M3-mAChR Stimulation Exerts Anti-apoptotic Effect Via Activating the HIF-1α/HO-1/VEGF Signaling Pathway in H9c2 Rat Ventricular Cells

Yang Hui, PhD,†‡ Yanli Zhao, MS,*§ Ning Ma, MS,† Yahui Peng, MS,*†‡ Zhenwei Pan, PhD,* Chaoxia Zou, PhD,*†‡ Pengxia Zhang, PhD,¶ and Zhimin Du, PhD*‖*

Background: The protective role of M_3-mAChR against apoptosis has been identified previously. However, the underlying mechanisms remain unclear. This study was performed to clarify the signaling pathways of the anti-apoptotic effect mediated by activation of M_3-mAChR in cultured cardiac H9c2 cells.

Methods: Both H9c2 rat ventricular cells and H9c2 cells with stable expression of M_3-mAChR were used.

Results: Activation of M_3-mAChR by cabarchol produced protective effect on etoposide-induced apoptosis in H9c2 cells. Forced overexpression of M_3-mAChR in H9c2 cells further enhanced this effect. Application of 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (inhibitor of M_3-mAChR), YC-1 [inhibitor of hypoxia-inducible factor 1, (HIF-1], or ZnPP (inhibitor of heme oxygenase-1)abrogated carbacol-induced cardioprotection, respectively. Moreover, the expression of HIF-1α, HO-1, and vascular endothelial growth factor (VEGF) were enhanced after the activation of M3-mAChR, and the induction of HO-1 and VEGF was reversed by HIF-1α inhibitor YC-1.

Conclusions: These findings indicated that M_3-mAChR upregulates HO-1 and VEGF expression likely through induction of HIF-1α, which at least partly underlies the cytoprotection of M_3-mAChR activation in H9c2 cells.

Key Words: acetylcholine receptors (muscarinic) M3, apoptosis, hypoxia inducible factor 1, heme oxygenase 1, vascular endothelial growth factor

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From the *Key Laboratory of Cardiovascular Medicine Research, Harbin Medical University, Ministry of Education, Harbin, China; †Department of Biochemistry and Molecular Biology, Harbin Medical University, Harbin, China; ‡State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Harbin, China; §Medical College of Qinghai University, Xining, Qinghai, China; ¶Jiamusi University, Jiamusi, China; and ‖Institute of Clinical Pharmacology of the Second Hospital, Harbin Medical University, Harbin, China.

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The authors Y. Hui and Y. Zhao contributed equally to this work.

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Reprints: Zhimin Du, PhD, Institute of Clinical Pharmacology of the Second Hospital, Harbin Medical University, 148 Baojian Road, Nangang Dist., Harbin 150086, P.R. China (e-mail: duzhiminhyd@126.com).

INTRODUCTION

Evidence has been emerging for the existence of the functional M_3-mAChR in heart tissue, which is of potential physiological, pathophysiological, and pharmacological significance.[1–6] Before the identification of cardiac M_3-mAChR, M_2-mAChR was considered the only one in charge of myocardial muscarinic transduction. Jaiswal et al[1] provided the first evidence for the functional existence of M_3-mAChR in mammalian heart. Subsequently, a host of functional and molecular studies revealed the expression of the M_3-mAChR transcript and protein in the heart tissues of various species including mouse, rat, guinea pig, cat, canine, and human.[7–11] In late 1990s, pharmacological data from radioligand-binding studies confirmed the functional M_3-mAChR subtypes in hearts.[12,13]

Since the existence of cardiac M_3-mAChR was recognized, great progress has been made in elucidating the pathophysiological roles of M_3-mAChR in the heart.[9,10,14] The function of cardiac M_3-mAChR includes the following: regulation of heart rate and cardiac repolarization, modulation of inotropic effects, protection of cardiac cells against ischemic injuries, regulation of cell-to-cell communication, participation in generation and maintenance of atrial fibrillation, and cytoprotection against apoptosis.[15]

The ability of M_3-mAChR to produce cytoprotection against apoptosis has been previously reported in several noncardiac cells. In cerebellar granule neurons, the M_3-mAChR–selective inhibitor 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (4-DAMP) was shown to abolish the cytoprotective action of carbachol (CCH) against apoptosis.[16,17] Budd et al[18,19] reported that the C-terminal tail of the M_3-mAChR possesses anti-apoptotic properties in Chinese hamster ovary cells, and Bcl-2 was involved in the M_3-mAChR signals to the anti-apoptotic pathway via both transcription and translation upregulation. Solyakov et al[20] reported that activation of M_3-mAChR inhibits the ability of the DNA-damaging chemotherapeutic agent etoposide to induce apoptosis by regulating p53 expression, modification, and localization. Studies performed in rat model of myocardial infarction and cultured rat myocytes also validated the protection against H_2O_2-induced apoptosis.[21]

However, previous studies mainly employ 4-DAMP a selective blocker of M_3-mAChR to explore the function of

M_3-mAChR. Considering the nonselective properties of pharmacological agents, we cannot completely rule out the influence of other on M-receptor subtypes when interpreting the data obtained by using 4-DAMP. Therefore, in this study, we tried to establish H9c2 cardiomyocytes stably transfected with the M_3-mAChR of human to validate the involvement of M_3-mAChR in apoptosis. Hirota et al[22] reported that M_3-mAChR signaling pathway activates hypoxia-inducible factor 1 (HIF)-1α in HEK293 cells. HIF is a transcription factor which can regulate many target genes such as HO-1 and vascular endothelial growth factor (VEGF), which produce anti-apoptotic action.[23,24] Thus, in this study, we further investigated the involvement of HIF-1α/HO-1/VEGF signaling pathway in M_3-mAChR–mediated anti-apoptotic effects in H9c2 cells.

MATERIALS AND METHODS

Materials

CCH, YC-1, protoporphyrin IX, zinc (Znpp), 4-DAMP, etoposide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Saint Louis, MO). G418 were purchased from Amresco (Amresco, OH). Anti-HIF-1α antibody, anti-VEGF antibody, anti-HO-1 antibody, horseradish peroxidase–conjugated anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were obtained from Fisher (King of Prussia, PA).

Cell Culture

Embryonic rat heart–derived cell line H9c2, obtained from the cell bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). H9c2 cells stably transfected with the M_3-mAChR subtypes of human are referred to as H9c2-M3, H9c2 cells stably transfected with empty vector are referred to as H9c2-PC, and stably transfected clones were obtained byG418 sulphate selection. Cells were cultured in Dulbecco modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Gibco), 3.7 g/L sodium bicarbonate. The cells were maintained at 37°C under a water-saturated atmosphere of 95% ambient air and 5% CO_2. Where indicated, cells were made quiescent by serum starvation for 24 hours before agonist addition.

MTT Assay

H9c2 cells grown in 96-well plates (Corning) were treated with the appropriate drugs, and MTT was added (0.5 mg/mL) to the plates. The plates were incubated at 37°C for 4 hours, and then throw away medium, 150 μL of DMSO was added. Ten minutes later, the absorbance was determined using a multiplate reader (Thermo, Anaheim, CA) at a wavelength of 490 nm.

Annexin V–FITC and Propidium Iodide Staining

H9c2 cells were plated on 60-mm plates. After incubation with the appropriate drugs, then 1×10^6 cells were collected by centrifugation. Resuspended cells in 500 μL of 1 × binding buffer, added 5 μL of Annexin V–FITC and 5 μL of propidium iodide, incubated at room temperature for 5 minutes in the dark. Annexin V–FITC and propidium iodide–stained cells were analyzed by flow cytometry.

Real-time Polymerase Chain Reaction

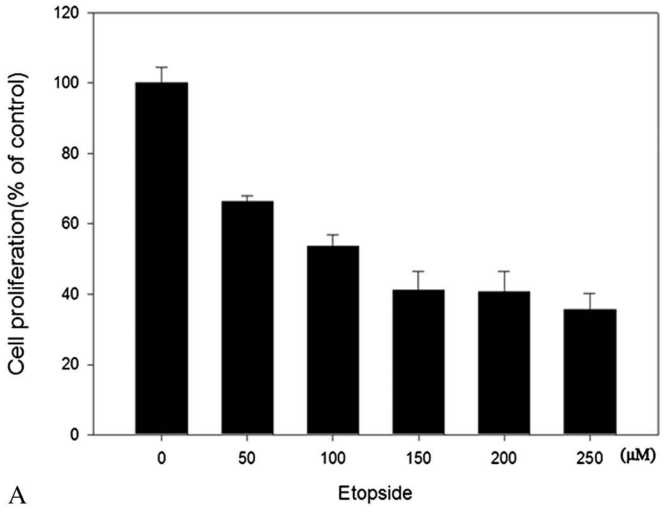
Total RNA was isolated from H9c2 and H9c2-m3 cells using Trizol reagent (Invitrogen) according to the manufacturer protocol. The first stranded complementary DNA was synthesized from 2 μg of total RNA using a reverse transcription SystemKit (Promega) according to the manufacturer protocol. Reverse transcription was carried out at 25°C for 5 minutes; the reaction is incubated at 42°C for up to 1 hour, after incubation at 70°C for 15 minutes. A total of 30 ng of cDNA, 200 nM both sense and antisense primers, and SYBR Green Supermix (Takala) in a final volume of 25 μL was used for polymerase chain reaction (PCR). Reactions were carried out on an ABI PRISM 7500 reverse transcription–polymerase chain reaction machine using optimized primers. Sequences of the primers are as follows:

HIF-1α sense, 5′-TGGGGTTGACTCAGTTTGAA-3′;
HIF-1α antisense, 5′-TCCACGTTGCTGACTTGATG-3′;
HO-1α sense, 5′-CACGCA TATACCCGCTACCT-3′;
HO-1α antisense, 5′-AAGGCGGTCTTAGCCTCTTC-3′;
VEGF sense, 5′-TATCTTCAAGCCGTCCTGTG-3′;
VEGF antisense, 5′-ACA AATGCTTTCTCCGCTCT-3′;
GAPDH sense, 5′-GTTGTCTCCTGCGACTTCA-3′;
GAPDH antisense, 5′-GGTGGTCCAGGGTTTCTTA-3′.

As an internal control, GAPDH primers were used for RNA template normalization. Fluorescent signals were normalized to an internal reference (Δ*R*n), and the threshold cycle (*C*t) was set within the exponential phase of the PCR. The relative gene expression was calculated by comparing cycle times for each target PCR. The target PCR *C*t values were normalized by subtracting the GAPDH *C*t value, which gives the Δ*C*t value. From this value, the relative expression level between treatments can be calculated using the following equation: relative gene expression = $2-[\Delta C\text{t (Sample)} - \Delta C\text{t (Control)}]$.

Immunoblot Assays

H9c2 cells and H9c2-m3 cells were washed twice in cold phosphate-buffered saline, whole cell lysates were prepared by incubating cells for 30 minutes in cold radioimmune precipitation assay (Lysis Buffer, biyuntian, Beijing, China). Samples were centrifuged at 10,000*g* to pellet cell debris. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Biocolors, Shanghai, China). A total of 50 μg protein of cellular lysates was then electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to polyvinylidine difluoride membranes by electroblotting. The membranes were incubated in 5% nonfat milk overnight, then incubated with rabbit anti-HIF-1α or anti-HO-1 or anti-VEGF (1:500) antibody for 2 hours, followed by incubation with horseradish peroxidase–conjugated anti-rabbit IgG



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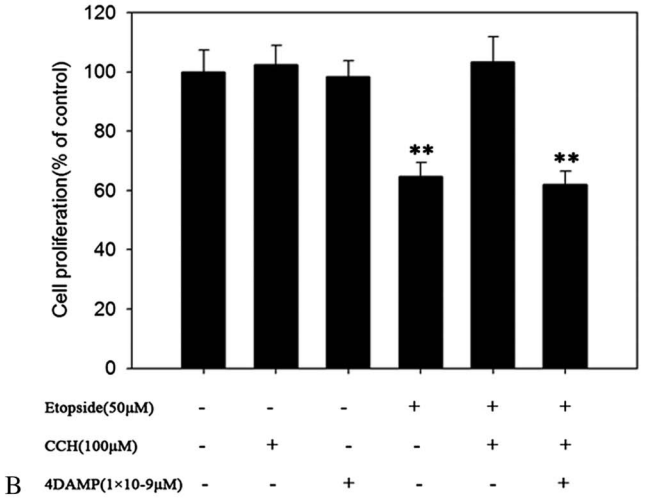

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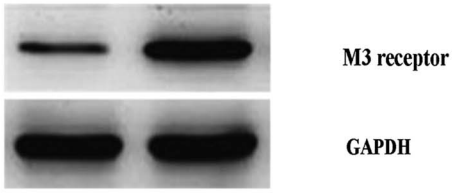
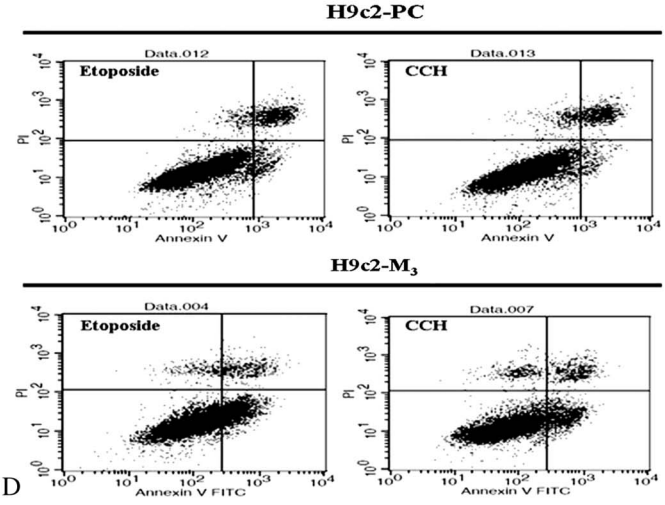


C H9c2/PC H9c2/M3



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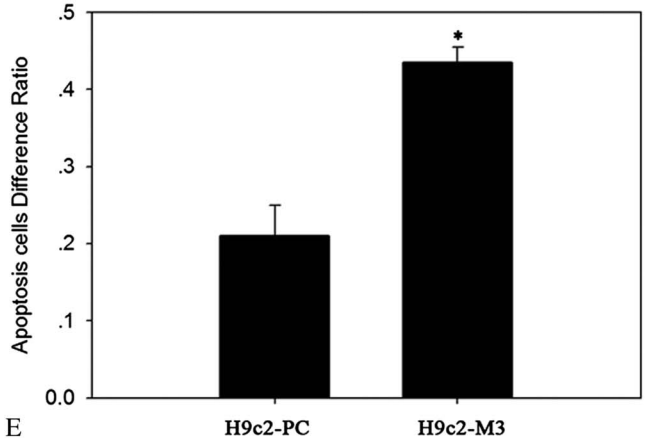

E

FIGURE 1. The protective effects of M_3 mAChR on etoposide-induced H9c2—cell apoptosis. In this experiment, etoposide (50 μM) was used to induce apoptosis, CCH (100 μM) was used to activate M_3–AchR, 4-DAMP (1×10^{-9} μM) was used to block M_3–AchR. H9c2 cells were incubated in media with various concentrations of etoposide for 48 hours, then percent cell survival was evaluated using MTT assay (A). H9c2-cells were pretreated with M_3–AchR agonist CCH (100 μM) or with M_3–AchR agonist CCH (100 μM) and M_3–AchR blocking pharmacon 4-DAMP (1×10^{-9} μM) together, then these cells were exposed to 50 μM etoposide for 48 hours. Percent cell survival was detected by MTT assay (B). H9c2-PC cells and H9c2-M_3 cells were collected, and the protein level of M_3 receptor was analyzed by immunoblotting (C). H9c2-M_3 cells and H9c2-PC cells were both divided into two groups, the left groups were exposed to 50 μM etoposide for 48 hours only, the right groups were exposed to 50 μM etoposide and CCH (100 μM) simultaneously for 48 hours, then the apoptosis determination was assessed by Annexin V–FITC and propidium iodide–staining flow cytometry (D). The apoptosis cells contained second and fourth areas. The difference of apoptosis cells ratio between the right group and the left group in H9c2-M_3 cells and H9c2-PC cells were contrasted (E). The results are expressed as means ± SEM, n = 3, separate wells of cells in at least 3 separate experiments. *$P < 0.05$, **$P < 0.01$, compared with control values.

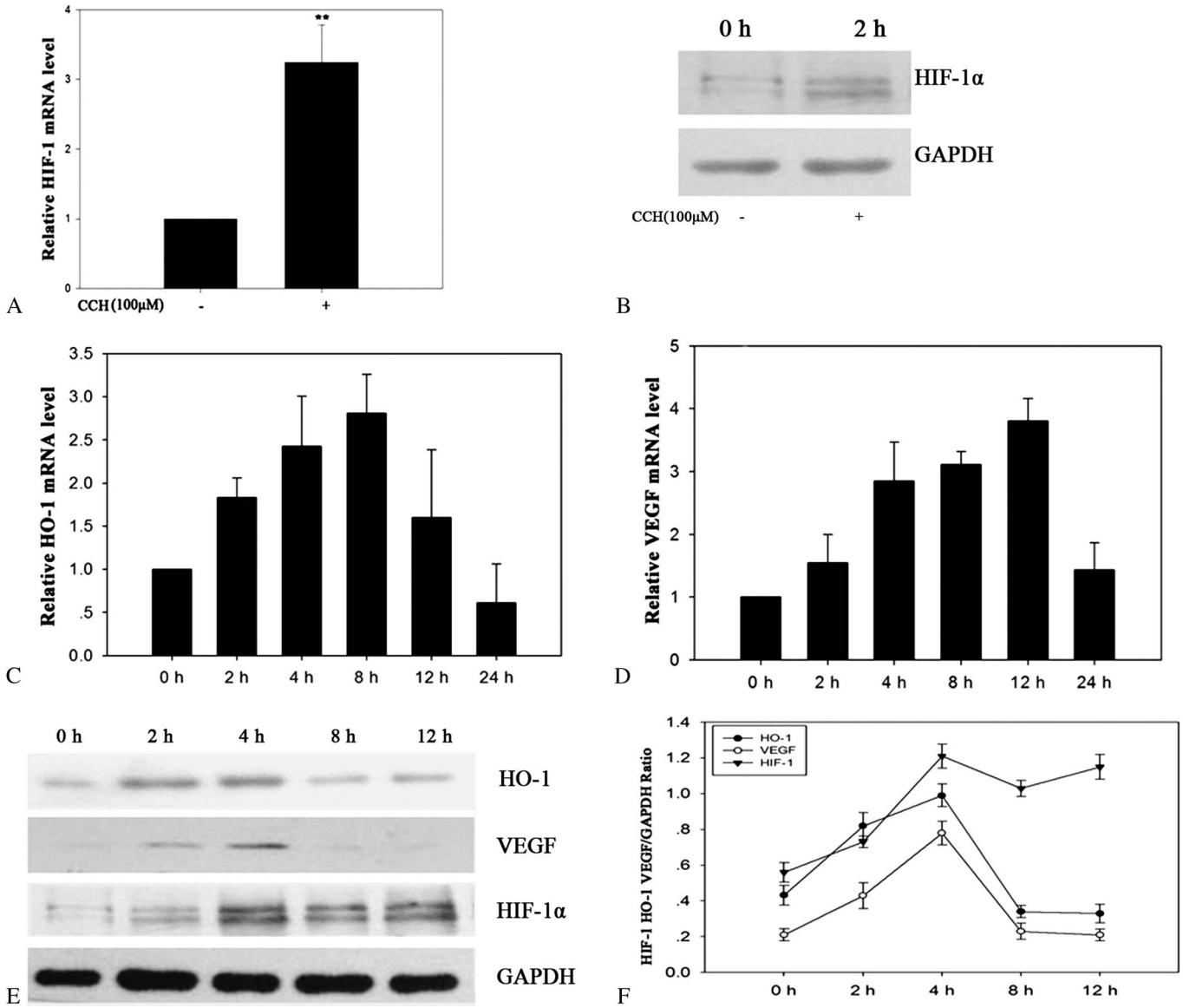

FIGURE 2. Activating M3-AchR with CCH-induced HIF-1α, HO-1, and VEGF expressing in a time-dependent manner. H9c2 cells were incubated in CCH (100 μM), then whole-cell lysates were collected for analyzing HIF-1α, HO-1 and VEGF by Real-time PCR and immune blotting. H9c2 cells were treated with100 μM CCH for 2 hours, the level of *Hif1a* and accumulation of HIF-1α protein were detected (A, B). H9c2 cells incubated in 100 μM CCH were collected at different time. HO-1 and VEGF mRNA were analyzed by real-time PCR (C, D). HIF-1α, HO-1, and VEGF protein were analyzed by immune blotting (E). The results are expressed as means ± SEM, n = 3, separate wells of cells in at least 3 separate experiments. $*P < 0.05$, $**P < 0.01$, compared with control values.

(1:10000) for 1 hour. Immunoreactive proteins were visualized using a commercially available enhanced chemiluminescence kit (pulilai, Beijing, China) with exposure of the transfer membrane to X-ray film (Kodak).

Statistical Analysis

The results are expressed as means ± SEM. The Sigmaplot 9.0 software was used for the statistical analyses. Comparisons between 2 groups were performed with the analysis of variance and Student 2-tailed *t* test for unpaired data. Differences between groups were considered significant when $P < 0.05$.

RESULTS

Effects of M3-mAChR Activation on Etoposide-Induced H9c2 Cell Apoptosis

To evaluate M_3-mAChR–mediated cytoprotection, H9c2 cells were exposed to 50 μM etoposide, a compound that can dose dependently inhibit cell survival (Fig. 1A) for 48 hours

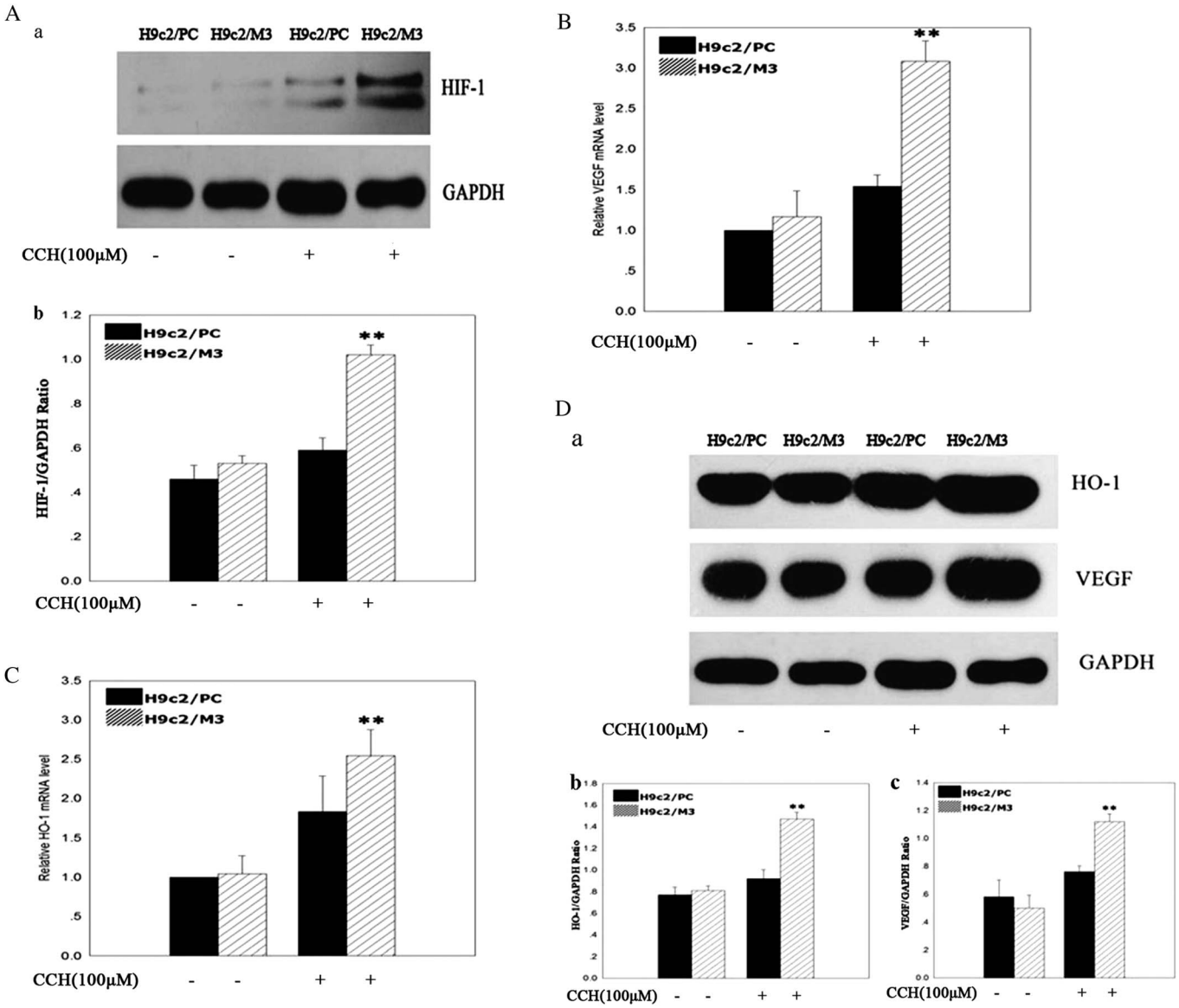

FIGURE 3. Validated the effects of M_3-AchR on HIF-1α, HO-1, and VEGF in H9c2-M_3 cells. H9c2-PC cells and H9c2-M_3 cells were incubated in 100 μM CCH for 2 hours, then these cells and the control groups were collected for real-time PCR and immunoblotting assay. HIF-1α protein in H9c2-PC cells and H9c2-M_3 cells were assayed by immunoblotting (Aa). The protein expression levels of HIF-1α in H9c2-cells and H9c2-M_3 cells were compared in histogram (Ab). The mRNA expression of VEGF and HO-1 were analyzed by real-time PCR (B, C). HO-1 and VEGF protein in H9c2 cells and H9c2-M_3 cells were assayed by immunoblotting (Da). The protein expression levels of HO-1 and VEGF in H9c2-cells and H9c2-M_3 cells were compared in histogram (Db, Dc). The results are expressed as means ± SEM, n = 3, separate wells of cells in at least 3 separate experiments. H9c2-M_3 cells versus H9c2-PC cells, $*P < 0.05$, $**P < 0.01$.

pretreated with M_3-mAChR agonist CCH (100 μM). CCH reversed the inhibition of etoposide on H9c2 cells viability, which was hampered by co-application of the selective M_3-mAChR blocker 4-DAMP (1×10^{-9} μM) (Fig. 1B). To further verify the anti-apoptotic effect of M_3-mAChR but not other subtypes, H9c2-cells stably expressed M_3-mAChR named H9c2-M3 cells were successfully established, as indicated by the significantly increased expression of M_3-mAChR protein (Fig. 1C). Activation of M_3-mAChR inhibited cell apoptosis more significantly in H9c2-M_3 cells than in H9c2-PC cells assessed by Annexin V–FITC and propidium iodide–staining flow cytometry (Fig. 1D, E), further confirming the involvement of M_3-mAChR in cardioprotection.

Effects of M3-AchR Activation on HIF-1α, HO-1, and VEGF Expression

M_3-mAChR signals was demonstrated to activate HIF-1 on both transcriptional activity and protein stabilization

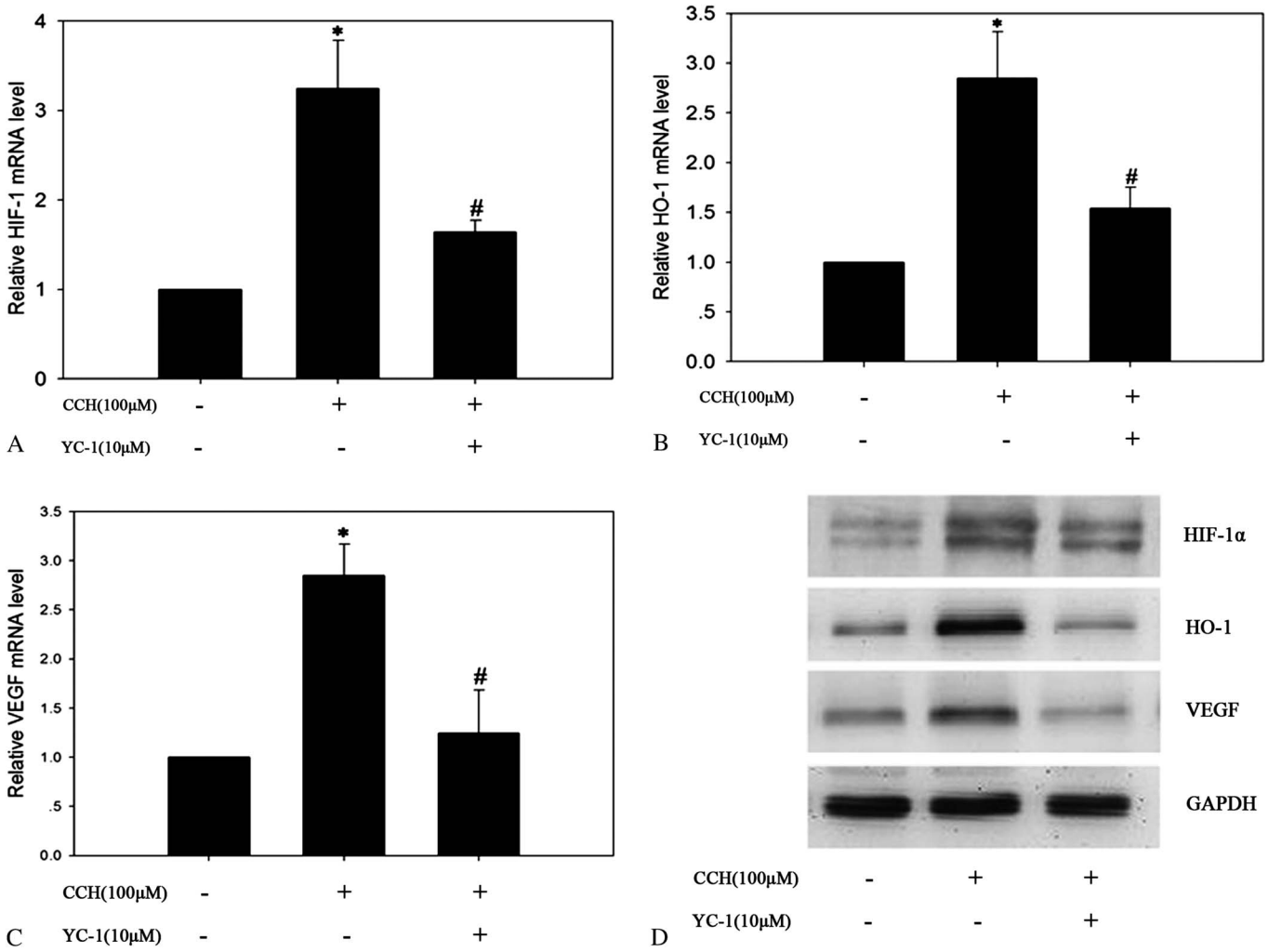

FIGURE 4. Stimulating M_3-AchR with CCH-induced HO-1 and VEGF depend on HIF-1α. H9c2 cells were pretreated with 10 μM YC-1 for 5 minutes, then incubated in 100 μM CCH for 2 hours. The whole-cell lysates were collected and analyzed by real-time PCR and immunoblotting. The mRNA expression of HIF-1α, HO-1, and VEGF in H9c2 cells were analyzed by real time PCR (A–C). The protein expression of HIF-1α, HO-1, and VEGF in H9c2 cells were analyzed by immunoblotting (D). The results are expressed as means ± SEM, n = 3, separate wells of cells in at least 3 separate experiments. *$P < 0.05$, compared with negative control; #$P < 0.05$, compared with CCH stimulating H9c2 cells.

and synthesis in HEK293 cells.[25] Consistently, in this study, we found when H9c2 cells were treated with100 μM CCH for 2 hours, the expressions of Hif1a mRNA and protein were both significantly increased (Figs. 2A, B). Moreover, in consistent with the induction of HIF-1α, we found CCH (100 μM) treatment also increased the expression of HO-1 and VEGF, which contain hypoxia-responsive element (HRE) in their encoded genes, at both mRNA and protein levels in H9c2 cell in a time-dependent manner (Figs. 2C–E). As CCH is a nonselective agonist of M-AChRs, we cannot exclude the involvement of other subtypes of M-AChRs besides M_3-mAChR when interpreting the above results. Therefore, we employed H9c2-M_3 cells to dissect the role of M_3-mAChR on HIF-1α, HO-1, and VEGF induction. We found in H9c2-M_3 cells CCH-activated HIF-1α, HO-1, and VEGF upregulation were more significant compared with H9c2-PC (PC: the mock transfected cells with the empty vector) cells (Fig. 3), which proved the ability of M_3-mAChR to induce HIF-1α, HO-1, and VEGF expression.

Furthermore, we found that HIF inhibitor YC-1 blocked CCH-induced upregulation of HO-1 and VEGF at both mRNA and protein levels in H9c2 cell (Fig. 4), suggesting that the induction of HO-1 and VEGF expression by M_3-mAChR was mediated by HIF-1α.

HO-1 and VEGF were Involved in M3-AChR-Mediated Anti-apoptotic Effects in H9c2 Cells

As VEGF and HO-1 have been reported to possess anti-apoptotic function, we proposed that they may be the factors mediating the anti-apoptotic effects of M_3-mAChR in H9c2 cell. We found carbacol treatment significantly increased the protein levels of HO-1 and VEGF in H9c2-PC cells and H9c2-M_3 cells when exposed to 50 μM etoposide (Fig. 5A).

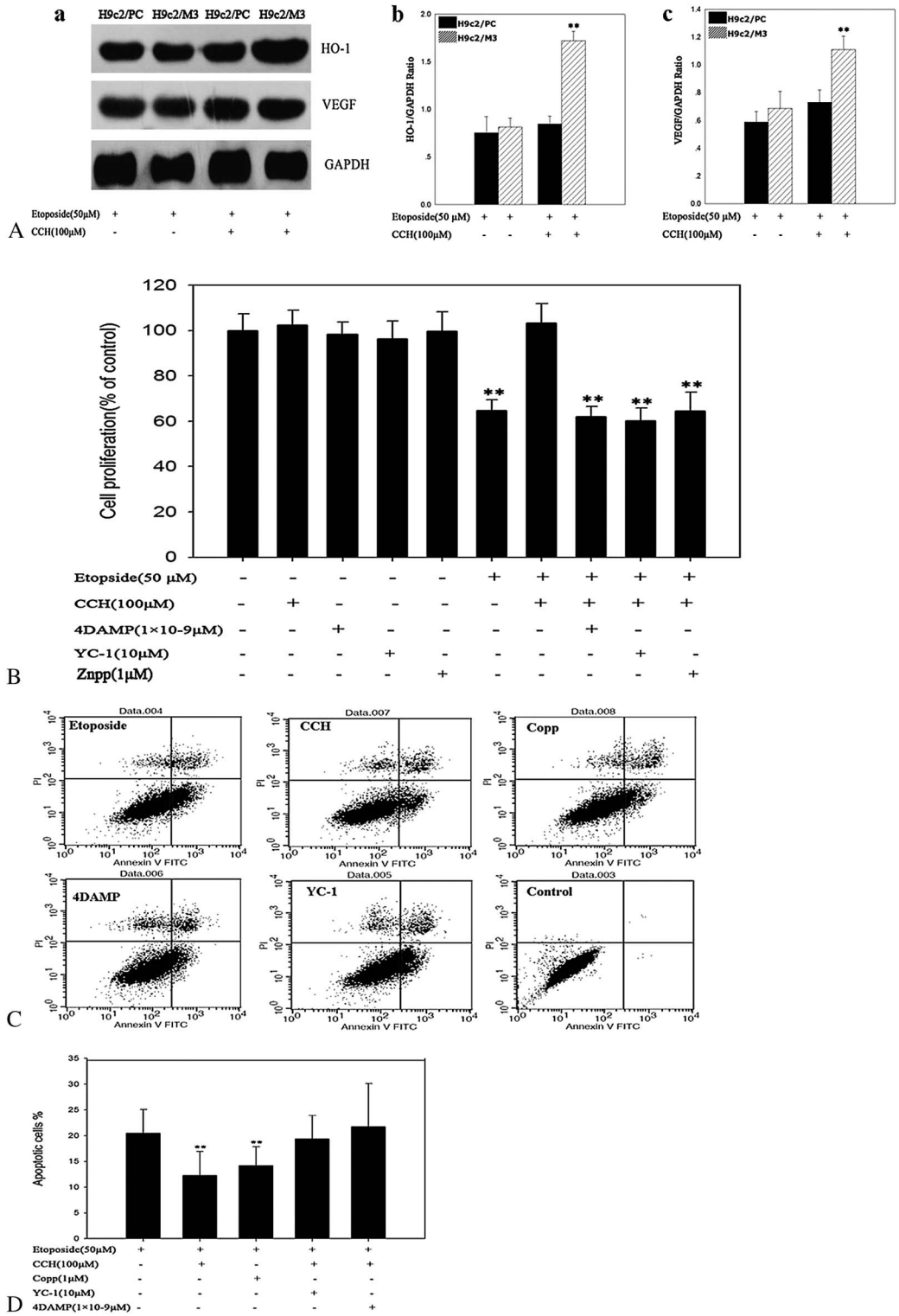

FIGURE 5. The anti-apoptosis of M_3-mAChR was at least partly from HIF-1α and HO-1. H9c2-M_3 cells and H9c2-PC cells were all exposed to 50 μM etoposide for 48 hours. Cells pretreated with CCH (100 μM) or not were collected to detect HO-1 and VEGF by immune blotting (A). H9c2-M_3 cells versus H9c2-PC cells, $^*P < 0.05$, $^{**}P < 0.01$. H9c2 cells exposed to etoposide were pretreated with CCH (100 μM) and 4-DAMP (1×10^{-9} μM) or YC-1 (10 μM) or ZnPP (1 μM), respectively. Percent cell survival was determined using the MTT assay (B). H9c2 cells exposed to etoposide were pretreated with CCH (100 μM) or CoPP (1 μM)or YC-1 (10 μM) or 4-DAMP (1×10^{-9} μM). Percent apoptosis cells were analysis by Annexin V–FITC and propidium iodide–staining flow cytometry (C). The results are expressed as means ± SEM, n = 3, separate wells of cells in at least 3 separate experiments. $^*P < 0.05$, $^{**}P < 0.01$, compared with etoposide-treating H9c2 cells.

Inhibition of HIF-1α or HO-1 by using YC-1 and ZnPP abolished M_3-mAChR activation–induced cell protection evaluated by MTT assay (Fig. 5B). Furthermore, the apoptosis of H9c2 cells greatly decreased in pretreatment with CCH or CoPP (an agonist of HO-1), whereas 4-DAMP and YC-1 reversed the effects (Fig. 5C). These results indicated that HIF-1α and HO-1 were involved in the protection of M_3-mAChR against apoptosis.

DISCUSSION

In this study, we confirmed the cytoprotection of M_3-mAChR against etoposide-induced apoptosis in H9c2 rat ventricular cells by using MMT assay and Annexin V–FITC and propidium iodide staining. H9c2 cells stably transfected with the M_3-mAChR subtype of human was used to exclude the influence of other subtypes of M-AChRs. Further studies showed activating M_3-mAChR promoted the transcription and translation of HIF-1α in H9c2 rat ventricular cells. And inhibition of HIF-1α by using YC-1 almost abolished M_3-AChR activation–induced cell protection. Results suggest the effect of M_3-mAChR against etoposide-induced apoptosis maybe mediated by HIF-1α in H9c2 cells.

HIF-1 as a transcription factor can regulate many genes by binding HRE, such as HO-1 and VEGF. In this study, HO-1 and VEGF can be induced by activating M_3-mAChR and reversed by the inhibitor of HIF-1α. Moreover, the induction of HO-1 strengthened, whereas inhibition of HO-1 suppressed the effect of M_3-mAChR against apoptosis. Results indicate HO-1 is involved in cytoprotection of M_3-mAChR against etoposide-induced apoptosis and maybe mediated by HIF-1α in H9c2 cells.

M_3-mAChRis coupled to G proteins of class Gq that upregulate phospholipase C and intracellular calcium as a signaling pathway.[26] Since the existence of cardiac M_3-mAChR was recognized, many pathophysiological roles of M_3-mAChR in heart are becoming recognized including the anti-apoptosis. Cardiac myocyte apoptosis may be a causal mechanism of heart failure. The anti-apoptotic mechanism underlying M_3-mAChR is still unknown. We deduce that HIF plays an important role in it.

Figure 6 shows a potential activation signal pathway of HIF by M_3-mAChR. It is reported that M_3-mAChR is coupled to activation of the phospholipase C/IP3 pathway and subsequent PKC activation.[27] PKC will trigger phosphorylation cascade reaction and subsequent transcription factor activation. In addition, an adhesion-dependent activation on MAPK and MEK by M_3-mAChR is reported.[25] It is demonstrated that M_3-mAChR–mediated signals promoted accumulation and transcriptional activation of HIF-1α dependent on tyrosine kinase, MAPK, and PI3K activity in HEK293 cells.[22] In this study, we supposed that CCH-activating M_3-mAChR is coupled to subsequent PKC, tyrosine kinase, or MAPK activation. Afterwards, HIF is activated in the following phosphorylation cascade reaction.

The HIF-1 as a transcription factor can bind to HRE located in the promoter and enhancer regions of hypoxia-regulated genes, causing their transactivation.[28–30] HIF can regulate some genes that encode critical proteins involved in the apoptotic pathway, such as some of the Bcl-2 family proteins. It was reported that the pro-apoptotic proteins Bnip3, Bad, Bax, and anti-apoptotic protein Bcl-2 can be regulated by HIF-1α.[31–33] HIF-1α exerts both anti-apoptotic and pro-apoptotic effects, depending on the cell type.[34] In this study, we put forward that M_3-mAChR–mediated signals induce HIF-1α expression under nonhypoxic conditions in H9c2 cell. HIF-1 is a heterodimer composed of a constitutively expressed HIF-1ß subunit and an inducibly expressed HIF-1α subunit.[35] Our results demonstrate that inhibition of HIF-1α nearly diminished the anti-apoptotic effects of M_3-mAChR in H9c2 cells, suggesting that HIF-1α is anti-apoptotic in this cardiac myocyte. And HIF maybe a major transcriptional factor to mediate the anti-apoptotic effect of M_3-mAChR in H9c2 cells (Fig. 6).

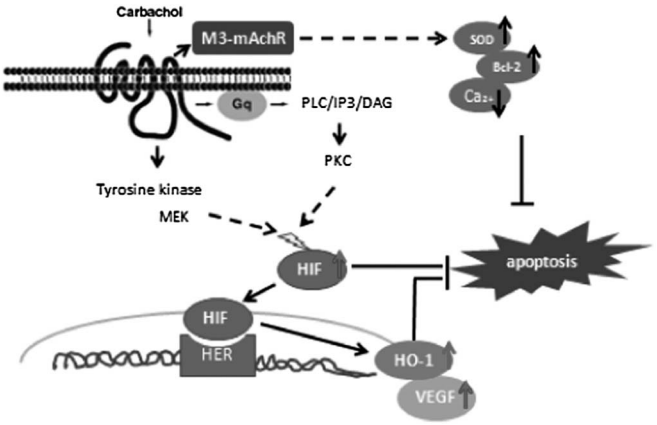

FIGURE 6. Schematic representation of the effect of M_3-mAchR signaling against apoptosis in cardiomyocyte. Activating M_3-mAChR signaling could protect cardiomyocyte against apoptosis. M_3-mAChR–induced HIF-1α on both transcription and translation levels. And HIF-1α could subsequently induce HO-1 and VEGF by binding HRE in them. During the anti-apoptosis of M_3-mAChR signaling pathway, the survival signaling molecules Bcl-2 and SOD increased, meanwhile apoptotic mediators such as intracellular Ca^{2+} decreased. Whether the changes of these survival signaling molecules and apoptotic mediators originated from HIF-1α and its downstream target genes such as HO-1 that induced by M_3-mAChR is yet to be elucidated.

In this study, we also detected other anti-apoptotic mediators downstream of HIF-1, such as HO-1and VEGF containing HRE (Fig. 6). Results indicate activating M_3-mAChR strongly induced the expression of HO-1 and VEGF. And YC-1 inhibited the induction of HO-1 and VEGF by M_3-mAChR. YC-1, an inhibitor of HIF-1α, was widely used both in vitro and in vivo studies[36–38] to downregulate HIF-1α and HIF-2α at the post-translational level.[39] This suggested that the induction of M_3-mAChR on HO-1 and VEGF depended on the activation of HIF-1α. Moreover induction of HO-1 strengthened, whereas inhibition of HO-1 suppressed the effect of M_3-mAChR against apoptosis. These results suggested that HO-1 maybe involved in the cytoprotection of M_3-mAChR against etoposide-induced apoptosis in H9c2 cells. Although VEGF can be induced in activating M_3-mAChR mediated by HIF-1α, the expression of VEGF

did not increase in etoposide-induced apoptosis. It seems that M_3-mAChR did not protect H9c2 cell against etoposide by inducing VEGF.

M_3-mAChR was previously reported to increase Bcl-2 and SOD, and decreased Fas and intracellular Ca^{2+} overload (Fig. 6).[21,40] We presume that M_3-mAChR produce anti-apoptotic action by increasing prosurvival signalling molecules and decreasing the pro-apoptotic mediators. M_3-mAChR and its downstream signal pathway maybe an important anti-apoptosis mechanism in heart. However, whether the changes of these survival signalling molecules and apoptotic mediators involved in M_3-mAChR–mediated anti-apoptosis is directly regulated by HIF-1α or its downstream target genes such as HO-1 requires further study.

Furthermore, except for anti-apoptosis HIF has effect on many other signal pathways. Enhanced expression of HIF-1 suggests that multiple genes maybe induced downstream signaling after M_3-mAChR activation in cardiac myocytes. For example HO-1 and VEGF is induced by this signaling which is demonstrated in this study. It means M_3-mAChR may be involved in more functional pathways in heart.

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