Whey protein hydrolysates decrease IL-8 secretion in lipopolysaccharide (LPS)-stimulated respiratory epithelial cells by affecting LPS binding to Toll-like receptor 4

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Abstract
Whey proteins (WP) exert anti-inflammatory and antioxidant effects. Hyperbaric pressurisation of whey increases its digestibility and changes the spectrum of peptides released during digestion. We have shown that dietary supplementation with pressurised whey improves nutritional status and systemic inflammation in patients with cystic fibrosis (CF). Both clinical indices are largely affected by airway processes, to which respiratory epithelial cells actively contribute. Here, we tested whether peptides released from the digestion of pressurised whey can attenuate the inflammatory responses of CF respiratory epithelial cells. Hydrolysates of pressurised WP (pWP) and native WP (nWP, control) were generated in vitro and tested for anti-inflammatory properties judged by the suppression of IL-8 production in CF and non-CF respiratory epithelial cell lines (CFTE29o- and 1HAEo-, respectively). We observed that, in both cell lines, pWP hydrolysate suppressed IL-8 production stimulated by lipopolysaccharide (LPS) to a greater magnitude compared with nWP hydrolysate. Neither hydrolysate suppressed IL-8 production induced by TNF-α or IL-1β, suggesting an effect on the Toll-like receptor (TLR) 4 pathway, the cellular sensor for LPS. Further, neither hydrolysate affected TLR4 expression or neutralised LPS. Both pWP and nWP hydrolysates similarly reduced LPS binding to surface TLR4, while pWP tended to more potently increase extracellular antioxidant capacity. In conclusion: (1) anti-inflammatory properties of whey are enhanced by pressurisation; (2) suppression of IL-8 production may contribute to the clinical effects of pressurised whey supplementation on CF; (3) this effect may be partly explained by a combination of reduced LPS binding to TLR4 and enhanced extracellular antioxidant capacity.

Key words: Pressurised whey: Inflammation: IL-8: Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, and affects an estimated one in 3600 live births in the USA. CF lung disease, which causes the majority of CF morbidity and mortality¹,², is characterised by pulmonary recruitment of inflammatory cells, primarily neutrophils, which secrete cytokines, oxidants and other pro-inflammatory factors³. Airway secretions in patients with CF are characterised by consistently elevated levels of IL-8⁴, the major neutrophil chemoattractant⁵. This perpetuates neutrophil recruitment and leads to a state of chronic inflammation and tissue injury⁶, further aggravated during exacerbations of CF lung disease.

Respiratory epithelial cells have increasingly been recognised as key participants of inflammatory responses in the airways. IL-8 is secreted by epithelial cells in response to pro-inflammatory stimuli, such as TNF-α, neutrophil elastase⁷ or bacterial products (e.g. lipopolysaccharide (LPS))⁸,⁹. The CF respiratory epithelium may be intrinsically pro-inflammatory¹⁰. Modulation of lung inflammation is a recognised therapeutic target in CF¹¹, yet current approaches have had limited success or undesirable adverse effects¹²,¹³. In addition to pulmonary inflammation, malnutrition has an important prognostic role in CF, and its correction is a central therapeutic goal¹⁴.

Abbreviations: CF, cystic fibrosis; DUOX1, dual oxidase 1; FRAP, ferric-reducing antioxidant power; LAL, Limulus amebocyte lysate; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; nWP, native whey protein; pWP, pressurised whey protein; ROS, reactive oxygen species; WP, whey protein.

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Whey proteins (WP), a by-product of the cheese-making industry, possess nutritional benefits as a source of protein of high biological value\(^{15}\). Whey products and whey-derived peptides have demonstrated a number of anti-inflammatory effects\(^{16,17}\). These anti-inflammatory effects include decreased cytokine release in rodent models of ischaemia–reperfusion\(^{16}\) and exposure to LPS\(^{19}\). In addition, individual whey constituents, such as lactoferrin\(^{20,21}\) or glycomacropeptide\(^{22}\), and peptides released from these by pepsin–pancreatin hydrolysis\(^{23}\) exhibit anti-inflammatory effects, such as suppression of tissue neutrophilia\(^{24}\) or inhibition of inflammatory cytokine release\(^{25}\).

The beneficial effects of whey are further potentiated by hyperbaric pressurisation, which induces conformational changes in WP\(^{26}\). These conformational changes expose peptide sequences normally embedded in the hydrophobic core, rendering them more accessible to enzymatic digestion and exposing concealed sulfhydryl groups\(^{27}\). Our group has demonstrated that pressurisation of WP improves their \textit{in vitro} digestibility, promotes the release of novel peptides by gastrointestinal digestive enzymes and enhances anti-inflammatory effects\(^{28}\).

These \textit{in vitro} findings were also confirmed in clinical studies. Thus, a 2-week supplementation with pressurised whey increased the levels of glutathione, a crucial low-molecular antioxidant, in peripheral blood mononuclear cells\(^{29}\). Further, we have reported that a 1-month dietary supplementation with pressurised whey improved nutritional status and markers of systemic inflammation in patients with CF\(^{30}\).

The aims of the present study were to: (1) investigate the potential anti-inflammatory and antioxidant effects of pressurised and native WP (nWP) hydrolysates in CF and non-CF respiratory epithelial cells and (2) explore the mechanisms by which pressurised and native whey exert their beneficial anti-inflammatory effects. We hypothesised that peptides available through intestinal absorption of digested whey act on respiratory epithelial cells to decrease IL-8 responses to pro-inflammatory and pathogenic stimuli, and that this inhibition is enhanced by the pressurisation of whey.

**Materials and methods**

**Reagents**

Inpro 90 Whey Protein Isolate, purchased from Vitalus Nutrition, had the following composition: protein (dry basis) $\geq 93\%$; $\beta$-lactoglobulin 43–48\%; glycomacropeptide 24–28\%; $\alpha$-lactalbumin 14–18\%; bovine serum albumin 1–2\%; Ig 1–3\%; lactoferrin $<1\%$. Pepsin from porcine stomach mucosa, porcine pancreatic trypsin, bovine pancreatic chymotrypsin, chymotrypsin and peptidase (all prepared in phosphate buffer, pH 7.0; enzyme:substrate ratios 1:200, 1:87 and 1:120) and pH was adjusted to 7.4, and the second-stage digestion was conducted for 60 min with pepsin, chymotrypsin and peptidase (all prepared in phosphate buffer, pH 7.0; enzyme:substrate ratios 1:200, 1:87 and 1:120, respectively). Afterwards, the enzymes were inactivated by the addition of $10\text{m}\text{-NaOH}$ (final pH 10.5).

**Isolation of peptides**

After digestion, pWP and nWP hydrolysates were subjected to ultrafiltration to remove high-molecular weight peptides, which are unlikely to be absorbed through the intestinal mucosa. A membrane filter with a molecular weight cut-off of 10 kDa (Millipore) was used in a stirred ultrafiltration membrane reactor (Amicon Ultrafiltration Cell, model 8050); the filtration was allowed to proceed at $4\text{°C}$ under a $N_2$ gas pressure of 40 pounds per square inch (psi). The peptides obtained through \textit{in vitro} digestion and filtration had an average molecular weight of less than 1 kDa (data not shown), which corresponds to the size of the peptides likely to be intestinally absorbed \textit{in vivo}\(^{32,33}\).

**Cell culture and experimental studies**

Immortalised human respiratory cell lines utilised in the present study were CFTE29o-\(^{\text{D}}\), which bears the most common CF mutation ($\Delta F 508$), and IHAEo- (non-CF cell line). Both cell lines were a kind gift from Dr D. Gruenert (University of California, San Fransisco). Basal cell culture conditions were as described in our previous studies\(^{34,35}\). For viability...
and IL-8 production experiments, cells were seeded at a density of $5 \times 10^5$ cells/ml in twenty-four-well cell culture plates and grown for 24 h in minimal essential medium supplemented with l-glutamine, penicillin/streptomycin and 10% heat-inactivated fetal bovine serum. The next day, cells became 100% confluent and were pre-incubated for 1 h with pWP or nWP hydrolysates (0–1000 μg/ml) in antibiotic-free minimal essential medium supplemented with 2% fetal bovine serum. Cells were then stimulated with LPS, TNF-α or IL-1β, as described in the Results section, along with a fresh preparation of hydrolysates. After incubation for indicated amounts of time, cell viability and IL-8 secretion were assessed as described below. For analysis of total glutathione, cells were seeded in 35 mm culture dishes, cultured and treated as above, and collected for glutathione analyses with a slight modification of our previous protocol.

Cell viability

Cell viability was assessed using the MTT assay, based on the reduction of MTT reagent into purple formazan crystals by viable metabolically active cells. Briefly, cells were washed with PBS and incubated with MTT solution (0.5 mg/ml) for 3 h. The formazan crystals were then dissolved in lysis solution containing 0.4 M-HCl and 100% isopropanol, and absorbances were measured at 540 nm. Backgrounds were assessed at 600 nm and subtracted from the 540 nm values. Cell viability was expressed as a percentage of untreated control.

IL-8 analysis

IL-8 secretion in cell-free supernatants was assessed with a commercial human IL-8 ELISA kit (BD Biosciences) according to the manufacturer’s instructions.

Limulus amebocyte lysate assay

LPS activity was assayed by the chromogenic Limulus amebocyte lysate (LAL) assay, using a commercial kit (Endochrome). Briefly, pWP hydrolysates at concentrations 500–2000 μg/ml in endotoxin-free water were incubated with LPS (2.5 μg/ml) for 30 min. Thereafter, 50 μl of the LPS hydrolysate solution were placed into ninety-six-well microtitre plates with an equal volume of LAL reagent and incubated at 37°C for 7 min. An addition of 100 μl of chromogenic substrate solution and further incubation for 5 min led to the development of a yellow colour. The reaction was stopped by the addition of 20% acetic acid, and absorbances were read on a microplate reader at 405 nm.

Analysis of Toll-like receptor 4 surface expression

Cells were seeded in 60 mm culture dishes and cultured until confluent, then treated with pWP hydrolysat (1000 μg/ml) and stimulated with LPS as above. After stimulation for 24 h, cells were washed with 50 mM-EDTA, washed with PBS and incubated with blocking solution (PBS supplemented with 1% bovine serum albumin) for 30 min. Cells were then washed, incubated with anti-TLR4 rabbit antibody for 1 h, washed again and incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody for 45 min. A FACScalibur flow cytometer coupled with CELLQuest software (BD Biosciences) was used to analyse cell fluorescence. Cell debris was excluded using forward and side scatter characteristics, and cell fluorescence documented in the FL1 (green) channel. Results are expressed as a percentage of cells with TLR4 surface expression.

Analysis of lipopolysaccharide binding

LPS binding to surface TLR4 was assessed with a modification of a published protocol. Briefly, cells were seeded in 60 mm culture dishes and pretreated with 1000 μg/ml of pWP hydrolysate. Cells were then incubated with 50 μM-EDTA, washed with 1000 μg/ml of hydrolysate and 2.5 μg/ml of FITC-LPS for 30 min at 4°C. FITC-LPS was dissolved in PBS (10% fetal bovine serum) as a source of LPS-binding protein. After washing, cells were resuspended in PBS, and cell fluorescence was analysed with a FACScalibur flow cytometer. Results are expressed as a percentage of cells with bound FITC-LPS.

Analysis of antioxidant capacity of cell-free supernatants

CFTE290o- and 1HAEo- cells were incubated with pWP or nWP hydrolysates for 24 h, following which cell-free supernatants were collected. The antioxidant capacity of these supernatants was assessed using the ferric-reducing antioxidant power (FRAP) assay. This assay is based on the reduction of the Fe³⁺–2,4,6-tripyridyl-3-triazine (TPTZ) complex to the ferrous (Fe²⁺) form. Briefly, FRAP reagent was prepared with sodium acetate buffer (300 mM), 2.5-mTPTZ solution (10 mM in 40 mM-HCl) and 2.5-mll ferric chloride solution (20 mM in double-distilled water) in a 10:1:1 ratio, respectively. Cell-free supernatants were incubated with FRAP reagent for 60 min, at a 1:20 ratio. A standard curve was constructed with bovine serum albumin, and absorbances were measured at 593 nm.

Analysis of intracellular total glutathione concentration

Glutathione was quantified using the enzymatic kinetic assay, adopted for a Cobas Mira S chemistry analyser. Reagent concentrations were from the previously published protocol. Protein content, measured using the bicinchoninic acid protein assay kit (Pierce) according to the manufacturer’s instructions, was utilised to normalise glutathione levels.

Statistical analysis

Data are presented as means with their standard errors. At least three independent experiments were conducted for each study. For cell culture assays, results were compared by one- or two-way ANOVA for each cell line, with Tukey’s post hoc test to determine statistically significant differences between the control and treatment groups. A P value of less than, or equal to, 0.05 was considered as significant. Statistical
analyses were performed using Sigma Stat version 2.03 (Systat Software, Inc.).

Results
Effects of whey protein hydrolysates on cell viability
We first verified that pWP or nWP hydrolysates are well tolerated over a wide range of concentrations by the respiratory epithelial cell lines. This was tested using the MTT assay, which showed that both pWP and nWP hydrolysates at concentrations of 12.5–1000 µg/ml were well tolerated by both cell lines (see Fig. S1, available online).

IL-8 secretion stimulated by lipopolysaccharide and TNF-α
The CF respiratory epithelium is exposed to bacterial products and pro-inflammatory cytokines. To exemplify these, CFTE29o- and 1HAEo- cells were incubated with LPS (2.5 µg/ml) and TNF-α (1 ng/ml) for 24 h to stimulate IL-8 secretion. Both stimuli significantly up-regulated IL-8 secretion in both cell lines; however, LPS was a weaker stimulus. Specifically, CFTE29o- and 1HAEo- cells up-regulated IL-8 in response to LPS by 3.8- and 5.7-fold, respectively, relative to their basal IL-8 secretion (P < 0.05 v. basal, both cell lines; Fig. 1(a)). In comparison, TNF-α stimulation led to a 21.2 (CFTE29o-) and 10.0 (1HAEo-) fold increase in IL-8 secretion (P < 0.05 v. basal, both cell lines; P < 0.01 v. LPS, both cell lines, data not shown). Since a 24 h exposure to TNF-α was overly hyperinflammatory, a shorter, 1 h exposure to this stimulus was tested. After the 1 h stimulation, the stimulus was removed, and cells were incubated for 23 h afterwards, at which point supernatants were collected for IL-8 ELISA. A shorter TNF-α stimulation resulted in a more modest IL-8 response, with CFTE29o- and 1HAEo- cells responding, respectively, more and less robustly than following 24 h exposure to LPS (CFTE29o-: 6.3-fold over basal; 1HAEo-: 2.1-fold over basal; P < 0.05 v. basal, both cell lines; P < 0.01 v. LPS, both cell lines, data not shown). Although these IL-8 responses were still significantly different from those following LPS stimulation, they were within the same order of magnitude of the response elicited by LPS. Therefore, in subsequent experiments, potential suppressing effects of WP hydrolysates were tested in respiratory epithelial cells stimulated with LPS for 24 h and TNF-α for 1 h.

Effects of whey protein hydrolysates on IL-8 secretion
The potential anti-inflammatory effects of pWP and nWP hydrolysates were first tested in respiratory epithelial cells under unstimulated conditions. Basal secretion of IL-8 in either cell line was not significantly affected by pWP or nWP hydrolysates, up to a concentration of 1000 µg/ml, although a trend towards reduced secretion was noted with pWP hydrolysates (see Fig. S2, available online). The effects of WP hydrolysates on the stimulated production of IL-8 were then evaluated. In both cell lines, the up-regulation of IL-8 by LPS was partially reverted by pretreatment with WP hydrolysates, and more so with pWP hydrolysate (Fig. 2). Specifically, in CFTE29o- cells, 500 and 1000 µg/ml of pWP hydrolysate significantly suppressed the LPS-stimulated IL-8 by 40 and 48%, respectively (P < 0.05 v. LPS alone, both comparisons; Fig. 2(a)). In contrast, the suppression caused by nWP was more modest (only 34% caused by nWP hydrolysate at a dose of 1000 µg/ml). While the latter decrease was still statistically significant (P < 0.05 v. LPS alone), it also tended to be lower when compared with the suppression by pWP at a

![Fig. 1. Stimulation of IL-8 secretion in CFTE29o- (■) and 1HAEo- (□) cell lines. Cells were stimulated with (a) 2.5 µg/ml of lipopolysaccharide (LPS) for 24 h, (b) 1 ng/ml of TNF-α for 1 h followed by 23 h incubation in stimulus-free culture medium, (c) 50 µg/ml of IL-1β for 24 h, (d) 50 pg/ml of IL-1β for 1 h followed by 23 h incubation in stimulus-free culture medium. IL-8 concentrations were assessed in cell supernatants by ELISA. IL-8 secretion in stimulated cells was expressed as a fold increase over that of basal cells. Values are means of three or more independent experiments, with their standard errors represented by vertical bars. * Mean value was significantly different relative to basal IL-8 secretion (P < 0.05).](https://www.cambridge.org/core/asset/10.1017/S0007114512004655/fig_1)
The LPS pathway upstream of NF-kB-activating kinases, which is the presumed target for WP hydrolysates, overlaps with the IL-1β pathway at MyD88, i.e. immediately after extracellular ligand binding to their respective receptors (Fig. 3). Should the anti-IL-8 effects of WP hydrolysates occur at the level or downstream of MyD88, then WP hydrolysates would suppress IL-8 stimulated by IL-1β. To test this, and to begin elucidating the possible mechanisms by which WP hydrolysates exert their anti-inflammatory effect, the following studies were devised.

Since we observed that 24 h stimulation with TNF-α leads to the overproduction of IL-8, we first tested IL-8 responses from cells stimulated with 1 ng/ml of IL-1β for 1 or 24 h. As in our previous studies (34, 35), we observed that IL-1β stimulation results in a very robust up-regulation of IL-8 secretion in CFTE290o-cells (71.8- and 15-fold for the 24 h and 1 h exposure, respectively, data not shown) and in 1HAEo- cells (51.5- and 12.3-fold, respectively, data not shown). Subsequently, the dose of IL-1β was decreased to 50 pg/ml in order to elicit a response comparable with that obtained with LPS stimulation. The 24 h exposure of CFTE290o- and 1HAEo- cells to 50 pg/ml of IL-1β resulted, respectively, in a 7.9- and 21.1-fold increase in IL-8 production (P < 0.05 v. basal; Fig. 1(c); P < 0.05 v. stimulation with LPS). In contrast to stimulation with LPS, neither WP hydrolysate, applied as above, affected IL-1β-induced IL-8 secretion (see Fig. S4, available online).

We also tested whether WP hydrolysates would be more effective against the 1 h exposure to 50 pg/ml of IL-1β. The 1 h stimulation was followed by the removal of IL-1β and the incubation of cells for 23 h in the stimulus-free culture medium, which, similar to a shorter TNF-α stimulation, led to a less pronounced increase in IL-8 secretion (4.4- and 4.2-fold in CFTE290o- and 1HAEo- cells, respectively; P < 0.05 v. basal; Fig. 1(d); P > 0.1 v. stimulation with LPS).

Still, WP hydrolysates were not effective against this weaker IL-1β stimulation (see Fig. S4, available online). This suggested that the effect of WP hydrolysates on LPS-stimulated IL-8 secretion was either due to the direct interaction with LPS, or occurred at the level of LPS binding to TLR4, the cellular sensor for LPS. In order to explore this hypothesis, the effects of WP hydrolysates on LPS activity, cell surface TLR4 expression and LPS–TLR4 binding were studied. In some of these experiments, only pWP was tested as the more potent of the whey hydrolysates.

**Lipoplycosecharide-neutralising activity of pressurised whey protein hydrolysate**

Potential direct LPS-neutralising activity of WP was tested using a quantitative chromogenic LAL assay. Inhibition of LPS-induced LAL activation by WP hydrolysates would indicate binding and neutralisation of the biological effects of LPS. No inhibition of LPS activity by 1000 μg/ml of pWP hydrolysate was observed, ruling out a direct interaction between WP hydrolysates and LPS (see Fig. S5, available online). Further, even a 2000 μg/ml concentration of the hydrolysate did not inhibit LPS-induced activation of the LAL (see Fig. S5, available online).
Effect of whey protein hydrolysates on the surface expression of Toll-like receptor 4

We next evaluated whether WP hydrolysates would affect TLR4 expression on the cell surface, as the down-regulation of TLR4 is likely to affect the magnitude of the LPS-stimulated IL-8 response. Flow cytometry was used to assess the surface expression of TLR4 in response to 24 h stimulation with LPS alone or in combination with pWP hydrolysate (1000 μg/ml). Neither LPS nor its combination with pWP hydrolysate affected surface TLR4 expression in either cell line (see Fig. S6, available online).

Effect of whey protein hydrolysates on lipopolysaccharide binding to surface Toll-like receptor 4

As neither LPS activity nor TLR4 cell surface expression was affected by pWP hydrolysate, we next examined the effect of WP hydrolysates on LPS binding to TLR4. Cells were pre-treated for 1 h with pWP or nWP hydrolysates (1000 μg/ml), and the ability of FITC-labelled LPS to bind to cell surface TLR4 was assessed by flow cytometry. Treatment with pWP hydrolysate resulted in a significant reduction in cell surface-bound FITC-LPS in both CFTE290- and 1HAEo- cells (27.3 and 31.5%, respectively; *P < 0.05* v. LPS alone; Fig. 4(a)).
DUOX1-induced ROS production, thereby affecting IL-8 secretion, and whether pWP hydrolysate would exhibit a greater ROS-suppressing potential. We first attempted to quantify intracellular ROS production directly in cells supplemented with WP hydrolysates. Unfortunately, this measurement was not reliable due to high inter-well variability (data not shown). To circumvent this limitation, we then evaluated extracellular H$_2$O$_2$ production indirectly, using the FRAP assay of cell culture medium following the 24h exposure of cells to pWP and nWP hydrolysates. In both cell lines, treatment with pWP and nWP hydrolysates (500 or 1000 µg/ml each) resulted in a dose-dependent increase in FRAP, suggesting an enhanced H$_2$O$_2$-scavenging capacity of extracellular microenvironment (Fig. 5). In both CFTE29o- and 1HAEo- cell lines, pWP hydrolysate tended to increase this antioxidative capacity of the cell culture medium more potently than nWP, although this did not reach statistical significance (Fig. 5).

Effect of lipopolysaccharide and/or whey protein hydrolysates on intracellular total glutathione concentration

Whey hydrolysates may also increase the intracellular antioxidant status and thus inhibit stimulated IL-8 responses (Fig. 6). To examine a potential effect of WP hydrolysates on changes in intracellular antioxidant status, intracellular total glutathione concentrations were studied. Whey, a mixture of cysteine-rich proteins, has been shown to affect

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in intracellular levels of glutathione, a crucial low-molecular weight antioxidant, which, in turn, has been reported to influence oxidant-sensitive pro-inflammatory transcription factors. Neither LPS nor WP hydrolysates had any significant effect on intracellular total glutathione concentrations (data not shown).

**Discussion**

The major original finding from the present study is that in vitro pretreatment of CF and non-CF respiratory epithelial cells with low-molecular weight peptide and amino acid products (<1kDa) from WP digestion significantly suppresses LPS-induced IL-8 secretion. The present findings are a first demonstration that whey-derived peptides can down-regulate the LPS-induced inflammatory response, which adds to previous findings showing the immunomodulatory effects of WP in animal models and in vitro. As some food-derived peptides may interact with cell surface receptors, we deemed it plausible that inhibition of LPS activity by WP hydrolysates could involve the inhibition of TLR4 activation. Indeed, the present results show that binding of LPS to TLR4 was decreased in the presence of WP hydrolysates. Lee et al. proposed that a direct inhibition of the binding of LPS to monocytes by lactoferrin is the mechanism by which lactoferrin feeding of neonatal piglets exerted anti-inflammatory effects following intravenous exposure to LPS. A variety of anti-bacterial peptides including whey-derived peptides have been shown to neutralise LPS via direct binding to the lipid A portion of LPS. Since the lipid A portion of LPS is responsible for both TLR and LAL activation, the lack of the effect of WP hydrolysates on LAL activation indicates that WP hydrolysates do not inactivate LPS. Taken together, our data suggest that the inhibitory effect of WP hydrolysates was most probably due to a direct interaction of the WP hydrolysates with the receptor, thereby preventing LPS recognition. We cannot eliminate, though, that WP hydrolysates also interfere with the recognition of LPS by CD14 or LPS-binding protein, events which are necessary for TLR4 activation.

Another possible mechanism by which WP hydrolysates may have modulated the inflammatory response is via improved antioxidant status either by provision of hydrogen-donating peptides or by contributing amino acids with antioxidant capacity, such as cysteine, tyrosine or tryptophan. Redox status is a known modulator of the inflammatory response, as oxidative stress up-regulates the production of inflammatory cytokines in vitro. WP have antioxidant properties in vivo and in vitro, and whey-derived peptides have been shown to exert free radical-scavenging activities both in vitro and in vivo. Moreover, the NADPH oxidase homologue DUOX1, in addition to its role in host defence via generating extracellular H$_2$O$_2$, has been attributed a role in cell signalling resulting in IL-8 secretion. It has also been shown that ROS production by DUOX1 is necessary for such signalling to occur. In that regard, we assessed the antioxidant capacity of cell culture medium following treatment of cells with WP hydrolysates, as an indirect indicator of extracellular H$_2$O$_2$ production. The present results show that WP hydrolysates significantly increased the antioxidant capacity of cell culture.
medium as assessed by the FRAP assay, suggesting an additional mechanism by which WP hydrolysates may exert their IL-8-suppressing effects. This antioxidant effect was somewhat enhanced with pressurisation of whey.

We did not find any effect of either LPS or WP hydrolysates on intracellular total glutathione concentrations. It is possible that either LPS or WP hydrolysates could have altered the relative proportions of oxidised and reduced glutathione, without affecting total glutathione concentrations, or altered intracellular redox status. These measurements would have contributed to a clearer understanding of these potential mechanisms. Nonetheless, we speculate that the superior effect of pWP hydrolysates in suppressing LPS-stimulated IL-8 responses is due to a combination of interference with LPS binding to TLR4 and altered redox status.

Previous results\(^{(28)}\) demonstrate that pressurisation results in qualitatively different peptide profiles from those obtained from native whey digestion as opposed to an increased number of available peptides. As pressurisation of WP potentiated the inhibitory effect of hydrolysates on the IL-8 response to LPS, as well as the enhancement of cell culture medium antioxidant capacity, this probably reflects a profile of relatively greater amounts of immunomodulatory and antioxidant peptides generated from the hydrolysis of pressurised WP. In that regard, hyperbaric pressure treatment of WP induces changes in their secondary and tertiary structures, exposing hidden and otherwise unavailable peptide sequences to enzymatic digestion. Indeed, WP are highly resistant to enzymatic digestion\(^{(63)}\), and undigested WP (namely β-lactoglobulin, α-lactalbumin, Ig and lactoferrin) have been found in the intestinal lumen\(^{(64)}\). Increasing the susceptibility of WP to digestion via pressurisation may alter the spectrum of absorbable peptides to increase the availability of bioactive peptides for intestinal absorption.

To our knowledge, the present findings demonstrate for the first time that whey-derived peptides could have an impact on inflammatory responses involving LPS-mediated IL-8 release and adds to clinical work showing a trend to IL-8 down-regulation with pressurised whey supplementation in CF patients, in addition to enhanced nutritional status\(^{(30)}\). There is increasing evidence that Gram-negative bacterial infections that lead to LPS-induced IL-8 release can exacerbate the inflammatory responses in chronic lung diseases such as CF\(^{(65)}\) and thereby contribute to lung pathophysiology. Although more studies are needed, the present study provides new insight and opens new avenues of research into the potential utilisation of WP in pro-inflammatory conditions involving the overproduction of IL-8 such as CF, particularly in relation to bacterial-induced inflammation. Effective suppression of IL-8 overproduction is a valid therapeutic target in CF\(^{(11)}\). Oral corticosteroids have unacceptable adverse side effects, inhaled corticosteroids have yet to prove long-term efficacy and high-dose ibuprofen has not gained popularity due to its varying bioavailability and potential adverse effects\(^{(12,13)}\). The IL-8-suppressing effects of WP hydrolysates demonstrated herein add an interesting possible therapeutic dimension to WP as a nutrition-based adjuvant to conventional therapy towards the restoration of a homeostatic anti-inflammatory balance in CF.

In conclusion, we demonstrate that hydrolysates of WP suppress LPS-stimulated IL-8 secretion \textit{in vitro} by affecting LPS binding to its receptor, TLR4, and by promoting enhanced extracellular antioxidant capacity. Pressurisation of whey tends to potentiate the latter mechanism, leading to a higher IL-8-suppressing activity.

**Supplementary material**

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114512004655

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