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***Quercus infectoria* Inhibits Set7/NF- κ B Inflammatory Pathway in Macrophages Exposed to a Diabetic Environment**

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Abstract

Chronic inflammation plays a key role in the pathogenesis of myriad complications associated with diabetes and thus anti-inflammatory therapies may ameliorate these complications. *Quercus infectoria* (Qi) extract has been shown to downregulate inflammatory processes; however, the molecular mechanisms of this anti-inflammatory activity remain unclear. The hypothesis of our study was that Qi extract exerts its anti-inflammatory effect by downregulating the Set7/NF- κ B pathway. Bone marrow-derived macrophages (BMM) were treated with high glucose plus palmitate medium (HG/Pa) to simulate the diabetic environment. Compared with control conditions, HG/Pa elevated expression Set7, expression and activity of NF- κ B along with expression of several inflammatory cytokines. These changes were associated with increased levels of intracellular reactive oxygen species (ROS). Moreover, similar alterations were demonstrated in BMM derived from mice fed a high fat diet (HFD) compared to those from lean mice, suggesting that HFD-induced changes in BM progenitors persist throughout differentiation and culture. Importantly, Qi extract dose-dependently reduced Set7, p65 and inflammatory cytokine expression relative to vehicle controls in both HG/Pa-and HFD-treated BMM. Finally, macrophages/monocytes isolated from wounds of diabetic mice that were treated with Qi solution exhibited lower expression of the inflammatory cytokines, IL-1 β and TNF- α , compared with vehicle treated wounds, demonstrating translation to the in vivo diabetic environment. Taken together, data from this study suggests that Qi downregulates diabetes-induced activity of the Set7/NF- κ B pathway.

Graphical abstract

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Keywords

Chronic inflammation; diabetes; macrophage; *Quercus infectoria*

1. Introduction

Diabetes is a significant health problem world-wide and is closely associated with myriad complications. Hyperglycemia is a hallmark of diabetes and is thought to contribute to the development of neuropathy, cardiovascular diseases, atherosclerosis, and chronic wounds. Hyperglycemia may promote diabetic complications in part by promoting inflammation via several pathways, especially those involving the transcription factor NF- κ B [1, 2]. NF- κ B can be considered a master regulator of inflammatory mediators and oxidative molecules [3, 4]. Thus, therapies that inhibit hyperglycemia-induced NF- κ B activity may ameliorate chronic inflammation and complications associated with diabetes.

Nutgall of *Quercus infectoria* (Qi) G. Olivier (Fagaceae) has been used for centuries in traditional medicine in several Asian countries for the treatment of infectious diseases and inflammatory disorders [5]. Previous researchers have identified a number of pharmacological activities of Qi extract including antimicrobial [6], anti-inflammatory [7, 8], and anti-oxidant activity [9]. Qi extract contains high concentrations of tannins such as gallic acid, ellagic acid, along with flavonoids that are known to have anti-inflammatory properties. Although many studies have attempted to identify the mechanism(s) by which Qi extract inhibits inflammation [7, 8], the pathways involved in this anti-inflammatory activity remain unclear.

Set7 is an enzyme that methylates lysine residues of both histone and non-histone proteins, including NF- κ B, and has been shown to contribute to hyperglycemia-induced inflammation in animal and cell culture models [10–12]. In humans, diabetes is associated with increased levels of Set7 in peripheral blood mononuclear cells, which in turn is associated with epigenetic modifications on the NF- κ B p65 promoter and upregulation of NF- κ B p65 along with target inflammatory cytokines [13]. Moreover, Set7 has been implicated in the

regulation of anti-oxidant enzyme expression and the accumulation of intracellular reactive oxygen species (ROS) [14]. Thus, the hypothesis of our study was that Qi extract exerts its anti-inflammatory effect by inhibiting the Set7/NF- κ B inflammatory pathway.

2. Materials and Methods

2.1. Animals

Diabetic db/db mice and non-diabetic wild-type C57Bl/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). Transgenic mice expressing *Photinus* luciferase cDNA under HIV-LTR (HLL) were used for luciferase assay [15]. For high fat diet-induced (HFD) mice, male C57Bl/6 mice were fed a high fat diet (60 kcal% fat diet, D12492 from Research Diets) for 18 weeks to induce obesity and insulin resistance. Experiments with db/db, normal chow- and HFD-fed C57Bl/6, and HLL mice were performed on 12–16 week-old mice. All experimental procedures were approved by the Animal Care Committee at the University of Illinois at Chicago.

2.2. Preparation of Qi extract and Qi solution

Nutgalls of *Quercus infectoria* were obtained from Thai Herbal shop, Songkhla, Thailand. The dry plant materials were extracted with 95% ethanol in the ratio 1:10 for 7 days. The solution was filtered and evaporated to dryness. Then, the extract was dissolved in 10% dimethylsulfoxide to obtain as a stock solution at a concentration 100 mg/ml. For in vivo experiment, Qi solution at 30% (w/v) of concentration was pharmaceutically formulated as a topical wound treatment including propylene glycol, polyethylene glycol 400, polysorbate 20, polysorbate 60, 95% ethanol, and distilled water.

2.3. Cell culture and treatment

Bone marrow-derived macrophages (BMM) were cultured as described previously [16]. Briefly, bone marrow cells were collected from femurs and tibias of mice and cultured (2×10^7 cells/well) in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 10% L-929 cell-conditioned medium (source of M-CSF), 2 mM L-glutamine, and 1% penicillin/streptomycin (Sigma Aldrich, St. Louis, MO, USA) in a humidified 10% CO₂ atmosphere at 37°C for 5 days. After incubation, medium was removed and replaced with normal glucose DMEM medium (NG: 1 g/l glucose) or high glucose DMEM medium (HG: 4.5 g/l glucose) or high glucose medium with different concentrations of palmitate (HG/Pa) (Sigma Aldrich, St. Louis, MO, USA) with/without different concentrations of Qi extract (1024 μ g/ml – 8 μ g/ml) for 24 h. Palmitate was prepared as a 100 mM stock solution in 70% ethanol/0.1 M NaOH with heating at 70°C in water bath [17]. For each experiment, palmitate was diluted freshly with 10% bovine serum albumin and then added into each well to obtain the indicated final concentrations. To determine the role of Set7 methyltransferase activity on HG/Pa-induced NF- κ B p65 expression, cells were treated with (*R*)-PFI-2 (Tocris, Minneapolis, MN, USA), a Set7 inhibitor (1–10 μ M) or vehicle (DMSO) [18]. For all experiments, NG or HG medium containing 10% bovine serum albumin served as a control.

2.4. Excisional wounding and treatment

Four full-thickness excisional wounds were created on the skin dorsum of each mouse using an 8 mm biopsy punch as described previously [19, 20]. Treatment with Qi solution was initiated on day 3 post-injury, to allow the early inflammatory response to proceed normally. Wounds were treated once a day for seven days; for each treatment, 15 µl of Qi solution or vehicle control (base solution of Qi solution) was applied to each wound and covered with Tegaderm dressing (3M, Minneapolis, MN). At the end of the experiment, wounds were harvested for cell isolation.

2.5. Cell isolation from wounds

Wounded cell isolation was performed according to previous study [19, 20]. Briefly, cells were dissociated from excisional wounds using an enzymatic digest with collagenase I, collagenase XI and hyaluronidase (Sigma Aldrich, St. Louis, MO, USA). Neutrophils, T cells, and B cells were marked by incubating cells for 15 min with fluorescein isothiocyanate-conjugated anti-Ly6G, anti-CD3, and anti-CD19. These cells were depleted from total cell population using anti-FITC magnetic beads following the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes and macrophages were then positively selected using anti-CD11b magnetic beads. Cell counts were performed using hemacytometer.

2.6. RNA isolation, RT-qPCR, and ELISA

Total RNA was isolated from samples using the RNeasy kit (Qiagen, Valencia, CA, USA) or the Cells to Ct kit (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed using the Thermoscript RT-PCR kit (Invitrogen, Carlsbad, CA, USA) or the Cells to Ct kit, and semi-quantitative PCR was performed with the primers in Table 1; primers selected based on established involvement in metabolic syndrome and diabetes [21]. All reactions were performed in triplicate, and cycle threshold values were averaged over triplicates. Relative mRNA expression was determined using the 2^{-CT} method, with GAPDH as the endogenous control gene. In addition, IL-6, TNF- α , and VEGF cytokine protein release under all tested conditions was measured by ELISA, as specified by manufacturer's instruction (R&D Systems, Minneapolis, MN, USA).

2.7. Cell lysate preparation and western blot analysis

Cells were harvested, wash with ice-cold Dulbecco's phosphate-buffer saline (DPBS), and gently lysed in ice-cold M-RER lysis buffer (Thermo Scientific, Waltham, MA, USA) containing protease inhibitor cocktail (1:100) and Na_3VO_4 (1:1000) (Sigma Aldrich, St. Louis, MO, USA). Cell lysates were centrifuged at $14,000\times g$ for 10 min at 4°C . The supernatant were collected for further analysis. Protein concentration was determined using Coomassie PlusTM assay kit (Thermo Scientific, Waltham, MA, USA).

For western blot analysis, the protein lysates from each sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane. After blocking with Tris-buffered saline based blocking solution (LI-COR, Lincoln, NE, USA) for 30 min, the membranes were incubated overnight at 4°C with primary antibodies against Set7 antibody or p65 (Cell signaling Technology, Danvers,

MA, USA). Immunoreactive bands were then detected by incubating with IRDye[®] 800CW Goat anti-Rabbit IgG (LI-COR, Lincoln, NE, USA) or IRDye[®] 680RD Goat anti-Mouse antibody (LI-COR, Lincoln, NE, USA) for 1 h and visualized using Odyssey[®] CLx Imaging system (LI-COR Lincoln, NE, USA).

2.8. Luciferase assay

For luciferase assay, cell lysates were collected by adding 300 µl of Luciferase Cell Culture Lysis Reagent (Promega, Fitchburg, WI, USA) into each well of 6-well plates. Cells were centrifuged at 12,000×g for 15 seconds at 4°C. The supernatant was collected for luciferase measurement. A volume of 20 µl of each cell lysate was added into a 1.5 ml-tubes containing 100 µl of Luciferase Assay Reagent (Promega, Fitchburg, WI, USA). Luciferase activity was immediately measured using GloMax[®] 20/20 luminometer (Promega, Fitchburg, WI, USA).

2.9. Measurement of intracellular ROS production

Intracellular ROS was measured using CellRoX Deep Red Reagent (Thermo Scientific, Waltham, MA, USA), a fluorogenic probes designed to measure ROS in live cells. Briefly, bone marrow cells from both WT and high fat diet (HFD) mice were differentiated to macrophages for 7 days as described above. Then, cells were detached from a petri dish by incubating with cold PBS and the same number of collected cells were cultured under normal glucose, high glucose, or high glucose plus palmitate with/without different concentrations of Qi extract for 24 h. CellRoX reagent was then added to each well at a final concentration of 5µM and incubated in the dark for 30 minutes at 37°C and then cells were fixed with 4% formaldehyde for 15 minutes. After permeabilization with ice-cold methanol for 10 minutes, a DyLight 800-conjugated β-actin monoclonal antibody (Invitrogen, Carlsbad, CA, USA) at a dilution of 1:100 was added as a cell number loading control. After 30 minutes of incubation, the fluorescent signals from cell populations in each well were quantified at 700 and 800 nm using Odyssey[®] CLx Imaging system (LI-COR, Lincoln, NE, USA).

2.10. Statistical analysis

Values are reported as means ± SE. All statistical comparisons were performed by SPSS 14.0 (SPSS Inc., Chicago, IL, USA) using t-tests. Differences between groups were considered significant if p < 0.05.

3. Results

3.1. Qi extract reduces inflammatory cytokine expression in macrophages exposed to simulated diabetic environment in vitro

To simulate the biochemical environment associated with chronic inflammation in diabetes, BMM were exposed to HG medium along with Pa at concentrations between 50–200 µM for 24 hours. NG medium and the vehicle for palmitate were used for control cells. Although neither HG nor Pa alone increased expression of IL-1β, TNF-α or IL-6 compared to control cells (Fig. 1A), their combination significantly increased expression of each inflammatory cytokine especially at palmitate concentrations of 100 and 200 µM.

Using this experiment as a foundation, we tested the effects of Qi extract on inflammatory cytokine mRNA expression in cultured BMM using the simulated diabetic environment with HG medium and Pa at 200 μ M. Qi extract induced a dose-dependent reduction in HG/Pa-induced inflammatory cytokine expression relative to vehicle control (Fig. 1B). Moreover, Qi extract also decreased cytokine protein release of TNF- α and IL-6 under HG/Pa treatment (Fig. 1C). Thus, Qi extract showed anti-inflammatory activity in vitro in a simulated diabetic environment.

3.2. Qi extract decreases NF- κ B activity in macrophages exposed to simulated diabetic environment in vitro

The NF- κ B pathway plays crucial roles in inflammatory responses by regulating transcription of inflammatory genes [22]. Since Qi extract reduced expression of inflammatory cytokines, we tested whether Qi also inhibited the NF- κ B pathway. HG/Pa stimulation for 24 h significantly increased mRNA expression of RelA/p65, a subunit of the NF- κ B signaling complex, compared with control cells (Fig. 2A). Importantly, Qi extract blocked the increased expression of RelA/p65 induced by HG/Pa at both the mRNA and protein levels (Fig. 2B, C).

To further ascertain whether Qi extract inhibits NF- κ B activity, we cultured BMM from the NF κ B reporter mice (HLL mice). These transgenic mice utilize the proximal 5' human immunodeficiency virus (HIV-1) long terminal repeat (LTR) to drive the expression of *Photinus* luciferase cDNA. The proximal HIV-LTR is a well-characterized NF κ B-responsive promoter that allows assessment of NF- κ B mediated transcriptional activity via measurement of luciferase activity [15]. In our experiments, HG/Pa significantly increased luciferase activity when compared with control cells indicating that the simulated diabetic environment increased NF- κ B transcriptional activity. Importantly, treatment with Qi extract produced a dose-dependent reduction in luciferase activity, suggesting that Qi may reduce inflammatory cytokine expression by inhibiting NF- κ B transcriptional activity (Fig. 2D).

3.3. Qi extract downregulates SET7 expression in macrophages exposed to simulated diabetic environment in vitro

Several studies have demonstrated that the diabetic environment increases levels of the methyltransferase Set7, which leads to increased NF- κ B p65 expression and activity, and increased expression of NF- κ B-dependent cytokines [11, 12]. In human peripheral blood mononuclear cells, diabetes is associated with increased levels of Set7, epigenetic modifications on the NF- κ B p65 promotor and upregulation of NF- κ B p65 along with target inflammatory cytokines [13]. Thus, we sought to determine the effects of Qi extract on Set7 levels. Importantly, both mRNA and protein expression of Set7 was increased in BMM incubated with HG/Pa compared to control cells (Fig. 3A). In addition, treatment with Qi extract at all tested concentrations blocked the HG/Pa-induced increase in mRNA and protein levels of Set7 (Fig. 3B, C). Furthermore, inhibition of Set7 methyltransferase activity by (*R*)-PFI-2 significantly decreased HG/Pa-induced p65 mRNA expression compared with vehicle control cells (Fig. 3D). Of note, (*R*)-PFI-2 at these concentrations did not change morphology of BMM nor Set7 transcript levels (data not shown). These data indicate that Qi

extract may exert its anti-inflammatory activity by reducing activity of the Set7/NF- κ B pathway.

3.4. Qi extract reduces HFD-induced dysregulation of Set7/NF- κ B pathway

To assess the effects of Qi extract on the Set7/NF- κ B pathway in a model of pre-diabetes, bone marrow cells were harvested from lean and HFD mice and differentiated to macrophages. BMM isolated from HFD mice exhibited significantly increased Set7 and NF- κ B p65 mRNA and protein levels compared to BMM isolated from lean mice, even in the absence of additional stimuli (Fig. 4A, B, E), indicating that increased Set7/NF- κ B p65 expression induced by HFD is stable, persisting throughout the generation of BMM from bone marrow progenitors in culture. Qi extract abrogated the HFD-induced expression of both Set7 and NF- κ B p65 mRNA and protein in a dose-dependent manner (Fig. 4C, D, E). In addition, Qi extract decreased the HFD-induced expression of NF- κ B-dependent genes, including IL-1 β , TNF- α , and IL-6 (Figure 4F). Thus, Qi extract can reverse stable changes in the Set7 pathway that are induced by HFD.

3.5. Qi extract decreases HFD- and high glucose/palmitate-induced ROS production

In addition to reducing activity of the NF- κ B pathway, inhibiting Set7 has been reported to increase expression of anti-oxidant enzymes and reduce accumulation of intracellular ROS [14]. Interestingly, a previous study demonstrated that Qi extract possesses anti-oxidant activity by scavenging of several free radicals including 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), hydrogen peroxide, and hydroxyl radicals [9]. Therefore, we decided to test whether Qi extract reduces ROS production induced by a pre-diabetic or diabetic environment. BMM incubated in medium containing HG/Pa showed significantly increased ROS levels compared to control cells (Fig. 5A). In addition, BMM isolated from HFD mice generated greater ROS levels than cells isolated from lean mice (Fig. 5B), suggesting that dysregulated ROS handling induced by HFD is passed down from progenitors to differentiated progeny in culture. Qi extract significantly reduced ROS production both in BMM incubated with HG/Pa incubation and in BMM isolated from HFD mice (Fig. 5). Thus, Qi extract can reverse elevated ROS production induced acutely by the diabetic environment or by stable changes that are induced by HFD.

3.6. Qi solution reduces expression of inflammatory cytokines in wound macrophages of diabetic mice

To study the effect of Qi solution on injury-induced inflammation in vivo in diabetic mice, we applied Qi solution or vehicle topically to skin wounds created in diabetic mice. Treatment was initiated on day three post-wounding and was applied daily for seven days. After seven days of treatment, monocytes/macrophages were isolated from wounds and the expression of inflammatory cytokines was assessed by real time PCR. Indeed, the expression of IL-1 β and TNF- α was significantly reduced after treatment with Qi solution compared with vehicle control (Fig. 6). In contrast, there was no significant effect of Qi solution on expression of IL-6. In short, Qi solution decreased expression of selected inflammatory cytokines in wound macrophages of diabetic mice.

4. Discussion

The findings of this study demonstrate that a simulated diabetic environment increased activity of the Set7/NF- κ B pathway in macrophages, increased levels of intracellular ROS and increased expression suggesting that this traditional medicine of several inflammatory cytokines. Importantly, Qi extract reduced the diabetic environment-induced activity of the Set7/NF- κ B pathway. Thus, Qi extract may inhibit inflammation [7, 8] and oxidative stress [9] via this pathway.

Diabetes is characterized by hyperglycemia, hyperlipidemia and chronic inflammation. Previous studies have shown that in addition to HG, elevated free fatty acids, especially Pa, may contribute to chronic inflammation in diabetes [23–25]. Pa, the most prevalent saturated free fatty acid in plasma, can activate NF- κ B signaling, resulting in increased inflammatory cytokine production in both human pancreatic beta and non-beta cells [23, 26, 27] as well as endoplasmic reticulum stress [17, 23]. Dasu and Jialal previously reported that HG/Pa amplified NF- κ B activity, increased pro-inflammatory cytokine expression and elevated ROS levels in human monocytic cells [24]. In our studies, we stimulated BMM with a combination of high glucose and palmitate. Levels of NF- κ B p65, the main subunit of the NF- κ B pathway, were significantly augmented by HG/Pa, associated with increased expression of inflammatory cytokines.

The diabetic environment may induce epigenetic modifications that, in turn, may contribute to the pathophysiology of diabetes and its complications [13, 28, 29]. For example, vascular smooth muscle cells derived from db/db mice and endothelial cells that were transiently exposed to HG exhibited sustained increases in the expression of NF- κ B p65 subunit, inflammatory cytokines, and oxidative stress molecules, even after re-establishing normal glucose levels [10, 30]. In addition, Set7, an enzyme that catalyzes methylation of both histone and non-histone proteins, has been reported to contribute to inflammatory responses in cell culture and animal models of diabetes as well as in diabetic humans [10–13]. Our results show that HG/Pa increased expression of Set7 in BMM associated with increased production of inflammatory cytokines and intracellular ROS. In addition, BMM isolated from mice subjected to HFD exhibit a similar phenotype, indicating that such alterations are stably induced by HFD as they are likely passed down from progenitors to differentiated progeny in culture.

Previous studies have shown that HG increased levels of Set7 in endothelial cells [10, 11] and in human peripheral blood mononuclear cells, associated with epigenetic modifications on the NF- κ B p65 promoter and upregulation of NF- κ B p65 along with target inflammatory cytokines [13]. Inhibition of Set7 also resulted in the attenuation of methylation on the p65 promoter [12], reduced transcription of inflammatory cytokines [12] and upregulated expression of enzymes involved in ROS scavenging, superoxide dismutase 2 and catalase, associated with reduced ROS levels [14]. In our studies, Qi extract inhibited the expression of Set7 in both acute HG/Pa- and chronic HFD-induced inflammatory responses, reduced NF- κ B activity, suppressed inflammatory cytokine expression, and reduced ROS levels. However, whether Set7/NF- κ B is the central pathway by which Qi extract exerts its anti-

inflammatory and anti-oxidant activity, and whether epigenetic modifications are involved, remains to be determined.

Quercus infectoria (Qi) contains high levels of polyphenols such as tannic acid, gallic acid, ellagic acid as well as flavonoids that may play important roles in the ability of Qi to inhibit production of inflammatory mediators such as histamine, serotonin, prostaglandin E2, and IL-6 [7, 8]. Qi extract dose-dependently reduced neutrophil degranulation induced by *N*-formylmethionyl-leucyl-phenylalanine that resulted in the inhibition of the release of lytic enzymes from neutrophils [7]. Importantly, oral administration of Qi extract (200–1,000 mg/kg animal body weight) diminished both carrageenan- and formalin-induced paw edema in rats indicating that it can inhibit both acute and chronic inflammation [31]. Moreover, Qi extract exhibits strong free radical scavenger activity, including DPPH, ATBS, hydrogen peroxide, hydroxyl radicals, nitric oxide, and superoxide [7, 9]. Although these studies established that Qi extract possesses anti-inflammatory and anti-oxidant activity, they did not elucidate the pathways involved. Our work indicates that suppression of the Set7/NF- κ B pathway may be involved in the ability of Qi extract to reduce the persistent inflammation and oxidative stress induced by the diabetic environment. These data suggest that Qi extract has potential to be utilized as an anti-inflammatory agent for treating of diabetic complications.

In summary, our findings indicate that Qi extract treatment ameliorated the inflammatory phenotype of BMM induced by pre-diabetic or diabetic environments, potentially by inhibiting the Set-7/NF- κ B pathway. Therefore, Qi extract can be considered as a potential agent for treatment of chronic inflammation associated with diabetes.

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Highlights

- Set7/NF- κ B activity increased in macrophages exposed to diabetic environment
- Qi extract inhibited diabetic environment-induced Set7/NF- κ B activity
- Qi extract also reduced diabetic environment-induced ROS levels
- Anti-inflammatory effects of Qi extract may be mediated by Set7/NF- κ B pathway

Fig. 1. High glucose/palmitate increases and Qi treatment reduces inflammatory cytokine expression in macrophages

(A) mRNA expression of inflammatory cytokines in bone marrow-derived macrophages (BMM) cultured with normal glucose (NG) or high glucose (HG) with/without palmitate at different concentrations for 24 h. (B) mRNA expression and (C) protein release of inflammatory cytokines in BMM cultured with HG plus palmitate at 200 μM with or without Qi extract at different concentrations or vehicle. All genes were normalized to GAPDH and then to vehicle control. Data shown as mean ± SE (n=6). * p<0.05 compared with control

Fig. 2. High glucose/palmitate increases and Qi treatment reduces NF-κB activity in macrophages

(A) NF-κB p65 mRNA expression in bone marrow-derived macrophages (BMM) after treatment with normal glucose (NG), high glucose (HG), and HG/Pa with 200 μM palmitate (B) NF-κB p65 mRNA expression after treatment with HG/Pa and different concentrations of Qi extract. (C) NF-κB p65 protein levels under same conditions as for B and normalized to levels of loading control (β-tubulin). (D) Luciferase activity indicating NF-κB promoter activity under same conditions as for C and D. mRNA expression values normalized to GAPDH and then to vehicle control. Data shown as mean ± SE (n=6). *, **, and *** mean value significantly different from that for NG, HG, or HG/Pa groups, respectively (p<0.05).

Fig. 3. High glucose/palmitate increases and Qi treatment reduces Set7 levels in macrophages
 (A) Expression of Set7 mRNA in bone marrow-derived macrophages (BMM) treated with normal glucose (NG), high glucose (HG), or high glucose and palmitate (HG/Pa). (B) Set7 mRNA expression after treatment HG/Pa and different concentrations of Qi extract. (C) Set7 protein levels under same conditions as for B. (D) NF-κB p65 mRNA expression in BMM cells treated with (R)-PFI-2, a Set7 methyltransferase activity inhibitor (1–10 μM). mRNA expression normalized to GAPDH and then to vehicle (DMSO) control. mRNA expression normalized to GAPDH and then to vehicle control. Data shown as mean ± SE (n=3–6). *, **, and *** mean value significantly different from that for NG, HG, and HG/Pa groups, respectively (p<0.05). # mean value significantly different between NG and HG/Pa for same concentration of inhibitor, ## and ### mean values significantly different those for vehicle treated HG/Pa or HG/Pa plus 1 μM of (R)-PFI-2, respectively (p<0.05).

Fig. 4. High fat diet induces stable increase in Set7 expression than can be reduced by Qi extract Bone marrow cells from mice fed normal chow (lean) or high fat diet (HFD) differentiated to macrophages (BMM) for 6 days prior to experiments. (A) Expression of Set7 and (B) NF-κB p65 mRNA in BMM from HFD and lean mice. (C) mRNA of Set7 and (D) NF-κB p65 on BMM cells after culture in normal glucose without palmitate and with different concentrations of Qi extract. (E) Protein levels of both Set7 and NF-κB p65 under the same conditions as for C and D. (F) Expression of inflammatory cytokines in BMM from HFD mice incubated in normal glucose and different concentrations of Qi extract. All genes were normalized to GAPDH and then to vehicle control. Data are mean ± SE (n=6). * and ** mean value significantly different from that for lean and HFD groups, respectively (p<0.05).

Fig. 5. Diabetic environment increases and Qi extract decreases reactive oxygen species in macrophages

Reactive oxygen species (ROS) production in bone marrow-derived macrophages (BMM) was detected using CellROX deep red dye. (A) ROS production in BMM treated with normal glucose (NG), high glucose (HG), HG plus palmitate (HG/Pa) with/without Qi extract at different concentrations. (B) ROS production in BMM from mice fed normal chow (lean) or high fat diet (HFD) with/without Qi extract at different concentrations. Data are mean $\pm$ SE (n=6). *, **, ***, and **** mean value significantly different from that for NG, HG, HG/Pa, and control groups, respectively ($p < 0.05$).

Fig. 6. Qi treatment reduces expression of inflammatory cytokines in monocytes/macrophages isolated from wounds of diabetic mice

Inflammatory cytokine expression in monocytes/macrophages (CD11b⁺) isolated from diabetic wounds treated with vehicle control or Qi solution for 7 days. All genes were normalized to GAPDH and then to vehicle control. * p<0.05 compared with control (n=6).

Table 1

PCR primers

	Forward	Reverse
GAPDH	TCTGACGTGCCGCCTGGAGA	GGGGTGGGTGGTCCAGGGTT
IL-1 β	ATGCCACCTTTTGACAGTGATG	CAGGTCAAAGGTTTGGAAGCA
TNF- α	TTCCAGATTCTTCCCTGAGGT	TAAGCAAAAGAGGAGGCAACA
IL-6	GCTGGTGACAACCACGGCCT	GGCATAACGCACTAGGTTTGCCG
NF- κ B p65	GGGCCGGGAACGGGA	GGCTGTTTGTCCGAGGC
SET7	CCGTGGAAGGGCACCT	GGAGTAGGTGACAGTGCAGA