Hemeoxygenase-1 expression in response to arecoline-induced oxidative stress in human umbilical vein endothelial cells

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Abstract

Background: Arecoline, the most abundant areca alkaloid, has been reported to stimulate reactive oxygen species (ROS) production in several cell types. Overproduction of ROS has been implicated in atherogenesis. Hemeoxygenase-1 (HO-1) has cytoprotective activities in vascular tissues. This study investigated the effect of arecoline on adhesion molecule expression and explored the role of HO-1 in this process.

Methods: Human umbilical vein endothelial cells (HUVECs) were treated with arecoline, then ROS levels and the expression of adhesion molecules and HO-1 were analyzed and potential signaling pathways investigated.

Results: After 2 h of arecoline treatment, ROS production was stimulated and reached a maximum at 12 h. Expression of the adhesion molecules ICAM and VCAM was also induced. Glutathione pretreatment completely blocked arecoline-stimulated ROS production and VCAM expression, but not ICAM expression. Arecoline also induced HO-1 expression and this effect was partly due by ROS stimulation. Inhibition of c-jun N-terminal kinase (JNK) by SP600125, p38 by SB 203580, or tyrosine kinase by genistein reduced arecoline-induced HO-1 expression. In contrast, inhibition of ERK (extracellular signal-related MAP kinase) by PD98059 had no effect. Transfection of HUVECs with the GFP/HO-1 gene, which resulted in a 5-fold increase in HO-1 activity, markedly, but not completely, inhibited the decrease in cell viability caused by arecoline.

Conclusions: This study demonstrates that, in HUVECs, arecoline stimulates ROS production and ICAM and VCAM expression. HO-1 expression is also upregulated through the ROS, tyrosine kinase, and MAPK (JNK and p38) signaling pathways.

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1. Introduction

It has been estimated that there are 200 to 600 million betel quid chewers in the world. Betel quid chewing is thought to cause oral submucous fibrosis and oral cancer. Betel quid usually comprises a piece of areca nut and lime with or without Piper betle leaves. Areca nut contains many polyphenols and several alkaloids arecoline being the major alkaloid. Recent evidence suggests that arecoline is cytotoxic and genotoxic for various kinds of cells [1,2]. It also produces reactive oxygen species (ROS) and depletes intracellular thiols in human peripheral blood lymphocytes [3]. Overproduction of intracellular ROS has been implicated in a variety of pathological conditions, including cancer, diabetes, and cardiovascular diseases, such as atherosclerosis. Atherosclerosis is a complex disease with a chronic inflammatory pathogenesis [4]. ROS contribute to the pathogenesis of atherosclerosis by altering endothelial functions, including causing injury to endothelial cells and increasing cell adhesion molecule-mediated leukocyte adhesion to endothelial cells [5]. In response to ROS, several stress proteins are regulated as a cytotoxic response to diminish cellular damage. Hemeoxygenase-1 (HO-1), a 32-kDa enzyme, is the rate-limiting enzyme in the conversion of heme into biliverdin, carbon monoxide, and free iron. HO-1 can be induced in both endothelial and vascular smooth muscle cells by several stimuli, including oxidized low-density lipoprotein [6,7], heavy metals [8], inflammatory cytokines [9,10], and oxidative stress [11]. There is evidence that HO-1 plays a key role in protection against oxidative stress [12] and that mitogen-activated protein kinases (MAPKs) as well as other kinases [13], including tyrosine kinases [14], are involved in HO-1 activation.

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Prospective cohort studies have indicated that the habit of chewing betel nut is an independent risk factor of cardiovascular disease in humans[15,16]; however, it is still unclear whether there is any direct relationship between betel quid chewing and the development of cardiovascular disease. Moreover, there have not been any studies on a possible association between arecoline and atherosclerosis. The aim of this study was to determine whether arecoline affected the process of atherosclerosis. We measured ROS production and cell adhesion molecule expression in arecoline-treated human umbilical vein endothelial cells (HUVECs) and investigated whether HO-1 played a protective role against arecoline-stimulated effects. The contribution of the activation of MAPKs and/or other kinases to arecoline-stimulated HO-1 induction was also examined.

2. Materials and methods

2.1. Cell culture and viability

HUVECs were isolated from human umbilical vein by digestion with 0.1% collagenase type IV (Sigma, St. Louis, MO, USA) as reported previously[17]. Cells at passages 3–5 were used for experiments and were cultured in 1.5% gelatin-coated dishes in Medium 199 (Gibco, Grand Island, NY, USA) containing 2 mM l-glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml), and 2% low serum growth supplement (50× LSGS, Cascade Biologics, Portland, OR, USA) at 37 °C in an atmosphere of 5% CO2/95% air. To evaluate viability, XTT labeling mixture (Boehringer Mannheim, Germany) was added to each well (final concentration of 0.3 mg/ml) and the cells incubated for 4 h, then the absorbance of the sample at 450 nm was measured using a microplate reader (EL312e; Bio-Tek Instruments, Winooski, VT, USA) and the viability calculated. Experiments were repeated for three times.

2.2. PBMC adhesion assay

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human whole blood using Ficoll-Paque (Invitrogen, Carlsbad, CA, USA) as reported previously[18]. The isolated PBMCs (3×10^6 cells/well) were added to cultured HUVECs that were untreated or had been treated with arecoline for 72 h, then, 2 h later, the non-adhesive PBMCs were removed and the adherent PBMCs and HUVECs washed gently washing with medium, then observed on an inverted microscope (Olympus CHX41; Olympus, Tokyo, Japan).

2.3. Intracellular ROS detection

2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA; Molecular Probes, Eugene, OR, USA) was used to measure intercellular ROS production. In brief, 10 μM H2DCF-DA was added to the culture medium 30 min before the end of treatment. At the end of treatment, the cells were harvested by trypsinization and resuspended in Hank’s balanced salt solution (HBSS; Gibco, Grand Island, NY, USA), then the fluorescence of the dichlorofluorescein formed from the oxidation of H2DCF-DA by cellular oxidants was measured using a FACScan flow cytometry (Beckman Coulter–Epics XL; Beckman Coulter Inc, Fullerton, CA, USA) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Data were analyzed using WinMDI 2.8 software. As a positive control, 100 μM H2O2 was added to HUVECs 2 h before cell harvesting.

2.4. ICAM-1 and VCAM-1 expression

After treatment, cells were harvested and washed twice with HBSS, then incubated for 30 min at 4 °C with monoclonal anti-human ICAM-1 or anti-human VCAM-1 antibodies (Santa Cruz Biotechnology, CA, USA). After washing, the cells were incubated with fluorescein isothiocyanate-labeled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then fixed in 4% paraformaldehyde and washed. ICAM-1 and VCAM-1 expression was detected by FACS cytometry (Becton Dickinson, San Jose, CA) and analyzed using WinMDI 2.8 software with a minimum of 1×10^4 cells/sample being evaluated in each case.

2.5. RT-PCR

RT-PCR was performed as described previously[18]. Total RNA was extracted using RNeasy C&T reagent (PORTech Technology, Taipei, Taiwan) and complementary DNA (cDNA) synthesized. Three micrograms of cDNA was used for PCR amplification (Promega, Madison, WI, USA) in a reaction volume of 50 μl containing 25 μl of 2×PCR Master Mix and 1 μl of each specific primer. The reaction mixture was heated to 94 °C for 5 min, then amplification was performed for 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min on a thermal cycler, then the reaction mixture was heated at 72 °C for 7 min. The primers used for PCR were 5′-CCACGGGCCCCAGCGTTCT-3′ and 5′-AAGCCTTACGGCCACAGTGC-3′ for HO-1 and 5′-GCTCGGATCAGCTAGCAG-3′ and 5′-ATAGCCCATCATCTGGG-3′ for β-actin. The amplified PCR products were separated by electrophoresis in a 2% agarose gel and the intensity of the HO-1 band calculated by densitometry and the results expressed as a percentage of the optical density of the corresponding β-actin band.

2.6. Western blot analysis

Western blotting was performed as described previously[19]. Cytosolic extracts were prepared using ice-cold lysis buffer and incubation on ice for 20 min. After centrifugation, the protein in the supernatants was quantified using a protein assay kit from Bio-Rad Laboratories (Hercules, CA, USA). Forty micrograms of protein per lane was electrophoresed on 10% or 12% SDS-polyacrylamide gels. After transfer of the protein from the gel to nitrocellulose membranes, the membranes were blocked at room temperature for 1 h in phosphate-buffered saline (PBS) plus 0.1% Tween 20 (PBS-T) containing 5% fat-free powdered milk, then incubated for 2 h at room temperature with monoclonal mouse anti-human ICAM-1 or VCAM-1 antibody or rabbit polyclonal anti-human HO-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the membranes were incubated at 25 °C for 60 min with the appropriate horseradish peroxidase-labeled secondary antibody (Pharmingen, San Diego, CA) and the proteins visualized by chemiluminescence detection (PerkinElmer Life Sciences, Inc, Madison, WI, USA). β-actin was used as the internal control.
2.7 Cloning of full length HO-1 cDNA

Based on the sequence of human HO-1 (NM_002133), we designed 5′ and 3′ gene-specific primers (5′-AAGCTTATGGAGCGTCCGCAACCC-3′; 5′-GGATCCTCACATGGCATTAAGCCCTAC-3′) to amplify a segment containing HindIII and BamHI restriction enzyme sites. Three micrograms of cDNA was used for PCR amplification (Promega, Madison, WI, USA) in a reaction volume of 50 μl containing 5 μl of 10×Pfu DNA Polymerase Buffer, 1 μl of dNTP (10 μM), 1 μl of each specific primer, and 0.6 μl of Pfu DNA Polymerase (2 units/μl) (Promega, Madison, WI, USA). The reaction mixture was heated to 94 °C for 5 min, then amplification was performed for 30 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 3 min with a thermal cycler, then the mixture was heated at 72 °C for 7 min. The HO-1 PCR segment was then digested with HindIII and

Fig. 2. Glutathione pretreatment reduced arecoline-induced ROS production and ICAM and VCAM expression. Cells were pretreated with reduced glutathione (163 μM; G163) or N-acetylcysteine (50 μM; N50) for 2 h, then incubated for 2 h or 72 h in the presence of the same agent with or without arecoline (50 μg/ml; A). Intracellular ROS production (A) or expression of ICAM (B) and VCAM (C) was measured by flow cytometry as described in Section 2. The gray peak represents the vehicle control; H2O2-treated cells were used as the positive control. The results are expressed as the mean±S.D. for three separate experiments.

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BamHI at 37 °C for 3 h (Promega, Madison, WI, USA) and the target sequence recovered from an agarose gel. The pEGFP-C1 vector (Clontech, Mountain View, CA, USA) was cleaved by the same enzymes and recovered from the agarose gel as the vector segment. These two recovered segments were ligated with T4 DNA ligase (Promega, Madison, WI, USA) at 16 °C for 16 h and the pEGFP-C1/HO-1 nucleotide sequence analyzed by gel electrophoresis.

2.8. Plasmid construction

The pEGFP-C1/HO-1 sequence was digested with HindIII and BamHI and the 867 bp segment recovered from an agarose gel. The segments were inserted into the HindIII and BamHI sites of the pEGFP-C1 vector to generate the pEGFP-C1/HO-1 vector. DH5α competent bacteria were transformed with the recombinant plasmid, which carries a kanamycin resistance gene. A number of independently transformed bacterial colonies were selected and grown in small-scale cultures. Recombinant plasmid pEGFP-C1/HO-1 was identified by restriction enzyme (HindIII/BamHI) analysis.

2.9. Cell transfection

Transfection was performed using lipofectamine2000 (Invitrogen, Life technologies, Carlsbad, CA, USA) and following the manufacturer’s protocol and the transfected

![Fig. 3. Arecoline induces PBMC adhesion to HUVECs. Human PBMCs cells isolated using Ficoll-Paque (PBMC; 3 × 10⁶ cells/well) were added to HUVECs that had been left untreated or treated with arecoline 50 μg/ml for 72 h, then, 2 h later, the non-adherent cells were removed and the adherent cells washed gently with medium and PBMC adhering to HUVECs observed by inverted microscopy.](image)

![Fig. 4. A HO-1 inhibitor increases arecoline-induced ROS production and ICAM and VCAM expression. Cells were pretreated for 30 min with the HO-1 inhibitor ZnPP (5 μM), then incubated for 2 h or 72 h in the presence of the inhibitor with arecoline (50 μg/ml; A50). Intracellular ROS production or ICAM and VCAM expression was measured by flow cytometry as described in Section 2. The gray peak represents the vehicle control. The results are expressed as the mean ± S.D. for three separate experiments.](image)

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cells incubated in fresh HUVEC growth medium. Cells transfected with empty vector served as controls.

2.10. Statistical analysis

All data are the mean ± S.D. The significance of differences was determined by one-way ANOVA followed by Fisher’s test. Statistical analyses were performed using SAS (version 6.011; SAS Institute Inc, Cary, NC, USA). P value < 0.05 was considered statistically significant.

3. Results

3.1. Arecoline induces ROS production

To determine whether arecoline caused ROS generation, HUVECs were treated with different concentrations of arecoline for 2 h or with 50 μg/ml (212 μM) of arecoline for different times. Using 2 h of treatment, ROS production was induced by arecoline in a dose-dependent manner (Fig. 1A), while, using 50 μg/ml of arecoline, the amount of ROS increased up to 12 h of treatment, then decreased slightly to a plateau from 24 h to 72 h (Fig. 1B). Treatment of HUVECs for 72 h, the IC50 value of arecoline was 50 μg/ml (data not shown) which was chosen for the evaluation of adhesion molecule expression and cell viability.

3.2. Arecoline induces ICAM and VCAM expression

To examine whether the arecoline-induced ROS production had an effect on adhesion molecule expression, HUVECs were treated with 50 μg/ml (212 μM) of arecoline for 2 h or 72 h, then ROS or ICAM and VCAM levels were determined by FACS cytometry with H2O2–treated HUVECs as the positive control. As shown in Fig. 2, after arecoline treatment, ROS production was induced (Fig. 2A, top left panel) or expression of ICAM (Fig. 2B, top left panel) and VCAM (Fig. 2C, top left panel) was increased. In addition, the adherence of PBMC to HUVECs was increased (Fig. 3).

3.3. Glutathione reduces arecoline-induced ICAM and VCAM expression

To examine whether glutathione could reverse these changes, HUVECs were pretreated with 163 μM of GSH (reduced glutathione) or 50 μM N-acetylcysteine (NAC), a GSH producer, for 2 h before and during arecoline treatment. GSH or NAC totally inhibited arecoline-induced ROS production (Fig. 2A). In addition, induction of VCAM expression was almost completely inhibited (Fig. 2C), while induction of ICAM expression was decreased, but not totally inhibited (Fig. 2B). These results suggest that ROS are partially responsible for the induction of VCAM and ICAM expression.

3.4. Arecoline induces HO-1 expression

HO-1 is known to be a cytoprotective protein involved in defense against oxidative stress-induced cellular damage, especially in the vascular system. To investigate the role of HO-1 in HUVECs during arecoline treatment, the cells were treated with 5 μM ZnPP, a HO-1 inhibitor, for 30 min, then were incubated for 2 h or 72 h with 50 μg/ml (212 μM) of arecoline in the continued presence of the inhibitor, and ROS production or ICAM and VCAM expression was measured. As shown in Fig. 4, ZnPP pretreatment induced ROS production and ICAM and VCAM expression even in the absence of arecoline treatment, while co-treatment with arecoline and ZnPP resulted in even higher levels of ROS, ICAM, and VCAM. These results suggest that HO-1 protects endothelial cells against injury caused by ROS and PBMC adherence induced by adhesion molecules.

HO-1 is reported to be expressed in response to ROS [11]. Since arecoline induced ROS production, we investigated whether HO-1 expression in HUVECs was changed by arecoline treatment. After 12 h of arecoline treatment, when ROS production is maximal (Fig. 1), HO-1 mRNA and protein levels were examined. Levels of HO-1 mRNA (Fig. 5A) and protein (Fig. 5B) were increased in a concentration-dependent manner by arecoline treatment, while co-treatment with arecoline and ZnPP resulted in even higher levels of HO-1. HO-1 expression was decreased, but not totally inhibited (Fig. 2B). These results suggest that ROS partially inhibit HO-1 expression.

![Fig. 5. Arecoline induces HO-1 expression. Cells were treated with different concentrations of arecoline for 12 h, then HO-1 mRNA levels were measured by RT-PCR (A) and protein levels measured by Western blotting (B). *: P < 0.05 compared to untreated controls.](image-url)

![Fig. 6. Glutathione decreases, but does not completely block, arecoline-induced HO-1 expression. Cells were pretreated for 2 h with reduced glutathione (163 μM, GSH) or N-acetylcysteine (50 μM, NAC), then incubated for 24 h in the presence of the same agent with arecoline (50 μg/ml, A50) and HO-1 expression was measured by Western blotting with β-actin as the internal control. After densitometric analysis, the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%. The results are expressed as the mean ± S.D. for three separate experiments. #: P < 0.05 compared to the arecoline-treated cells.](image-url)
3.5. HO-1 expression is not only due to ROS

To examine whether arecoline-induced HO-1 expression was due solely to ROS production, HUVECs were pretreated for 2 h with GSH 163 μM or NAC 50 μM at the same concentration as in Fig. 2 which completely inhibited arecoline-induced ROS production, then were treated with 50 μg/ml of arecoline for 24 h, when HO-1 expression was induced. Fig. 6 shows that either GSH or NAC inhibited almost all of the arecoline-induced HO-1 expression, but not completely, indicating that arecoline still induced some HO-1 expression even when ROS production was completely blocked. This shows that arecoline-induced HO-1 expression was not only due to ROS and that other factors were involved.

3.6. MAP kinase pathways in the regulation of HO-1 expression by arecoline

MAP kinases contribute to the regulation of HO-1 expression in many cell types [20,21], but the relative contribution of different pathways to HO-1 upregulation by arecoline in HUVECs is not known. Since arecoline treatment (50 μg/ml) strongly increased HO-1 expression at 24 h, these conditions were used to investigate the signaling pathways involved in HO-1 induction using selective pharmacological agents. HUVECs were pretreated with selective pharmacological agents for 2 h, then with arecoline (50 μg/ml) in the presence of the same agent for 24 h. Fig. 7A shows that inhibition of p38 with SB 203580 (SB) or of c-jun N-terminal kinase (JNK) with SP600125 (SP) reduced arecoline-induced HO-1 expression, indicating involvement of these pathways. Addition of genistein (Gen), a tyrosine kinase inhibitor, also reduced arecoline-induced HO-1 expression, indicating tyrosine kinase activation was involved. In contrast, inactivation of ERK (extracellular signal-related MAP kinase) with PD98059 (PD), an inhibitor of the ERK upstream activators MAPK kinase MKK1 and MKK2, had no effect (Fig. 7B). In accordance with the cytoprotective role of HO-1 and these findings, ICAM and VCAM expression was increased by SB 203580, SP600125, or genistein pretreatment of arecoline-treated HUVECs, while HO-1 expression was reduced (Fig. 7A) and PD98059 again had no effect (data not shown). These data show that HO-1 upregulation by arecoline occurs via the JNK and p38 MAPK pathways, but not the ERK pathway, and also via tyrosine kinase activation.

3.7. Effect of HO-1 overexpression on arecoline cytotoxicity

To investigate the role of HO-1 in the arecoline-induced cytotoxicity for HUVECs, cells were incubated with or without 5 μM ZnPP for 30 min, then were treated with 50 μg/ml (212 μM) arecoline for 72 h, when cell viability was examined. As shown in Fig. 8A, ZnPP pretreatment alone decreased cell viability by 30% and arecoline alone decreased viability by 47% compared to the control group, while co-treatment resulted in a decrease of 62%, indicating that HO-1 acted as a cytoprotective agent against arecoline-induced toxicity. Furthermore, we performed a gene transfer experiment in which HO-1 protein fused in-frame to the C-terminal portion of GFP (GFP/HO-1) or GFP protein alone was expressed under the regulation of the CMV promoter in HUVECs. Transfected cells could be detected by GFP fluorescence (Fig. 8B). Cells transfected with

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HO-1 transfection, but not GFP transfection, inhibited the decrease in cell viability caused by arecoline. However, the damage caused by arecoline was not completely prevented even when HO-1 was overexpressed 5-fold, suggesting that multiple cytoprotective factors are needed to completely prevent arecoline-induced damage of HUVECs.

4. Discussion

In this study, we found that arecoline stimulated ROS production in HUVECs and that this was related to ICAM and VCAM expression. Glutathione pretreatment completely inhibited arecoline-stimulated ROS production and VCAM expression, but not ICAM expression. Simultaneously, arecoline treatment increased HO-1 expression. Inhibition of c-jun N-terminal kinase (JNK), p38, or tyrosine kinase induced the inhibition of HO-1 expression, suggesting that arecoline induced the upregulation of HO-1 via these signaling pathways. Overexpression of HO-1 as a result of gene transfer attenuated arecoline-induced cell damage, but not completely, indicating that other cytoprotective mechanisms were needed to overcome arecoline cytotoxicity for HUVECs.

There are a variety of intracellular sources of ROS in vascular cells, including mitochondrial respiration, NADPH oxidase, lipoxygenase, cyclooxygenases, xanthine oxidase, and the uncoupling of nitric oxide synthesis [22]. Arecoline is reported to markedly increase mitochondrial membrane potential hyperpolarization in KB epithelial cells [2], to induce cyclooxygenase-2 expression in human sperm cell, buccal mucosal fibroblasts and primary oral keratinocytes [23,24], and to increase eNOS expression in HUVECs [25], showing that arecoline can induce ROS generation in several ways. ROS plays both deleterious and beneficial roles. Interestingly, various ROS-mediated actions protect cells against ROS-induced oxidative stress and re-establish or maintain the “redox balance”, also termed “redox homeostasis”. This “two-faced” character of ROS is clearly substantiated [26,27]. Studies have also revealed roles for hemeoxygenase in addition to heme metabolism. The HO-1 isoform is involved in healing, psoriasis, keratinocyte proliferation, and, in its role as a heat shock protein, protection against cellular oxidative stress [28–30].

This study showed that ROS induced HO-1 expression, which protected against ROS-induced oxidative stress, such as the increase in ICAM and VCAM expression in arecoline-treated HUVECs. ROS production was induced by arecoline after 2 h of treatment and was maximal at 12 h, a time at which increased HO-1 expression was seen, followed by attenuation of the enhancement of ICAM and VCAM expression. However, HO-1 expression alone was not sufficient to completely prevent arecoline-induced damage. Given that HUVECs transfected with the HO-1 gene showing 5-fold higher HO-1 activity were not completely protected from the damage caused by arecoline, it is clear that other cytoprotective pathways are needed to block the effect of arecoline in HUVECs. In addition, arecoline probably has other effects on HUVECs. For example, arecoline induces cell cycle arrest at the M/G2 phase and changes apoptotic-related protein expression in HUVECs (our unpublished data).

The MAP kinases, ERK, p38, and JNK, and ROS are upstream activators of transcription factors, such as AP-1, NF-kB, and Nrf2, which are implicated in HO-1 expression [21,31]. MAP kinases regulate HO-1 expression in many cell types [20,21], but the roles of ROS and the MAP kinase cascade in arecoline-stimulated HO-1 induction in HUVECs have not been previously reported. Our results showed that glutathione completely blocked arecoline-stimulated ROS production, but only partially inhibited HO-1 expression, indicating that factors other than ROS were also involved. Inhibition of JNK, p38, or tyrosine kinase activity impaired arecoline-induced HO-1 expression, indicating that these proteins were involved in this process. However, it is not known whether HO-1 induction is an adaptive and/or persistent response to arecoline and further studies, such as the use of si-RNA or knockout mice, are required to fully clarify...
the role of HO-1 in HUVECs and the significance of HO-1 induction in arecoline-associated atherosclerosis.

In conclusion, this study shows that arecoline stimulates ROS production in HUVECs and that this is associated with ICAM and VCAM expression. In addition, HO-1 expression is upregulated by arecoline-induced oxidative stress through ROS and the tyrosine kinase and MAPK (JNK and p38) signaling pathways.

Conflict of interest

None.

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