Equol Inhibits LPS-Induced Oxidative Stress and Enhances the Immune Response in Chicken HD11 Macrophages

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Key Words
Oxidative stress • Lipopolysaccharide (LPS) • Equol • Immune response • Chicken macrophage

Abstract
Background/Aims: There has been increasing recent attention on the antioxidative capacity of equol. This study tested the effect of equol on oxidative stress induced by lipopolysaccharide (LPS) and regulation of immunity in chicken macrophages. Methods: Chicken HD11 macrophages were challenged with LPS (100 ng/mL) alone or with LPS (100 ng/mL) and (+)-equol (10, 20, 40, 80, 160 μmol/L) together for 24h. Evaluated responses included the contents of malondialdehyde (MDA) and reduced glutathione (GSH), activities of total superoxide dismutase (T-SOD) and inducible nitric oxide synthase (iNOS), transcript abundance of superoxide dismutase 2 (SOD2), catalase (CAT), glutathione transferase (GST), Toll-like receptor 4 (TLR4), tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β), and contents of the cytokines TNFα, IL-1β, interleukin-2 (IL-2) and interferon beta (IFNβ). Results: Exposure to LPS induced oxidative stress as contents of MDA increased and GSH decreased in LPS-treated cells (P < 0.05) compared to those in control cells. Compared to LPS alone, co-treatment with equol (20 μmol/L, 40 μmol/L or 80 μmol/L) reduced contents of MDA and increased those of GSH (both P < 0.05). Activity of T-SOD increased (P < 0.05) in cells treated with the higher concentration of equol (80 μmol/L or 160 μmol/L), however, all concentrations (20 μmol/L to 160 μmol/L) increased activity of iNOS (P < 0.05). The highest concentration of equol (160 μmol/L) increased SOD2 and GST transcripts (P < 0.05). Equol treatment increased transcripts of TLR4, TNFα and IL-1β (P < 0.05). And there were similar changes in contents of IL-1β, IL-2, IFNβ and TNFα in the cells (P < 0.05). Conclusions: It concluded that equol can protect chicken HD11 macrophages from oxidative stress induced by LPS through reducing lipid peroxidation products and enhancing contents of antioxidants, and activities of relevant antioxidant enzymes; effects were also seen in gene expression related to the immune response and increased contents of cytokines. The optimal concentration of equol on antioxidation and immune enhancement in chicken macrophages was 40 μmol/L.
**Introduction**

An imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage, is termed 'oxidative stress' [1]. Oxidants are formed as a normal product of aerobic metabolism but can be produced at elevated rates under pathophysiological conditions. Lipopolysaccharide (LPS) is a component of cell walls and the major virulence factor of gram-negative bacteria. Oxidative stress can be induced by LPS in mouse macrophages [2, 3] and in rats [4].

Equol, the product of daidzein degradation by intestinal bacterial flora [5], shows strong antioxidative properties, as well as ability to regulate cell cycles [6]. Equol exists in 2 enantiomeric forms, R-(+)equol and S-(-)equol [7]. Both (±)equol enantiomers can be readily synthesized from daidzein by catalytic hydrogenation [8]. (±)equol is the form that has been commercially available and mostly utilized in studies of its biological properties [9] although S-(-)equol is a natural diastereoisomer, exclusively produced by intestinal bacteria [7] and it preferentially activates estrogen receptor type β [10, 11]. Equol may also have beneficial effects on bone health [12-14] and has been used for preventing and treating diseases such as cardiovascular disease [15], breast cancer [16] and climacteric symptoms [17]. Among these varied biological activities, there has been increasing recent attention on the antioxidative capacity of equol. Antioxidant protection by equol in muscle cells of embryonic broilers [18] has been demonstrated, but little else is known of equol’s function in chickens.

Macrophages play a pivotal role in innate and adaptive immunity by killing foreign organisms, presenting antigens, secreting chemical mediators, phagocytizing foreign invaders, and undergoing oxidative stress. They interact with cells such as T cells, B cells, natural killer cells, dendritic cells, neutrophils, and fibroblasts. Activated macrophages produce a number of multifunctional compounds including cytokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as nitric oxide (NO) [19].

Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and ferrous sulfate (FeSO\textsubscript{4}) have been used to induce oxidative stress in chick skeletal muscle cells [20] and intestinal epithelial cells but were not effective in chicken macrophages (unpublished data). There are reports [21-23] that 1 μg/mL of LPS induces oxidative stress in macrophages of mice. Preliminary testing with different concentrations of LPS (0, 100, 400 ng/mL) to induce oxidative stress in chicken macrophages found that 100 ng/mL of LPS was the most effective (data not shown). Therefore, it was hypothesized that LPS would be effective in inducing oxidative stress in chicken macrophages, and that equol may play a role in antioxidation and regulation of immune function.

**Materials and Methods**

*Equol and HD11 Cell Line*

(±)Equol was purchased from Abcam (Cambridge, UK). The chicken macrophage cell line HD11 was a gift from Dr S.J. Lamont (Iowa State University, Ames, IA).

*Materials*

Fetal bovine serum (FBS), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), sodium pyruvate, nonessential amino acids, glutamine, 2-mercaptoethanol, penicillin-streptomycin, trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) and RPMI 1640 medium were purchased from Gibco (Grand Island, NY) or Invitrogen (Carlsbad, CA, USA). Lipopolysaccharide (LPS; from *Salmonella enterica* serotype *enteritidis*), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were purchased from Sigma-Aldrich (St. Louis, MO).

*Cell Culture*

HD11 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 10.0 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, 2.0 mmol/L glutamine, 100 U/mL penicillin-streptomycin, and 2.0 mmol/L 2-mercaptoethanol.
penicillin, 100 μg/mL streptomycin and 5 × 10⁻⁵ mol/L 2-mercaptoethanol. The cells were maintained in a humidified incubator at 37°C under 5% CO₂ in air. The cells were sub-cultured every 3 to 4 days to maintain logarithmic growth. Cell concentration was adjusted to 4 × 10⁵ cells/mL and wells of 6-well plates were seeded with 2 mL (1 mL per well in 12-well plates). Cells were allowed to grow for 24 h before treatments were applied. LPS and equol were dissolved in ddH₂O and dimethylsulfoxide (DMSO) respectively as stock solutions. Challenge with LPS (100 ng/mL) used a 1000-fold dilution into the culture medium, and equol stock solution was diluted 1000-fold to 16,000-fold; control cells received the same volume of ddH₂O and DMSO. The cells were treated for an additional 24 h.

**Effects on number of live cells**

HD11 cells were plated in 12-well plates. After 24 h, cells were exposed to LPS (0 or 100 ng/mL) and LPS-treated cells were co-incubated with equol (0, 10, 20, 40, 80 and 160 μmol/L) for another 24 h. A standard MTT assay [24] was used to quantify live cells.

**Contents of malondialdehyde and reduced glutathione and activities of total superoxide dismutase and inducible nitric oxide synthase**

HD11 cells were treated as above to test effects of treatment on contents of malondialdehyde (MDA) and glutathione (GSH), and activities of total superoxide dismutase (T-SOD) and inducible nitric oxide synthase [25]. After removing media, cells were harvested, washed twice with phosphate buffered saline (PBS), homogenized by sonication in 2 mL PBS and protein was measured with bicinchoninic acid (BCA). The thiobarbituric acid reactive substances (TBARS) method [26] was used to estimate cellular MDA content and GSH was measured with dithio-bis-nitrobenzoic acid (DTNB); both were expressed as nanomoles per milligram protein. Activities of T-SOD and iNOS were measured using kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), previously validated for use, and expressed as units/mg protein.

**Quantitative RT-PCR (qPCR)**

HD11 cells were incubated for the second 24-h period in the control medium or that containing LPS (100 ng/mL), alone or with moderate (40 μmol/L) or high (160 μmol/L) concentrations of equol. Total RNA was extracted with Trizol reagent (Invitrogen) and adjusted to 500 ng/mL for all samples then first-strand cDNA was synthesized (Promega, Beijing, China). Specific transcripts were quantified by qPCR with an ABI 7500 Real-time Detection System (Applied Biosystems, Carlsbad, CA) using a SYBR® Premix Ex Taq™ II kit (Takara, Dalian, China); the primers used (Sangon Biological Engineering, Shanghai, China), based on chicken sequences, are listed in Table 1. The amplification was performed in a total volume of 25 μL, containing 12.5 μL of 2X SYBR® Premix, 0.5 μL internal reference dye 6-carboxy-X-rhodamine (ROX), 2.5 μL of the 5 × diluted cDNA, 1.25 μL of each primer (10 mmol/L), and 7 μL ddH₂O. The real-time PCR program started with denaturing at 95°C for 1 min, followed by 35 cycles of 95°C for 15 s and 60°C for 60 s. Dissociation analysis of amplification products was performed after each PCR to confirm that only one PCR product was amplified and detected. Data were analyzed with ABI 7500 SDS software (Applied Biosysytems) with the baseline being set automatically by the software and values of average ΔCt (normalized using β-actin) were used to calculate relative expression by the comparative Ct method, calculated as 2⁻ΔΔCt. Results are expressed as relative abundance, being log (2⁻ΔΔCt).

**Enzyme-linked immunosorbent assay for cytokines**

HD11 cells, treated exactly as described above for the qPCRs, were harvested and homogenates were prepared as described earlier. Concentrations of tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), interleukin-2 (IL-2) and interferon beta (IFNβ) were measured with relevant chicken-specific ELISA kits (Shanghai Lianshuo Biological Technology, Shanghai, China).

| Gene name | Sequence | GenBank No. |
|-----------|----------|-------------|
| SOD2      | f-5'-TGGTGTTCACAAGGATCAGGC-3' | NM_204211 |
|           | r-5'-CCCCCAATGTGGAGAAGCA-3'  |             |
| CAT       | f-5'-AGAGTCTGCGATCGAAGAG-3'  | NM_00103215 |
|           | r-5'-ACCCAGATTCCTCCACAACA-3' |             |
| GST       | f-5'-GATGAAACGTGCTCCAACACG-3' | NM_00100177 |
|           | r-5'-TCACTCCTGTTGCTCCTCAA-3' |             |
| TLR4      | f-5'-AGCTGAATTTGCTGAGCTCAAT-3' | NM_00103693 |
|           | r-5'-GGCAGTTAAGCAGGAAAG-3'   | NM_204267  |
| TNFa      | f-5'-AATTTGACAGCGTTTCTGCG-3' |             |
|           | r-5'-TATGAAAGGTGCTCAGATG-3'  |             |
| IL-1β     | f-5'-GAAGCTCGTTGCTGCAGAT-3'  | NM_204524  |
|           | r-5'-ACTGACATCTGCGACATTG-3'  |             |
| β-actin   | f-5'-GAGAAATTTGGGCTGACACA-3' | NM_205518  |
|           | r-5'-CCTGAATCTCTCATTGCGA-3'  |             |
Statistical Analysis

The effects of treatments, LPS or LPS plus equol, were analyzed by the GLM procedure of SAS (version 8.0, SAS Institute, 1998). Multiple comparisons of means were performed using Least Significant Difference (LSD) tests and the SE derived from the Error Mean Squares for n = 6. Significance was set at p < 0.05.

Results

Effects of Equol on Cell Survival

As shown in Figure 1, there was no effect of treatment with 100 ng/ml LPS on the number of live HD11 macrophages after 24 h. Cell survival was unaffected by co-incubation (LPS plus equol), except when the highest concentration of equol (160 μmol/L) was used, but it was still 78% of the controls.

Effects of Equol on contents of MDA and GSH

The anti-oxidative activity of equol was examined using LPS-stimulated macrophages. Concentrations of MDA increased by 29.9% and GSH decreased by 20.5% (both P < 0.05) in cells treated just with LPS, compared to untreated controls (Fig. 2). The increase in MDA induced by LPS was offset by 20 to 80 μmol/L equol with 40 μmol/L being most effective. All concentrations of equol resulted in MDA contents that did not differ from untreated control cells. In contrast, the LPS-provoked decrease in GSH was reversed, in a concentration-dependent manner, by all tested levels of equol; the maximal effect, with 160 μmol/L actually increased GSH above that in untreated control cells.

Effect of equol on activities of T-SOD and iNOS

Figure 3 shows that the activities of T-SOD and iNOS in LPS-treated cells were not different (P > 0.05) to those in untreated cells. In the presence of LPS, higher levels of equol (80 and 160 μmol/L) resulted in elevated T-SOD (P < 0.05). In the case of iNOS activity, all equol plus LPS treatments stimulated the enzyme above activities measured in controls or cells treated with LPS alone (P < 0.05).

Effects of equol on transcript abundance of SOD2, CAT and GST

Transcripts of these genes, the products of which are directly involved in oxidative and anti-oxidative dynamics, were examined using qPCR (Fig. 4). The relative expression of SOD2 in macrophages was increased, only in cells exposed to LPS together with equol at the higher concentration (160 μmol/L). The relative expression of CAT (P < 0.05) increased after treatment with LPS and LPS plus 40 μmol/L equol but not with the higher concentration. Indeed, co-treatment with 160 μmol/L equol strongly suppressed expression of CAT, to a level that was less than in untreated control cells. Differing from expression of SOD2 and CAT,
that of \textit{GST} increased with exposure to LPS or LPS plus the moderate concentration of equol and increased further when 160 μmol/L equol was used.
Enhanced expression of TLR4, TNFα and IL-1β genes, and contents of TNFα, IL-1β, IL-2 and IFNβ occur in equol-treated macrophages

Indices of immune function of chicken HD11 macrophages were examined by selected gene expression of TLR4 and cytokines (Fig. 5), and contents of cytokines (Fig. 6) in LPS-induced or LPS-induced and equol-treated cells. Stimulation with LPS for 24 h caused a small increase in TLR4 transcripts and this response was greatest in the presence of 40 μmol/L equol and somewhat less with the higher concentration (160 μmol/L). Expression of TNFα was not affected by LPS alone but increased (P < 0.05) when LPS was combined with equol; the induction was greater with the higher concentration. The abundance of IL-1β transcripts increased (P < 0.05) with LPS treatment and further increased from co-treatment, with maximal expression occurring in the presence of 160 μmol/L equol. The contents of IL-1β, IL-2 and IFNβ were unaffected by LPS alone but that of TNFα decreased (P < 0.05) in the challenged macrophages. With exception of TNFα in cells exposed to LPS plus 40 μmol/L, all cytokines increased when LPS was combined with equol; the pattern of changes with 0, 40 or 160 μmol/L was very similar for IL-1β, IL-2 and IFNβ. Note that the changes in contents of interleukins (Fig. 6) were very similar to those of the IL-1β transcripts (Fig. 5).

Discussion

A wide range concentrations of LPS (10-10000 ng/mL) [21, 27, 28] can induce oxidative stress in macrophage of mice. LPS from different bacterial sources will not always elicit identical biological responses, especially when tested in different target species. Specific LPS signaling pathways are likely to differ between tissues [29]. Related studies of LPS-induced oxidative stress in chicken macrophages have not been reported. The concentration of 100 ng/mL LPS has most often been used to induce immune or inflammatory responses in
chicken HD11 macrophages [30, 31], therefore, 0, 100 and 400 ng/mL Salmonella LPS were screened on chicken macrophages and 100 ng/mL was found to be most effective.

As MDA and GSH are critical indicators of oxidative stress, these were measured in LPS-challenged macrophages to determine oxidative stress and to assess the anti-oxidative activity of equol. The results found here are consistent with others’ findings that MDA contents increase with LPS induction [3, 32-34]. The present work demonstrates successful construction of an oxidative stress model with LPS in chicken macrophages; previous emphasis on LPS-challenged macrophages has been on immune responses. The reduction in GSH in LPS-treated chicken macrophages shown here is consistent with Qiao et al [35] who found that, in mice, LPS induced a significant decrease of GSH levels in all tested tissues including liver, lung and kidney; similarly, Kheir-Eldin et al [36] showed that oxidative stress in rats caused cellular GSH-depletion. In the new model developed here, co-incubation with equol counteracted the oxidative damage from LPS alone by decreasing MDA and increasing GSH content.

The antioxidant enzymes SOD, CAT and GST play important roles in maintaining the redox homeostasis in cells. Impaired activity of SOD with augmented the C-reactive protein level in blood when oxidative stress occurred in mice [37]. Pretreatment with low-dosage equol (1 μmol/L) significantly increased T-SOD activity compared with H₂O₂ treatment alone in chicken muscle cells [18]. Oxidative stress induced here in macrophages by LPS did not deplete T-SOD, but the higher concentrations of equol enhanced T-SOD activity and the highest concentration increased the abundance of SOD2 transcripts. It would seem that the effect of equol on SOD activity varies with cell type and treatment. Compounds with antioxidant functions generally increase activities of antioxidant-related enzymes [38, 39], but activities can be down-regulated in some antioxidant-treated cells because the compounds may have directly reduced the prevailing oxidative stress [40, 41].

Inducible nitric oxide synthase is mainly expressed in macrophages [42] and is a key enzyme that participates in inflammatory progression [3]. The expression of Heme oxygenase-1 (HO-1), another important enzyme of antioxidation, can be downregulated
by inhibition of iNOS in macrophages of mice [43]. The activity of iNOS increased with all tested concentrations of equol used here with chicken macrophages, contrasting with equol inhibiting nitric oxide content and iNOS gene expression in murine macrophages [42].

Immunocytes stimulated with LPS are known to release cytokines, in a time-dependent manner, as part of the immune response. Cytokine release began 2 h after LPS addition and was maximal between 6 and 8 h [44]. The contents of cytokines, measured here at 24 h after LPS, were the same as those in control cells or were even lower in the case of TNFα. The contents of cytokines such as TNFα [3, 45] and IL-1β [46, 47] increased when oxidative stress occurred. The present results demonstrated that oxidative stress still existed at 24 h, despite the lack of increase in cytokines but their levels, and that of TLR4, were increased in the presence of equol. The levels of the cytokines were consistent with the relative levels of their gene transcripts.

Cell growth and survival were suppressed here by equol at 160 μmol/L, similar to previous findings with equol in different cell lines [48, 49], including some cancer cell lines [48, 50]. Some studies [51, 52] indicated that equol may act as a pro-oxidant, as well as an antioxidant. This may lead to equol exhibiting antioxidant activities at the same time as inducing apoptosis [50], even death. The dead cells (suspended) in wells treated with 160 μmol/L equol were removed when live cells were collected and they showed that equol exhibited potent antioxidative ability and immunostimulatory responses in the surviving cells. Similar results [48] for high concentrations of equol (100 or 200 μmol/L) exhibiting growth inhibition along with potent antioxidation were obtained in human HepG2 cells. More interesting was the absence of effect on cell survival of the range of concentrations of equol, up to 80 μmol/L, despite very variable levels of markers of the antioxidative system and inflammatory process. Taken together, the results indicate that the optimal concentration of equol for these endpoints in chicken macrophages was 40 μmol/L.

It is concluded that equol protects chicken HD11 macrophages from LPS-induced oxidative stress by reducing contents of lipid peroxidation products such as MDA, enhancing the content of the antioxidant GSH, increasing antioxidase activities (T-SOD and iNOS) and stimulating expression of SOD2 and GST. Equol also increased immune function by increasing expression of TLR4 and genes encoding cytokines. The optimal concentration of equol on antioxidation and immune enhancement in chicken macrophages was 40 μmol/L.

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Disclosure Statement

None.

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