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Hypaconitine protects H9c2 cells from oxidative stress-induced apoptosis

Zhi-Hui Li¹, Wanhong Xu², Guo-Hui Li¹, Ling Shen³, Min-Jie Mao¹, Zhi-Gang Wu³, Xue-Ting Shao³ and Kun Fang¹*

¹Department of Intensive Care Unit, Hangzhou Red Cross Hospital, Zhejiang Chinese Medical University, China.
²Hangzhou High Throughput Drug Screening Center, ACEA Bio, Hangzhou, Zhejiang, China.
³State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, Zhejiang University School of Medicine, China.

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The aim of the present study was to investigate the protective effect of hypaconitine on apoptosis induced by H₂O₂ and the underlying molecular mechanism in cardiac myocytes. First, cardiac myocytes were pretreated with different concentrations (0, 62.5, 125, and 250 ng/ml) of hypaconitine before exposure to 100 µM H₂O₂. Cell viability, apoptosis, and activation of caspase-3 and -9, p38 mitogen-activated protein kinases (MAPK), and nuclear factor κB (NF-κB) p65 protein were examined. In our study, H₂O₂ treatment resulted in a dose-dependent increase in the number of apoptotic cells. In addition, caspase-3 and -9, total and phospho-p38 MAPK and phospho-NF-κB p65, measured by western blot, were markedly activated by H₂O₂ treatment and, apoptosis induced by H₂O₂ was significantly reduced by pretreatment with hypaconitine in a dose-dependent manner. Similarly, the activation of caspase-3 and -9, phospho-p38 MAPK, and phospho-NF-κB p65 was blocked by hypaconitine; the strongest effect was observed at 250 ng/ml. In conclusion, in this study, we first demonstrated that hypaconitine protects cardiac myocytes from apoptosis triggered by H₂O₂ in a dose-dependent manner ranging from 62.5 to 250 ng/ml. In addition, our results, at least partially, showed that hypaconitine inhibited cell apoptosis via blocking the activation of 3 important signaling pathways, MAPK pathway, NF-κB pathway, and caspase pathway, mediated by H₂O₂.

Key words: Oxidative stress, heart failure, H9c2, apoptosis, hypaconitine.

INTRODUCTION

Heart failure is a leading cause of morbidity and mortality worldwide, and is accompanied by progressive left ventricular remodeling characterized by hypertrophy of the myocytes, impaired vascularization in the heart, abnormal extracellular matrix composition (fibrosis), and elevated cardiac myocytes cell death (Eguchi et al., 2008). In heart failure after pressure-overload-induced cardiac hypertrophy, apoptosis of cardiac myocytes is proposed to be a critical point in the transition between compensatory hypertrophy and heart failure (Yamaguchi et al., 2003). Much attention has therefore focused on the role of apoptosis and/or necrosis of contractile cardiac myocytes in the development of cardiac pathologies (Clerk et al., 2007).

Oxidative stress is considered a major apoptotic stimulus in many cardiovascular diseases, and reactive oxygen species (ROS) can, in fact, trigger myocyte apoptosis by upregulating proapoptotic genes, which are inhibited by antioxidants (Xu and Wang, 2008). In vitro and in vivo studies have demonstrated that generation of reactive oxygen species (ROS) may activate necrosis, apoptosis, even hypertrophy in cardiac myocytes (Sheng et al., 2007). Furthermore, caspase, nuclear factor κB (NF-κB), and p38 mitogen-activated protein kinases (MAPK) signaling pathways are suggested to be involved in cardiac myocytes apoptosis (Zhuang et al., 2007;...
Wang et al., 1998). Convincing data has been accumulated in the treatment of oxidative stress-induced cell injury by using natural products or plant extracts. For example, the investigations by Tuo et al. (2004) have demonstrated that onychin could exhibit a protective effect against lysophosphatidylcholine-mediated cardiovascular injury by preserving endothelium-dependent relaxation of rabbit aortic rings. Hypaconitine is a constituent of the aconite root, a traditional Chinese medicine that has been frequently prescribed to relieve muscular pain. In addition, it was concluded that hypaconitine, not aconitine, was the main constituent responsible for the action of the aconite root (Kimura et al., 1998; Muroi et al., 1990). Because higher doses of hypaconitine exhibit neuromuscular blocking effects that help relieve muscular pain, many studies focused their attention on its nerve toxicity (Kimura et al., 1998; Muroi et al., 1990), and few studies have focused on its beneficial effect on cardiac myocytes and the underlying mechanism.

In the present report, we first investigated whether hypaconitine was involved in the protection of cardiac myocytes from H₂O₂-induced apoptosis. To explore the molecular mechanisms involved in the antiapoptotic effect of aconitine, the present study further examined how hypaconitine modulated the activity of MAP kinases and NF-κB p65 and the expression of cleaved caspase-3 or -9 involved in oxidant-triggered apoptosis.

MATERIALS AND METHODS

All cell culture medium components were purchased from Invitrogen Life Technologies unless otherwise noted. H₂O₂ was purchased from Sigma (St. Louis, MO, USA) and prepared immediately in phosphate buffered saline (PBS) at 10, 25, 50, 100, 200, or 400 µM before use. Hypaconitine (Lot number 6798-9403, Beijing Drug Administration, Beijing, China, purity=98%) was dissolved in Dulbecco’s modified Eagle’s medium (DMEM) at 2000 ng/ml. It was diluted to 62.5, 125, 250, 500, 1000, and 2000 ng/ml, respectively as final concentrations. Annexin V/FITC Kit was purchased from Bender MedSystems GmbH (Vienna, Austria). Antibodies used in the western blot analysis were rabbit anti-caspase-3 and caspase-9 polyclonal antibodies (Chemicon, CA, USA) recognizing only the cleaved large subunits (17 kDa of caspase-3 and 37 kDa of caspase-9) and rabbit polyclonal antibody against β-actin was purchased from Santa Cruz Biotechnology, Inc., USA (43 kDa). Phospho-p38 MAPK (38 kDa), total p38 MAPK (38 kDa) and phospho-NF-κB p65 (65 kDa, recognizing phosphorylation at the Ser536 position) were from Cell Signal (Beverly, MA, USA).

Cell culture

H9c2 cells, a clonal line of cardiac myocytes derived from embryonic rat heart tissue, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Following the protocol provided by ATCC, the cells were cultured in complete medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 1% (v/v) penicillin and streptomycin. The cultures were maintained at 37°C in a 5% CO₂ humidified atmosphere.

Cell viability assay (MTS)

Cell viability was quantified on the basis of metabolic activity with the MTS assay (Promega, Madison, WI) according to the manufacturer’s protocol. Cardiac myocytes were seeded at a density of 5 × 10⁴/well in a 96-well plate in DMEM with 5% FBS. The cultures were incubated for 3 h at 37°C in serum-free medium containing 20 μl/well of the MTS tetrazolium compound. The absorbance of formazan products was photometrically measured at 490 nm with a microplate reader. The cell viability was expressed as absorbance (OD).

Flow cytometry analysis

Recovery of the cells was monitored by examining the levels of apoptosis. Annexin V binding and propidium iodine (PI) staining was determined by flow cytometry. The cells were washed with ice-cold PBS and double stained with FITC-coupled Annexin V protein and PI for 20 min. Flow cytometry was performed with a 488-nm laser coupled to a cell sorter (FacsCalibur; BD Biosciences, San Jose, CA). Cells stained with both PI and Annexin V were considered necrotic and cells stained only with Annexin V were considered apoptotic.

Western blot analysis

The expression of caspase-3, caspase-9, phospho-p38 MAPK, total p38 MAPK, and phospho-NF-κB p65 was detected by western blot. Cells were washed with PBS (4°C) and harvested under non-denaturing conditions by incubation (4°C/5 minutes) with lysis buffer as described above. Western blot was performed as described above. The immuno-blot was incubated for 16 h with monoclonal anti-β-actin antibody, rabbit anti-cleaved caspase-3 and -9, p38 MAPK, antiphospho-p38 MAPK antibody, and rabbit antiphospho-NF-κB p65 antibody in PBS-Tween. The membrane was washed and incubated for 2 h at room temperature with a peroxidase-labeled goat anti-rabbit or anti-mouse immunoglobulin. After further washing, the proteins were detected by ECL chemiluminescence (from Amersham International, England). For the semi-quantitative determination of protein expression, western blotting images of some experiments were scanned on a flatbed scanner and the density of the bands were quantitated using ImageQuant software (Molecular Dynamic, Sunnyvale, CA). Densitometry results were reported as percentages of medium control after normalization with the average arbitrary integrated values of the β-actin signal.

Statistical analysis

The results were expressed as the mean ± SD. For multiple comparisons, data were subjected to one-way ANOVA followed by Fisher’s multiple comparison test. p < 0.05 was considered statistically significant.

RESULTS

Effects of H₂O₂ on apoptosis of cardiac myocytes

A number of studies have shown that H₂O₂, as an
example of oxidative stress, induces apoptosis of cardiac myocyte in vitro (Aoki et al., 2002). Moreover, cell apoptosis measurement determined the optimum concentration of \( \text{H}_2\text{O}_2 \) that induces apoptosis in our cell model. In the initial study, H9c2 cells were exposed to increasing concentrations (0~400 \( \mu \)M) of \( \text{H}_2\text{O}_2 \) for 24 h. As shown in Figure 1, the percentage of apoptotic cells was 1.51% in the absence of \( \text{H}_2\text{O}_2 \), and steadily increased to 13.26% when the cells were exposed to 25 \( \mu \)M; 94.2% of cells were apoptotic when treated with 400 \( \mu \)M. At 100 \( \mu \)M, the percentage of apoptotic cells was 43.72%.

**Effect of hypaconitine on the viability of cardiac myocytes**

In the initial experiments, 100 \( \mu \)M \( \text{H}_2\text{O}_2 \) was the optimal concentration. Next, we wanted to identify the safe dosage of hypaconitine in cardiac myocytes. The cytotoxic effects of hypaconitine were evaluated by the MTS assay 24 h after the treatment of H9c2 cells with concentrations of hypaconitine ranging from 0 to 2000 ng/ml. Although no cytotoxicity was evident at concentrations below 250 ng/ml, there was a significant \( (p = 0.00) \) dose-dependent decrease in cell number at concentrations above 250 ng/ml; 2000 ng/ml resulted in more than 50% loss in cell viability compared to the basal level (Figure 2). In the end, we used hypaconitine in our following experiments at concentrations of 62.5, 125, and 250 ng/ml.

**Hypaconitine blocked H9c2 cells apoptosis and the decrease in viability induced by \( \text{H}_2\text{O}_2 \)**

To assess the effect of hypaconitine on \( \text{H}_2\text{O}_2 \)-induced decreased viability and apoptosis, cells were randomly divided into 6 study groups and then incubated for 24 h (Table 1). In this part, the exposure of H9c2 cells to 100 \( \mu \)M \( \text{H}_2\text{O}_2 \) for 24 h resulted in an obvious decrease in cell viability as measured by MTS, \( p=0.000 \) vs group1 (Figure 3). When the cells were pretreated with hypaconitine (0~250 ng/ml) for 2 h, the cell damage was significantly attenuated in a concentration-dependent manner \( (p = 0.000 \text{ every group vs the group2}) \) and reached the best protective effect at 250 ng/ml (Figure 3). In other words, pretreatment with hypaconitine (0~250 ng/ml) dose-dependently inhibited \( \text{H}_2\text{O}_2 \)-induced H9c2 cell injury. When H9c2 cell apoptosis was quantified by flow cytometry, the control group had 2.54± 0.23% of cells were in the early phase of apoptosis (Annexin V-positive and PI-negative) (Figure. 4). An increase in the number of apoptotic cells (45.98± 4.94%) was observed in the \( \text{H}_2\text{O}_2 \) alone-treated group with a lower number of living cells. Pretreatment with hypaconitine (62.5, 125, 250 ng/ml) for
Figure 2. Confirmation of the safe dose of hypaconitone in H9c2 cells.

Table 1. Groups in different culture conditions in this study.

<table>
<thead>
<tr>
<th>Condition/group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂ (µM)</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Hypaconitone (ng/ml)</td>
<td>0</td>
<td>0</td>
<td>62.5</td>
<td>125</td>
<td>250</td>
<td>250</td>
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Figure 3. Cytoprotective effect of hypaconitone on decreased H₂O₂-induced cell viability.
2 h decreased the apoptotic rate to 32.07±3.92, 20.72±3.69, and 7.85±0.91%, respectively (Figure 4). Moreover, administration of hypaconitine (250 ng/ml) alone caused not only no alteration in cell viability but also no changes in the percentage of living and apoptotic cells compared to normal control values.

Mechanism of hypaconitine defending against oxidative injury of H9c2 induced by H2O2

Cell fate in response to severe stress will depend on the balance between antiapoptotic and proapoptotic factors (Cieslak and Lazou, 2007). Our foregoing data showed that hypaconitine pretreatment enhanced the H2O2-induced suppressed viability and decreased apoptosis. To further confirm that hypaconitine possesses antiapoptotic properties in cardiac myocytes under oxidative stress and to explore its signaling pathways, cleaved caspase-3 and -9, and phosphorylation of p38 MAPK and NF-κB p65 were examined by western blotting. Figure 5A to C show that H2O2 stimulation triggered the activation of the above signaling molecules, as assessed by their protein levels using specific antibodies. These data indicated that apoptosis increasing was induced by H2O2 in a dose-dependent manner and it followed the activity increasing of the caspase-, p38 MAPK-, and NF-κB-dependent pathway in H9c2 cells. However, the expression of caspase-9 and -3 decreased significantly when hypaconitine was used before treatment with H2O2 (Figure 5A), which showed that hypaconitine could protect against oxidative injury, at least partially, via reducing cleavage of caspase-3 and -9.

In agreement with the data of caspase-3 and -9, protein expression studies showed that hypaconitine reduced the enhancement of phospho-NF-κB p65 protein levels (Figure 5C) and the phospho-p38/p38 MAPK ratio (Figure 5B) induced by H2O2 in cardiac myocytes in a dose-dependent manner. In addition, hypaconitine treatment alone did not influence these protein levels (Figure 5A to C). These data suggest that hypaconitine will also exert its antiapoptotic effect by decreasing phospho-NF-κB p65.
DISCUSSION

Heart failure is a common end-stage event resulting from various cardiovascular diseases, and it is now well established that apoptosis of cardiac myocytes is an important component of cardiac remodeling, ultimately leading to heart failure (Eguchi et al., 2008). Oxidative stress has been shown to trigger apoptosis of cardiac myocytes in myocardial infarction, ischemia-reperfusion injury, cardiomyopathy, atherosclerosis, and heart failure (Wang et al., 2005; Haunstetter and Izumo, 1998). This study was undertaken to examine the effect of hypaconitine on H$_2$O$_2$-induced apoptosis of H9c2 cells and the possible molecular mechanisms involved.

It is widely accepted that high concentrations of H$_2$O$_2$ or high levels of oxidative stress promote cardiac myocyte death, but some groups have reported that lower, nontoxic concentrations of H$_2$O$_2$ promote cytoprotection or growth (Clerk et al., 2007). Similarly, in our study, when cardiac myocytes were exposed to increasing concentrations of H$_2$O$_2$ ranging from 25 to 400 µM for 24 h, the percentage of apoptotic cells (assessed by annexin V binding and PI staining) was significantly increased from 13.26 to 94.2% in a dose-dependent manner. As shown in Figure 1, H$_2$O$_2$ at 10 µM had no effect on cell survival (1.87% apoptotic cells vs. 1.51% H$_2$O$_2$-untreated cells), and we did not observe any cell-growth promoting effect. Maybe, this effect will be noticed at a concentration below 10 µM. H$_2$O$_2$ used at a concentration of 100 µM in this study, which has been reported to be in the range that mainly induces cell apoptosis but little necrosis (Xu and Wang, 2008), caused 43.72% apoptosis of cardiac myocytes in our study. Thus, in our subsequent study, 100 µM was chosen as the optimal concentration.

Traditionally, hypaconitine from Fuzi is decocted with other herbal medicines, such as Panax ginseng, and is generally used in heart failure and other kinds of heart diseases (Luo et al., 2008). In the present study demonstrated, for the first time, a direct antiapoptotic effect of hypaconitine in cultured H9c2 cells. In H$_2$O$_2$-induced cell injury, when H9c2 cells were preincubated with hypaconitine for 2 h, the safe concentration of 62.5-250 ng/ml of hypaconitine considerably boosted cell viability determined by the MTS assay. Meanwhile, hypaconitine significantly reduced the apoptotic rate of H9c2 cells as demonstrated in Figures 3 and 4. This effect became even more apparent when the concentration of hypaconitine increased from 62.5 to 250 ng/ml, which caused significant reduction of cell death compared with the cells treated with H$_2$O$_2$ only.

Furthermore, we showed that the 3 important signaling pathways involving p38 MAPK, NF-κB, and caspase activated by H$_2$O$_2$ were inhibited by hypaconitine in oxidatively stressed cardiac myocytes. Functionally, caspase-3 is a critical effector caspase of the apoptotic process and caspase-9 is an initiator of caspase-3 in the mitochondria-dependent pathway (Jiang et al., 2005). In our present study, H$_2$O$_2$ treatment significantly activated caspase-9 and caspase-3 in H9c2 cells. However, western blot analysis also showed that hypaconitine pretreatment remarkably suppressed proteolytic activation of caspase-3 and caspase-9 (Figure 5A). Further, oxidants can trigger the activation of multiple signaling pathways including the phosphorylation cascades leading to the activation of MAPKs, NF-κB (Wang et al., 1998) and the expression enhancing of Bax (Wu and Hu, 2011). Previous studies have shown that p38 MAPK is a downstream effector of the H$_2$O$_2$-induced apoptotic process (Tuo et al., 2004). Several groups observed increased p38 MAPK activity with apoptosis in stressed H9c2 cells, neonatal cardiac myocytes, adult

![Figure 5. Effect of hypaconitine on H$_2$O$_2$ induced early intracellular signaling events.](image-url)
myocytes, and reperfused hearts. These results suggest that p38 MAPK may play a critical role in the development of cardiac apoptosis (He et al., 1999; Hreniuk et al., 2001; Yue et al., 2000). Consistent with these results, in our study, robust phospho-p38 MAPK was activated by H₂O₂ in H9c2 cells (Figure 5B). Among the potential MAPK-regulated transcription factors, NF-κB is known to be activated in response to oxidative stress (Wang et al., 1998). Although most published reports have provided evidence suggesting that NF-κB is antiapoptotic, whether this transcription factor acts as a proapoptotic or antiapoptotic factor may depend on the cell type and the activating pathway (Wang et al., 1998).

It was therefore of interest to determine the effect of NF-κB on the cellular response to H₂O₂ in our model. In keeping with our previous data, treatment with H₂O₂ also resulted in the remarkable activation of phospho-NF-κB p65 in our cells as shown in Figure 5C and, both the activation of phospho-p38 MAPK and phospho-NF-κB p65 were significantly suppressed by hypaconitine pretreatment in a dosage-dependent manner. The level of phospho-p38 MAPK and phospho-NF-κB p65 returned almost completely to the normal level when the concentration of hypaconitine was 250 ng/ml (Figure 5B and C). Further, hypaconitine treatment alone did not affect the protein level of cleaved caspase-3 and -9, the ratio of phospho-p38/p38 MAPK, and the protein level of phospho-NF-κB p65 (Figure 5A to C).

In conclusion, the results of our study demonstrated that hypaconitine protects cardiac myocytes in a dose-dependent manner against H₂O₂-mediated cytotoxicity and apoptosis. We clearly showed that hypaconitine inhibited apoptotic cell death in part mediated via activation of 3 important signaling pathways, MAPK pathway, NF-κB pathway, and caspase pathway, which are triggered by H₂O₂. Thus, hypaconitine may be an effective drug for protection cardiac myocytes from apoptosis in treating heart failure.

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