsistencies observed in the current findings.

LIMITATIONS AND SUGGESTIONS

Previously published research found mismatches in myocardial oxygen demand and supply, as well as a decrease in oxygen uptake in ISO-induced MI, but this was not validated in our study (Poderoso et al. 1995; Radhakrishnan et al. 2023). Instead, we used circulating troponin T, a gold standard marker for cardiac injury (Ali et al. 2019), to validate our ISO-induced MI model. Further research should include circulating oxygen levels or myocardial oxygen demand parameters. Second limitation was the lack of an ISO-induced MI model *in vitro* to investigate the inflammatory activity. Numerous studies have used LPS-induced macrophage activation to investigate the anti-inflammatory effects of α7nAchR, but little evidence exists to use the OGD/R-induced method. We anticipate that the OGD/R activation method *in vitro* will be more comparable to the ISO-induced MI model *in vivo* because both methods result in oxygen demand disruption. The third limitation is that isolated cardiac macrophages from rats would have been preferable. However, due to technical difficulties encountered during the isolation process and the ethical consideration of minimizing the number of animals used in the study, RAW264.7 cell lines were chosen. Fourth, the young animals used in this study may not accurately have represented true MI epidemiology, including that of myocardial infarction and takotsubo cardiomyopathy, which are commonly associated with the elderly (Greulich et al. 2019; Rroku et al. 2022). Nonetheless, ISO induction does show an ischemia-like lesion, a hallmark of myocardial infarction and takotsubo cardiomyopathy (Ali et al. 2020; Forte et al. 2021).

CONCLUSION

ISO-induced MI adversely affects the heart, as indicated by the deterioration of left ventricular function in Langendorff's isolated heart preparation. However, daily activation of the α7nAchR via TTS was able to improve the left ventricular function. This cardioprotection was linked to lower levels of circulating MI markers cTnT, inflammatory TNFα and collagen deposition in the left ventricle. The observed effects were attenuated in the sham control and during pharmacological inhibition of α7nAchR, suggesting its role in cardioprotection *in vivo*. Further investigation on the anti-inflammatory role of α7nAchR the in macrophages conditioned to hypoxia showed a reduction of pro-inflammatory cytokines (TNFα) and the expression of the NFκB gene but did not influence the production of anti-inflammatory cytokines. The findings of this study indicate that TTS may have potential as an additional treatment option for MI by targeting macrophage pro-inflammatory activity via the TNFα/NFκB pathway.

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