Acetylcholine Attenuated TNF-α-Induced Apoptosis in H9c2 Cells: Role of Calpain and the p38-MAPK Pathway

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Key Words
Acetylcholine • Muscarinic receptor • Calpain • Calpastatin • p38-MAPK • Apoptosis

Abstract
Background: Previous studies have shown that inflammation is associated with excessive activation of calpains. Acetylcholine (ACh) has been reported to inhibit pro-inflammatory cytokine release and protect against cardiomyocyte injury. However, there is no direct evidence regarding whether ACh can regulate calpains to exert cardioprotection. To this end, we investigated the effect of ACh on tumour necrosis factor alpha (TNF-α)-induced cardiomyocyte injury and further explored the underlying mechanism. Methods: Flow cytometry and transmission electron microscopy were performed to evaluate apoptosis and cellular ultrastructure. Western blotting was performed to assess changes in protein expression. siRNA was employed to silence specific proteins. Results: TNF-α treatment increased the expression of cleaved caspase-3, calpain-1 and p38-mitogen-activated protein kinase (p38-MAPK). The calpain inhibitor PD150606 and the p38-MAPK inhibitor SB203580 inhibited apoptosis induced by TNF-α. Moreover, SB203580 decreased the expression and activity of calpain-1, possibly related to the up-regulation of calpastatin. ACh significantly inhibited TNF-α-induced cell apoptosis, as evidenced by decreases in caspase-3 cleavage, p38-MAPK phosphorylation, and calpain-1 expression and activity as well as increases in calpastatin expression. These beneficial effects of ACh were abolished by atropine or M\textsubscript{2}AChR siRNA. Conclusion: Our results suggest that ACh ameliorated TNF-α-induced calpain activation by decreasing p38-MAPK phosphorylation and enhancing calpastatin expression, indicating that calpain may be an important link between inflammatory factors and myocardial cell apoptosis.

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Introduction

Tumour necrosis factor-alpha (TNF-α), a well-known inflammatory cytokine, is an important factor in the pathogenesis of cardiovascular injury [1]. Previous studies have shown that TNF-α production was elevated after exposure to hypoxia in H9c2 cardiomyocytes [2]. In patients with acute myocardial infarction (AMI), the plasma level of TNF-α increased, which could be a marker of cell death and recurrent ischaemia following AMI [3]. In a large-animal study, coronary microembolization increased infarction size and TNF-α expression in the myocardium [4]. Therapeutic approaches blocking TNF-α production might have prophylactic value against secondary myocardial ischaemic injury; such an approach may reduce myocardial infarction and improve cardiac function, accompanied by a decrease in cardiomyocyte apoptosis [5].

Several lines of evidence suggest that vagus nerve stimulation (VNS) decreases infarct size and inflammation marker expression and prevents myocardial remodelling induced by ischaemia/reperfusion (I/R) [6]. Additionally, acetylcholine (ACh) elicits an anti-apoptotic effect through the activation of the muscarinic ACh receptor (MACHR) and the activation of anti-oxidant systems [7]. Previous studies in our lab have found that ACh protects cardiomyocytes by reducing TNF-α production in both post-infarction hearts and cultured H9c2 cells exposed to hypoxia [8]. Interestingly, Bajaj and Sharma showed that calpain inhibition suppressed TNF-α-induced apoptosis in a cardiac muscle cell line [9]. However, the effects of ACh on the impact of calpain activity and the balance of the calpain/calpastatin system are not yet fully understood.

Calpains are a family of calcium-dependent cysteine proteases with pro-apoptotic properties in the context of Ca\(^{2+}\) overload [10, 11]. The myocardium contains modest amounts of the ubiquitous calpain-1 and calpain-2, which are present in the cytosol as inactive proenzymes [12]. Excessive calpain activity plays a critical role in mitochondrial damage and oxidative phosphorylation during cardiac I/R injury [13] and vascular injury in diabetics [14]. Several other studies have also demonstrated that the inhibition of calpain activity during I/R may reduce apoptosis and infarct size [15]. In addition, calpain is also specifically controlled by the endogenous inhibitor calpastatin. Over-expression of calpastatin inhibits calpain-1 and calpain-2 activity in vitro. A recently report showed that calpains increased apoptosis, and calpastatin had the opposite effect [16]. Obviously, calpain inhibition may represent a new avenue for modulating damage to the heart. In this study, we aimed to investigate the effect of ACh on TNF-α-induced apoptosis and the role of calpain in cardiomyocytes. The inhibition of the p38-mitogen-activated protein kinase (p38-MAPK) signalling pathway as a potential mechanism mediated by ACh was also examined.

Materials and Methods

Materials

ACh, atropine, SB203580 and PD150606 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), calpain-1, calpastatin, p38-MAPK, phosphorylated p38-MAPK, caspase-3, Bcl-2 and Bax primary antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Infrared secondary antibodies were purchased from Odyssey (Li-Cor Biosciences, Lincoln, NE, USA). The calpain activity assay kit was purchased from Genmed Scientifics Inc. (Wilmington, DE, USA). Drug concentrations are expressed as their final molar concentrations within the culture dish.

Cardiomyocyte culture and treatment protocol

H9c2 cells, which are derived from rat embryonic ventricular cardiomyocytes, were seeded at 1×10^5 cells/cm\(^2\) in 6-well plates with high-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and were cultured in a 5% CO\(_2\) incubator at 37°C.

The day after the cells were seeded, the cardiomyocytes were starved in FBS-free DMEM for 6 h and were then randomly divided into the following four groups: (1) control group (C): cardiomyocytes
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continuously cultured for 12 h in FBS-free DMEM; (2) TNF-α group (T): cardiomyocytes treated with 20 ng/mL TNF-α for 12 h; (3) ACh group (A): cardiomyocytes treated with 10⁻⁶ mol/L ACh for 14 h; and (4) ACh pre-treatment group (A+T): cardiomyocytes pre-incubated with 10⁻⁶ mol/L ACh for 2 h and then co-incubated with ACh and TNF-α for 12 h. All drugs were dissolved in pre-warmed FBS-free DMEM and were added directly to the cardiomyocytes. In the control groups, equivalent volumes of medium were added.

Assessment of apoptosis

Apoptosis was evaluated using flow cytometry. Apoptotic and necrotic cells were double-stained with annexin V and propidium iodide (PI). At the end of the experiment, cells grown adherently were gently mechanically detached and resuspended in 100 μL binding buffer. Subsequently, the cells were incubated for 15 min in the dark with 5 μL Annexin V and 100 μL PI solution (BD PharMingen, San Diego, CA) at 25°C, and then 400 μL of binding buffer was added. Flow cytometry analysis was performed using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) equipped with a 488-nm argon laser. The CellQuest software (Becton Dickinson) was used for data acquisition.

Cell survival assay

Cell viability was examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assay. At the end of the experiment, the cells were incubated in the MTT solution (0.5 mg/mL) for 4 h at 37°C and were then solubilized with 150 μL DMSO. The absorbance was read at 492 nm using a multi-well scanning spectrophotometer. Cells in the control group were considered to be 100% viable.

Transmission electron microscopy (TEM)

Harvested cells were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2–7.4) for 2 h at 4°C, embedded in propylene oxide and epoxy resin overnight at 37°C, and then embedded and cut into ultrathin sections (1–2 μm), double-stained with uranyl acetate and lead citrate, and examined by electron microscopy (H-7650; HITACHI, Japan). Technical support and materials were provided by the electron microscopy centre of Xi’an Jiaotong University.

Caspase-3 activity assay

Caspase-3 activity was determined using the caspase-3 activity assay kit (Bestbio, Shanghai, China). Cells were harvested and extracted on ice in lysis buffer for 15 min. The lysates were centrifuged for 5 min at 500 × g, and the supernatants were assessed for protein content. Subsequently, supernatant samples containing 50 μg protein were incubated with 90 μL reaction buffer and 10 μL caspase-3 substrate (containing 40 μmol/L N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide) at 37°C for 2 h in the dark. The OD values were measured at 405 nm with a spectrophotometer.

Calpain activity assay

Calpain activity was assayed using the Calpain Activity Assay kit (Genmed Scientifics Inc., Wilmington, DE, USA). Briefly, cells were suspended in 50 μL of extraction buffer on ice for 30 min and then centrifuged at 10,000 × g for 5 min at 4°C. The cell lysates (~50 μg) were then diluted in extraction buffer, which contained reaction buffer and calpain substrate. The samples were incubated at 37°C for 1 h, and the absorbance of each sample was read in a fluorescence spectrometer equipped with a 360-nm excitation filter and a 520-nm emission filter (POLARstar Omega and FLUOstar Omega microplate readers, BMG Labtech, Germany).

Western Blotting

The cardiomyocytes were prepared for immunoblotting as described previously [17]. The protein concentration was quantified using the bicinchoninic acid (BCA) protein assay (Beyotime Biotechnology, Jiangsu, China). Proteins (30 μg/lane) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then electrotransferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies at 4°C overnight and then incubated with IRDye 800CW secondary antibodies for 45 min at room temperature. The bands were then visualized with an Odyssey infrared-imaging system (Li-Cor Biosciences, Lincoln, NE, USA) and quantified by scanning densitometry.
siRNA knockdown of M_2AChR

Small interfering RNA (siRNA) was performed to knock down M_2AChR in H9c2 cells. The siRNA oligonucleotides were synthesized by GenePharma Co. Ltd (Shanghai, China). The siRNA sequence targeting M_2AChR is as follows: 5’-GUGCUCAUCAAACUUCUTTAGAAAGUAUUGAUGAGCATTT-3’. A scrambled non-target siRNA was also used as a negative control. The detailed method was described previously [18]. Transfections were performed with 100 nM of each siRNA using TurboFect siRNA Transfection Reagent (Thermo Fisher Scientific, Cleveland, OH, USA) according to the manufacturer’s instructions. Forty-eight hours after transfection, the efficiency of siRNA-mediated M_2AChR knockdown was determined by Western blotting.

Statistical analysis

All results are expressed as means ± SEM. P < 0.05 was considered statistically significant. Comparisons of the results were performed one-way ANOVA followed by Tukey's post hoc test or Student's t test using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). All Figs were prepared using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

TNF-α promoted cell apoptosis in H9c2 cells

H9c2 cells were treated with various concentrations of TNF-α (2.5-40 ng/mL) for 12 h, which was consistent with a previous study [19]. As shown in Fig. 1A, ultrastructural analysis of the cardiomyocytes indicated that cells treated with TNF-α for 12 h began to show

![Fig. 1. TNF-α induced ultrastructural injury and apoptosis of H9c2 cells in a dose-dependent manner. TEM analysis of cells exposed to TNF-α (2.5-40 ng/mL) for 12 h showing cytoplasmic vacuoles (arrows), apoptotic bodies (arrowheads) and cell membranes dissolution (A). Caspase-3 activity (B) and the percentage of apoptotic cells were evaluated using a caspase-3 activity assay kit and flow cytometry. Data represent the means ± SEM (n=3). *P < 0.05 and **P < 0.01 vs. the control group; *P < 0.05 and **P < 0.01 vs. the TNF-α group. Scale bar = 5 µm.](image-url)
characteristics of apoptosis, including the presence of cytoplasmic vacuoles, cell membrane dissolution, chromatin condensation, and the formation of apoptotic bodies. Based on the caspase-3 activity (Fig. 1B), the percentages of cardiomyocytes in Q4 (early apoptosis) and Q2 (late apoptosis), and the overall percentage of apoptotic cells (Fig. 1C) increased gradually with TNF-α concentration.

ACh increased cell viability and decreased TNF-α-induced apoptosis in H9c2 cells
To determine the effect of ACh on cell viability and whether ACh prevents TNF-α-induced apoptosis, H9c2 cells were pretreated with different concentrations of ACh (10⁻⁸–10⁻⁵ mol/L) for 2 h and then treated with TNF-α for 12 h. ACh ameliorated apoptosis (Fig. 2A) and the decrease in cell viability (Fig. 2B) induced by TNF-α. The TNF-α-induced increase in cleaved caspase-3 expression was attenuated by ACh in a dose-dependent manner (Fig. 2C), with ACh (10⁻⁶ mol/L) resulting in significant protection of cardiomyocytes. Therefore, we chose 10⁻⁶ mol/L ACh for the following experiments.

The effect of ACh on calpain-1 and calpastatin expression in cardiomyocytes treated with TNF-α
H9c2 cells were treated with different concentrations of TNF-α (2.5-40 ng/mL) for 12 h. Western blots showed that TNF-α increased calpain-1 expression, although it decreased calpastatin expression in a dose-dependent manner (Fig. 3A). Increasing concentrations of ACh (10⁻⁶–10⁻⁵ mol/L) gradually reversed the changes in the calpain-1 and calpastatin protein levels induced by TNF-α (Fig. 3C). These results suggested that the calpain/calpastatin system is involved in TNF-α-induced cell injury.

Calpain and p38-MAPK signals participated in TNF-α-induced apoptosis
H9c2 cells were co-treated with PD150606 (an inhibitor of calpains, 10⁻⁶ mol/L) or SB203580 (an inhibitor of p38-MAPK, 10⁻⁶ mol/L) in the presence of TNF-α. The Bcl-2/
Bax ratio (Fig. 4A) and the level of cleaved caspase-3 protein (Fig. 4B) were assessed by Western blotting. The decrease in the Bcl-2/Bax ratio and the increase in cleaved caspase-3 expression induced by TNF-α were reversed after treatment with PD150606 or SB203580 compared with the TNF-α group.

To investigate the role of p38-MAPK in the TNF-α-induced up-regulation of calpain-1, H9c2 cells were co-treated with SB203580 and TNF-α, and calpain-1 and calpastatin expression, as well as calpain activity, were assessed. SB203580 inhibited the TNF-α-induced up-regulation of calpain-1 and down-regulation of calpastatin in H9c2 cells (Fig. 4C, D and E).
Muscarinic receptors were responsible for ACh-mediated cardioprotection

ACh acts on either muscarinic or nicotinic ACh receptors (AChR) to exert its cardioprotective effects [20]. To investigate the cholinergic receptors that play predominant roles in the ACh-mediated suppression of cell injury, atropine (a non-selective muscarinic receptor antagonist, 10^{-6} mol/L) and hexamethonium (a non-selective nicotinic receptor antagonist, 10^{-6} mol/L) were individually combined with TNF-α, following pretreatment of H9c2 cardiomyocytes (10^{-6} mol/L) with ACh. As shown in Fig. 5, ACh pretreatment significantly inhibited the apoptosis rate in cardiomyocyte, up-regulated the cleaved caspase-3 protein levels and increased the Bcl-2/Bax ratio. These protective effects of ACh were eliminated by atropine but not hexamethonium. Our results suggest that the muscarinic receptor may be responsible for the protective effects of ACh in H9c2 cells.

Effects of ACh on p38-MAPK and calpain-1 expression

We determined the effects of ACh on p38-MAPK levels and calpain-1 expression and activity in cardiomyocytes using Western blotting and a calpain activity assay kit. ACh significantly inhibited the TNF-α-induced increases in the level of p38-MAPK protein and calpain expression and activity. Interestingly, these effects of ACh were eliminated by atropine, but not hexamethonium, which was in agreement with the apoptosis data (Fig. 6).

Fig. 4. Calpain-1 and p38-MAPK participates in TNF-α-induced apoptosis. (A) Ratio of Bcl-2/Bax protein expression. (B) Cleaved caspase-3 protein levels. (C) Calpain-1 protein expression. (D) Calpain activity. (E) Calpastatin protein expression. *P < 0.05, **P < 0.01 vs. the control group. #P < 0.05 and ##P < 0.01 vs. the TNF-α group.
Knockdown of M₂AChR abolished the anti-apoptotic effect of ACh via calpain-1 and the p38-MAPK signalling pathway

To further determine whether the M₂AChR is responsible for the beneficial effects of ACh on the calpain-dependent apoptotic pathway after TNF-α stimulation, we suppressed M₂AChR expression by transfecting siRNA in H9c2 cells. As shown in Fig. 7A, M₂AChR protein expression was down-regulated compared with the corresponding siRNA. The M₂AChR siRNA efficiently abolished the increase in the Bcl-2/Bax ratio (Fig. 7B) and the attenuation of cleaved caspase-3 expression (Fig. 7C) in the presence of ACh. Furthermore, we assessed the effect of M₂AChR on the expression of calpain-1 and p38-MAPK in TNF-α-stimulated H9c2 cells. Compared with the negative control siRNA group, M₂AChR-depleted cells displayed higher levels of p38-MAPK phosphorylation (Fig. 7D) and calpain-1 expression and activity (Fig. 7E and 7F). These results indicated that the beneficial effects of ACh in the context of TNF-α in H9c2 cells were mainly mediated through M₂AChR.

**Fig. 5.** The muscarinic receptor participates in ACh-mediated cardioprotective effects. (A) Flow cytometry analysis of cardiomyocyte apoptosis. (B) Bcl-2, Bax and cleaved caspase-3 protein expression. *P < 0.05, **P < 0.01 vs. the control group. ^P < 0.05 and ^^P < 0.01 vs. the TNF-α group. $$$P < 0.01 vs. the ACh-treated group.
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Findings from the present study demonstrated that ACh attenuated TNF-α-induced calpain activation by decreasing p38-MAPK phosphorylation and regulating the activity of calpain.

**Discussion**

Findings from the present study demonstrated that ACh attenuated TNF-α-induced calpain activation by decreasing p38-MAPK phosphorylation and regulating the activity of calpain.
of the calpain/calpastatin system. Our data showed that: (i) TNF-α enhanced calpain-1 expression and activity, reduced calpastatin expression, and increased cleaved caspase-3 protein levels; (ii) the p38-MAPK inhibitor SB203580 decreased calpain-1 expression and increased calpastatin expression, decreased the Bcl-2/Bax ratio and enhanced the amount of cleaved caspase-3; (iii) pretreatment with ACh abolished the increases in p38-MAPK phosphorylation, calpain-1 expression and activity, and apoptosis induced by TNF-α; (iv) the protective effects of ACh were abolished by atropine and M₃AChR siRNA, but hexamethonium failed to cause any changes. Our findings have revealed a novel pathway underlying the cardioprotection elicited by ACh, suggesting that calpain appears to be an important link between inflammatory factors and the control of myocardial cell apoptosis (Fig. 8).

The multifunctional cytokine TNF-α has been implicated in complications arising subsequent to the ischaemic injury of cardiac tissue and diabetic peripheral neuropathy [21, 22]. Sustained TNF-α over-expression may be maladaptive, provoking the induction of cardiomyocyte apoptosis, which was related to the activation of TNF-α receptor 1 [23, 24]. The most widely studied form of intrinsic apoptosis is initiated by the release of cytochrome C from the mitochondria, resulting in the formation of the apoptosome. The executioner caspase-3 is activated by the apoptosome, and pro-apoptotic members of the Bcl-2 protein family are activated and act on the mitochondria to induce the release of cytochrome C [25]. Therefore, we attempted to determine whether ACh exerted an effect on the expressions of caspase-3, Bcl-2 and Bax. The present study indicated that TNF-α induced cardiomyocyte apoptosis in a dose-dependent manner. Pre-treatment with ACh dramatically attenuated the up-regulated expression of caspase-3 and Bax, as well as up-regulating Bcl-2 expression induced by TNF-α.

Autonomic dysfunction during cardiovascular disease has serious clinical consequences; thus, improved vagal tone may be a new therapeutic target for the treatment of cardiovascular diseases [26]. Our previous studies reported that vagal stimulation decreased infarct size, improved heart function and ameliorated regional cardiac I/R-induced endothelial dysfunction in mesenteric arteries through the direct effects of ACh [8, 27]. Administration of ACh prior to hypoxia or reoxygenation reduced apoptosis and the level of inflammatory cytokines in H9c2 cells [2, 6, 18]. In cardiomyocytes, MACHr plays a crucial role in targeting apoptosis inhibition to specific receptor sites [28, 29]. Otherwise, MACHr induces the activation of survival cascades, increased endogenous anti-oxidant reserves, and the reduction of apoptotic mediators such as p38-MAPK during I/R [30]. A recent study showed that an M₃ACHr inhibitor reduced the TNF-α-activated endoplasmic reticulum apoptotic pathway via epidermal growth factor receptor-phosphatidyl inositol 3-kinase signalling in cardiomyocytes [19]. Therefore, we co-treated cells with ACh and atropine or hexamethonium in the present study and found that, compared with the ACh-treated group, atropine but not hexamethonium augmented cleaved caspase-3 expression and decreased both the Bcl-2/Bax ratio and cell injury. Moreover, M₃ACHr siRNA abrogated the beneficial effects of ACh, supporting the role of M₃ACHr in the anti-apoptotic effects of ACh in H9c2 cells. The results demonstrated that TNF-α-induced apoptosis was inhibited by ACh through M₃ACHr.

Calpains belong to a family of calcium-dependent thiol-proteases that has been implicated in several acute inflammatory disorders of the cardiovascular system [29]. A recent study showed that calpain-1 over-expression enhanced myocardial injury and dysfunction within 4 days after coronary occlusion [31]. However, calpain-1 was activated in cardiomyocytes during LPS stimulation, which was inhibited by over-expression of calpastatin and prevented cardiac hypertrophy in angiotensin II-induced hypertension [16]. We detected a marked increase in calpain activity and protein expression and a decrease in calpastatin protein expression in H9c2 cells after TNF-α stimulation. In addition, pharmacological inhibition of calpain (PD150606) prevented cell death in TNF-α-stimulated H9c2 cardiomyocytes. It should be noted that calpain activity can be regulated through phosphorylation by MAPKs, protein kinase C, and protein kinase A [32-34]. MAPKs represent a highly conserved superfamily of serine/threonine protein kinases. There are three well-characterized MAPK
subfamilies: extracellular signal-regulated kinases (ERKs), c-Jun-N-terminal kinases (JNKs) and p38-MAPK [35]. In particular, p38-MAPK is activated by elevated reactive oxygen species (ROS) generation in response to angiotensin II, I/R or hypoxia [36-38]. Chen et al. have reported that pretreatment of cells with TNF-α increased basal and \( \text{H}_2\text{O}_2 \)-stimulated p38-MAPK expression and cardiomyocyte apoptosis, which was inhibited by SB203580 [39]. Consistent with previous studies, our data showed that TNF-α enhanced the phosphorylation of p38-MAPK and increased calpain protein levels. SB203580 decreased calpain-1 expression and activity, increased the Bcl-2/Bax ratio and calpastatin expression, and decreased the levels of cleaved caspase-3. Our previous studies reported that ACh prevents Ang II-induced apoptosis in H9c2 cells through inhibition of ROS-mediated p38 MAPK activation [7]. Skok et al. thought the α7 nicotinic ACh receptor plays an important role in inhibiting p38-MAPK pathway activation [40]. The data presented here indicated that ACh depressed p38-MAPK-dependent intracellular signalling resulting in calpain inactivated via \( \text{M}_2\text{AChR} \).

In summary, ACh protected cardiomyocytes against TNF-α-induced apoptosis by inhibiting p38-MAPK signalling and balancing the activity of the calpain/calpastatin system. Our current studies have shown that ACh elicited beneficial effects via \( \text{M}_2\text{AChR} \) in H9c2 cardiomyocytes, confirming the anti-apoptotic effect of ACh in the presence of TNF-α (Fig. 8). The present study provided initial evidence that calpain activation stimulates myocardial apoptosis during inflammation and further revealed that that vagal modulation may represent a promising strategy for the treatment of cardiovascular diseases.

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**Disclosure Statement**

None.
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