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A comparative study of the effects of quercetin and its glucuronide and sulfate metabolites on human neutrophil function in vitro

Sunita Suri [a,*], *Moira A. Taylor* [a], *Anne Verity* [a], *Sandra Tribolo* [b], *Paul W. Needs* [b], *Paul A. Kroon* [b], *David A. Hughes* [b], *Vincent G. Wilson* [a]

[a] *Centre for Integrated Systems Biology and Medicine, School of Biomedical Sciences, Medical School, University of Nottingham, Nottingham NG7 2UH, UK*

[b] *Phytochemicals and Health Programme, Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK*

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ABSTRACT

Exposure of neutrophils to either lipopolysaccharide (LPS) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) is associated with changes in the expression of cell adhesion molecules and elevation of intracellular calcium ions. Although dietary flavonoids are reported to possess anti-inflammatory properties, little is known regarding the effect of their metabolites. We have investigated the effects of quercetin and its major metabolites on LPS and fMLP-stimulated human neutrophils using concentrations comparable to those reported in feeding studies on human volunteers. The metabolite quercetin 3-glucuronide caused a significant reduction in fMLP-evoked calcium influx in human neutrophils (approximately 35%), while neither quercetin 3′-sulfate nor quercetin produced a similar change. Acute exposure of human neutrophils to LPS altered cell shape and surface expression of CD16, but neither of these events were significantly altered by quercetin, quercetin 3-glucuronide nor quercetin 3′-sulfate. In addition, LPS caused a fivefold up-regulation in the expression of β_2-integrin (CD11b/Mac 1) and a concomitant 70% down-regulation of L-selectin (CD62L) adhesion molecule expression in human neutrophils. Neither effect was altered by quercetin, quercetin 3-glucuronide or quercetin 3′-sulfate. In conclusion, we found that acute exposure to quercetin and quercetin 3′-sulfate does not affect either expression of cell adhesion molecules or the elevation of intracellular calcium ions in response to LPS and fMLP in human neutrophils. However, quercetin 3-glucuronide reduced fMLP-evoked calcium responses. While this study highlights that metabolites of quercetin may possess different biological properties, dietary ingestion of quercetin is unlikely to exert a major effect on neutrophil function in vivo.

* *Corresponding author*. Tel.: +44 115 823 0141; fax: +44 115 823 0142.
E-mail address: sunita.suri@nottingham.ac.uk (S. Suri).

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium; CD11b/Mac 1,β_2-integrin/membrane-activated complex-1; CD62L, L-selectin; DMSO, dimethylsulfoxide; FITC, fluorescein isothiocyanate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FS, forward scatter; LKS, low potassium salt; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PE, phycoerthythrin; Q, quercetin; Q3GlcA, quercetin 3-glucuronide; Q3′S, quercetin 3′-sulfate.

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1. Introduction

Neutrophils constitute the first line of defence during microbial infection. Early events in inflammation involve the recruitment of neutrophils to the site of injury or damage where changes in intracellular calcium, $[Ca^{2+}]_i$ can cause the activation of proinflammatory mediators from neutrophils including superoxide generation, degranulation, release of IL-8 and adhesion to vascular endothelium [1,2]. Due to the crucial dependence of calcium flux on the proinflammatory properties of neutrophils, the ability of activated neutrophils to restore calcium homeostasis is of prime importance. Stimulation of neutrophils with various agonists including the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) causes an increase in intracellular calcium that can be measured by the use of fluorescent Ca^{2+} indicator dyes such as Fluo-3 [3]. In addition, bacterial lipopolysaccharide (LPS), an essential component of the surface of Gram-negative bacteria [4] can directly affect neutrophil function by causing shape change as a prerequisite for cellular migration [5], activate neutrophils as measured by increases in the surface expression of CD16 (FcγRIII) [6] and modulate the expression of adhesion molecule receptors along with delaying neutrophil apoptosis [7].

Quercetin is a flavonoid found in large quantities in many plant foods, including apples, tea and onions, that forms a significant part of the daily dietary intake of polyphenols [8]. It has been the subject of numerous studies regarding its biological actions, chiefly because epidemiological evidence indicates that regular ingestion of fruit and vegetables is associated with decreased risk of cardiovascular diseases [9–13]. In humans, quercetin is rapidly absorbed from the gastrointestinal tract and metabolized to sulfate and glucuronide conjugates, but only slowly eliminated via the kidneys [8,14]. Several intervention studies with either onions or quercetin-containing capsules have established that the plasma levels of quercetin rarely exceed 0.1 μM, but the metabolites (sulfate and glucuronide) can be detected at 30-fold higher levels [15–18].

Numerous studies have reported the anti-inflammatory effects of dietary flavonoids in neutrophils. These have included effects of quercetin on reactive oxygen species [19], IL-6 production [20], and delayed apoptosis of neutrophils [21]. In addition, quercetin has been reported to suppress other rapid events in neutrophils including tyrosine phosphorylation [22], changes in surface expression of L-selectin (CD62L) and β_2-integrin (CD11b/Mac 1) [21] and the generation of inflammatory eicosanoids [23], but only with concentrations in excess of 10 μM. It appears that quercetin has the potential to alter neutrophil function, which may be important in modifying inflammatory events associated with the development of inflammatory diseases. With the exception of the study by de Pascual-Teresa et al. [24], all of the in vitro studies on neutrophils to date have focused on the action of quercetin alone and not on the action of its human metabolites.

In the present study we have compared the effect of quercetin, quercetin 3′-sulfate and quercetin 3-glucuronide against acute changes in human neutrophil function induced by LPS and fMLP to see whether $[Ca^{2+}]_i$ flux and the expression of the adhesion molecules CD62L and CD11b/Mac 1 are affected. In addition, we examined the effect of quercetin and its major human metabolites on neutrophil shape change and expression of CD16 following stimulation with LPS. These experiments have been undertaken with concentrations of the agents not exceeding those achieved by normal dietary intake of foods containing the flavonoid.

2. Materials and methods

2.1. Blood collection and neutrophil isolation

Venous peripheral blood was obtained from healthy human volunteers into polypropylene tubes containing EDTA as anticoagulant. Individual samples for whole blood studies were routinely kept at 37 °C for 45 min and incubated in the presence or absence of quercetin or quercetin metabolite prior to experimentation or immediately processed to isolate neutrophils. For the latter purpose, neutrophils were isolated using a sequential sedimentation and centrifugation method from individual blood samples. Firstly, blood was mixed with 6% dextran in 0.9% NaCl at a ratio of 4:1(v/v). Erythrocytes were allowed to settle for 30 min, then the supernatant containing plasma and cells was carefully drawn off into fresh polypropylene centrifuge tubes. After centrifugation at 100 × *g* for 10 min at 4 °C, the supernatant was discarded and the remaining cells were gently resuspended. Erythrocytes were lysed with 0.2% ice cold NaCl in a volume equivalent to the original volume of plasma and the tube was inverted and left for 1 min. Osmolarity was reconstituted with the same volume of 1.6% ice cold NaCl and the tube inverted. Cells were then centrifuged at 200 × *g* at 4 °C for 10 min. The cell pellet was resuspended in 5 ml low potassium salt (LKS) buffer supplemented with 1.8 mM calcium (LKS buffer: NaCl 118 mM, KCl 5 mM, $MgSO_4$ 0.8 mM, D-glucose 5.5 mM, Na_2CO_3 8.5 mM, BSA 0.1%, HEPES 20 mM, $CaCl_2$ 1.8 mM, adjusted to pH 7.4). Cell viability was >95%, assessed by trypan blue dye exclusion.

2.2. Flow cytometry

Flow cytometry was performed on a Beckman Coulter® Epics XL-MCL flow cytometer equipped with an argon laser (488 nm emission) able to excite fluorochromes emitting at 525 nm (FL1 channel: FITC, Alexa Fluor 488, Fluo-3) and 575 nm (FL2 channel: PE). The flow cytometer was connected to a XL data management workstation computer interfaced with System II™ software version 3.0 (Beckman Coulter). Neutrophils were recognised on the basis of forward angle light scatter (FS) and side angle light scatter (SS) which identified neutrophils and excluded dead cells and other cell types (lymphocytes and erythrocytes). Gates were drawn around the neutrophil population and concomitantly plotted as frequency histograms to depict the expression of CD16, CD11b/Mac 1 or CD62L. These were recorded as median forward scatter and median fluorescence. Kinetics of intracellular calcium mobilisation was evaluated in isolated neutrophil samples loaded with Fluo-3. Neutrophil populations were gated and concomitantly plotted as fluorescence against time.

2.3. Measurement of intracellular calcium mobilisation in human neutrophils

Isolated neutrophils suspended in LKS buffer were incubated with 10 μM quercetin, 10 μM quercetin 3-glucuronide (Q3GlcA) or 10 μM quercetin 3′-sulfate (Q3′S) and vehicle (DMSO for quercetin; H_2O for the metabolites) and maintained at 37 °C for 45 min followed by loading with 5 mM Fluo-3 for 20 min at 37 °C with occasional gentle tube inversion. Cells were then kept on ice. Fluo-3-loaded neutrophil cell fluorescence was measured by flow cytometry.

50 μl of the cell suspension was added to 450 μl LKS buffer (supplemented with 1.8 mM calcium), vortexed and immediately analysed by flow cytometry with a set data acquisition time of 100 s. Cells were gated by forward scatter and side scatter characteristics into monocytes, neutrophils and lymphocytes. For measurement of fMLP-agonist evoked increases in intracellular calcium we used a stopped-flow technique where after a 30-s run to determine baseline Fluo-3 fluorescence, cell acquisition was paused, 1 μM fMLP was added and flow acquisition resumed for the remaining 90 s. For each plot, rectangular analysis boxes were drawn over the time axis and divided into two segments. The lower segment before addition of agonist depicted normal unstimulated cells (time gate E, Fig. 2). The upper segment depicted stimulated cells with elevated intracellular calcium after the addition of agonist (time gate F). Lower and upper segments were drawn both before and after addition of agonist to illustrate the elevation of intracellular calcium over time (time gates D and G). The number of cells in each of the time gates was quantified and agonist response was measured as the percentage of cells showing an increase in intracellular calcium. Results are expressed as the percentage of cells showing an increase in intracellular calcium for each condition and shown as the mean ± S.E.M. of six investigations.

2.4. Shape change assay using CD16 marker expression

Whole blood (198 μl) that had been preincubated at 37 °C for 45 min with either 10 μM quercetin, 10 μM quercetin 3-glucuronide, quercetin 3′-sulfate or vehicle (DMSO for quercetin; H_2O for the metabolites) was further incubated at 37 °C for 30 min in the presence or absence of 1 μg/ml LPS. Cells were then stained with CD16-FITC antibody for 30 min on ice, followed by lysis of contaminating erythrocytes and fixation with Optilyse C for 30 min at room temperature. Lysed cells were diluted with an equal volume of PBS and left for 30 min before measurement of forward scatter and CD16-FITC by flow cytometry. Data were acquired for 5000 immunofluorescent gated $CD16^+$ neutrophil events. Results are expressed as the median forward scatter and fluorescence for each condition and shown as the mean ± S.E.M. of six to eight investigations.

2.5. Analysis of CD11b/Mac 1 and CD62L adhesion molecule expression

In a similar method to the CD16 marker expression assay, two aliquots of whole blood (198 μl each) that had been preincubated at 37 °C for 45 min with either 2 μM or 10 μM quercetin, and 10 μM quercetin 3-glucuronide or 10 μM quercetin 3′-sulfate and vehicle (DMSO for quercetin; H_2O for the metabolites) were further incubated at 37 °C for 30 min with 1 ng/ml or 1 μg/ml LPS. Cells were then stained either with CD11b/Mac 1-Alexa Fluor 488 antibody or CD62L-PE antibody for 30 min on ice, followed by lysis of contaminating erythrocytes and fixation with Optilyse C for 30 min at room temperature. Lysed cells were diluted with an equal volume of PBS and left for 30 min before measurement of forward scatter and CD11b/Mac 1-Alexa Fluor 488 and CD62L-PE immunofluorescence by flow cytometry. Data were acquired for at least 5000 immunofluorescent gated neutrophil events over 60 s. Results are expressed as the median forward scatter and fluorescence for each condition and shown as the mean ± S.E.M. of four investigations.

2.6. Reagents and antibodies

Quercetin, Dextran 500, lipopolysaccharide (from *E. coli* 0111:B4), *N*-formyl-methionyl-leucyl-phenylalanine and all other laboratory reagents unless otherwise specified were from Sigma–Aldrich (UK). Trypan blue dye was from Invitrogen, Paisley, UK. Quercetin metabolites (quercetin 3-glucuronide and quercetin 3′-sulfate) were chemically synthesised by previously described methods [25]. Fluo-3 was from Molecular Probes, Invitrogen, CD16-FITC was from Caltag, Paisley, UK and Optilyse C was from Beckman Coulter, High Wycombe, UK. CD11b/Mac 1-Alexa Fluor 488 antibody and CD62L-PE antibody were from BD Biosciences Pharmingen, Oxford, UK.

2.7. Data analysis

Data are presented as the mean ± S.E.M. from a number (n) of independent experiments each using cells from different donors. Graphs were plotted using Kaleidagraph software and statistical analysis was performed using GraphPad Prism software. Differences in mean forward scatter values for neutrophils or median fluorescence values were assessed by Student's paired two-tailed t-test. When there was more than one treatment condition, values were assessed by ANOVA followed by Dunnett's post hoc test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Concentration-dependent effects of fMLP and LPS on neutrophil forward scatter and intracellular calcium

In preliminary experiments, fMLP (0.1–100 nM) caused a concentration-dependent rise in the fraction of cells exhibiting elevated intracellular calcium (Fig. 1a) within 30 s of exposure to the stimulant. This response to fMLP was not sustained but declined towards baseline over a 3-min period. In contrast, LPS (10 ng/ml to 10 μg/ml) failed to mimic this response (Fig. 1a). Both fMLP and LPS caused a concentration-dependent increase in forward scatter of neutrophils in whole blood after 30 min (Fig. 1b), but fMLP was approximately 10-fold more potent than LPS. In subsequent experiments, fMLP was used to assess calcium-associated events in neutrophils, while LPS was used to monitor calcium-independent changes in neutrophil function.

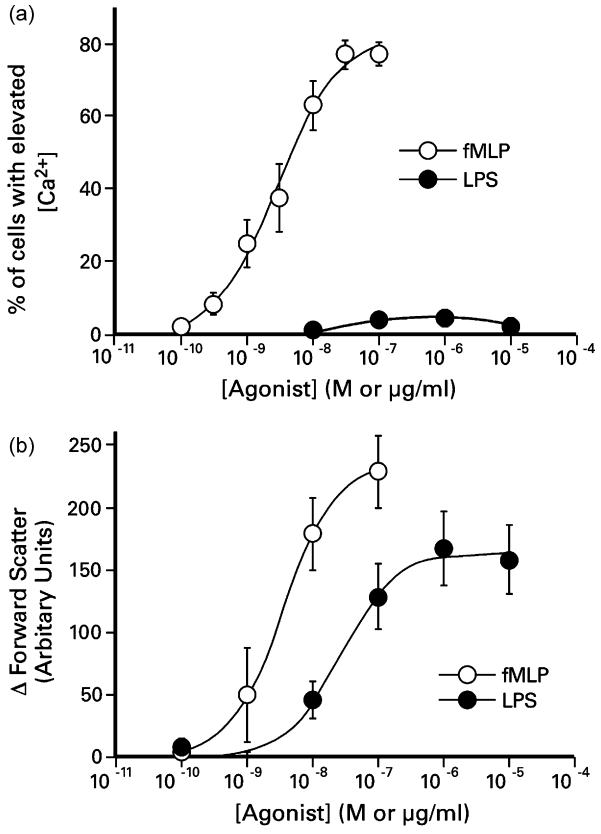

Fig. 1 – Effect of varying concentrations of fMLP and LPS on forward scatter in human neutrophils (a) ($n = 6$) and the percentage of cells with elevated $[Ca^{2+}]_i$ after the addition of fMLP ($n = 6$) or LPS ($n = 3$) in human neutrophils (b). Results shown are the mean ± S.E.M. of three to six investigations (as indicated).

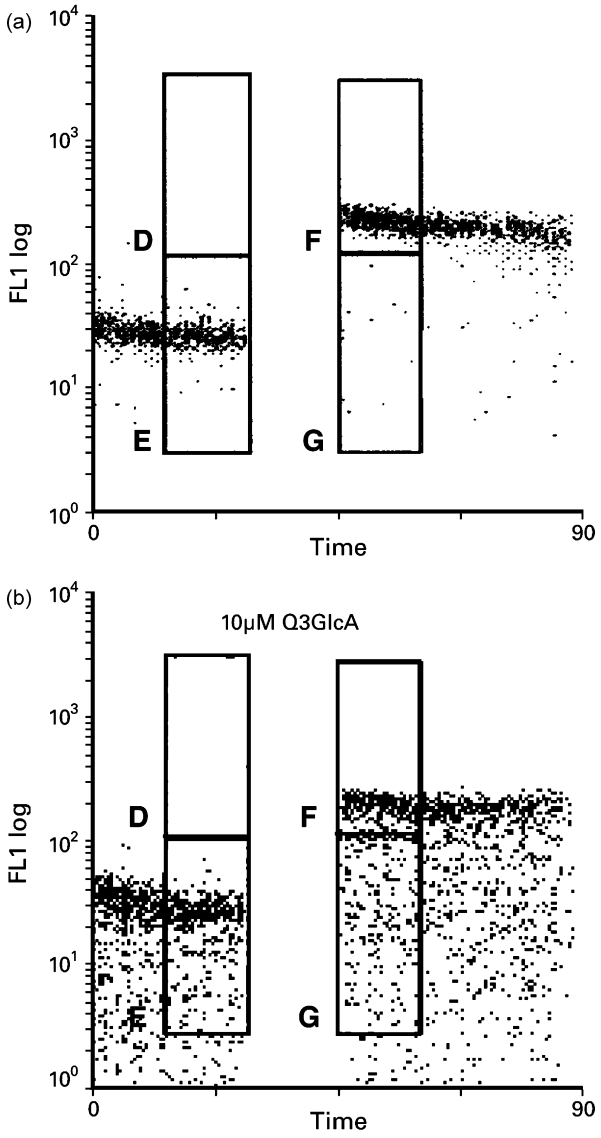

Fig. 2 – Representative flow cytometry fluorescence intensity time plots of fluo-3 loaded neutrophils. (a) Neutrophils incubated with vehicle, H_2O. (b) Neutrophils incubated with 10 μM quercetin 3-glucuronide (Q3Glc A). Resting cells were monitored for 30 s (resting state: box E) before stimulation with 1 μM fMLP (gap between boxes D/E: F/G) when flow analysis was recommenced (calcium-activated state: box F).

3.2. *Effect of quercetin and quercetin metabolites on fMLP-evoked calcium influx in Fluo-3-loaded human neutrophils*

Intracellular calcium in isolated neutrophils was measured by flow cytometry using the fluorescent calcium indicator Fluo-3. Fig. 2 illustrates representative flow cytometry data of fMLP-induced calcium uptake into neutrophils. After the addition of 1 μM fMLP, 73.7 ± 7.5% of cells ($n = 6$) showed an increase in intracellular calcium (Figs. 2a and 3). This effect was not significantly affected by prior exposure to 10 μM quercetin (Fig. 3). In contrast, quercetin 3-glucuronide caused a significant inhibition (47.0 ± 6.62%, $n = 6$) of the response to 1 μM fMLP (Figs. 2b and 3). Quercetin 3′-sulfate (10 μM) also reduced the response of 1 μM fMLP to 57.3 ± 7.67% ($n = 6$), but this did not reach statistical significance (Fig. 3).

3.3. *Effect of quercetin and quercetin metabolites on shape change and CD16 expression in LPS-stimulated human neutrophils*

Whole blood assays were used to determine the effect of LPS on neutrophils. Neutrophils were identified and gated by their higher forward scatter compared with monocytes and lymphocytes. The shape change of human neutrophils was measured as the percentage increase in forward scatter following stimulation with 1 ng/ml LPS or 1 μg/ml LPS for 30 min. Exposure to a higher concentration of LPS (1 μg/ml) for 30 min caused a significant dose-dependent increase of 22.6 ± 2.6% ($n = 6$) in forward scatter and a 1.48 ± 0.1-fold increase ($n = 6$) in CD16-FITC expression (Fig. 4a–d). Neither quercetin, quercetin 3-glucuronide nor 10 μM quercetin 3′-sulfate affected LPS-induced changes in forward scatter or surface expression of CD16 (Fig. 4a–d). Exposure to 1 ng/ml LPS caused a significant 8.3 ± 2.1% ($n = 6$) increase in forward scatter and a 1.18 ± 0.1-fold increase ($n = 6$) in CD16-FITC expression. Neither 10 μM quercetin, 10 μM quercetin

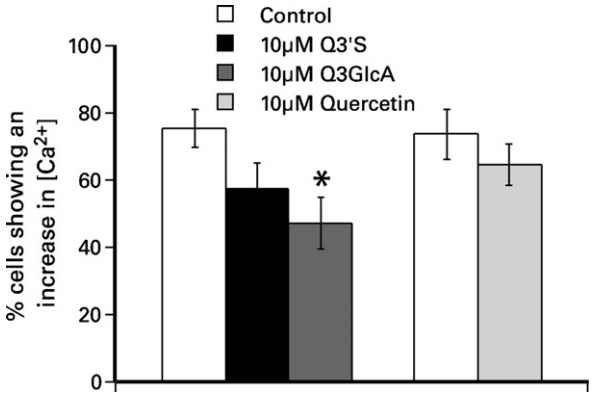

Fig. 3 – Effect of fMLP on $[Ca^{2+}]_i$ in fluo-3-loaded human neutrophils in the presence of external calcium. The percentage of cells showing an increase in change in $[Ca^{2+}]_i$ after the 30-s timed addition of 1 μM fMLP was calculated in human neutrophils that were preincubated with or without 10 μM quercetin, 10 μM quercetin 3-glucuronide (Q3GlcA) or 10 μM quercetin 3′-sulfate (Q3′S) (control for quercetin was DMSO). Results shown are the mean ± S.E.M. of six investigations. *($P < 0.05$) indicated a significant difference by Dunnett's multiple comparison test.

3-glucuronide nor 10 μM quercetin 3′-sulfate affected basal values of forward scatter or CD16 expression (data not shown).

3.4. *Effect of quercetin and quercetin metabolites on the surface expression of adhesion molecules in LPS-stimulated human neutrophils*

Whole blood CD11b/Mac 1 up-regulation and CD62L down-regulation assays were used to determine the effect of LPS on neutrophils from healthy volunteers. Representative flow cytometry histograms are shown in Fig. 5a and b illustrating the shifts in the fluorescence intensity peaks following exposure of neutrophils to LPS. CD11b/Mac 1 peaks shift to the right after exposure to LPS showing an up-regulation in the surface expression of the adhesion molecule (Fig. 5a) and simultaneously in parallel, a leftward shift was observed with CD62L peaks following shedding or down-regulation of the adhesion molecule (Fig. 5b). The presence of quercetin and its metabolites did not alter the expression patterns of CD11b/Mac 1 or CD62L.

CD11b/Mac 1 and CD62L expression were quantified and measured as the log fluorescence compared to control and shown as the mean of four individual investigations. Resting neutrophils that had not been exposed to LPS (untreated control) showed a low surface expression of CD11b/Mac 1 (Table 1; Fig. 5c and e) and a high surface expression of CD62L (Fig. 5d and f). After a 30-min exposure to 1 μg/ml LPS, CD11b/Mac 1 expression had significantly increased by 55.2 ± 0.9% (control; $n = 4$) (Table 1; Fig. 5c and e) and CD62L expression in contrast had concomitantly decreased by 67.0 ± 4.4% (control; $n = 4$) (Fig. 5d and f). Neither quercetin (2 μM and 10 μM), quercetin 3-glucuronide (10 (M), nor quercetin 3′-sulfate (10 (M) affected basal values of CD11b/Mac 1 (Table 1; Fig. 5c and e) and CD62L expression (Fig. 5d–f). Similarly the flavonoids did not alter LPS-induced up-regulation of CD11b/Mac 1 (Table 1; Fig. 5c and e) or the down-regulation of CD62L adhesion molecules (Fig. 5d and f). A submaximal concentration of LPS (1 ng/ml) caused a 42.2 ± 4.3% up-regulation of CD11b/Mac 1 that was also neither affected by quercetin, quercetin 3-glucuronide, nor quercetin 3′-sulfate (Table 1).

4. Discussion

Using isolated human neutrophils or neutrophils in whole blood, we undertook a comparative study of the effect of quercetin and two of its major human metabolites on fMLP and LPS-induced inflammatory changes. The key observation from this study is that quercetin was devoid of any effect on fMLP and LPS-changes in human neutrophils even when used at concentrations 30-fold greater (10 μM) than that known to occur in vivo [17,18]. Similarly, the principal metabolites found in man, quercetin 3′-sulfate and quercetin 3-glucuronide, were ineffective against LPS-induced changes. In this case, the maximum concentration employed was closer to (but did not exceed) the peak concentrations reported in vivo (3–10 μM) [17,18]. In effect, quercetin and its metabolites did not modulate acute events of inflammation in neutrophils to a significant degree. This conclusion is consistent with a recent report which shows that while daily ingestion of quercetin (1000 mg) increase plasma quercetin levels; it failed to modify exercise-induced changes in plasma levels of various cytokines, hormones and inflammation [26].

This is the first study of the effects of physiological ranges of quercetin and its metabolites on calcium flux and adhesion molecule expression in inflamed human neutrophils. Interestingly, quercetin-3-glucuronide (and to a lesser extent, the sulfate metabolite) significantly reduced fMLP-induced changes in intracellular calcium in human neutrophils. This finding highlights that metabolites of quercetin may possess biological activity that is different from the parent aglycone and, in this instance, has a selective effect on calcium-associated activation of neutrophils. Loke et al. [27] have shown that there are different structural requirements for antioxidant and anti-inflammatory activity of quercetin. They found that metabolic transformation amounting to specific structural requirements for anti-inflammatory activity of quercetin was not related to structural requirements for antioxidant activity in neutrophils [27]. Results from the current studies may also be related to metabolic transformation of the metabolite compounds from its parent aglycone form.

The rolling and activation of neutrophils along the endothelium and vascular junction during inflammation involves the expression of members of the adhesion molecule family including L-Selectin (CD62L) and β_2 integrin (CD11b/Mac 1) [28]. The CD11b/Mac 1 complex is one of three β_2 integrins, expressed primarily on neutrophils, monocytes/macrophages and NK cells [28]. fMLP is a potent chemoattractant known to activate neutrophils by specific G protein-coupled receptors linked to elevation of intracellular calcium [2] and to generate reactive oxygen species [29]. LPS causes a

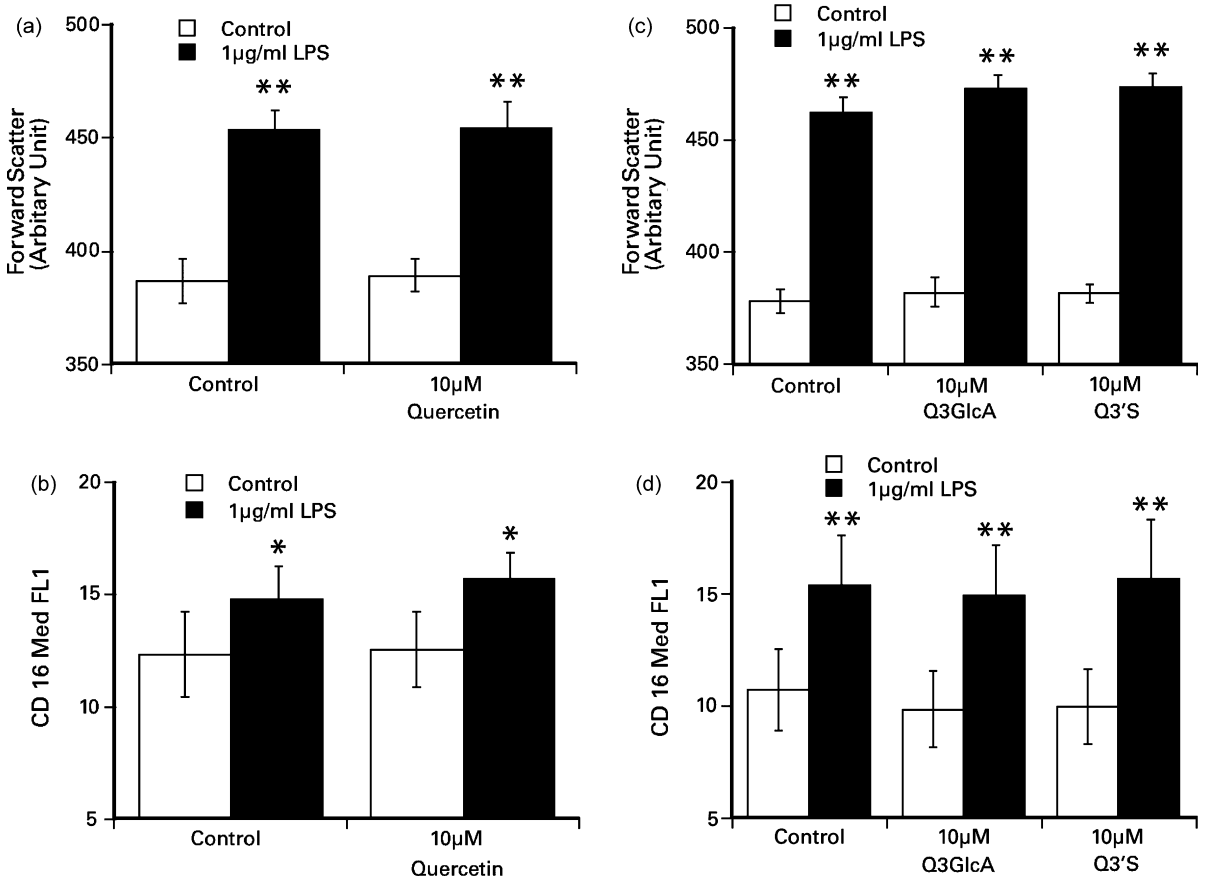

Fig. 4 – Effect of LPS on shape change in human neutrophils in the absence (control) or presence of 10 μM quercetin (a), or 10 μM quercetin 3-glucuronide (Q3GlcA), quercetin 3′-sulfate (Q3′S) (c) (control for quercetin was DMSO). Shape change was expressed as forward scatter in arbitrary units and shown as the mean ± S.E.M. of six to eight investigations. Effect of LPS on surface expression of CD16 in human neutrophils in the absence (control) or presence of 10 μM quercetin (b), or 10 μM quercetin 3-glucuronide, quercetin 3′-sulfate (d) (control for quercetin was DMSO). CD16-FITC was expressed as the log fluorescence (CD16 Med FL1) compared to control and shown as the mean ± S.E.M. of six to eight investigations. *($P < 0.05$) and **($P < 0.01$) indicated a significant difference from control or vehicle by Dunnett's multiple comparison test.

calcium-independent increase in the surface expression of CD11b/Mac 1 and CD16, involving the movement of secretory and special granules to the cell surface [30], together with a decrease (shedding) in the surface expression of CD62L.

Based on the changes in forward scatter in $CD16^+$ neutrophils, both fMLP and LPS were capable of activating the cells but, as judged by the rapid changes in cellular calcium (Fig. 2), appear to mediate these effects by different mechanisms. Results form the current studies suggest that quercetin and its metabolites have no effect on adhesion molecule expression in inflamed conditions in human neutrophils although intracellular calcium effects are decreased with quercetin 3-glucuronide, but not with quercetin-3′-sulfate or quercetin. These findings contrast with numerous other studies using supra-dietary concentrations of quercetin (>10 μM) which suggest that inhibitory effects on human isolated neutrophils in vitro involves multiple targets [20,21,31,32].

In order to better understand the beneficial effect of dietary flavonoids in inflammation and cardiovascular health, it is crucial to establish a functional correlate between in vitro and ex vivo findings. Importantly, as pointed out by Kroon et al. [33], the metabolites of the dietary flavonoid in vivo should be determined and then used in in vitro experiments at the appropriate concentration. For example, while there is extensive information on the properties of the quercetin on immune and haematopoietic cells in vitro, the significance is questionable, as the intake of quercetin in man is very low. However, other studies have been done on numerous cells of the immune and vascular system including endothelial cells [34,35], monocytes [27,35,36], lymphocytes [24], macrophages [37] and neutrophils [20,21,35,38] using physiological ranges of quercetin and its metabolites. There clearly are effects of quercetin and its metabolites on cells of the immune and vascular system that are seen at realistic physiological concentrations.

A novel monoclonal antibody targeting quercetin 3-glucuronide has identified that quercetin 3-glucuronide is taken up by foam-laden macrophages in human atherosclerotic lesions indicating that a target of the metabolites appears to be in activated macrophages in injured arteries [37]. In addition, using in vitro models, this study has shown that

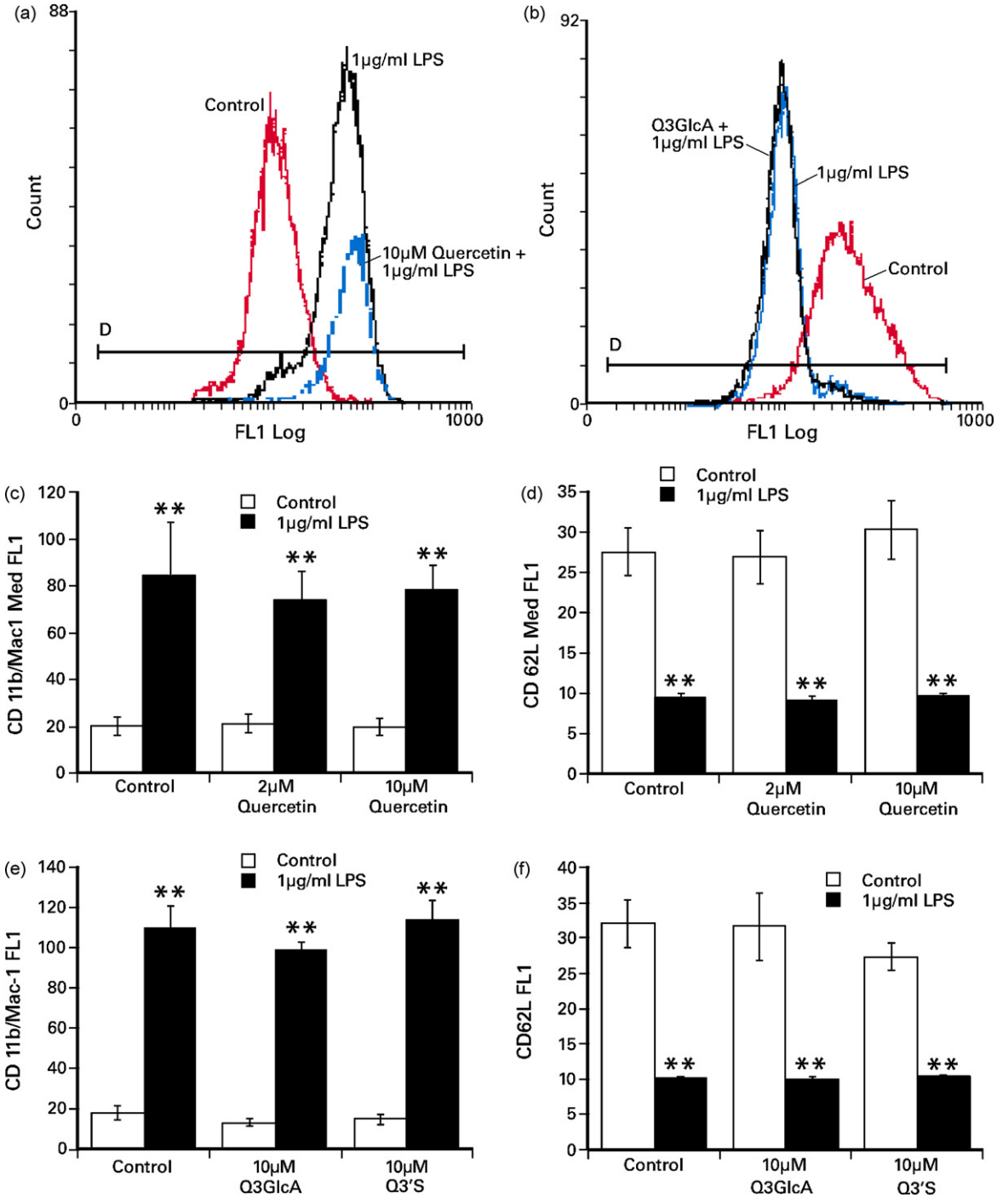

Fig. 5 – Effect of LPS on surface expression of CD11b/Mac 1 and CD62L in human neutrophils. Representative flow cytometry histogram plots illustrate CD11b/Mac 1 expression in human neutrophils (a) in untreated cells (control, red peak), in the presence of 1 μg/ml LPS (black peak) and in the presence of both 10 μM quercetin and 1 μg/ml LPS (blue peak). CD62L expression in human neutrophils is shown on the right plot (b) with untreated cells (control, red peak), in the presence of 1 μg/ml LPS (black peak) and in the presence of both 10 μM quercetin 3-glucuronide (Q3GlcA) and 1 μg/ml LPS (blue peak). Quantified histograms are illustrated in c–f. CD11b/Mac 1 and CD62L expression in human neutrophils in the absence (control) or presence of 2 μM or 10 μM quercetin (c and d), or 10 μM quercetin 3-glucuronide, quercetin 3′-sulfate (e and f) (control for quercetin was DMSO). CD11b/Mac1-Alexa Fluor 488 was expressed as the log fluorescence (CD11b/Mac1 Med FL1) compared to control and shown as the mean ± S.E.M. of four investigations. CD62L-PE was expressed as the log fluorescence (CD62L Med FL1) compared to control and shown as the mean ± S.E.M. of four investigations. **($P < 0.01$) indicated a significant difference from control or vehicle by Dunnett's multiple comparison test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1 – Dose effect of LPS on CD11b/Mac 1 expression in human neutrophils preincubated with 2 μM, 10 μM quercetin or 10 μM quercetin metabolites

	CD11b/Mac1 Med FL1		
	Untreated	1 ng/ml LPS	1 μg/ml LPS
Control (DMSO)	20.1 ± 4.1	57.4 ± 17.8	84.4 ± 22.7*
2 μM quercetin	21.2 ± 4.0	63.3 ± 13.5	74.1 ± 11.9*
10 μM quercetin	19.9 ± 3.8	56.5 ± 16.2	78.2 ± 10.4*
Control (H_2O)	18.1 ± 3.7	91.6 ± 16.3*	110.1 ± 11.0*
10 μM quercetin 3-glucuronide	13.1 ± 1.9	82.4 ± 9.5*	100.0 ± 3.4*
10 μM quercetin 3′-sulfate	14.7 ± 2.7	79.0 ± 11.6*	114.3 ± 9.2*

Values shown are the log fluorescence (Med FL1) and are the mean ± S.E.M. of four observations.
* ($P < 0.01$) indicated a significant difference from control for the metabolites (H_2O) or control for quercetin (DMSO) by ANOVA followed by Dunnett's multiple comparison test.

the metabolites are taken up by a murine macrophage cell line and subsequently converted (deconjugated) back to the aglycone form [37]. Another study has demonstrated the deglucuronidation of another flavonoid, luteolin monoglucuronide to its aglycone, luteolin during inflammation in neutrophils [38]. These observations are important because they indicate that it is feasible for conjugated quercetin metabolites to be converted to aglycones at certain (inflamed) sites, and that in vitro studies to assess the effects of quercetin aglycone have relevance if they are performed at appropriate (high nanomolar–low micromolar) concentrations.

An important factor in attempting to establish a functional correlate between in vitro and in vivo observations is the assay system adopted. In human volunteers, the ingestion of onions (115–300 mg quercetin content) failed to modify the expression of either COX-2 mRNA in human lymphocytes [24] or interfere with platelet aggregation ex vivo [39]. However, Hubbard et al. [40] demonstrated that ingestion of 500 mg quercetin-3-glucoside (in capsule form) by human subjects was associated with a significant inhibition of collagen-stimulated platelet aggregation ex vivo. In a separate in vitro study, these investigators presented evidence that quercetin inhibited collagen-induced platelet aggregation by an action involving several different kinases in the clotting cascade [41], including phosphoinositide 3-kinase and tyrosine kinase Syk. The advantage of the ex vivo assessment of human platelet function is that isolation of the cells and aggregation assay are relatively rapid, allowing direct comparison with in vitro studies [40,41]. From an experimental point of view, neutrophils have similar advantages to platelets in so much that ex vivo assessment of function can be undertaken quickly following isolation or even in whole blood.

Homeostasis of the immune system is normally tightly regulated. It must be remembered that neutrophils are essentially the first cells to arrive at the site of injury [2,42] and any excessive modulation of their response by normal dietary flavonoid intake may have undesirable consequences. Studies that have shown effects of flavonoids and their metabolites on cells that are recruited during the course of prolonged or chronic inflammation such as macrophages and endothelial cells are possibly a better target for health benefits of flavonoids. Compared to the existing literature regarding quercetin and neutrophil function [19,20,21,27,38], the relatively limited effects described here indicate that key cellular components in innate immunity are not affected by dietary quercetin.

We have shown that physiological ranges of quercetin and its major human metabolites do not affect shape change and adhesion molecule expression in neutrophils under inflammatory conditions. Quercetin 3-glucuronide reduces intracellular calcium influx when stimulated with fMLP and this may be attributed to a structural difference to its parent compound. Taken together, the principal conclusion from this study is that dietary ingestion of quercetin is unlikely to exert a major effect on neutrophil function in vivo.

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