A Bovine Whey Protein Extract Can Enhance Innate Immunity by Priming Normal Human Blood Neutrophils¹–³

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Abstract

Bovine milk-derived products, in particular whey proteins, exhibit beneficial properties for human health, including the acquired immune response. However, their effects on innate immunity have received little attention. Neutrophils are key cells of innate defenses through their primary functions of chemotaxis, phagocytosis, oxidative burst, and degranulation. A whey protein extract (WPE) purified from bovine lactoserum was evaluated for its direct and indirect effects on these primary functions of normal human blood neutrophils in vitro. Although WPE had no direct effects on primary functions, a 24-h pretreatment of neutrophils with WPE was associated with a significant and dose-dependent increase of their chemotaxis, superoxide production, and degranulation in response to N-formyl-methionine-leucine-phenylalanine, as well as of their phagocytosis of bioparticles. The pretreatment increased the surface expression of CD11b, CD16B, and CD32A receptors. The major WPE protein components β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) were the main active fractions having an additive effect on human neutrophils that became more responsive to a subsequent stimulation. This effect on NADPH oxidase activity was associated with translocation of p47phox to plasma membrane. Glycomacropeptide, a peptide present in measurable amounts in WPE products, was able to enhance the individual effect of β-LG or α-LA on neutrophils. The present data suggest that WPE, through β-LG and α-LA, has the capacity to enhance or “prime” human neutrophil responses to a subsequent stimulation, an effect that could be associated with increased innate defenses in vivo. J. Nutr. 139: 386–393, 2009.

Introduction

Caseins and whey proteins are the 2 major protein fractions of cow milk. Whey proteins are separated from casein curds during the cheese-making process. The major proteins present in bovine whey come from the mammary gland that secretes β-lactoglobulin (β-LG), α-lactalbumin (α-LA), and glycomacropeptide (GMP), and from serum, like IgG1 and IgG2, IgA, IgE, and IgM and albumin. Besides their use in functional foods, whey protein products, and more specifically whey protein-derived products, have been shown to be efficient in certain pathologies. For instance, whey proteins inhibited gastric ulcerative lesions induced by ethanol or indomethacin, inhibited chemical-induced malignancy in mice, improved bone loss of ovariectomized rats, and reduced hyperglycemia in type 2 diabetic patients (1–5). Moreover, in vitro and in vivo studies have demonstrated modulation of immune functions by several whey protein-derived products (6,7). As examples, β-LG, the most abundant protein in whey (55–65% of total whey proteins), stimulates the proliferation of murine spleen cells and lamina propia lymphocytes (8,9). It is also useful to stress that researchers have shown that probiotics expressing β-LG can be used to manage food allergy (10). The 2nd most abundant whey protein, α-LA (15–25% of total whey proteins), modulates macrophage and B- and T-lymphocyte functions (11). Moreover, the α-LA–derived peptide f51–53 directly affects neutrophils (12). The 3rd most abundant whey peptide component, GMP, can affect immunity and attenuate inflammatory colitis in rats (6,13,14). At optimal concentrations, the other bioactive whey-derived proteins like Ig and lactoferrin present in whey protein extract (WPE) can also exert immune modulatory functions (6,7).
On the other hand, data regarding the specific effect of whey proteins on innate immunity are still in infancy. Whey proteins can reduce rotavirus-induced diarrhea and improve immune status in HIV infection. Certain mechanisms of protective action of these whey proteins in infections have been suspected, such as increases of natural killer cells in neonate rats and of plasma glutathione concentrations in HIV-infected patients (15,16). However, efficient innate immunity is critically dependent upon the capability of neutrophils to be activated rapidly in the face of an acute threat. They are the most abundant white blood cells that play a crucial role in infections and acute inflammation (17).

These polymorphonuclear leukocytes are also implicated in chronic inflammatory diseases (18,19). Neutrophil phagocytosis of microorganisms is indispensable, as demonstrated by fatal infections that occur in patients with neutropenia or with impaired neutrophil functions (20,21). Bovine whey was reported to increase neutrophil counts in rats and sheep (22,23). The repertoire of defense mechanisms deployed by neutrophils includes phagocytosis of pathogens, production of reactive oxygen species, and release of proteolytic enzymes. Interestingly, factors like granulocyte-macrophage colony-stimulating factor (GM-CSF) can enhance innate immunity by increasing neutrophil functions like antimicrobial activity (24). Colostral whey proteins from immunized cows activate phagocytosis of pathogenic microbes by human neutrophils, an effect related to specific antibodies in whey (25). However, the role of whey proteins on innate functions led by human neutrophils has not been elucidated.

The goal of this study was to determine whether a novel patented WPE that contained bovine transforming growth factor-β2 (TGF-β2) could modulate innate functions of defense supported by human blood neutrophils. We therefore set out to test whether WPE would have direct or indirect effects on neutrophil production of superoxide anions, exocytosis of myeloperoxidase (MPO), chemotaxis in response to N-formyl-methionine-leucine-phenylalanine (fMLP), phagocytosis of zymosan bioparticles, and expression of surface receptors associated with cell adhesion and phagocytosis. We also tested whether components of WPE like β-LG, α-LA, GMP, Ig, serum albumin, lactoferrin, and TGF-β2 could be responsible for the effect of WPE.

Materials and Methods

Reagents. Ficol-Paque, RPMI 1640, and fetal bovine serum (FBS) were obtained from Wisent, Fluorescein-5-isothiocyanate (FITC)-conjugated annexin-V and propidium iodide (PI) were from Biosource International. The bacterial peptide fMLP, purified human MPO, cytosolassan B, β-LG (L2506, no. 095H7000), and α-LA (L5385, no. 0012k7048) were obtained from Sigma Chemical. Solutions of purified bovine β-LG and α-LA were endotoxin free as evaluated with the limulus assay (50–647U, lot FL0036, Cambrex). GMP (JE 003–6-940) was from Davisco. Calcein/annexin-V and propidium iodide (PI) were from Biosource International. Bovine Ig, serum albumin, lactoferrin, and TGF-β2 were endotoxin free as evaluated with the reverse phase-HPLC, and 11.5 μg/g TGF-β2 (ELISA kit from R&D Systems). Dilutions were carried out with the incubation medium.

Preparation of neutrophils and culture conditions. The institutional review board of the Université Laval (Québec, QC) approved the present study and healthy adult volunteers signed a consent form. Neutrophils were prepared in sterile conditions at room temperature (RT), as previously described (27). Briefly, blood with anticoagulant solution was centrifuged (250 × g; 10 min, RT) and the platelet-rich plasma removed. After sedimentation of erythrocytes in sterile Dextran T-500 (2%) 30 min at RT, neutrophils were purified by centrifugation over a Ficoll-Paque cushion (450 × g; 20 min, RT). Neutrophils were collected at the bottom and contaminating erythrocytes were removed by hypotonic lysis (15 s, RT). After 2 washes, neutrophils were counted and resuspended in Mg2+-free Hanks’ balanced salt solution (HBBS) containing 1.6 mmol/L CaCl2. Cell viability was routinely assessed by Trypan blue dye exclusion test in each experiment (>99%). Neutrophils were incubated at 1010 cells/L in sterile polypropylene tubes (1 mL/tube) at 37°C, 5% CO2 for 24 h. The incubation medium for pretreatment of neutrophils was RPMI 1640 + 1% FBS + 1% pen/strep.

Production of superoxide anions. The production of superoxide anions (O2−) by neutrophils was evaluated, as previously described (28). After removal of pretreatment incubation medium, neutrophils (108 cells/L) were resuspended in HBSS with calcium. Freshly prepared cytochrome c (125 mg/L) in HBSS was added to neutrophils for incubation (37°C, 5 min) with 0.1 μmol/L fMLP. Controls were performed with added DMSO, the vehicle of fMLP. The reactions were stopped on ice and tubes were centrifuged at 1000 × g for 2 min at RT. Detection of O2− was based on its ability to reduce cytochrome c. The difference between reduced and oxidized cytochrome c at 550 nm was measured with a Beckman DU spectrophotometer. The change in OD was converted to nanomoles of O2− released into supernatants using the extinction coefficient E255 (reduced-oxidized) = 21 × 10−3 L·mol−1·cm−1.

Exocytosis of primary granules. Release of MPO was measured, as previously described (29). Neutrophils (1010 cells/L) were incubated 2 min with 10 μmol/L cytochalasin B and then stimulated 5 min with 0.1 μmol/L fMLP (vehicle or control). The reaction was stopped on ice and cells were lysed in 1 mL of 50 mmol/L potassium phosphate buffer (pH 6.0) to which 0.5% hexadecyltrimethylammonium bromide was added. Supernatant or lysed cells (0.1 mL) was mixed with 2.4 mL of 50 mmol/L potassium phosphate buffer containing 0.2 g/L o-dianisidine dihydrochloride and 0.5 mL of 0.003% hydrogen peroxide. The change in absorbance at 460 nm was measured by spectrophotometry. MPO content was calculated using a human MPO calibration curve created with known dilutions of pure human MPO.

Chemotaxis. Neutrophil chemotaxis was assayed by the fluorescence-based neutrophil transpolycarbonate migration method using micro-chamber 96-well plates (30). Neutrophils pretreated with WPE were resuspended in RPMI 1640 + 10% FBS and were loaded with 5 μg of calcein/AM for 30 min at 37°C. Loaded neutrophils were washed twice and resuspended in RPMI + 10% FBS (3 × 106 cells/L). Total fluorescence from a known number of neutrophils was obtained by placing into the bottom chamber 30 μL of 0.05, 0.5, 1, 2, 3, and 5 × 106 cells/L. Wells of plate lower chamber were filled with 30 μL of the control medium (HBBS) or with 30 μL of fMLP (1 mmol/L). The polycarbonate filter (3-μm pores) was positioned on the upper chamber plate before neutrophils (30 μL; 3 × 105 cells/L) were placed on the filter and allowed to migrate for 1 h in the dark (37°C and 5% CO2). Each experiment was performed in triplicate. Nonmigrating cells were removed and cell migration was measured with a microplate fluorescence reader (FL600; Bio-Tek Instruments) with bottom-read configuration (excitation, 485 nm; emission, 530 nm). Results are expressed in percentages (mean ± SEM) of fluorescent cells that migrated through the filters with respect to total number of fluorescent cells loaded.

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Phagocytosis of bioparticles. Phagocytic activity of WPE-pretreated neutrophils was measured using fluorescent zymosan A bioparticles, according to the manufacturer’s instructions. Fluorescent particles were preopsonized in PBS + 50% normal pooled human serum for 1 h at 37°C. Zymosan was washed 3 times before addition to 2.5 × 10⁷ neutrophils (ratio cell:particles = 1:10) in RPMI + 0.1% FBS for 10 min at 37°C. After washing and resuspension in 300 μL RPMI + 0.1% FBS containing 10% trypan blue (added to quench the fluorescence of bioparticle conjugates bound to the surface, but not internalized). In each sample, 10,000 cells were analyzed by flow cytometry. Because the fluorescence intensity of a given cell was proportional to the number of ingested bioparticles, the mean fluorescence intensity of a given sample reflected the overall uptake of zymosan. The phagocytic index, calculated as percentage of cells that phagocytized bioparticles × mean fluorescence intensity, reflects phagocytic capacity of cells that internalized zymosan.

Expression of CD11b, CD16b, and CD32A receptors. Neutrophils pretreated with WPE (1–100 mg/L) were resuspended in cold PBS + 1% FBS. As a first step, 1.5 × 10⁶ cells were blocked with 10% decomplemented human serum (30 min, 4°C). Cells were washed twice in PBS + 1% FBS before incubation (30 min, 4°C) with anti-FcyRIIA (CD32A) mAb, anti-CD11b mAb, or with isotype IgG2a or IgG2b mAb, respectively. Cells were washed twice, treated with FITC-conjugated goat anti-mouse antibody, and incubated for 30 min at 4°C. For CD16b expression, neutrophils were directly incubated (30 min, 4°C) with PE-conjugated anti-FcyRIIB mAb or with isotype PE-conjugated mouse IgG1. After washing and resuspension in PBS + 1% FBS, cells were analyzed by flow cytometry (Beckman Coulter EPICS XL). Results were expressed as mean fluorescence intensity of 10,000 cells with the isotype control subtraction. Autofluorescence was measured by incubating cells with buffer alone.

Immunoblotting of p47phox. After incubation, neutrophils were centrifuged and pooled in 1 mL of buffer containing anti-proteases. After sonication and elimination of nuclei (600 × g; 2 min at 4°C), supernatants were purified by Percoll gradient centrifugation (37000 × g; 30 min). Plasma membranes were collected from the upper band after centrifugation (100,000 × g; 45 min) and stored at −80°C. The purified membranes were incubated in 1 mL of binding buffer (RT), supernatants containing membranes from 28.3 × 10⁶ neutrophils were separated on 10% SDSPAGE before transfer on polyvinylidine difluoride membranes. After blocking (5% Biotto), membranes were probed with a polyclonal antibody against p47phox with secondary peroxidase-conjugated goat anti-rabbit antibody, before detection by the enhanced chemiluminescence system and visualization on Kodak Biomax MR film. After stripping, membranes were blocked in gelatin (2%) and reprobed with an mAb against Lyn, a tyrosine kinase present in the membrane fraction of neutrophils and used as a loading control, followed by a peroxidase-conjugated goat anti-mouse antibody (31).

Analysis of viability. WPE-pretreated neutrophils were labeled with FITC-conjugated Annexin V (apoptosis marker) and PI (necrosis marker) according to manufacturer’s protocol, and analyzed by flow cytometry. Briefly, neutrophils washed with HBSS were resuspended in 100 μL 1× binding buffer. The cell suspension was incubated with 5 μL FITC-conjugated annexin V and PI (5 min, RT, in the dark). A volume of 400 μL of binding buffer 1× was added to each tube before analysis using a Beckman Coulter EPICS XL.

Statistical analysis. Results are expressed as means ± SEM. For O₂⁻, MPO, phagocytosing cells, phagocytic index, and apoptotic cells, neutrophils from each donor were randomly assigned the pretreatment with all the concentrations of WPE or of its components α-LA, β-LG, GMP. The donors represent experimental blocks. The analysis was performed by using a 1-way blocked ANOVA. Due to experimental conditions of chemotaxis, neutrophils were not treated at the same time by all the WPE concentrations, and thus the analysis required an ordinary 1-way ANOVA. Studies on O₂⁻, MPO, and chemotaxis compared fMLP-stimulated cells pretreated with WPE with fMLP-stimulated cells pretreated with vehicle. Studies on phagocytosis, apoptosis, and surface expression of CD11b, CD16b, and CD32A compared cells without fMLP stimulation. To compare the effect of WPE with the additive effects of α-LA and β-LG, the sum of the effect of each protein was compared with the effect of the 2 proteins added together, and to the effect of WPE. Means were compared using the post hoc Tukey-Kramer test. Neutrophil phagocytosis was also analyzed for the global linear trend of the WPE effect. Statistical analyses were performed using GraphPad Instat software 3.0 (GraphPad Software). Statistical significance was fixed at P < 0.05.

Results

Neutrophil functions modified by WPE. The effect of WPE on neutrophil viability was first evaluated. WPE increased significantly nonapoptotic cells (absence of labeling with annexin V and PI). WPE from 25 to 100 mg/L reduced early (annexin V-positive, PI-negative cells) and late (annexin V-positive, PI-positive cells) apoptosis of neutrophils (Supplemental Fig. 1). WPE at 25 mg/L (vs. medium alone) diminished the number of early and late apoptotic cells by 21.8% (P < 0.01) and 39.3% (P < 0.001), respectively. WPE at 100 mg/L decreased early and late apoptotic neutrophils by 31.2% (P < 0.001) and 52.2% (P < 0.001), respectively. As a consequence of reduced apoptosis, viability (absence of labeling with annexin V and PI) of WPE-treated neutrophils was improved. Treatment of neutrophils with WPE from 25 to 100 mg/L was associated with an increase in the number of viable neutrophils ranging from 1.38- (P < 0.01) to 1.54-fold (P < 0.001) that in untreated neutrophils. On the other hand, WPE did not affect necrotic (PI-positive cells only) neutrophils (data not shown).

Production of superoxide anions. Direct and indirect effects of graded concentrations of WPE (1–100 mg/L) were studied on neutrophil production of O₂⁻ in vitro. WPE had no direct influence on O₂⁻ production by neutrophils incubated up to 24 h (data not shown). However, WPE had indirect effects, like cell priming, on neutrophil production of O₂⁻ in the presence of a subsequent stimulus. The time course of neutrophil priming by WPE up to 24 h showed a gradual priming effect that began at 4 h with a maximal effect at 24 h (data not shown). Hence, all further experiments were carried out with a 24-h period of neutrophil priming with WPE. In response to stimulation by fMLP, WPE-pretreated neutrophils dose-dependently produced more O₂⁻ than did neutrophils preincubated for 24 h in incubation medium alone (Fig. 1). Preincubation of neutrophils with WPE from 10 to 100 mg/L was associated with increased O₂⁻ production from 2.9- to 6.5-fold that in untreated neutrophils.

Although WPE was aseptically produced, some preparative steps could be associated with contamination by lipopolysaccharide (LPS). The LPS content in 100 mg/L WPE was evaluated at 0.1 μg/L LPS, as measured by the limulus assay. Because LPS can prime and enhance neutrophil production of O₂⁻, WPE effects were studied in the presence of neutralizing anti-CD14, -TLR4, and -CD18 mAbs against LPS receptors (32–34). In combination, the 3 mAb did not influence the priming effect of WPE at 100 mg/L, whereas they completely abrogated production of O₂⁻ induced by LPS (0111:B4 serotype from Escherichia coli) at a concentration up to 100 times that present in 100 mg/L WPE (data not shown). These experiments indicated that LPS was not involved in WPE effects on neutrophils.

Release of MPO. WPE-pretreated neutrophils released more MPO than control neutrophils in response to fMLP (Fig. 2A).
FIGURE 1 WPE primes the production of superoxide anions by human blood neutrophils. Cells were preincubated in the incubation medium alone (Ctl) or with WPE for 24 h. Superoxide production was subsequently measured in the absence (WPE alone) or in the presence of fMLP. Values are means ± SEM, n = 6 means of duplicates. Within a treatment, means without a common letter differ, P < 0.05.

The dose-dependent release of MPO reached a maximum of 44% of MPO released/total MPO at 100 mg/L WPE. At this latter concentration of WPE, the fMLP-induced release of MPO by neutrophils was 1.8-fold that in untreated neutrophils stimulated by fMLP. WPE alone did not affect the release of MPO by neutrophils (Fig. 2A).

Chemotaxis. WPE-pretreated neutrophils in the absence of fMLP did not modify their migration. However, WPE-pretreated neutrophils had an increased migration toward 10⁻⁹ mol/L of fMLP (Fig. 2B). The percentage of cells (pretreated with 100 mg/L WPE) that migrated in the presence of fMLP increased to 3.4-fold that in untreated cells.

Phagocytosis. Bioparticles were ingested by 4935 ± 524 freshly isolated neutrophils compared with 2933 ± 436 neutrophils pretreated by control medium alone for 24 h, with a similar phagocytic index for both populations (317 ± 56 and 605 ± 87, respectively). Pretreatment of neutrophils with WPE significantly increased the number of cells that engulfed zymosan (Fig. 2C). After a pretreatment with 50 mg/L WPE, the number of neutrophils with ingested zymosan increased to 1.9-fold that in untreated neutrophils. In parallel, the number of bioparticles internalized by neutrophils was augmented with a phagocytic index that increased to 1.7-fold that in untreated neutrophils. (Fig. 2D). When compared with freshly isolated neutrophils, the number of WPE (50 mg/L)-pretreated neutrophils that phagocitized zymosan and their phagocytic index increased to 1.2- and 1.9-fold that in freshly isolated cells, respectively. Note that WPE from 1 to 100 mg/L linearly increased the number of phagocytosing neutrophils and their phagocytic index (P < 0.0001).

Expression of CD11b, CD16B, and CD32A receptors by WPE-pretreated neutrophils. Associated with the enhancement of innate functions of neutrophils, priming of neutrophils increases adhesions molecules like CD11b and receptors for phagocytosis such as FcγRIIIB (CD16B) and FcγRIIA (CD32A) (33). Pretreatment of neutrophils with WPE dose dependently increased the expression of CD11b, CD16B (right + left peaks), and CD32A (Table 1). WPE was not fluorescent by itself and did not interfere with flow cytometry analysis. At 50 mg/L WPE, the expression of CD11b, CD16B, and CD32A increased to 1.6-, 2.3-, and 1.5-fold that in untreated neutrophils, respectively (Table 1; Fig. 3). After 24-h incubation, neutrophils were characterized by a low (left peak) and a high (right peak) expression of CD16B (Fig. 3B), as previously reported (36). However, 50 mg/L WPE enhanced the high expression of CD16B (160 vs. 99 mean fluorescence intensity for WPE-treated vs. control neutrophils, respectively), whereas the low expression of CD16B was not modified (8 vs. 7 mean fluorescence intensity for WPE vs. control neutrophils, respectively). These results suggest that pretreatment of neutrophils with WPE primed neutrophils to an enhanced expression of adhesion molecules and receptors for phagocytosis.

Identification of active compounds from WPE. The 3 major components were tested individually at 37 mg/L β-LG-L and 8 mg/L GMP (concentrations equivalent to their content in 100 mg/L WPE). β-LG and α-LA alone primed neutrophils to a subsequent response to fMLP (Fig. 4A). Thus, β-LG- and α-LA-pretreated neutrophils significantly produced more superoxide anions in response to fMLP than did neutrophils preincubated in control medium. This increased O₂⁻ production was similar for β-LG- and α-LA-pretreated neutrophils and corresponded to ~50% of that of WPE-pretreated neutrophils. Cells pretreated simultaneously with β-LG + α-LA...
had a response to fMLP similar to that of WPE-pretreated neutrophils (Table 2; Fig. 4A). The comparison between the arithmetic sum of the effect of β-LG and α-LA alone, the effect of the 2 proteins added together, and WPE was not significant (P = 0.43). This analysis indicated that β-LG- and α-LA added simultaneously were responsible for the effect of WPE. The addition of GMP to β-LG and α-LA did not increase the priming effect of β-LG + α-LA. However, the addition of GMP to α-LA alone was associated with a slight, but significant, priming effect that increased to 1.3-fold that in cells pretreated with α-LA alone (Table 2). Because bovine GMP can have dose-dependent effects on human cells (14), graded concentrations of GMP added to β-LG and α-LA (at concentrations equivalent to their content in 100 mg/L WPE) were used to prime neutrophils (Fig. 4B, C). GMP dose dependently increased the priming effects of β-LG and α-LA on the O$_2^-$ production by fMLP-stimulated neutrophils. The priming effect of β-LG and α-LA with GMP at 240 mg/L increased to 2.2- and 1.8-fold that in cells pretreated with β-LG and α-LA alone, respectively. A priming effect (P < 0.05) by GMP alone was obtained only at the highest concentration tested. Bovine lactoferrin, IgG, serum albumin, and TGF-β2 (at concentrations equivalent to their content in 100 mg/L WPE) had no direct and no priming effects on neutrophils (data not shown). Collectively, these results indicate that WPE primed human blood neutrophils mainly through the simultaneous action of its 2 major protein constituents β-LG and α-LA. However, GMP could have also a priming effect on neutrophils depending on its concentration in WPE.

Translocation of p47phox. Priming of neutrophils translocates p47phox subunit of the NADPH-oxidase from cytosol to plasma membrane (37). Analysis of membranes of neutrophils incubated with WPE or β-LG + α-LA had an important increase of p47phox subunit, compared with membranes of control neutrophils incubated in medium alone (Fig. 4D). These data indicate that, in term of NADPH-oxidase activity, WPE or its active components primed neutrophils through at least in part translocation of the oxidase p47phox subunit to plasma membranes.

Discussion

Although whey proteins from bovine milk have multiple beneficial effects on human health, in particular on innate and acquired immunity (6,38), the exact cellular and molecular mechanisms of action of whey proteins remain fragmentary and poorly understood. To this end, we studied, direct and indirect effects of a bovine WPE on normal human blood neutrophil functions associated with innate defenses. Interestingly, besides the absence of direct effects of WPE on primary functions of human blood neutrophils required for innate immunity like chemotaxis, phagocytosis, oxidative metabolism, and degranulation, this product was shown to exert a significant priming effect on neutrophils that become more responsive to a subse-

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Surface expression of CD11b, CD16B, and CD32A by neutrophils in the presence of WPE</th>
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</thead>
<tbody>
<tr>
<td>WPE, mg/L</td>
<td>CD11b$^1$</td>
</tr>
<tr>
<td>0 (control)</td>
<td>11.6 ± 0.7$^a$</td>
</tr>
<tr>
<td>1</td>
<td>13.3 ± 2.5$^b$</td>
</tr>
<tr>
<td>5</td>
<td>14.5 ± 2.7$^{ab}$</td>
</tr>
<tr>
<td>10</td>
<td>15.6 ± 1.8$^{ab}$</td>
</tr>
<tr>
<td>25</td>
<td>19.1 ± 2.7$^b$</td>
</tr>
<tr>
<td>50</td>
<td>18.8 ± 0.6$^b$</td>
</tr>
<tr>
<td>100</td>
<td>18.6 ± 2.8$^b$</td>
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</tbody>
</table>

$^1$ Results are means ± SEM. Means in a column without a common letter differ, P < 0.05.

$^2$ n = 3 donors.

$^3$ n = 5.

$^4$ Values represent means ± SEM of the low and high expressions.
quent stimulation by a bacterial chemoattractant peptide such as fMLP. It is noteworthy that acute inflammation is an obligatory innate response of the host to an infectious and noninfectious injury (39–41). The physiological endpoint of such an inflammatory response is helping eradicate the agent(s) of the injury and restore homeostasis. Moreover, this physiological inflammatory response can be favorably enhanced by priming agents, like GM-CSF and G-CSF (24,42). These agents can increase neutrophil primary functions associated with innate immunity such as adherence and migration, phagocytosis, the respiratory burst and degranulation leading, for instance, to increased microbicidal capacity in vitro, ex vivo, and in vivo (43). From this point of view, WPE can be considered as a complete priming agent that enhances primary functions of normal human blood neutrophils (Figs. 1–2). As a corollary, WPE can be easily compared with known priming agents like GM-CSF and tumor necrosis factor-α that, similarly to WPE (Fig. 4D), activate the translocation of NADPH-oxidase subunits to plasma membrane of neutrophils (37). Moreover, this priming effect of WPE could be efficient in vivo. Indeed, it is useful to know that such a WPE ingested orally by patients has an antinflammatory capacity without any side effects, as we reported recently (44).

WPE increased not only neutrophil chemotaxis but also the surface expression of CD11b (Fig. 3A), a mediator of neutrophil adhesion to endothelial cells (45). Of note, physiological inflammation is associated with an increased expression of CD11b and inversely an impaired expression of CD11b in neonates is associated with an infectious susceptibility (46). On the other hand, modification of CD16 expression induced by WPE has a dual meaning. First, the increase of CD16 high expressing neutrophils (Fig. 3B) has been related to an increase of nonapoptotic neutrophils (36). The increase of neutrophils survival induced by WPE corroborated this fact. Second, the WPE-induced increase of CD32A-expressing neutrophils (Fig. 3C) associated with the shift of CD16 low to CD16 high expressing neutrophils indicate that CD32A in the presence of WPE could be converted to a high state of activation with the cumulative effect of β-LG and α-LA (at concentrations found in WPE) represent the presence of 3 different donors.

**TABLE 2** GMP primes neutrophils to produce superoxide anions

<table>
<thead>
<tr>
<th>Without fMLP</th>
<th>With fMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>O$_2^-$, nmol/10$^6$ cells</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>WPE</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>GMP</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>α-LA</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>α-LA + GMP</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>β-LG</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>β-LG + GMP</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>α-LA + β-LG + GMP</td>
<td>1.4 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n = 9 means of duplicates. Labeled means in a column with superscripts without a common letter differ, P < 0.05.

WPE increased not only neutrophil chemotaxis but also the surface expression of CD11b (Fig. 3A), a mediator of neutrophil adhesion to endothelial cells (45). Of note, physiological inflammation is associated with an increased expression of CD11b and inversely an impaired expression of CD11b in neonates is associated with an infectious susceptibility (46). On the other hand, modification of CD16 expression induced by WPE has a dual meaning. First, the increase of CD16 high expressing neutrophils (Fig. 3B) has been related to an increase of nonapoptotic neutrophils (36). The increase of neutrophils survival induced by WPE corroborated this fact. Second, the WPE-induced increase of CD32A-expressing neutrophils (Fig. 3C) associated with the shift of CD16 low to CD16 high expressing neutrophils indicate that CD32A in the presence of WPE could be converted to a high state of activation with the cumulative effect of β-LG and α-LA (at concentrations found in WPE) represent the presence of 3 different donors.

**FIGURE 4** β-LG, α-LA, and GMP prime neutrophils to produce superoxide anions. Cells were preincubated for 24 h in the incubation medium alone (Ctl), with 100 mg/L WPE or 37 mg/L β-LG and 2.5 mg/L α-LA (A), or with or without 8, 40, 80, or 240 mg/L GMP in the presence of 37 mg/L β-LG (B) and 2.5 mg/L α-LA (C). Superoxide production was then measured in the absence (A) or in the presence (A–C) of fMLP. Results are means ± SEM, n = 9 (A), 3 (B), or 6 (C). Each measurement was performed in duplicate. Within a treatment, means without a common letter differ, P < 0.05 (A–C). (D) Translocation of p47$^{\text{phox}}$ to plasma membrane of WPE-treated neutrophils. Cells were incubated 24 h in medium alone (Ctl), with 100 mg/L WPE, or with 37 mg/L β-LG + 2.5 mg/L α-LA. Membranes were probed with anti-p47$^{\text{phox}}$ and anti-Lyn antibodies. The data shown are representative of 3 different donors.
neutrophils, graded concentrations of GMP associated with a dose-dependent priming effect when added to β-LG and α-LA (Fig. 4B,C) could suggest to increase GMP concentrations in future WPE preparations. Bovine GMP therefore has the capacity to reduce, similarly to sulphasalazine, the hapten-induced colitis in rats, a model of inflammation related to local infiltration of neutrophils (13,49).

In conclusion, bovine WPE reduces apoptosis of human blood neutrophils and exerts a dose-dependent priming effect on these cells that, when stimulated subsequently, have increased functions of adherence, chemotaxis, phagocytosis, oxidative burst, and degranulation. Its mechanism of action in vitro is mainly related to the presence of efficient concentrations of β-LG and α-LA in this WPE. However, knowing that β-LG, more than α-LA or GMP, is resistant to gastric digestion and remains intact after absorption in vivo (50), WPE priming effects on neutrophils in vivo could be mainly related to β-LG. The addition of WPE to the diet of healthy and diseased humans could be useful to increase the physiological innate response of their neutrophils.

Literature Cited


