

## Full-length article

## Prevention of lipopolysaccharide-induced injury by 3,5-dicaffeoylquinic acid in endothelial cells

Ruo-peng ZHA<sup>1,2</sup>, Wei XU<sup>1,2</sup>, Wen-yi WANG<sup>1,2</sup>, Li DONG<sup>1</sup>, Yi-ping WANG<sup>1,3</sup><sup>1</sup>State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China; <sup>2</sup>Graduate School of the Chinese Academy of Sciences, Shanghai 201203, China

### Key words

3,5-dicaffeoylquinic acid; lipopolysaccharide; human dermal microvascular endothelial cells; reactive oxygen species; apoptosis; caspase-3

<sup>3</sup> Correspondence to Dr Yi-ping WANG.  
Phn 86-21-5080-6733.  
Fax 86-21-5080-7088.  
E-mail ypwang@mail.shnc.ac.cn

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### Abstract

**Aim:** To investigate the effect of 3,5-dicaffeoylquinic acid (3,5-diCQA) on lipopolysaccharide (LPS)-induced injury in human dermal microvascular endothelial cells (HMEC-1). **Methods:** The anti-oxidant effect was detected using the malondialdehyde (MDA) assay in a rat liver microsome model of lipid peroxidation. Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell lipid peroxide injury was measured by lactate dehydrogenase (LDH) release. Apoptotic cells were detected by flow cytometry, and confirmed by DNA fragmentation analysis. Caspase-3 activity was measured using a specific assay kit. The level of intracellular reactive oxygen species (ROS) was determined by flow cytometry with a 2,7-dichlorodihydrofluorescein diacetate fluorescence probe. **Results:** The exposure of microsomes to ascorbate-Fe<sup>2+</sup> resulted in lipoperoxidation according to an increase in the level of MDA. MDA formation decreased in a dose-dependent manner on treatment with 5, 10, or 50 µmol/L 3,5-diCQA. Treatment with LPS for 16 h resulted in a 60% decrease in cell viability and an increase in LDH release from 47.6% to 61.5%. DNA laddering was observed by agarose gel electrophoresis. The level of apoptotic cells peaked at 27% after treatment with LPS for 12 h. Following treatment with LPS for 12 h, intracellular ROS and caspase-3 activity increased. Pretreatment with 3,5-diCQA at 5, 10, or 50 µmol/L for 1 h attenuated LPS-mediated endothelial cell injury. The anti-apoptotic action of 3,5-diCQA was partially dependent on its capacity for anti-oxidation and the suppression of caspase-3 activity. **Conclusion:** 3,5-diCQA displays anti-oxidative and anti-apoptotic activity in HMEC-1 due to scavenging of intracellular ROS induced by LPS, and the suppression of caspase-3 activity.

### Introduction

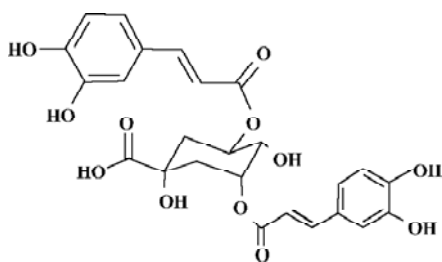
The vascular endothelium serves as the key barrier between the intravascular compartment and extravascular tissues, and plays a critical role in a large number of physiological and pathological processes<sup>[1]</sup>. The progressive dysfunction of the endothelium is regarded as a common denominator of impaired organ function after a variety of pathological tissue alterations such as ischemia, shock, or sepsis<sup>[2]</sup>.

Lipopolysaccharide (LPS) is a critical glycolipid component of the outer wall of Gram-negative bacteria. LPS can induce apoptosis in bovine and ovine endothelial cells *in*

*vitro*. When the expression of new genes is inhibited using cycloheximide (CHX), human endothelial cells become responsive to the apoptotic signals induced by LPS<sup>[6]</sup>. The induction of reactive oxygen species (ROS) by LPS correlates with the onset of apoptosis, and agents that inhibit the formation of ROS are known to protect against LPS-induced endothelial cell apoptosis<sup>[1,3]</sup>.

The phenylethanoid 3,5-dicaffeoylquinic acid (3,5-diCQA), a white powder,  $M_w$  516.44, purified by CC and HPLC) is present in a variety of plants, including green coffee beans (*Lactuca indica*)<sup>[4,5]</sup>, and is an important

component of the Chinese herb *Erigeron breviscapus*. Apart from functioning as a free radical scavenger, 3,5-diCQA inhibits tyrosinase activity and has a neuroprotective capacity<sup>[5,14]</sup>. Anti-oxidant or anti-apoptotic activity has not previously been described for 3,5-diCQA in endothelial cells. In the present study, we investigated the effect of 3,5-diCQA on lipid peroxides in microsomes, LPS-stimulated ROS generation, and LPS-induced injury in human dermal microvascular endothelial cells (HMEC-1).



**Figure 1.** Chemical structure of 3,5-diCQA.

## Materials and methods

**Reagents** 3,5-diCQA was a gift from the Kunming Institute of Botany (Chinese Academy of Sciences, Kunming Shanghai, China). The purity of the compounds was greater than 95%, as verified by HPLC. LPS (*Escherichia coli* 0111:B4), CHX, BSA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and MCDB-131 medium were purchased from Sigma (St Louis, MO, USA). 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Beyotime Institute of Biotechnology (Haimen, China). The lactate dehydrogenase (LDH) and malondialdehyde (MDA) assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The CaspACE assay system colorimetric kit was from Promega (USA). All other reagents or drugs were of analytical grade.

**Preparation of microsomes and assay of lipid peroxides** The microsomes were prepared from rat livers. The liver was cut into small pieces and homogenized in cold buffer A [(0.1 mol/L sucrose, 0.05 mol/L KCl, 0.04 mol/L KH<sub>2</sub>PO<sub>4</sub>, and 0.03 mol/L EDTA (pH 7.2)]. After centrifugation (1000×g, 20 min), the supernatant was transferred to another tube and recentrifuged at 105 000×g for 60 min. The sediment was washed and resuspended in buffer A. All operations were carried out at 4 °C<sup>[9]</sup>. Lipid peroxidation of microsomes was measured as described previously<sup>[17]</sup> with the following modifications: the membranes, at a concentration of 1.5 mg protein, were incubated at 37 °C with 0.05 mol/L phosphate (pH 7.4) and 0.4 mmol/L ascorbic acid. The final volume was

1.5 mL. Phosphate buffer was contaminated with sufficient iron to provide the necessary ferric iron for lipid peroxidation. Control membrane preparations were performed in parallel in the absence of ascorbate. Lipid peroxidation was initiated by incubating the membrane preparations in a water bath at 37 °C for 30 min. Lipid peroxidation was assessed by detection of thiobarbituric acid-reactive MDA, an end product of the peroxidation of polyunsaturated fatty acids and related esters. The level of MDA was determined using the MDA assay kit. The results were normalized to the level of MDA equivalents/mg of protein.

**Cell culture and drug treatment** HMEC-1 were maintained in a 5% CO<sub>2</sub> atmosphere at 37 °C in MCDB-131 medium supplemented with 10% FBS and 10 µg/L human recombinant epidermal growth factor. The cells treated with CHX (50 mg/L) were used as controls unless otherwise specified. Cell injury was induced by incubation with LPS (100 µg/L) and CHX (50 mg/L). 3,5-diCQA was added to the cells 1 h prior the addition of LPS and CHX. All experiments were carried out on confluent cultures.

**Assay of intracellular ROS** The measurement of intracellular ROS was based on the ROS-mediated conversion of non-fluorescent DCFH-DA into DCFH. DCFH is membrane impermeable and rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein in the presence of intracellular ROS<sup>[23]</sup>. The intensity of fluorescence reflects the level of oxidative stress. Intracellular ROS was measured after incubation in LPS and/or CHX for 12 h by replacing the medium with 50 mmol/L phosphate buffer (pH 7.4) containing 10 µmol/L DCFH-DA, and incubating in the dark for 1 h at 37 °C. The level of DCFH fluorescence was measured at an emission wavelength of 530 nm and an excitation wavelength of 485 nm by flow cytometry<sup>[19]</sup>. The results were expressed as the percentage of control fluorescence intensity.

**Assay of cell survival and cell damage** To quantitate the proportion of viable cells, HMEC-1 were seeded onto 96-well plates at a density of 40 000 cells/well. The cells were incubated in LPS and/or CHX for 16 h. The viable cell number was estimated using the MTT assay. Briefly, the medium was removed and replaced with Krebs (120 mmol/L NaCl, 5.6 mmol/L KCl, 1.2 mmol/L MgSO<sub>4</sub>, 1.2 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 25 mmol/L NaHCO<sub>3</sub>, 10 mmol/L glucose, and 2.5 mmol/L CaCl<sub>2</sub>) containing 1% BSA and 1 g/L MTT, and then incubated for 5 h. The medium was aspirated and the formazan product was solubilized with DMSO. Absorbance at 630 nm (back-ground absorbance) was subtracted from absorbance at 570 nm for each well. Viability was expressed as a proportion of the viable cell number incubated without LPS<sup>[6,15]</sup>. Both released and total LDH concentrations were

determined. The release of LDH from cells is a pathological manifestation of increased plasma membrane permeability. The total LDH was determined after incubation in the presence and absence of LPS for 16 h by releasing the compound from the cells with 1% Triton-X 100 and incubating for 30 min at 37 °C. The level of LDH was determined in samples of culture medium (30 µL) with or without Triton-X 100 using the LDH detection kit. The activity of LDH was corrected for the volume, and released LDH activity was expressed as a percentage of total cellular LDH<sup>[8]</sup>.

**DNA fragmentation assay** After incubation in the presence or absence of LPS for 16 h, the cells ( $5 \times 10^5$ ) were harvested by centrifugation and washed twice with phosphate buffered solution (PBS). The cells were then lysed by incubating in lysis buffer [20 mmol/L EDTA, 100 mmol/L Tris (pH 8.0), and 0.8% (w/v) SDS] with RNaseA/T1 (RNase A 500 U/mL, RNase T1 20 kU/mL (Amesco) for 2 h at 37 °C. The lysed cells were then incubated in the presence of proteinase K (20 mg/mL) for 4 h at 55 °C. DNA samples were loaded onto 2% horizontal agarose gels containing ethidium bromide. The gels were run at 35 V for 6 h, and the DNA fragments were visualized using UV illumination<sup>[16]</sup>.

**PI apoptosis assay** For the flow cytometric analysis of fragmented DNA,  $5 \times 10^5$  cells/well on the 6-well plates were harvested after incubation in the presence or absence of LPS for 12 h, incubated with 1 mL of 75% cold ethanol for 2 h at -20 °C, and then washed with PBS. The cell pellets were incubated with 50 mg/L PI and 100 mg/L RNase A at 37 °C for 30 min. Sub G<sub>1</sub> cell counts were determined on a FACSsort flow cytometer using the Cell Quest analysis program<sup>[7]</sup>.

**Caspase-3 activity assay** Caspase-3 activity was evaluated using the CaspACE assay system according to the protocol provided by the manufacturer. The cells plated in the flasks were treated with LPS and/or CHX for 8 h, were then harvested, washed, and lysed by 4 cycles of freezing and thawing, followed by incubation on ice for 15 min. The samples were then centrifuged at 15000×g for 20 min at 4 °C. The cell lysate supernatant was aliquoted into the wells of a 96-well plate, and 32 µL buffer, 2 µL DMSO, 10 µL DTT (100 mmol/L), and 2 µL of DEVD-pNA (10 mmol/L) were added to a final volume of 100 µL. The samples were incubated for 4 h at 37 °C. The absorbance at 405 nm was measured and enzyme activity was calculated as pmol pNA liberated per hour/µg protein according to the formula provided by the manufacturer.

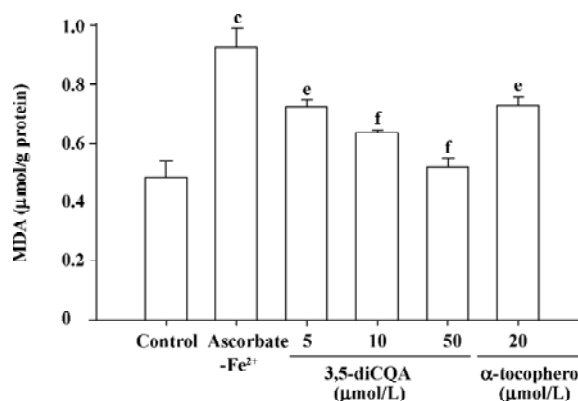
**Statistical analysis** All experiments were repeated at least 3 times. All data were expressed as mean±SD. Statistical analysis was performed by the Student's *t*-test using the software SPSS 11.0 for Windows (SPSS, Chicago, USA).

*P*<0.05 was considered statistically significant.

## Results

### Effect of 3,5-diCQA on microsome lipid peroxidation

The anti-oxidative activity of 3,5-diCQA was assessed by measuring its effect on microsome lipid peroxidation. The exposure of microsomes to ascorbate-Fe<sup>2+</sup> led to an increase in the level of MDA from the control level of 0.49 µmol/g protein to 0.93 µmol/g protein (*P*<0.01) indicating lipoperoxidation. A concentration-dependent decrease in ascorbate-Fe<sup>2+</sup>-induced MDA formation to 0.72, 0.64, and 0.52 µmol/g protein was observed for 3,5-diCQA at 5, 10, and 50 µmol/L, respectively (Figure 2). Treatment with 20 µmol/L α-tocopherol, a well known anti-oxidant, gave an MDA value of 0.73 µmol/g protein (*P*<0.01).



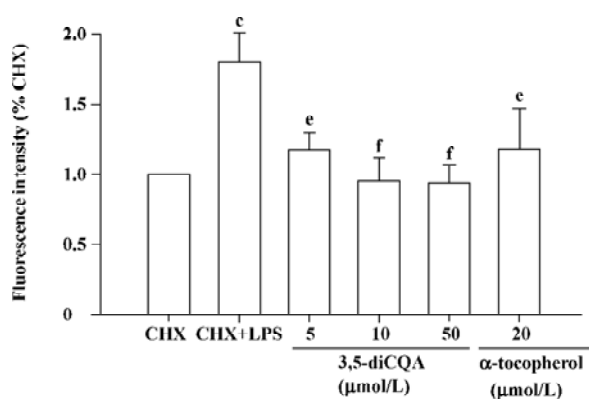
**Figure 2.** Effect of 3,5-diCQA on microsome lipid peroxidation. *n*=3. Mean±SD. <sup>c</sup>*P*<0.01 vs control. <sup>e</sup>*P*<0.05, <sup>f</sup>*P*<0.01 vs ascorbate-Fe<sup>2+</sup>.

### Inhibitory effect of 3,5-diCQA on ROS formation

According to the result in the model of the microsome above, we further determined intracellular ROS levels to assess the anti-oxidative activity of 3,5-diCQA in LPS-stimulated HMEC-1. Cells treated with CHX and LPS for 12 h gave an increase in intensity of fluorescence indicative of ROS formation, 1.8-fold the intensity observed for the control cells treated with CHX alone. Pretreatment with 3,5-diCQA (5, 10, or 50 µmol/L) or α-tocopherol (20 µmol/L) significantly decreased the intensity of fluorescence (Figure 3).

### Effect of 3,5-diCQA on cell viability and damage figure

Consistent with previously reported data, and supported by the predictive assay presented in the present report, LPS did not induce injury in HMEC-1 alone. Injury was induced when HMEC-1 were treated with LPS together with CHX. The



**Figure 3.** Inhibitory effect of 3,5-diCQA on ROS formation.  $^{\circ}P < 0.01$  vs control.  $^{\circ}P < 0.05$ ,  $^{\dagger}P < 0.01$  vs CHX+LPS.

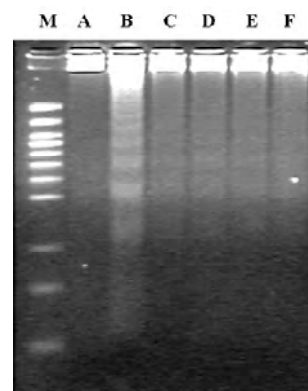
effect of 3,5-diCQA on LPS-induced apoptosis in HMEC-1 in the presence of CHX was examined. After 16 h incubation with both LPS and CHX, cell viability was reduced to 60% of the level of the control group treated with CHX alone. Pre-treatment with 3,5-diCQA (5, 10, or 50  $\mu\text{mol/L}$ ) decreased the cell death rate to 19%, 14%, and 12%, respectively (Table 1). The release of LDH after LPS treatment for 16 h was significantly higher than the cells treated with CHX alone. Treatment with 3,5-diCQA at 5, 10, or 50  $\mu\text{mol/L}$  decreased LDH release in a concentration-dependent manner.  $\alpha$ -Tocopherol (20  $\mu\text{mol/L}$ ) also showed significant suppression of LPS-induced cell death and LPS-induced LDH release (Table 1).

**Table 1.** Effect of 3,5-diCQA on HMEC-1 viability and LDH release. Experimental values are expressed as mean $\pm$ SD from 3 independent experiments.  $^{\circ}P < 0.01$  vs CHX.  $^{\circ}P < 0.05$ ,  $^{\dagger}P < 0.01$  vs CHX+LPS.

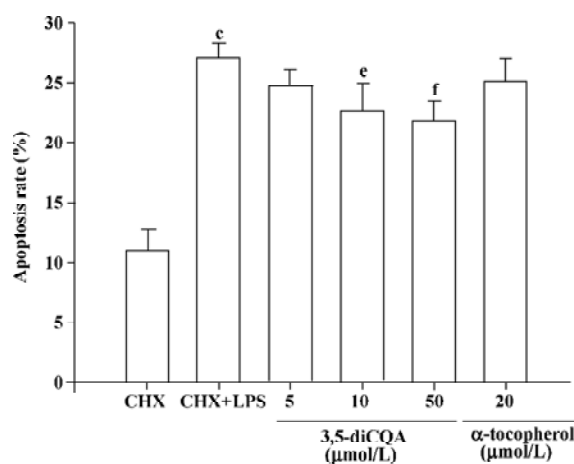
Group	Dose ( $\mu\text{mol/L}$ )	Cell viability (% of CHX group)	LDH release (% of total)
CHX		100 $\pm$ 0	47.6 $\pm$ 5.8
CHX+LPS		60.1 $\pm$ 10.0 $^{\circ}$	61.5 $\pm$ 5.0 $^{\circ}$
3,5-diCQA	5	81.1 $\pm$ 9.5 $^{\circ}$	56.8 $\pm$ 5.9 $^{\circ}$
	10	86.4 $\pm$ 8.5 $^{\dagger}$	53.8 $\pm$ 5.9 $^{\circ}$
	50	88.2 $\pm$ 4.6 $^{\dagger}$	51.2 $\pm$ 5.4 $^{\dagger}$
$\alpha$ -Tocopherol	20	81.9 $\pm$ 6.3 $^{\circ}$	54.7 $\pm$ 5.3 $^{\circ}$

**Effect of 3,5-diCQA on cell apoptosis** Apoptosis induced by LPS in the presence of CHX was confirmed by the DNA fragmentation assay. CHX alone did not induce fragmentation of chromosomal DNA. Treatment with 3,5-diCQA or  $\alpha$ -tocopherol suppressed LPS-induced DNA laddering (Figure

4). Flow cytometric analysis was performed after PI staining. DNA fragmentation was observed in 27% of cells with hypodiploid DNA in LPS-induced apoptosis. The percentage of cells with DNA fragmentation decreased significantly on treatment with 10 or 50  $\mu\text{mol/L}$  3,5-diCQA, while 5  $\mu\text{mol/L}$  3,5-diCQA and 20  $\mu\text{mol/L}$   $\alpha$ -tocopherol had no effect (Figure 5).

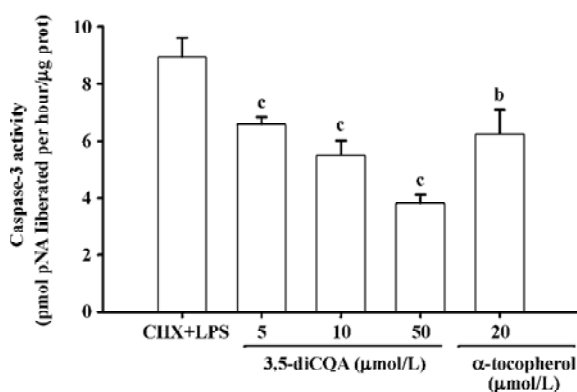


**Figure 4.** DNA laddering after 3,5-diCQA treatment. (A) CHX; (B) CHX+LPS; (C, D, E) CHX+LPS+3,5-diCQA 5, 10, 50  $\mu\text{mol/L}$ . (F)  $\alpha$ -tocopherol 20  $\mu\text{mol/L}$ .



**Figure 5.** Effect of 3,5-diCQA on cell apoptosis.  $n=3$ . Mean $\pm$ SD.  $^{\circ}P < 0.01$  vs control.  $^{\circ}P < 0.05$ ,  $^{\dagger}P < 0.01$  vs CHX+LPS.

**Effect of 3,5-diCQA on caspase-3 activity** Caspase-3 activity in HMEC-1 was 8.92 pmol pNA liberated/hour in LPS-induced apoptosis. In the cells treated with CHX alone, the caspase-3 activity was similar to the control group (data not shown). Caspase-3 activity decreased in a dose-dependent manner on treatment with 3,5-diCQA. Caspase-3 activity induced by LPS was suppressed by 20  $\mu\text{mol/L}$   $\alpha$ -tocopherol (Figure 6).



**Figure 6.** Effect of 3,5-diCQA on caspase-3 activity. *n*=3. Mean±SD. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs CHX+LPS.

## Discussion

In the present study, 3,5-diCQA effectively suppressed the generation of oxide free radicals in the microsome model, reduced the production of intracellular ROS induced by LPS in HMEC-1, protected HMEC-1 from LPS-induced cell injury, and inhibited the activity of caspase-3.

LPS is a bacterial endotoxin that functions as a pro-inflammatory mediator inducing significant levels of endogenous TNF- $\alpha$  and mediating endothelial cell damage<sup>[11]</sup>. LPS induces apoptosis in bovine and ovine endothelial cells *in vitro* and elicits apoptosis in human endothelial cells in the absence of new gene expression. The induction of apoptotic cell death by LPS is confirmed by several criteria, including morphological changes, DNA laddering, TdT-mediated dUTP nick end labeling, the activation of caspase, and poly (ADP-ribose) polymerase cleavage<sup>[1]</sup>.

In the present study, LPS-induced cytotoxicity of HMEC-1 was suppressed by treatment of the cells with 3,5-diCQA or  $\alpha$ -tocopherol. Cell injury, determined by the release of LDH, was also suppressed by treatment with 3,5-diCQA. The DNA fragmentation associated with LPS-induced cell death was partially inhibited by treatment with 3,5-diCQA.

Oxidants or pro-oxidants are significant regulators of apoptosis and can induce the apoptotic process<sup>[24,25]</sup>. The significance of oxidative stress in apoptosis is strongly supported by the capacity of various cellular anti-oxidants to block apoptosis induced by a diverse number of agents<sup>[25,26]</sup>. Oxidative stress is a common factor in apoptosis and is induced by stimuli including LPS and TNF- $\alpha$  exposure<sup>[3,19,27-29]</sup>. Several phenylethanoids also possess free radical scavenging properties and protect cells against oxidative stress-induced injury<sup>[30-32]</sup>. In the present study, we investigated the anti-oxidant capacity of the phenylethanoid 3,5-diCQA

and found that both lipid peroxidation of microsomes and intracellular ROS accumulation induced by LPS were significantly suppressed. These results suggest that 3,5-diCQA may protect endothelial cells against apoptosis by directly scavenging intracellular ROS.

The signaling pathways induced by apoptosis converge to the caspase pathway to execute the final phase of the apoptotic process<sup>[12]</sup>. The caspase family of proteases consists of at least 14 members in mammals that are constitutively expressed in almost all cell types as inactive pro-enzymes (zymogens) that become processed and activated in response to a variety of pro-apoptotic stimuli<sup>[13]</sup>. Caspase-3 is a downstream member of the caspase cascade and acts as a central effector in the execution phase, the proteolytic events of which can lead to apoptosis and contribute to DNA fragmentation, nuclear morphological changes, and eventual cell death. The effect of LPS-induced caspase activation on endothelial cells is dramatic, resulting in the cleavage of nuclear proteins and structural proteins that mediate cell-cell and cell-substrate adhesion<sup>[18,21]</sup>. *In vivo* studies support a role for caspases in mediating LPS-induced EC apoptosis<sup>[20,22]</sup>. Thus, substances that inhibit the activity of caspase-3 may protect cells from apoptosis. The anti-apoptotic capacity of 3,5-diCQA was identified in the present study by the marked suppression of caspase-3 activity in LPS-treated cells.

In summary, 3,5-diCQA displays anti-oxidative and anti-apoptotic activity in HMEC-1 due to the scavenging of intracellular ROS and the reduction of caspase-3 activity. The anti-oxidative and anti-apoptotic capacity of 3,5-diCQA provides clinical potential for the treatment of diseases involving the progressive dysfunction of the endothelium, including ischemia, shock, or sepsis.

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