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The pro-apoptotic effect of hydroquinone in human neutrophils and eosinophils

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ABSTRACT

Hydroquinone (HQ) is a benzene metabolite that is involved in hematopoiesis via its accumulation into bone marrow. HQ also acts as a toxic agent that influences various immune responses. Both neutrophils and eosinophils function as important leukocytes in immunological regulation and immune diseases. In this study, we examined the toxic effects of HQ on the apoptosis of human neutrophils and eosinophils isolated from the blood of healthy donors. HQ markedly increased the apoptosis of neutrophils and eosinophils in a concentration- and a time-dependent manner. The pro-apoptotic effect is involved in activation of caspase 9 and caspase 3. Reactive oxygen species (ROS) production was enhanced after HQ treatment in a dose-dependent manner. In addition, HQ upregulated the release of IL-8 and MCP-1 from neutrophils and eosinophils, respectively. Taken together, the results of this study demonstrated that HQ strongly induces the apoptosis of neutrophils and eosinophils through the caspase 9/3-dependent pathway and the increased ROS production. HQ exerts a cytotoxic effect in human neutrophils and eosinophils and may impair the regulation of immune responses.

1. Introduction

Volatile organic compounds (VOCs) are off-gases from various building materials, leading to induce indoor air pollution and possibly to sick building syndrome (Bernstein et al., 2008; Franchi et al., 2006; Otto et al., 1992). Sick building syndrome is characterized by various allergic/non-allergic-related symptoms, such as nausea, eye or throat irritation, itchy skin and lung inflammation (Bernstein et al., 2008; Thorn, 1998). Hydroquinone (HQ) is a major metabolite of benzene that is further oxidized by peroxidase in the bone marrow (Schlosser and Kalf, 1989; Schrenk et al., 1996; Snyder, 2000). HQ has been shown to be a potential toxic agent that influences immune cell responses. In antigen-primed mice, HQ enhances allergic immune responses via an increase in interleukin (IL)-4 production and immunoglobulin E (IgE) levels (Lee et al., 2002). HQ also increases the proliferation of granulocyte–macrophage progenitor cells, after which it induces the terminal granulocytic differentiation of murine myeloblasts via inhibition of apoptosis (Hazel et al., 1996a,b; Hazel and Kalf, 1996). Conversely, HQ induces the apoptosis of human Jurkat T cells (Kim et al., 2009) and impairs lymphocyte proliferation by suppression of DNA synthesis and cytokine production (Li et al., 1996; McCue et al., 2000; Poirier et al., 2002).

Neutrophils are the most abundant leukocytes that participate in innate immunity, responding to chemotatic factors and inflammatory mediators (Fitzharris et al., 1987). Eosinophils are recruited into the injured site and act as important inflammatory cells by inducing the release of specific granule proteins and reactive oxygen species (ROS) (Elsner and Kapp, 1999; Palmqvist et al., 2007). Both neutrophils and eosinophils have a short life span due to constitutive apoptosis, which is an important mechanism for maintaining the number of cells (Rothenberg and Hogan, 2006; Savill et al., 1989). Granulocyte apoptosis is controlled by various mechanisms, including activation of intrinsic apoptotic signaling protein such as caspase, and secretion of cytokine such as IL-6, IL-8, and MCP-1 (Bianchi et al., 2006; Colotta et al., 1992). The dysregulation of apoptosis enhances the inflammatory response or induces an unusual inhibitory effect on the immune response.

Although HQ has toxic effects on various cell types in the immune system, these effects are still controversial and the role of HQ on human mature granulocytes is not well understood. To understand the toxic effect of HQ on granulocytes, we examined alteration of the apoptosis of human neutrophils and eosinophils and the involved pro-apoptotic signaling after the treatment with HQ. We also demonstrated that ROS production and cytokine release were triggered by HQ in these cells.

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2. Materials and methods

2.1. Reagents

Ficoll-Hypaque solution was purchased from Amersham Phamacia biotechnology (Buckinghamshire, UK). CD16 microbeads magnetic cell sorting kit was obtained from Miltenyi Biotechnology (Bergisch Gladbach, Germany). RPMI-1640 medium and FBS were purchased from Life Technologies, Inc. (Gaithersburg, MD). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit, BD OptEIA™ Set Human IL-8, BD OptEIA™ Set Human MCP-1 and an enhanced chemiluminescence detection system were purchased from BD biosciences (San Diego, CA). Anti-procaspase 3, anti-procaspase 9 and anti-ERK2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HQ, benzene, phenol and 2′,7′-dichlorofluorescein diacetate (DCFDA) were purchased from Sigma-Korea (Seoul, Korea).

2.2. Neutrophil and eosinophil isolation

Human neutrophils and eosinophils were isolated from heparinized peripheral blood of healthy volunteers using Ficoll-Hypaque gradient centrifugation. Erythrocytes were removed by hypotonic lysis, and then the granulocytes were divided into neutrophils and eosinophils using magnetic cell sorting kit with CD16 microbeads. The CD16-positive cells were neutrophils and the unlabeled cells were eosinophils. The cells were washed three times by PBS and resuspended at 3×10^6/ml in an RPMI-1640 medium with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cell viability was at least 90% as evaluated by the trypan blue exclusion test. Purity of neutrophils and eosinophils was above 97% as assessed by counting the cells by cytospin. This study was approved by the Institutional Review Board of Eulji University for normal volunteers.

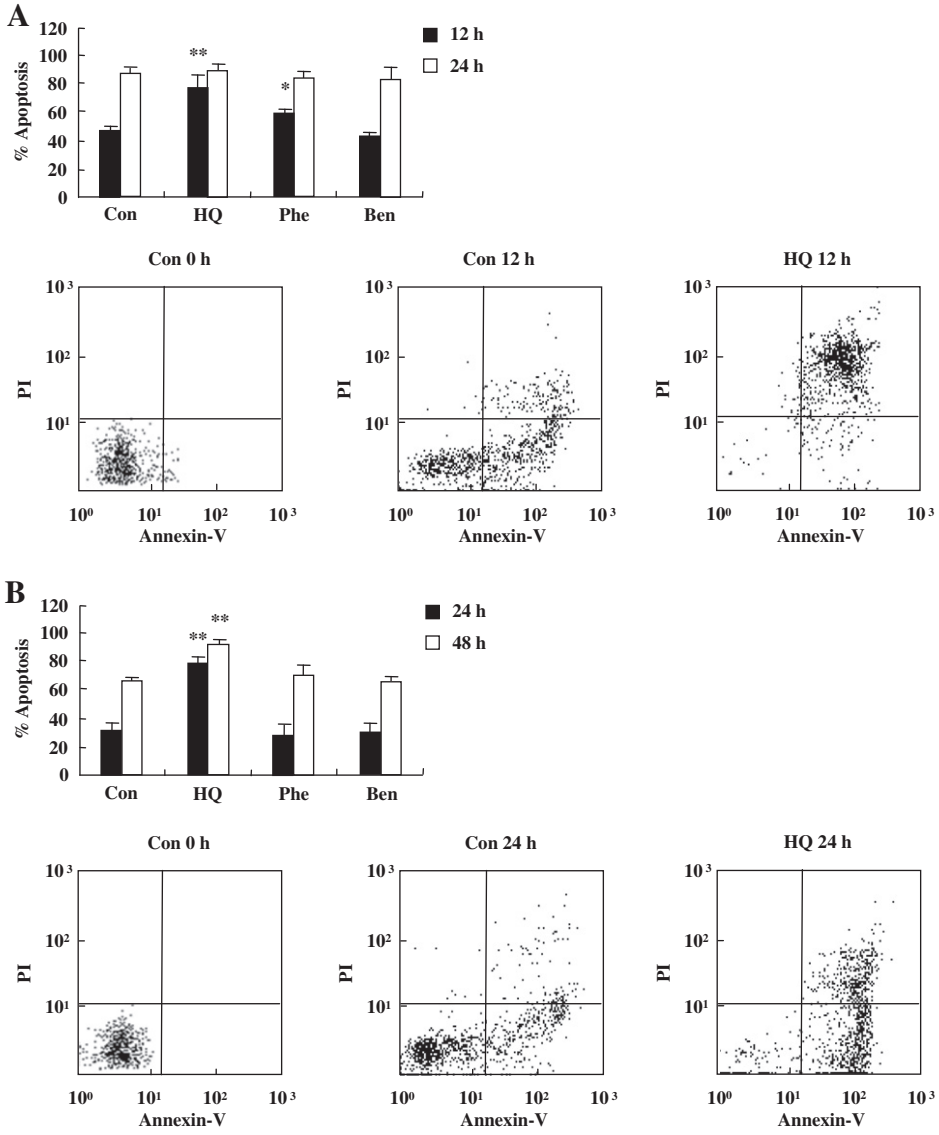

Fig. 1. HQ enhances the constitutive apoptosis of neutrophils and eosinophils. Neutrophils and eosinophils were isolated from human peripheral blood. Neutrophils (A) and eosinophils (B) were incubated for 12 and 24 h in the absence (Con) and presence of 50 μM HQ, phenol (Phe) or benzene (Ben). The apoptosis of these cells was analyzed by measuring the binding of annexin V-FITC and PI using flow cytometry as described in the Section 2. The lower panels of (A) and (B) represent the dot plot data. Fresh isolated cells were considered a negative control (Con 0 h). The percentage of apoptotic cells represents the percentage of all annexin V- binding cells in the total cell population. Data are expressed as the means ± S.E.M. in four individual experiments (A) or in three individual experiments (B). $^*p < 0.05$ and $^{**}p < 0.01$ indicate a significant difference between the untreated group and the chemical-treated group at the same incubation time.

2.3. Detection of apoptosis of neutrophils and eosinophils

Neutrophils and eosinophils were isolated from human peripheral blood. Neutrophils and eosinophils were incubated for 12 and 24 h in the absence and presence of 50 μM HQ, phenol or benzene. The apoptosis of these cells was analyzed using an annexin V–fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Diego, CA). Isolated neutrophils were incubated with an FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature. Finally, apoptotic neutrophils were analyzed using a FACSCalibur with CellQuest software (BD bioscience) and were determined as the percentage of cells showing annexin V+/PI− and annexin V+/PI+. Ten thousand events were collected for each experiment. For the morphological estimation of apoptosis of neutrophils and eosinophils, the cells were cytocentrifuged and stained with Wright staining solution.

2.4. Western blotting

The neutrophils and eosinophils were washed with ice-cold PBS and lysed with lysis buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.5 mM PMSF, 0.1 mM DTT, 0.1 mM Na_3VO_4, and protease inhibitors). The cell lysates were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membrane. The transferred membranes were incubated with anti-procaspase 3 or anti-procaspase 9 antibodies (1:3000) and were developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech.). The same blot was stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.4 mM Tris HCl, pH 6.8), and reprobed with anti-ERK2 antibodies (1:1000) for use as internal control. Quantification of Western blots was performed using Quantity One software (Bio-Rad Laboratory, Inc.). Integrated density values for each protein were normalized to ERK2.

2.5. ROS production

Both neutrophils and eosinophils were treated with 100 ng/ml of phorbol-12-myristate-13-acetate (PMA) or HQ. After incubation for 30 min, these cells were washed and resuspended at 1×10^7/ml in pre-warmed PBS, respectively. 3.3 μM of DCFDA was added to label the intracellular ROS and then incubated for 10 min at room temperature. The labeled cells were immediately observed using fluorescence-activated cell sorting (FACS) analysis (BD Biosciences).

2.6. Enzyme-linked immunosorbent assay

Isolated neutrophils and eosinophils were incubated without or with 50 μM HQ and the supernatants were collected. To determine the concentrations of inflammatory mediators in the supernatant of the cells, we performed a sandwich ELISA using OptEIA™ Set Human MCP-1, and IL-8 according to the manufacturer's instructions. All assays were performed in triplicate. The concentration of each protein was calculated from the standard curve.

2.7. Statistical analysis

All data were expressed as the means ± S.E.M. Data were analyzed by Student's *t*-test using SPSS statistical software package (Version 10.0, Chicago, IL). A p value less than 0.05 was considered statistically significant.

3. Results

3.1. HQ promotes the constitutive apoptosis of human neutrophils and eosinophils

To determine if HQ affects the cell death of neutrophils and eosinophils by its toxic activity, we first examined the apoptosis and necrosis of the cells induced by benzene and benzene metabolites such as HQ and phenol. Cell apoptosis and necrosis were assessed by FITC-conjugated annexin V and PI staining using flow cytometry. In neutrophils, the percentage of apoptotic cells (annexin V-positive cells) at 12 and 24 h were 49.5% and 88.2%, respectively (Fig. 1A). Apoptotic neutrophils were significantly increased at 12 h after HQ treatment when compared with medium alone (Fig. 1A). Necrotic neutrophils were rarely induced by HQ. As shown in Fig. 1B, the levels of apoptotic eosinophils at 24 and 48 h were 35.9% and 65.1%, respectively. The addition of HQ led to a marked increase of eosinophil apoptosis at 24 and 48 h. Although phenol induced the alternation of neutrophil apoptosis, the effects of phenol and benzene on neutrophils and eosinophils were not comparable to that of HQ. We next performed dose-dependent experiments to confirm the pro-apoptotic effect of HQ. As shown in Fig. 2A, HQ considerably enhanced the constitutive apoptosis of neutrophils at 12 h in a concentration-dependent manner. The constitutive apoptosis of eosinophils was significantly increased at 24 and 48 h depending on the HQ concentration (Fig. 2B). These results indicate that HQ increases the constitutive apoptosis of neutrophils and eosinophils.

3.2. HQ induces the activation of caspase 9 and caspase 3 in neutrophils and eosinophils

To understand how HQ increases the constitutive apoptosis in neutrophils and eosinophils, we examined the protein levels of pro-apoptotic signal protein such as procaspase 9 and procaspase 3. In constitutive apoptosis of neutrophils, the expression of procaspase 9 and procaspase 3 were decreased in a time-dependent manner (Fig. 3A). Moreover, both proteins in eosinophils were

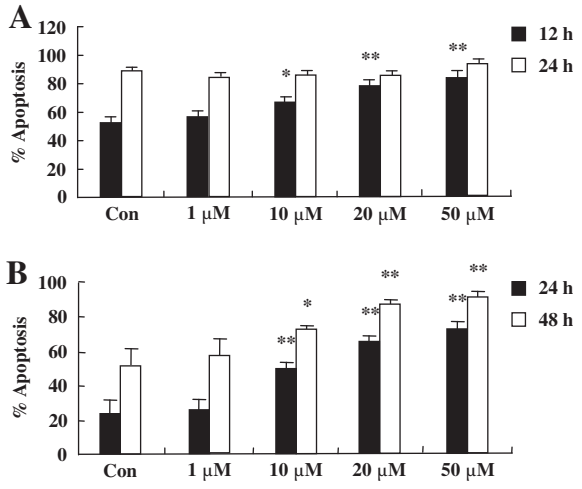

Fig. 2. HQ increases the apoptosis of neutrophils and eosinophils in a concentration-dependent manner. Neutrophils (A) and eosinophils (B) were incubated for the indicated period in the absence (Con) and presence of HQ at the indicated concentration. The apoptosis of these cells was analyzed by measuring the binding of annexin V-FITC and PI using flow cytometry as described in the Section 2. Data are expressed as the means ± S.E.M. in four individual experiments (A) or in three individual experiments (B). $^*p < 0.05$ and $^{**}p < 0.01$ indicate a significant difference between the untreated group and the HQ-treated group at the same incubation time.

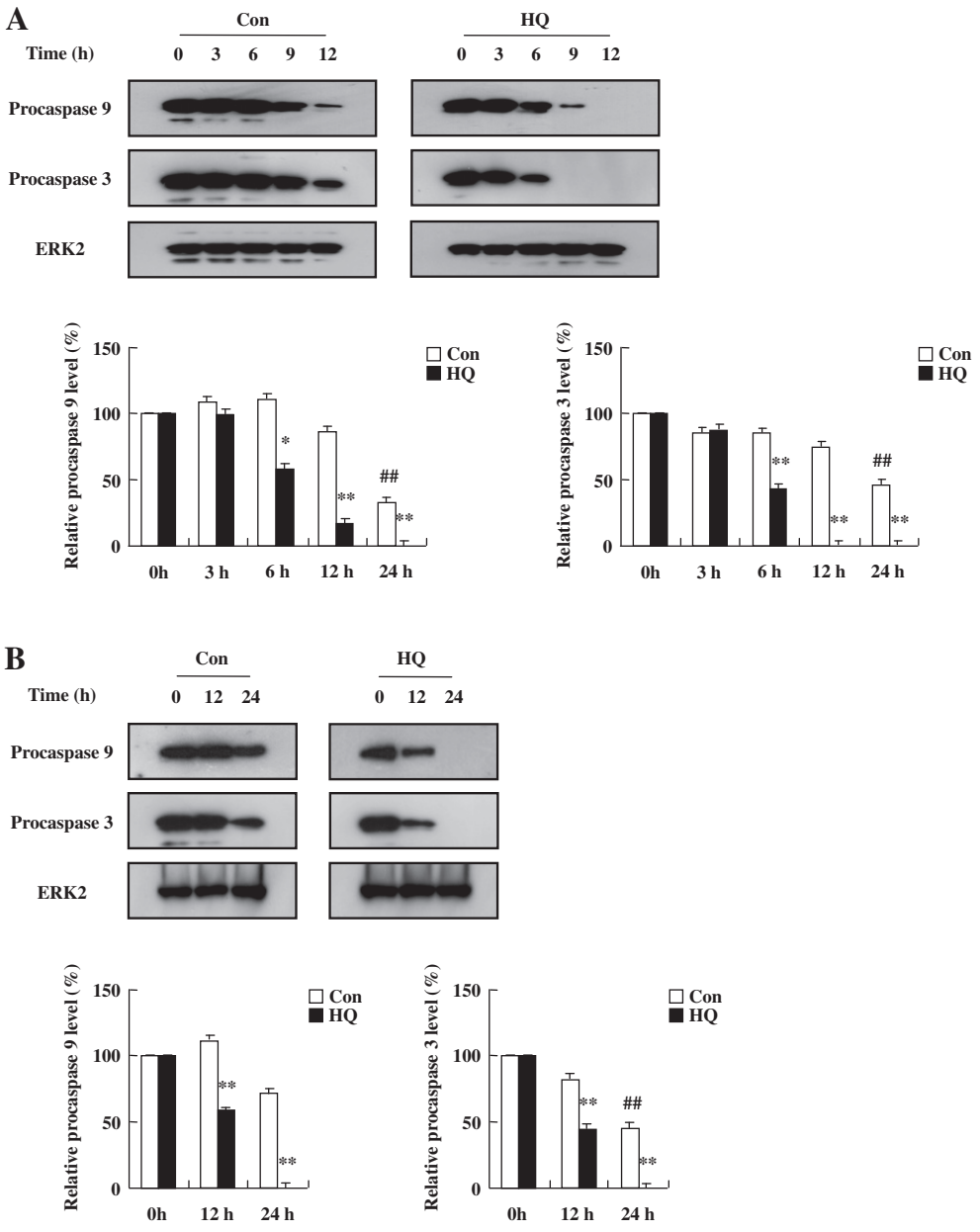

Fig. 3. HQ promotes the activation of caspase 9 and caspase 3 in human granulocytes. Isolated neutrophils (A) and eosinophils (B) were incubated without (Con) or with 50 μM HQ for the indicated time. Harvested cells were lysed and the lysates were then used for Western blotting as described in the Section 2. The expression of procaspase 9 and procaspase 3 was detected with anti-procaspase 9 or anti-procaspase 3 antibody. The membrane was reprobed with anti-ERK2 antibody as an internal control. Densitometric analysis of Western blots in A and B was illustrated as bar graph under each Westerns blot image. Data are expressed as the means ± S.E.M and are presented in relation to negative control (0 h), which was set at 100%. ##$p < 0.01$ indicate a significant difference between the untreated 0 h group and the untreated time group.*$p < 0.05$ and **$p < 0.01$ indicate a statistically significant difference between the untreated group and the HQ-treated group.

diminished during constitutive apoptosis (Fig. 3B). These data indicate that the increased apoptosis of these cells due to HQ is associated with the increased cleavage of procaspase 9 and procaspase 3.

3.3. HQ increases ROS production in neutrophils and eosinophils

To investigate the further mechanism of HQ-induced apoptosis on the granulocytes, intracellular ROS levels were measured by DCFDA labeling of the cells. PMA (100 ng/ml) was used as a positive control for ROS production in the cells. PMA enhanced ROS production and HQ increased the ROS levels of PMA-treated neutrophils and eosinophils in a concentration-dependent manner, and significant differences were observed among groups treated with different concentrations of these compounds (Fig. 4). Treatment with HQ alone increased ROS production in neutrophils and eosinophils.

3.4. HQ alters the cytokine levels during constitutive apoptosis of neutrophils and eosinophils

Extracellular stimulators including toxic chemicals modulated the release of inflammatory cytokines and pro-apoptotic or anti-apoptotic mediators in these cells. Therefore, we evaluated the

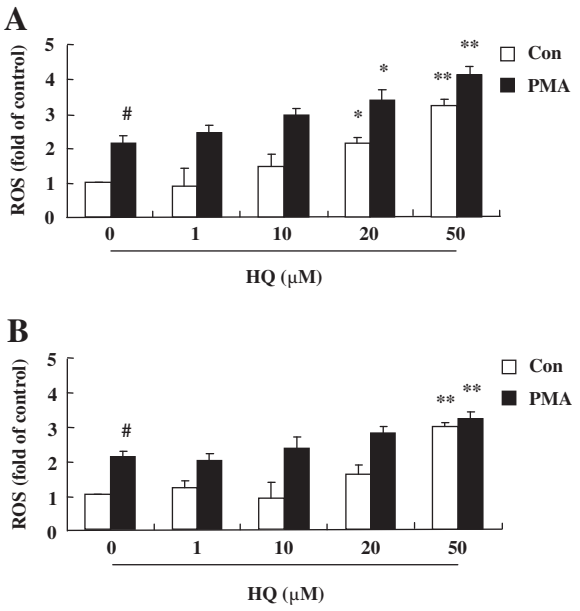

Fig. 4. Q increases the ROS production of human granulocytes. To measure the ROS levels, neutrophils (A) and eosinophils (B) were incubated for 30 min in the absence (Con) and presence of 100 ng/ml of PMA with HQ at the indicated concentrations. After the incubation time, these cells were labeled with 5 μM of DCFDA and then observed by FACS analysis. #$p < 0.05$ indicate a significant difference between the untreated group and the PMA-treated group. *$p < 0.05$ and **$p < 0.01$ indicate a significant difference between the untreated group and the HQ-treated group and between the PMA-treated group and the PMA plus HQ group at the same incubation time.

protein released in the supernatant of the cells using a sandwich ELISA kit. In neutrophils, the secretion of IL-8, IL-6 and MCP-1 was significantly upregulated during the progression of apoptosis. HQ markedly increased the release of IL-8, but decreased the release of IL-6 and MCP-1 (Fig. 5A). In eosinophils, HQ suppressed the increased IL-8 expression, but increased MCP-1 secretion (Fig. 5B). These results indicate that HQ differentially regulates the cytokine secretion between neutrophils and eosinophils, despite the pro-apoptotic effect of HQ on neutrophils and eosinophils. We next examined the alteration of cytokine release to determine if it is associated with a pro-apoptotic effect of HQ in neutrophils and eosinophils. At 24 h after HQ stimulation of human neutrophils and eosinophils, cell-free supernatants were collected and added to freshly isolated neutrophils and eosinophils. The supernatant from the neutrophils and eosinophils after HQ treatment had no effect on the apoptosis of neutrophils or eosinophils, respectively (Fig. 5C and D). These data indicate that cytokine alteration and unknown soluble factors were not related to the increased apoptosis of neutrophils and eosinophils that occurred due to HQ.

4. Discussion

The proliferation and granulocytic differentiation of murine myeloblasts are increased by the suppression of apoptosis after exposure of HQ (Hazel et al., 1996a; Hazel and Kalf, 1996). Granulocyte–macrophage progenitor cells are increased and accumulate into bone marrow due to HQ (Henschler et al., 1996). Conversely, HQ blocks both cell cycle and cytokine production, and induces lymphocyte apoptosis (McCue et al., 2000). Although the effects of HQ were examined in various cell types, its effects on granulocyte, especially neutrophils and eosinophils, have not been fully elucidated. In this study, we investigated the cytotoxic role of HQ in neutrophils and eosinophils isolated from the blood of healthy donors.

Because neutrophils and eosinophils are important effector cells in a variety of immune responses, the delayed or enhanced apoptosis of these cells leads to imbalance of the immune systems, including the development of inflammation and suppression of immune response in host defense (Louis and Djukanovic, 2006; Simon, 2003). We demonstrated that HQ strongly induces the apoptosis of human neutrophils and eosinophils via the activation of caspase 9 and caspase 3 (Figs. 1 and 3). As shown in Figs. 1 and 2, HQ significantly inhibited the apoptosis of human neutrophils at 12 h. A significant inhibition of HQ is not shown at 24 h because apoptotic neutrophils were considerably increased at 24 h without HQ. The spontaneous apoptosis of eosinophils is slower than that of neutrophils. HQ significantly suppressed the apoptosis of eosinophils at 24 and 48 h. In a previous study, the apoptosis of human Jurkat T cells was found to be induced by HQ and occur predominantly via a caspase-dependent process (Inayat-Hussain and Ross, 2005). HQ also induces the progression of apoptosis in HL-60 cells through ERK phosphorylation and activation of caspase 9 and caspase 3 (Kim et al., 2009). In hematopoietic progenitor cells, HQ increases TNF-α-induced apoptosis via the inhibition of NF-κB (Kerzic et al., 2003). Therefore, we evaluated HQ to determine if it modulated any intracellular signal molecules in apoptotic neutrophils and eosinophils. A variety of inhibitors of specific signal molecules, including PP2, AG490, U73122, rottlerin, Ro-31-8425, Ly294002, PD98059, SB202190, SP 600125 and BAY-11-7985, were used for determination of the intracellular pro-apoptotic pathway. Neither neutrophil nor eosinophil apoptosis induced by HQ was altered by treatment with these inhibitors (data not shown), indicating that the Src family protein kinases, JAK, PLC, PKCδ, classical PKC family proteins, PI-3 kinase, ERK, p38 MAPK, JNK and NF-κB are not activated by HQ. The signaling molecules stimulated by HQ in neutrophils and eosinophils are different from those in other cell types. In future studies, we need to determine another signaling pathway of HQ-induced apoptosis of human neutrophils and eosinophils.

HQ-induced apoptosis is primarily caused by the production of intracellular ROS in different cell types. HQ induced increased intracellular ROS production in human neutrophils and eosinophils (Fig. 4A and B). In HL-60 cells, high levels of ROS mediate DNA damage or activation of the caspase pathway (Ishihama et al., 2008; Kim et al., 2009; Terasaka et al., 2005). Based on previous reports, the increase in ROS level in response to HQ may be related to the induction of cell apoptosis. As shown in Fig. 5A and B, both the IL-8 level in neutrophils and the MCP-1 level in eosinophils are upregulated by HQ. IL-8 and MCP-1 are known to be chemotatic factors of neutrophils and monocytes. These chemokines regulate the proliferation and survival of neutrophils and eosinophils in inflammatory diseases (Shakoory et al., 2004), and are released by intracellular ROS production in inflammatory responses (Brzozowski et al., 2003; Kimura et al., 2003). However, HQ decreased the expression of IL-6 and MCP-1 in neutrophils and IL-8 expression in eosinophils. The change in the secreted cytokines due to HQ is not responsible for HQ-induced apoptosis as shown in Fig. 5C and D. HQ differentially regulates the measured cytokines between neutrophils and eosinophils, although it is not known if this alteration finally induces either pro-inflammatory or anti-inflammatory response.

In conclusion, HQ significantly elevated the apoptosis of neutrophils and eosinophils via the caspase 9/3-dependent pathway. HQ increases the intracellular ROS level and regulates cytokine secretion. These results indicate that HQ has a cytotoxicity in human neutrophils and eosinophils. Thus, HQ may act as a risk factor in the regulation of apoptosis in inflammatory cells.

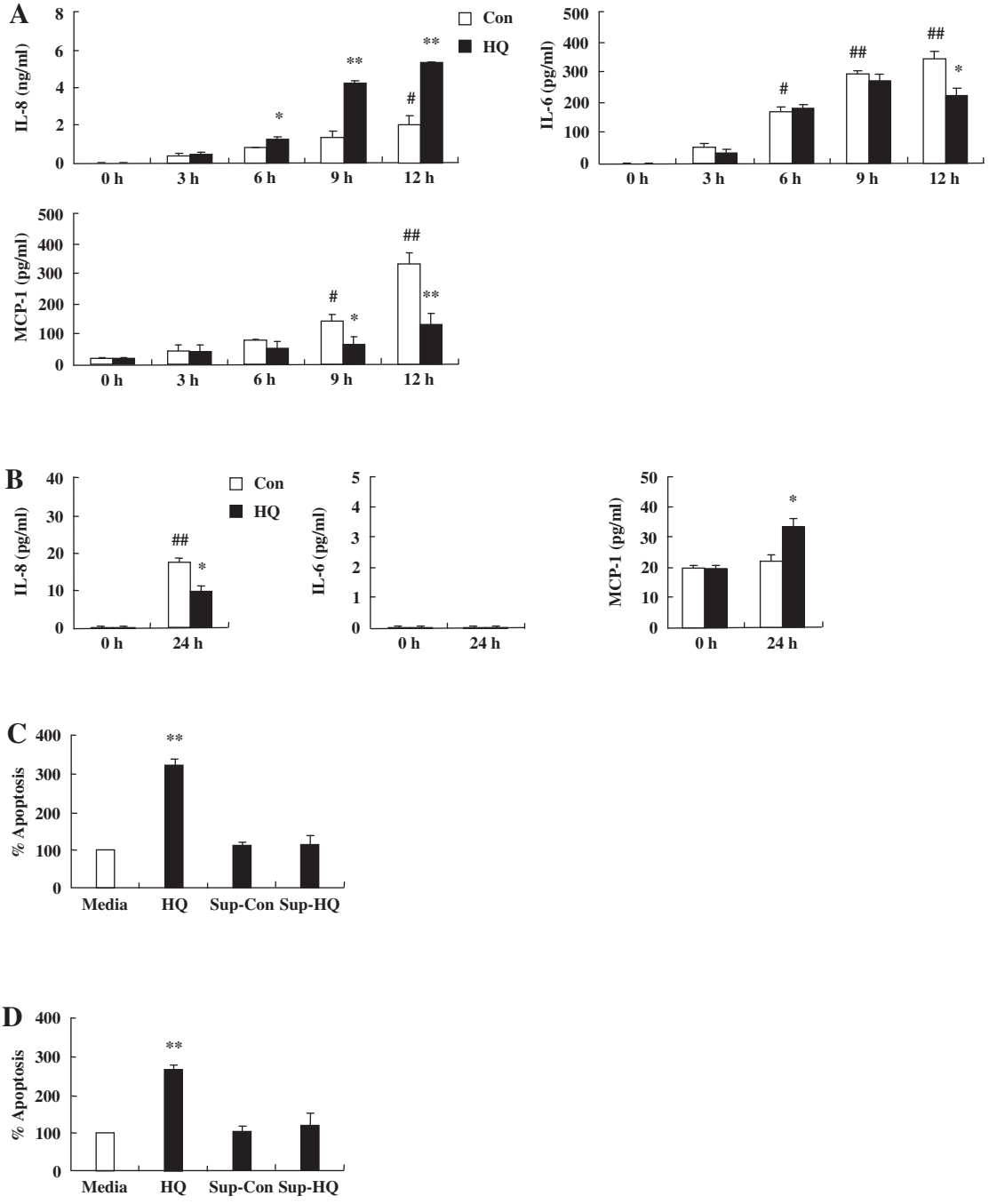

Fig. 5. HQ alters the release of cytokines during constitutive apoptosis of human granulocytes. Isolated neutrophils (A) and eosinophils (B) were incubated without (Con) or with 50 μM HQ for the indicated time. After the indicated period, the collected supernatants were used for determination of the IL-8, IL-6 and MCP-1 expression by ELISA as described in the Section 2. Data were expressed as the means ± S.E.M. of three independent experiments. #$p < 0.05$ and ##$p < 0.01$ indicate a significant difference between the untreated 0 h group and the untreated time group. *$p < 0.05$ and **$p < 0.01$ indicate a significant difference between the untreated group and the HQ-treated group at the same incubation time. C and D, isolated neutrophils (C) and eosinophils (D) were incubated with or without 50 μM HQ for 12 and 24 h, respectively. The supernatant without (Sup-Con) or with the treatment of HQ (Sup-HQ) of neutrophils or eosinophils was collected and added to the fresh isolated neutrophils and eosinophils for 12 and 24 h, respectively. The RPMI-1640 medium supplemented with 10% FBS (Media) or 50 μM HQ was added to each cell for a negative control and a positive control, respectively. Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI using flow cytometry. Data are expressed as the means ± S.E.M in five individual experiments (C) or in three individual experiments (D), and are presented in relation to the media group, which was set at 100%. **$p < 0.01$ indicate a significant difference between the media group and the HQ-treated group and between the media group and the Sup-treated group.

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