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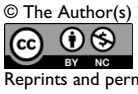

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Aleglitazar, a dual peroxisome proliferator-activated receptor-α and -γ agonist, protects cardiomyocytes against the adverse effects of hyperglycaemia

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Abstract

Purpose: To assess the effects of Aleglitazar on hyperglycaemia-induced apoptosis.

Methods: We incubated human cardiomyocytes, cardiomyocytes from cardiac-specific peroxisome proliferator-activated receptor-γ knockout or wild-type mice in normoglycaemic or hyperglycaemic conditions (glucose 25 mM). Cells were treated with different concentrations of Aleglitazar for 48 h. We measured viability, apoptosis, caspase-3 activity, cytochrome-C release, total antioxidant capacity and reactive oxygen species formation in the treated cardiomyocytes. Human cardiomyocytes were transfected with short interfering RNA against peroxisome proliferator-activated receptor-α or peroxisome proliferator-activated receptor-γ.

Results: Aleglitazar attenuated hyperglycaemia-induced apoptosis, caspase-3 activity and cytochrome-C release and increased viability in human cardiomyocyte, cardiomyocytes from cardiac-specific peroxisome proliferator-activated receptor-γ knockout and wild-type mice. Hyperglycaemia reduced the antioxidant capacity and Aleglitazar significantly blunted this effect. Hyperglycaemia-induced reactive oxygen species production was attenuated by Aleglitazar in both human cardiomyocyte and wild-type mice cardiomyocytes. Aleglitazar improved cell viability in cells exposed to hyperglycaemia. The protective effect was partially blocked by short interfering RNA against peroxisome proliferator-activated receptor-α alone and short interfering RNA against peroxisome proliferator-activated receptor-γ alone and completely blocked by short interfering RNA to both peroxisome proliferator-activated receptor-α and peroxisome proliferator-activated receptor-γ.

Conclusion: Aleglitazar protects cardiomyocytes against hyperglycaemia-induced apoptosis by combined activation of both peroxisome proliferator-activated receptor-α and peroxisome proliferator-activated receptor-γ in a short-term vitro model.

Keywords

Aleglitazar, peroxisome proliferator-activated receptor-α and peroxisome proliferator-activated receptor-γ agonist, hyperglycaemia, cardiomyocytes, apoptosis

Introduction

Type 2 diabetes mellitus (T2DM) is associated with an increased incidence of myocardial infarction and diabetic cardiomyopathy.[1–4] Clinical guidelines state that the increased cardiovascular risk in patients with T2DM can be reduced by controlling dyslipidemia, blood pressure, body weight and hyperglycaemia.[5] However, most patients still do not achieve recommended goals for these risk factors.[6] This highlights the need for further decreasing the cardiovascular risk in individuals with T2DM. Diabetic cardiomyopathy, characterized by left ventricular hypertrophy, myocardial apoptosis, fibrosis and dysfunction, is a common complication of T2DM.[3,4] T2DM is associated with poorer outcomes

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following myocardial infarction.[7] Cardiac hypertrophy is associated with deactivation of peroxisome proliferator-activated receptor-α ($PPAR_\alpha$).[8] We and others have shown that peroxisome proliferator-activated receptor-γ ($PPAR_\gamma$) agonists, especially of the thiazolidinedione family, attenuate myocardial infarct size and post-ischaemic dysfunction in normal and diabetic animals.[9–12] However, the protective effects of pioglitazone were reported to be independent of cardiomyocyte $PPAR_\gamma$ activation.[9] There are data showing that $PPAR_\alpha$ agonists also protect against ischaemia-reperfusion injury.[13–17] Aleglitazar (Ale) is a non-thiazolidinedione dual $PPAR_\alpha$ and $PPAR_\gamma$ agonist that has glucose-lowering and lipid-modifying effects.[18,19] Ale was developed for the potential treatment of hyperglycaemia and dyslipidemia in patients with T2DM. It was shown that Ale protects the pancreas, kidneys and eyes of male Zucker diabetic fatty (ZDF) rats against diabetic complications.[20] Studies suggested that Ale could minimize PPAR-related adverse effects and weight gain in the treatment of patients with T2DM.[18] Preclinical and phase I studies have shown favourable effects of Ale on glycaemic control, insulin sensitivity and dyslipidemia.[19] The aim of this study was to assess whether dual activation of $PPAR_\alpha$ and $PPAR_\gamma$ by Ale would have beneficial anti-apoptosis effects in human and murine cardiomyocytes exposed to hyperglycaemia. The data generated from these in vitro experiments might have potential therapeutic implications for the treatment of diabetic cardiomyopathy. During the conduction of the study, however, Roche announced that following the results of a regular safety review of the Ale AleCardio phase III trial,[21,22] the independent Data and Safety Monitoring Board (DSMB) has recommended halting the trial due to safety signals and lack of efficacy with respect to reduction in cardiovascular events and mortality. Based on this recommendation, the manufacturer (F. Hoffmann-La Roche Ltd, Basel, Switzerland) has decided to terminate the AleCardio trial and all other trials involving Ale (http://www.roche.com/media/store/releases/med-cor-2013-07-10.htm). However, some aspects of the drug have not been adequately evaluated. Evidence from several recent large, randomized, controlled trials suggests that improved glycaemic control is associated with a reduced risk of cardiovascular disease (CVD), but that this effect may be greater in individuals with a shorter duration of diabetes and without CVD, and that it may take many years of good glycaemic control to translate into cardiovascular (CV) risk reduction.[23–25] So, Ale may reduce cardiomyocyte apoptosis induced by high glucose of the newly diagnosed patients with diabetes and dyslipidemia and attenuate the progression of atherosclerosis.

Material and methods

Isolation of cardiomyocytes from cardiac-specific $PPAR_\gamma$ knockout mice

Cardiac-specific inducible $PPAR_\gamma$ KO ($PPAR_\gamma^{CKO}$) mice were generated by crossing B6.Cg-Tg(Myh6-cre/Esr1)1Jmk/J mice with B6.129-Ppargtm2Rev/J mice in our laboratory, as described before.[9] Mice carrying both *loxp* and *Cre* transgene were selected to perform the studies. $PPAR_\gamma$ deletion in cardiomyocytes was achieved after 7 days of tamoxifen administration (TAM; 20 mg/kg/day intraperitoneal injections). Cardiomyocytes from 3-month-old $PPAR_\gamma^{CKO}$ or wild-type (WT) mice were isolated using methods previously described with minor modification.[9] Mice were heparinized (5 IU/g) and anaesthetized with ketamine (60 mg/kg) and xylazine (6 mg/kg). Hearts were excised and cannulated via the aorta and connected to the perfusion apparatus. Hearts were perfused for 3 min at a rate of 3 mL/min with calcium-free media. Hearts were perfused with the modified Eagle's medium (MEM) containing Type-II collagenase (1 mg/mL) and 20 μM $CaCl_2$ at a rate of 3 mL/min for 15 min. After perfusion, atria were removed and ventricles were cut into small pieces and minced in collagenase solution for 6 min. Myocytes were washed and plated on laminin-coated plate in media 199 with 4% fetal bovine serum (FBS) and 100 U penicillin and returned to the incubator for attachment (1 h). After attachment, non-attached, rounded cells were washed away and the media was replaced with fresh serum-free medium to remove all non-myocytes. After additional 16 h, the medium was replaced with normal culture medium (MEM) with Hanks' Balanced Salt Solution, supplemented with 0.1 mg/mL bovine serum albumin and penicillin 100 U/mL at 37°C in a 2% CO_2 incubator. After 24 h, when the 88% of the cultured myocytes showed rod-shaped, and viability was 90%, we started the experiments. Knocking out of $PPAR_\gamma$ in the cardiomyocytes was confirmed by immunoblotting and reverse transcription polymerase chain reaction (RT-PCR).

Human cardiomyocytes

Primary human cardiomyocytes (HCM) are cultured cardiac myocytes. These cells have been used as a model for drug development and predictive toxicity testing. HCMs have been used in studies of human gene expression,[26] oxidative stress,[27] diabetes and apoptosis.[28] HCMs are isolated from human adult heart tissue[29] and were purchased from ScienCell Research Laboratories (USA).

Determine the dosage range of Ale in cardiomyocytes culture

At first, we determined the concentration range of Ale that will not cause toxicity in vitro. Lactate dehydrogenase (LDH) was measured as an indicator of cell viability [LDH release detection kit (Roche)]. HCMs and mCM-WT cardiomyocytes were seeded in 200 μL medium for 96-well plate with a density of 8×10^2 cells/mm^2 and then treated with or without Ale at different concentrations (0–40 nM) for different time points (12, 24 or 48 h).[30] Ale was dissolved in 0.1% dimethyl sulfoxide (DMSO). LDH activity

was measured with two replicates for each condition at an absorbance of 490 nm. WT cardiomyocytes not exposed to Ale served as a control. Myocytes from one mouse were used for each experiment. In total, six $PPAR_{\gamma}^{CKO}$ mice and six WT mice were used. Cytoplasmic enzyme release was shown as a ratio of the released LDH into the media to the total LDH (release plus cellular content) at the end of treatment.

To determine the effect of Ale on apoptosis, caspase-3 activity and mitochondrial cytochrome-C release

To explore the effect of Ale on cardiomyocytes apoptosis and leakage of cytochrome-C (Cyt-c) induced by hyperglycaemia (HG, glucose 25 mM), we incubated cardiomyocytes from human (HCMs) or mice ($PPAR_{\gamma}^{CKO}$ or WT) in normoglycaemic condition (NG, glucose 5.5 mM) or hyperglycaemic conditions (HG, glucose 25 mM). Cells were treated with different concentrations of Ale for 48 h. The degree of intracellular DNA fragmentation (apoptosis) was quantified using the Cell Death Detection $ELISA^{PLUS}$ kit (Roche, Hertfordshire, UK) per manufacturer instruction.[30] Caspase-3 activity was performed using a colorimetric activity assay kit according to the manufacturer's instructions. The cells were harvested and then suspended in the cell lysis buffer to obtain cell lysate. Protein concentration was determined using Lowry Protein Assay, and 200 mg protein of cell lysate was incubated in 100 mL of reaction buffer containing 5 mL of caspase-3 substrate (4 mM DEVD-pNA) in 96-well plates. The reaction buffer contained 1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM NaCl and 10% glycerol. The samples were incubated in the dark and caspase-3 activity was evaluated using a spectrophotometer at 405 nm.[9] We also measured translocation of cytochrome-C from mitochondria into cytosol during apoptosis. Cytosolic fractions were obtained at the end of the treatments and cell lysate was prepared. The release of mitochondrial cytochrome-C into the cytosol was determined by ELISA (MBL International, Woburn, MA), per the manufacturer instructions. The change in colour was monitored at a wavelength of 450 nm using a plate reader (Molecular Devices, Sunnyvale, CA). Measurements were performed in duplicate, and the Cyt-c content was expressed as OD450 per mg protein.[9] A total of 6 $PPAR_{\gamma}^{CKO}$ mice and 6 WT were used.

Viability assays

Cells were pretreated with different concentrations of Ale, and cell death was induced by exposure of cells to HG. Cells incubated in NG condition served as a control. The viability of cells (with and without exposure to HG) was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit [R&D systems (Minneapolis, MN, USA)]. The HG-induced cell death was examined by counting trypan-blue stained cells, and the results were expressed as a percentage of the total cells counted, based on the ability of live cells to exclude trypan blue. For each well, we counted 250–300 cells. 3-[4,5-yl]-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) was used to treat the cells for 4 h at 37°C. The attached cells were lysed in 2-isopropanol containing 0.04 M HCl, and the amount of metabolized MTT was determined using a microplate reader.[9] A total of 4 $PPAR_{\gamma}^{CKO}$ mice and 4 WT mice were used.

Total antioxidant capacity assay

The total antioxidant capacity of Ale in cells was evaluated by Trolox equivalent antioxidant capacity assay using a standard antioxidant assay kit (Cayman, Ann Arbor, MI, USA). Briefly, the cells were homogenized on ice in a buffer containing 5 mM potassium phosphate; 0.9% NaCl; and 0.1% glucose, pH 7.4. The samples were centrifuged, and the supernatant were collected for the assay according to manufactory instruction, and the absorbance was read at 405 nm using a microplate reader. A total of 6 $PPAR_{\gamma}^{CKO}$ mice and 6 WT mice were used.

Detection of reactive oxygen species generation

To determine the effect of Ale on reactive oxygen species (ROS) formation, cardiomyocytes were treated as above. 50 μM 2′7′-dichlorofluorescin (DCF)-diacetate was added for 30 min at 37°C. The intracellular ROS concentration was quantified by the measurement of DCF fluorescence intensity using an excitation wavelength of 485 nm and emission wavelength of 524 nm. A total of 5 $PPAR_{\gamma}^{CKO}$ mice and 5 WT mice were used.

Statistical analysis

Results are reported as mean ± standard error (SE). Data were compared using analysis of variance (ANOVA) with Sidak corrections for multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of Ale on cytotoxicity

At first we looked for the effects of escalating concentrations of Ale on LDH release to assess for potential toxicity in HCM and wild-type mouse cardiomyocytes (mCM-WT). Cells were incubated for 12, 24 or 48 h with escalating concentrations of Ale, and the percent difference in LDH release was calculated (Figure 1). Ale at concentrations of 0.1 to 20 μM did not significantly increase LDH release compared to vehicle alone. However, at concentrations of 30 and 40 μM, there was a significant increase in

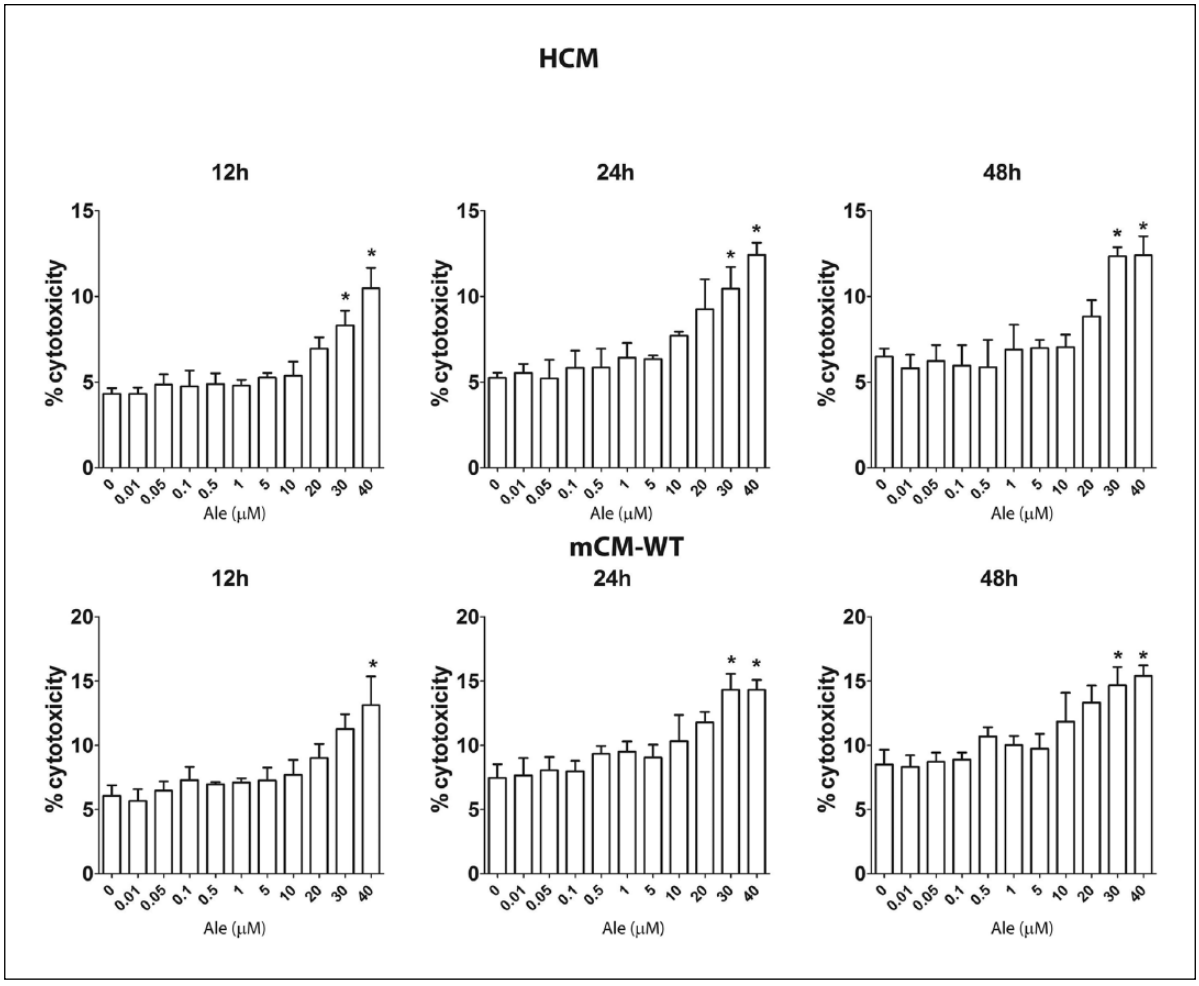

Figure 1. Effects of Aleglitazar on cytotoxicity in human cardiomyocytes (HCM) and wild-type mice cardiomyocytes (mCM-WT). The cells were incubated with different concentrations of Aleglitazar (0–40 μM) for 12, 24 or 48 h. *$p < 0.05$ versus 0.0 μM Ale group (n = 4 in each group).

LDH release. Therefore, in the next experiments, we used Ale at concentration up to 20 μM. The reported through blood levels of Ale in humans are 0.0017–0.01 μM.[31]

Effects of Ale on HG-induced apoptosis, caspase-3 activity and cytochrome-C release

Cells were incubated at normoglycaemic condition (NG, glucose 5.5 mM) or hyperglycaemic conditions (HG, glucose 25 mM) with escalating concentration of Ale (0.01–20 μM) for 48 h (Figure 2). To evaluate the effect of Ale on HG-induced apoptosis, we measured DNA fragmentation (a late marker of apoptosis) and caspase-3 activity (an early marker of apoptosis).[32] HG increased apoptosis, caspase-3 activity and cytochrome-C release in HCM, mCM-WT and mCM-PPAR$_\gamma$ KO cardiomyocytes. Ale, dose dependently, decreased apoptosis, caspase-3 activity and cytochrome-C release. The effects were significantly different from HG without Ale at 1.0–20 μM for HCM and mCM-WT and 10–20 μM for the mCM-PPAR$_\gamma$ KO cardiomyocytes, suggesting that at lower concentrations most of the protective effects of Ale are PPAR$_\gamma$ dependent (Figure 2), whereas at higher concentration, PPAR$_\alpha$ activation partially mediates the protective effects.

Cell viability (MTT)

HCM, mCM-WT and mCM-PPAR$_\gamma$-KO cardiomyocytes were incubated in NG or HG without or with escalating concentrations of Ale (Figure 3). There was a significant decrease in cell viability in the cultures exposed to HG without Ale. Ale at concentrations of 0.5–20, 1.0–20 and 10–20 μM increased cell viability in the HCM, mCM-WT and mCM-PPAR$_\gamma$-KO cardiomyocytes, respectively. Yet, at 20 μM, the protective effect of Ale was smaller in the

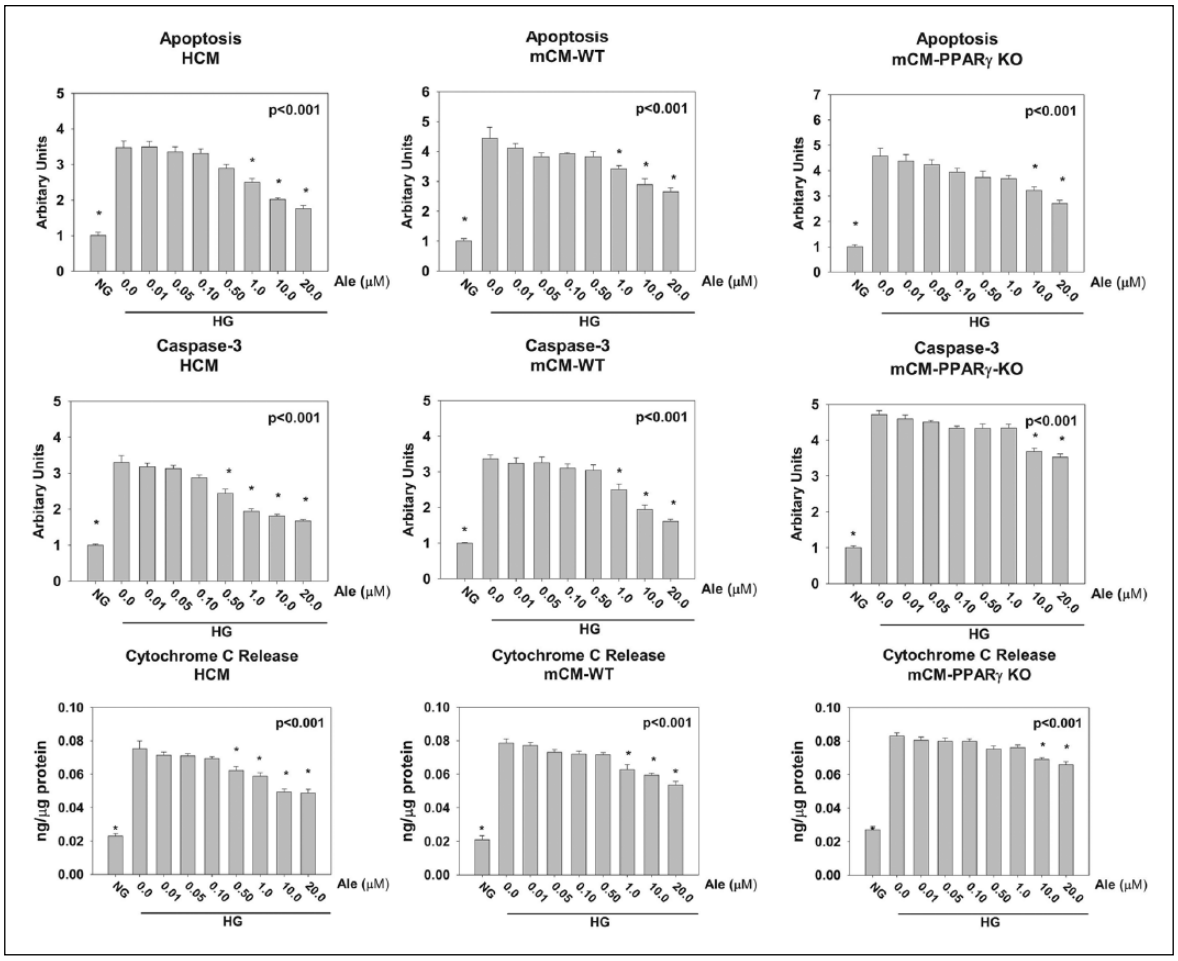

Figure 2. Effects of Aleglitazar on apoptosis, caspase-3 activity and cytochrome-C induced by HG in human cardiomyocytes (HCM), wild-type mouse cardiomyocytes (mCM-WT) and cardiac-specific inducible $PPAR_{\gamma}$ KO mice cardiomyocytes (mCM-$PPAR_{\gamma}$ KO). Caspase-3 activity is expressed as a ratio of the activity in each group/the activity in the normoglycaemic (NG) group. *$p < 0.05$ versus 0.0 μM Ale group (n = 6 in each group).
NG: normoglycaemia; HG: hyperglycaemia.

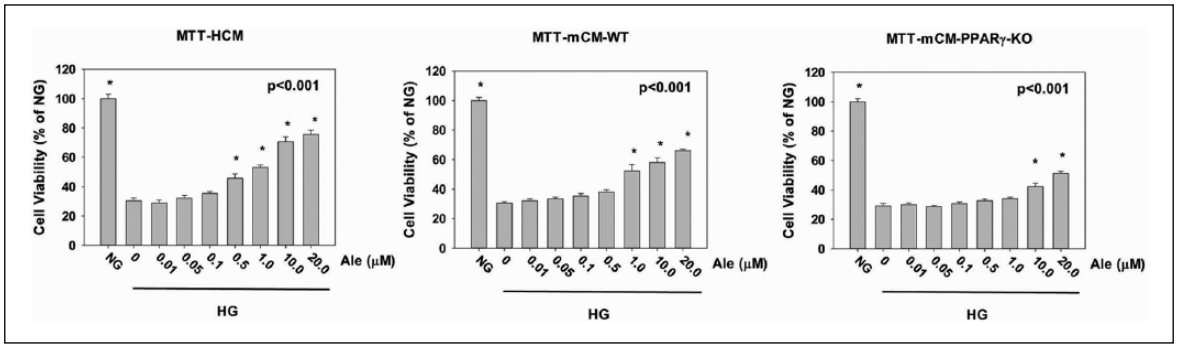

Figure 3. Cell viability (MTT) in cells exposed to hyperglycaemia (HG) without and with escalating concentrations of Ale. Results are expressed as percentage of the normoglycaemia (NG) group. *$p < 0.001$ versus 0.0 μM Ale group (n = 6 in each group).

$mCM\text{-}PPAR_{\gamma}$-KO cardiomyocytes than in the HCM and mCM-WT cardiomyocytes. These findings suggest that the protection effect exerted by Ale at lower concentrations (1.0 μM) is $PPAR_{\gamma}$ dependent, whereas at higher concentrations (10 and 20 μM) it is mediated by both $PPAR_{\alpha}$ and $PPAR_{\gamma}$ activation (Figure 3).

Antioxidant capacity

Exposure to HG for 48 h reduced the antioxidant capacity of the cardiomyocytes. Ale, dose dependently, blunted the effects of HG. Again in mCM-PPAR$_\gamma$-KO cardiomyocytes, Ale had a significant effect only at concentrations of 10 and 20 μM. In contrast, Ale had a significant effect at 1.0 μM in HCM and mCM-WT, suggesting that at lower concentrations the effect of Ale is mainly PPAR$_\gamma$ dependent (Figure 4).

ROS generation

Treatment of cardiomyocytes with HG for 48 h significantly increased ROS levels. This effect of HG on ROS production was significantly attenuated by 1.0, 10 and 20 μM of Ale in both HCM and mCM-WT cardiomyocytes, whereas only at 10 and 20 μM of Ale in mCM-PPAR$_\gamma$ KO cardiomyocytes, suggesting again that at lower concentrations, the effect of Ale is PPAR$_\gamma$ dependent. Moreover, at 10 and 20 μm, more attenuation was seen in the HCM and mCM-WT cardiomyocytes than in the mCM-PPAR$_\gamma$ KO cardiomyocytes, suggesting involvement of both the PPAR$_\alpha$ and PPAR$_\gamma$ receptors (Figure 5).

The effects of PPAR$_\gamma$ and PPAR$_\alpha$ silencing on the protective effect of Ale on cell viability in cardiomyocytes exposed to HG

HG reduced cell viability. Ale (20 μM) improved viability. The protective effect was partially blocked by siRNA to PPAR$_\alpha$ (siPPAR$_\alpha$) or PPAR$_\gamma$ (siPPAR$_\gamma$). When both PPAR$_\alpha$ and PPAR$_\gamma$ were silenced, the protective effect was completely lost. These experiments show that the protective effect in both HCM and mCM-WT is mediated via combined activation of PPAR$_\alpha$ and PPAR$_\gamma$ (Figure 6).

Discussion

In this study, we found that activation of both PPAR$_\alpha$ and PPAR$_\gamma$ by Ale increased cell viability and decreased HG-induced increased apoptosis, caspase-3 activity and Cyt-c release in a dose-dependent manner. HG reduced the antioxidant capacity of the cardiomyocytes and increased ROS concentrations, whereas Ale attenuated these adverse effects of HG. It seems that at low concentration, the protective effects of Ale are mediated mainly by PPAR$_\gamma$ activation, whereas at higher concentration both PPAR$_\alpha$ and PPAR$_\gamma$ are involved. An alternative explanation is that PPARα activation facilitates or has synergistic effects with the effects of PPAR$_\gamma$ activation. siRNA against PPAR$_\alpha$ or PPAR$_\gamma$ partially blocked the protective effect. However, silencing both PPAR$_\alpha$ and PPAR$_\gamma$ completely blocked the effect of Ale.

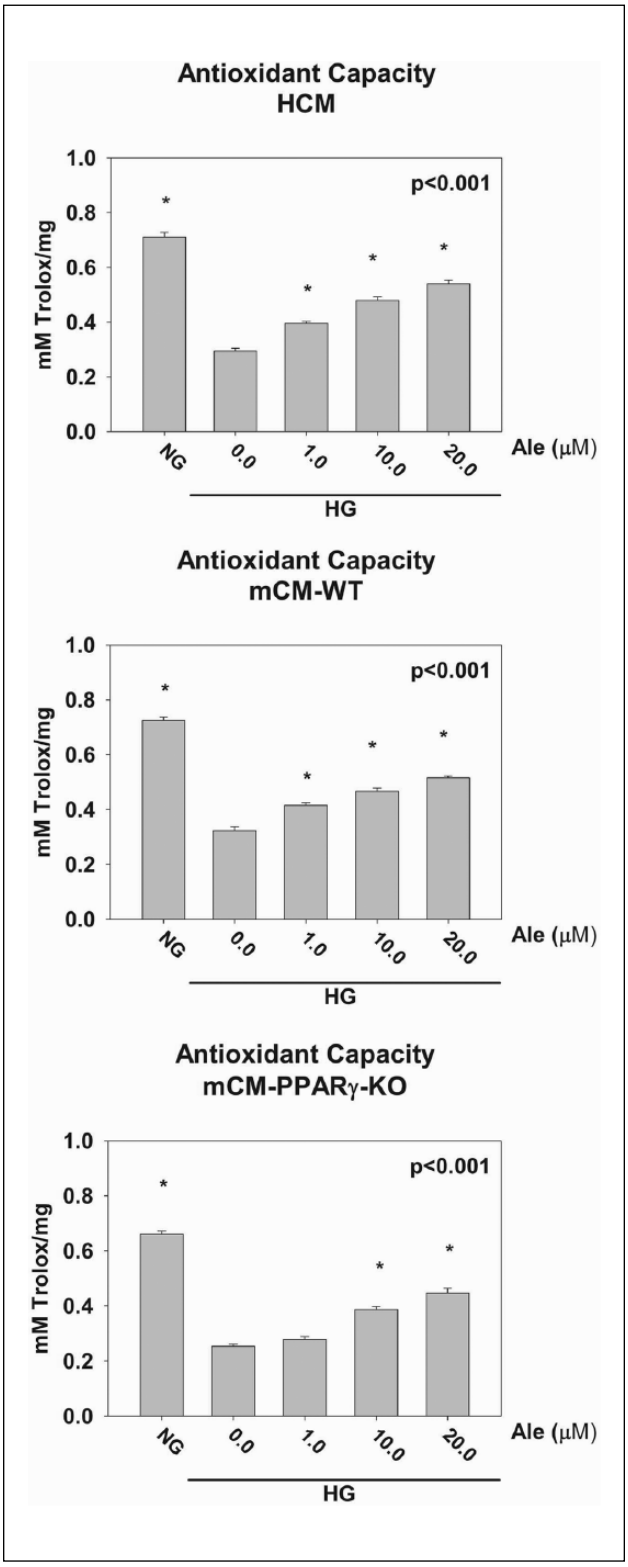

Figure 4. Exposure to HG for 48 h reduced the antioxidant capacity of the cardiomyocytes. Ale dose dependently blunted the effects of HG. *$p < 0.05$ versus 0.0 M Ale group (n = 6 per group).
NG: normoglycaemia; HG: hyperglycaemia.

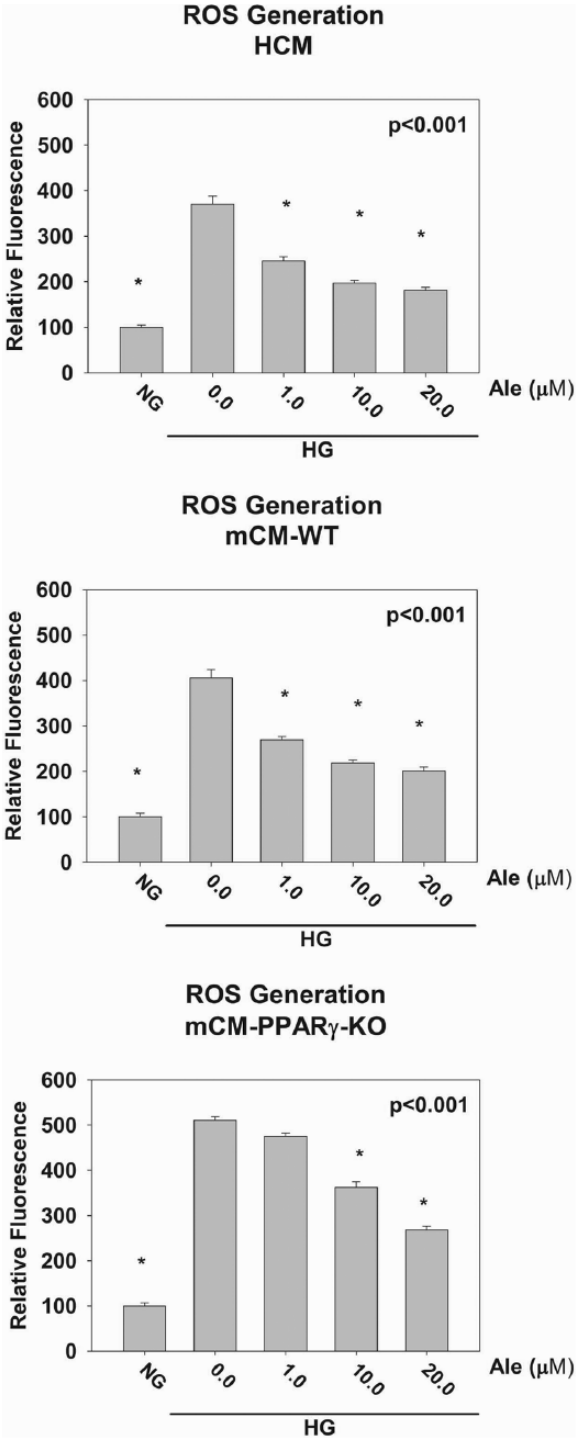

Figure 5. Effects of Aleglitazar on ROS generation caused by HG. *$p < 0.05$ versus 0.0 μM Ale group (n = 6 per group).

HG induces myocardial apoptosis, leading to diabetic cardiomyopathy.[33] HG induces Cyt-c release.[33] The leakage of Cyt-c from mitochondria into the cytoplasm is known to activate caspases and initiate apoptosis.[34] Previous studies revealed that ischaemia/reperfusion promotes myocardial apoptosis via mitochondrial Cyt-c-mediated caspase-3 activation and is dependent on ROS production.[33] Werner et al.[35] reported that Ale reduces oxidative stress-induced apoptosis in circulating angiogenic cells. In this study, HG-induced cardiomyocyte apoptosis, caspase-3 activation and Cyt-c release were dose dependently attenuated by Ale. Furthermore, Ale improved viability of the cells exposed to HG.

The protective effects of metformin against ischaemia-reperfusion injury are $PPAR_{\alpha}$-dependent.[36] Metformin attenuated the decrease in state 3 respiration and complexes I and II and decreased mitochondrial permeability transition pore (PTP) opening. It was suggested that $PPAR_{\alpha}$ activation by metformin abolishes oxidative stress-induced physical interactions between $PPAR_{\alpha}$ and cyclophilin D and thus with inhibition of PTP formation.[37] On the other hand, some of the cardioprotective effects of $PPAR_{\alpha}$ activation are related to activation of adenosine monophosphate-activated protein (AMP) kinase,[37] stimulation of fatty acid β-oxidation with improved adenosine triphosphate (ATP) production,[38,39] and to its anti-inflammatory properties.[40] Therefore, by activating $PPAR_{\alpha}$, Ale can attenuate mitochondria-mediated cell death (as shown in this study as attenuation of Cyt-c release). Others identified insulin-like growth factor-1 as the downstream target of $PPAR_{\alpha}$ activation that mediates the protective effect.[15] Moreover, it has been shown that $PPAR_{\alpha}$ agonists protect the heart against ischaemia-reperfusion injury by upregulating the prosurvival mediators PI3-Kinase/Akt, endothelial nitric oxide synthase and endothelin-1.[11,13,14]

The protective effects of $PPAR_{\gamma}$ agonists are also ascribed to activation of the prosurvival mediators PI3-Kinase/Akt and cyclooxygenase 2 (COX2).[9–11] Thus, by activation of both the $PPAR_{\alpha}$ and $PPAR_{\gamma}$ receptors, additive effects on the activation of the prosurvival pathways are expected.

A direct association between hyperglycaemia and oxidative stress in diabetic injuries has been shown.[41,42] Ho et al.[43] demonstrated that exposure of human umbilical vein endothelial cells in vitro to HG caused a significant increase in ROS formation in association with caspase-3 activation and apoptosis. Addition of ascorbic acid as an antioxidant agent to the cell culture suppressed the activation of caspase-3 and the induction of apoptotic cell death.[43] Mitochondria from diabetic hearts have morphological and functional damage coupled with lipid oxidation and ROS generation.[44,45] Furthermore, ROS formation induced by hyperglycaemia can ultimately cause cardiomyocyte apoptosis.[28,46,47] In this study, the effects of HG on ROS production and antioxidant capacity were significantly attenuated by 1.0, 10 and 20 μM of Ale in both the HCM and mCM-WT, whereas only at 10 and 20 μM of Ale in the mCM-$PPAR_{\gamma}$ KO cardiomyocytes. Ale was significantly effective

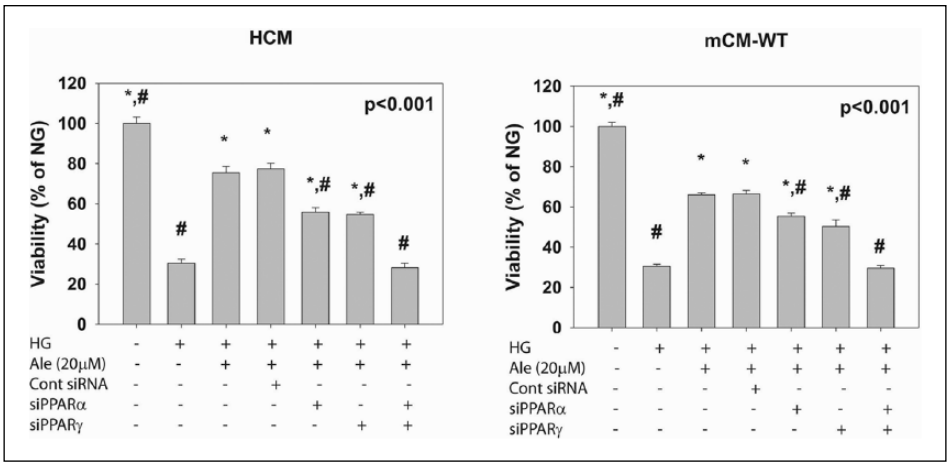

Figure 6. The effects of $PPAR_\gamma$ and $PPAR_\alpha$ silencing on the protective effect of Ale on cell viability in cardiomyocytes exposed to HG. *$p < 0.05$ versus HG; #$p < 0.05$ versus HG + Ale (n = 6 per group).

in improving cell viability at concentrations higher than 0.5, 1.0 and 10.0 μM in HCM, mCM-WT and mCM-$PPAR_\gamma$-KO, respectively. The findings revealed that the protection effects exerted by Ale at lower concentrations are probably $PPAR_\gamma$ dependent, whereas at higher concentration, $PPAR_\alpha$ activation partially mediates the effect. Our experiments using siRNAs to $PPAR_\alpha$ and $PPAR_\gamma$ further support this observation. In a previous study, we showed that Ale increased the DNA binding capacity of both $PPAR_\alpha$ and $PPAR_\gamma$ (relatively more increase in $PPAR_\alpha$ than $PPAR_\gamma$ in HCM, whereas more increase in $PPAR_\gamma$ than $PPAR_\alpha$ in mCM-WT).[30] Silencing $PPAR_\alpha$ alone or $PPAR_\gamma$ alone partially blocked the effect of Ale on cell viability in both HCM and mCM-WT, whereas silencing both $PPAR_\alpha$ and $PPAR_\gamma$ completely blocked the protective effect. This suggests that in contrast to pioglitazone,[9] Ale does not have PPAR-independent effects.

In contrast to our encouraging results with short-term in vitro exposure, a recent clinical study found that long-term Ale did not reduce the risk of cardiovascular events and mortality in patients with T2DM and recent acute coronary syndrome.[22] Benardeau et al.[20] also showed that Ale improved glycaemic control and protected the pancreatic beta-cells and the kidneys of ZDF rats. Many agents that have shown promising results in in vitro and in vivo animal models failed to show beneficial effects in the clinical setting. The potential factors that could explain these discrepancies have been discussed.[48,49] These factors include the animal models, length of therapy, dose and concentrations of drugs and interactions with other agents commonly administrated in the clinical setting. Specifically, here we assessed the short-term protective effects of Ale against high concentrations of glucose (25 mM = 450 mg/dL) in vitro. These short-term protective effects may not be translated to long-term protective effects in the clinical setting, especially as most of the diabetic patients are not exposed to such high glucose concentrations with the concomitant use of anti-diabetic medications. Another important issue is the drug concentration. Wright et al.[50] have shown that the effects of both Ale and muraglitazar on the expression of various genes in primary HCM change at different drug concentrations. Similar findings have been described in primary human hepatocytes.[51] Ale had a more potent effect on $PPAR_\alpha$ than on $PPAR_\gamma$ activation in HCM, whereas in the mCM-WT, $PPAR_\gamma$ activation was more prominent, it might be that in the concentrations achieved in the clinical setting, these favourable effects cannot be reached. We used drug concentrations slightly higher than the reported through levels in patients (0.0017–0.01 μM).[31] Thus, the long-term effects of Ale in the clinical setting could be different from those seen in our short-term experiments. And finally, we cannot exclude that long-term exposure to the drug may induce adverse effects or drug–drug interactions that could potentially offset the protective effects against high glucose levels. An additional explanation could be the duration of diabetes before exposure to the drug. Evidence from several recent large, randomized, controlled trials suggests that improved glycaemic control is associated with a reduced risk for CVD, but that this effect may be greater in individuals with a shorter duration of diabetes and without CVD, and that it may take many years of good glycaemic control to translate into CV risk reduction.[52,53] It remains unknown whether or not Ale can improve cardiovascular outcomes in patients with short-term T2DM. Unfortunately, further clinical studies and the use of Ale in the clinical setting were terminated (http://www.roche.com/media/store/releases/med-cor-2013-07-10.htm).

The investigational agent, muraglitazar, was associated with an apparent excess of adverse cardiac ischaemic events in an aggregate analysis of small phase-2 and phase-3 trials,[54] whereas development of another dual agonist, tesaglitazar, was also terminated due to renal toxicity.[55] Yet, newer dual $PPAR_{\alpha/\gamma}$ agonists are being developed.[56–58]

Thus, overcoming drug interactions and adverse effects, dual $PPAR_{\alpha/\gamma}$ might still show efficacy in reducing cardiovascular morbidity and mortality in patients with T2DM. However, the exact spectrum and magnitude of gene activation by each of the specific dual $PPAR_{\alpha/\gamma}$ agonists differ and is also affected by the drug concentration.[50,51] Thus, results, obtained with one dual $PPAR_{\alpha/\gamma}$ agonist at a specific concentration, cannot be inferred to the other $PPAR_{\alpha/\gamma}$ agonist agents.

The reported serum trough levels of Ale in humans are 0.0017–0.01 μM.[31] The concentrations that were associated with a significant effect in our in vitro experiments are much higher. Therefore, it is unclear whether similar protective effects can be seen in in vivo animal models or in the clinical setting. Yet, we found that at 5 mg/kg/day, Ale reduced experimental infarct size in db/db mice exposed to 30 min coronary artery occlusion followed by 24 h reperfusion.[30]

The limitation of the study is that no in vivo experiments were done to clarify whether the favourable antioxidant, anti-hypertrophic and cardioprotective effects of Ale, observed in short-term in vitro exposure, can be seen in diabetic animals. Future studies are warranted to address these issues. Furthermore, our findings cannot be extrapolated to the clinical practice.

Conclusion

Ale protects cardiomyocytes against hyperglycaemia-induced apoptosis by combined activation of both $PPAR_{\alpha}$ and $PPAR_{\gamma}$ in an in vitro short-term model.

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Yan Chen and Hongmei Chen contributed equally to this work.

Declaration of conflicting interests

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