Two major bovine milk whey proteins induce distinct responses in IEC-6 intestinal cells

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Abstract

α-Lactalbumin (α-LA) and β-lactoglobulin (β-LG) are major whey proteins in bovine milk. We studied the effects of these molecules on the intestinal cell response by comparing the native form with the denatured form containing oligomers obtained by treatment with 2,2,2-trifluoroethanol (TFE). We previously reported that proteins in native and TFE-treated forms exhibited cell growth stimulation and cytotoxicity, respectively, in undifferentiated rat crypt IEC-6 and human colon Caco-2 cells. However, neither whey protein showed cytotoxicity even in the TFE-treated form in differentiated Caco-2 cells. Only undifferentiated immature intestinal cells can distinguish between these native and denatured proteins. Moreover, α-LA and β-LG exhibited different oligomer formation characteristics during the TFE treatment. In the present study, we compared the effects of native and TFE-treated whey proteins on IEC-6 cells in more detail. The native forms of both whey proteins exhibited cell proliferative effects in a concentration-dependent manner. For the TFE-treated forms, α-LA showed rapid and potent cytotoxicity, whereas β-LG altered cell responses depending on its concentration and exposure time; lower concentration/shorter exposure and higher concentration/longer exposure induced cell growth stimulation and cytotoxicity, respectively. Pre-treatment of the cell membrane with cholesterol suppressed the effects on the cell response only in TFE-treated β-LG (TFE-β-LG). In a preliminary examination using inhibitors of signal transduction, TFE-treated α-LA acted on the intrinsic apoptosis pathway *via* Bcl-2-associated X and p53, whereas the action of TFE-LG did not require this pathway. Tyrosine phosphorylation is necessary for the cell proliferation effect of both native whey proteins; however, native α-LA, but not native β-LG, also required activation of the pathway with selective epidermal growth factor receptor tyrosine kinase and Janus kinase 2/3. In summary, the two major bovine milk whey proteins induced similar yet discrete responses in undifferentiated intestinal cells. Even when oligomers are formed, β-LG may be much less hazardous to immature intestinal cells than α-LA.

α-Lactalbumin (α-LA) and β-lactoglobulin (β-LG) are major whey proteins in bovine milk. α-LA, a monomeric globular protein with a molecular mass of approximately 14.2 kDa, is present in the milk of most mammals (Lönnerdal and Lien, 2003) and is essential for lactose synthesis (Hill and Brew, 1975). β-LG, a dimeric globular protein with a molecular mass of approximately 18.3 kDa, is present in the milk of ruminants, but not in that of humans (Hambling *et al*., 1992; Sawyer, 2021). Interestingly, bovine colostrum contains a much higher concentration of β-LG than mature milk (Pérez *et al*., 1990; Levieux and Ollier, 1999). However, the physiological significance of β-LG remains unclear.

In our previous studies using rat intestinal crypt IEC-6 cells (Xu *et al*., 2005a, 2005b; Xijier *et al*., 2012; Inagaki *et al*., 2017), it was found that both whey proteins exerted opposing effects on cell growth depending on their molecular forms: promotion in the case of the native form and inhibition in the case of denatured forms containing oligomers. These results can be interpreted as the formation of misfolded molecules, which cause the aggregation of protein molecules and are toxic to living systems (Bucciantini *et al*., 2002; Dobson, 2003). The ability of polypeptide chains to form misfolded toxic structures seems to be a generic feature of polypeptide chains, not the structures in which they usually function in vivo (Stefani and Dobson, 2003). In our studies, a denatured oligomeric state was obtained when the native molecule was exposed for a given period to 30% 22,2-trifluoroethanol (TFE), which is a conformational denaturant for proteins and peptides (Buck, 1998; Bucciantini *et al*., 2002; Naeem *et al*., 2015). Toxic α-LA-containing oligomers are found in many commercial α-LA protein products (Xu *et al*., 2005a). Oligomer formation in α-LA and β-LG has been observed in commercially

available milk, albeit in small amounts, which occurs during pasteurisation and sterilisation (Inagaki *et al.*, 2017; Liu *et al.*, 2019).

Recently, we investigated the effects of the native and denatured states of these proteins on cell growth using intestinal Caco-2 cells at various stages of differentiation (Kobayashi *et al.*, 2021). In undifferentiated cells, we observed similar patterns of IEC-6 cell proliferation: stimulation in the native state and inhibition in the TFE-treated denatured state. In contrast, in differentiated mature Caco-2 cells, cell barrier function was enhanced by both native and denatured whey proteins. No adverse effects were observed in mature cells or living neonatal mice, even with denatured whey proteins. However, the mechanisms underlying these contrasting findings remain unclear. Based on these observations, only undifferentiated immature intestinal cells could distinguish between the proteins in the native and TFE-treated denatured states.

In this latest study, we compared the effects of two globular bovine milk whey proteins on the growth of undifferentiated IEC-6 intestinal cells. The results showed that the two proteins induced similar yet discrete responses in IEC-6 intestinal cells. The denatured β-LG may be much less hazardous for immature intestinal cells than its α-LA counterpart. Based on the results, we also discuss a possible function of β-LG in bovine colostrum.

Materials and methods

A detailed account of methodologies is provided in the online Supplementary File.

Reagents

Bcl-2 associated X protein (Bax)-inhibiting peptide V5 (BIP-V5), tyrosine kinase inhibitor genistein, and Janus kinase (JAK) 2/3 tyrosine kinase inhibitor AG490 were purchased from Merck Millipore (Burlington, MA, USA). Pifithrin-α (PFT-α) and water-soluble cholesterol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Inhibitor stock solutions in dimethylsulphoxide (DMSO) were kept at $-30°C$.

Purification of native whey proteins and TFE treatment

Native α-LA and β-LG were purified from each commercial whey protein (Davisco Foods International, Eden Prairie, MN, USA). Purification was performed as described by Kobayashi *et al.* (2021). The purified native α-LA monomer was dissolved in 50 mM acetate buffer containing 30% (v/v) TFE buffer at 0.5 mg/ml (Bucciantini *et al.*, 2002). The purified native β-LG dimer was dissolved in 25 mM Tris-HCl buffer containing 25% (v/v) TFE buffer at 0.5 mg/ml. Each solution was filtered through a 0.22 μm sterilised filter and incubated at 37°C for 2 d while slowly rotating at 12 rpm on a Versatile Tube Rotator (Miltenyi Biotec, Gladbach, Germany). After dialysis, the solution was lyophilised.

Cell line

IEC-6, a crypt cell line derived from the rat small intestine, was obtained from the American Type Culture Collection (Rockville, MD, USA). The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) (ICN Biomedicals, Aurora, OH, USA) supplemented with 10% foetal calf serum (FCS) and cultured at 37°C in 5% CO_2.

Cell proliferation assay

The water-soluble tetrazolium-1 (WST-1) assay was performed using a cell counting kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, 100 μl of cell suspension prepared at 0.7×10^5 cells/ml with the culture medium including 10% FCS were seeded into 96-well plates and incubated for 24 h. Then, the medium was removed and replaced with 100 μl of the FCS-free experimental medium containing the whey protein sample, and the culture was incubated at 37°C in 5% CO_2. The experimental medium was then removed and cell growth was detected using the WST-1 reagent. Absorbance was measured at 450 nm.

Addition of cholesterol to the cell culture

Water-soluble cholesterol was used to alter the cholesterol content in the cell membrane. This treatment was performed during pre-culture. Briefly, 100 μl of a cell suspension prepared at 0.7×10^5 cells/ml with culture medium containing 10% FCS was seeded into 96-well plates and incubated for 22 h. Then, the medium was removed and replaced with 10% FCS containing 0.3 mM water-soluble cholesterol. After incubation for 2 h, the medium was removed and the cells were washed with a medium without water-soluble cholesterol. The medium was then removed, and FCS-free experimental medium containing protein specimens was added for 24 h. Cell proliferation was measured using WST-1 assays.

Inhibitor experiments

For the experiments, the stock solution of the inhibitor was diluted in FCS-free medium. The highest concentration of DMSO used was 0.1% (v/v). The control used was 0.1% DMSO only. The prepared inhibitor was sterilised by filtration through a 0.22 μm filter (Merck Millipore). Cell suspension (100 μl) prepared at 0.7×10^5 cells/ml with the culture medium including 10% FCS were seeded into 96-well plates and incubated for 24 h. The medium was replaced with an FCS-free experimental medium containing protein specimens and/or drugs. After incubation for 24 h, cell proliferation was measured using WST-1 assays.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance, followed by Dunnett's test (Figs 1–3) and Tukey's multiple test (Fig. 4) using GraphPad Prism 9.0. A value of $P < 0.05$ was considered statistically significant.

Results

Concentration-dependent effects of native and TFE-treated whey proteins

We examined the concentration-dependent effects of a 24-h exposure to native and TFE-treated whey proteins on IEC-6 cells using WST-1 assays (Fig. 1). Both native forms enhanced cell growth in a concentration-dependent manner, and the cell proliferation activity of native β-LG was much greater than that of native α-LA. After TFE treatment, α-LA showed cytotoxicity. Intriguingly, the TFE-treated β-LG (TFE-β-LG) exhibited opposite effects depending on concentration; cell proliferation was enhanced
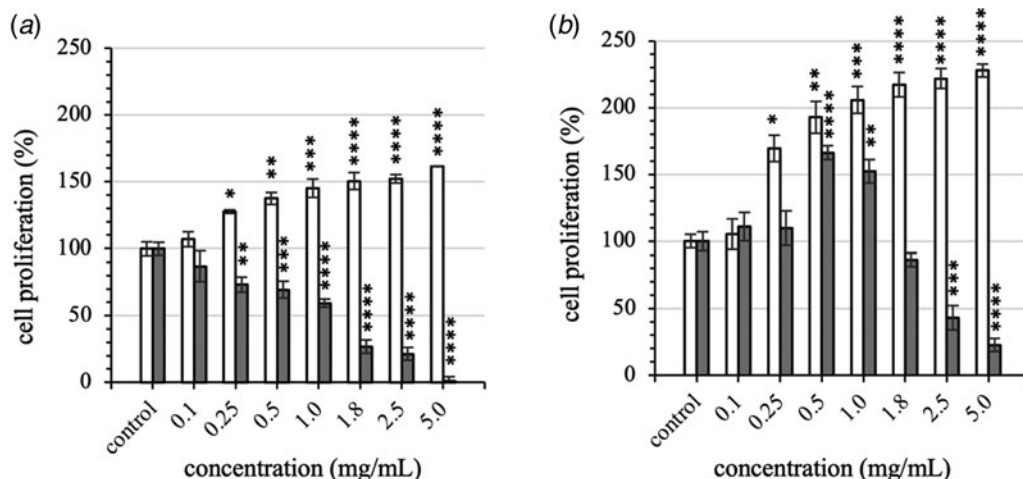
Figure 1. Effects of native and TFE-treated whey proteins on cell proliferation in rat crypt IEC-6 cells. Cells were seeded and incubated for 24 h. The experimental medium without FCS was supplemented with the protein specimens, and cells were cultured for 24 h at 37°C in 5% CO_2. Cell growth was measured using WST-1 assays as described in Material and methods. Control was cultured in the medium without the protein specimens. Cell proliferation was determined as a per cent of the corresponding control. Each bar is expressed as mean ± sd ($n = 4$). Asterisks indicate significant differences as determined by one-way ANOVA followed by Dunnett's multiple comparisons: ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$. (a) α-LA (b) β-LG; white and grey bars are native and the TFE-treated whey proteins, respectively.

at lower concentrations (0.5–1.0 mg/ml), whereas inhibiting activity was observed at higher concentrations (2.5–5.0 mg/ml).

Exposure time-dependent effects of TFE-treated whey proteins

The observed cytotoxicity of TFE-treated α-LA (TFE-α-LA) was robust, whereas that of TFE-β-LG counterpart was modest (Fig. 1). To further characterise the differences between them, we investigated the time-dependent effects of exposure. Proliferation activity was measured after culturing the cells for a given time in MEM containing 5.0 mg/ml of the TFE-treated protein (Fig. 2a). In the case of medium without FCS (as a control), the cells exhibited slow growth, and the absorbance after 24 h of incubation approximately doubled that of 3 h of incubation. Potent cytotoxicity was observed in the medium containing TFE-LA, even after 3 h of culture. In contrast, TFE-β-LG exhibited cytotoxicity 12 h after the addition of the experimental medium. Intriguingly, it functioned to increase cell proliferation for 3 and 6 h after addition.

To understand the persistence of the impact of whey proteins on cells at different periods of incubation, we removed the medium containing the whey proteins, further cultured the cells for 12 h in medium containing 10% FCS, and measured cell proliferation activity (Fig. 2b). Control cells cultured in a medium without whey proteins grew rapidly after exposure to a medium containing 10% FCS. The effects of TFE-α-LA persisted after its removal from the medium, inducing almost complete cell growth arrest. Even 3 h of exposure resulted in scarce proliferation activity after 12 h of cultivation in medium containing 10% FCS. In contrast, exposure to TFE-β-LG for 3 h significantly promoted cell
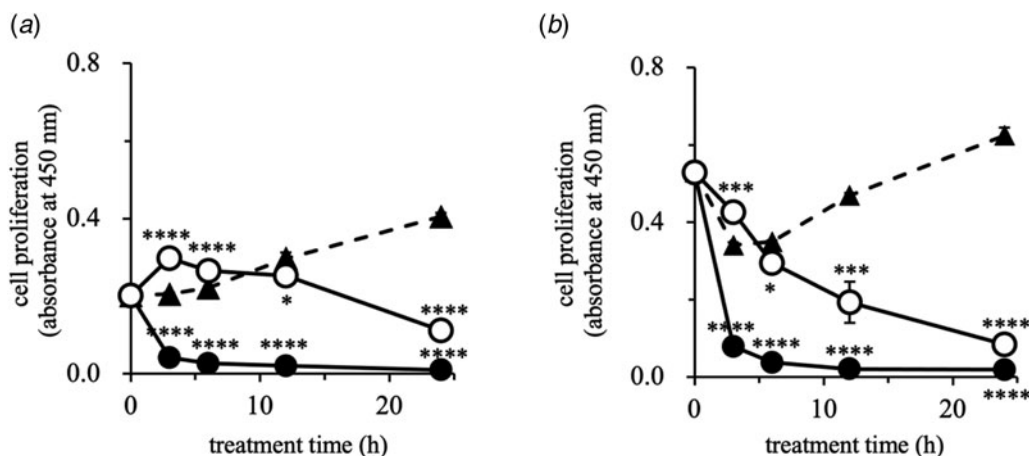

Figure 2. Exposure time-dependent effects of TFE-treated whey proteins (a) and the persistence of their impact (b). Cells were seeded and incubated for 24 h. Cells were cultured in the experimental medium supplemented with TFE-α-LA (closed circles and solid line), TFE-β-LG (open circles and solid line) at 5.0 mg/ml, and without the protein sample (control, triangles and dotted line), and cultured for 3 h, 6 h, 12 h, and 24 h at 37°C in 5% CO_2. Cell growth was measured at a given time using WST-1 assays (a), or the inoculum was removed, further incubated for 12 h in DMEM supplemented with 10% FCS, and then cell growth was measured using WST-1 assays (b). Each value is expressed as mean ± sd ($n = 4$). Asterisks indicate significant differences at the same time as determined by one-way ANOVA followed by Dunnett's multiple comparisons: ****$P < 0.0001$, ***$P < 0.001$, and *$P < 0.05$.
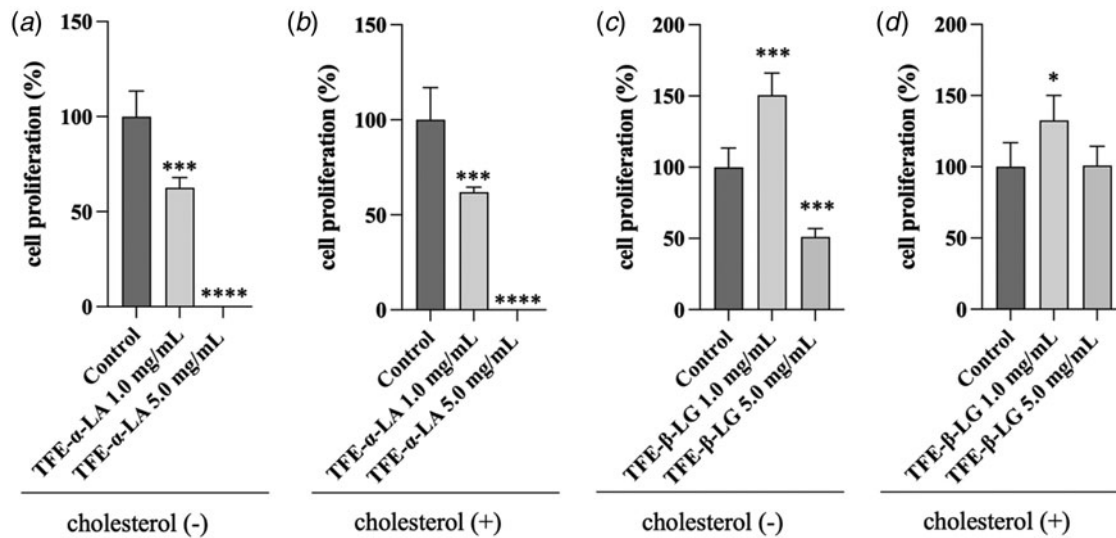
Figure 3. Effects of membrane cholesterol levels on cell proliferation. Cells were seeded and incubated for 22 h. The medium was replaced with DMEM supplemented with 10% FCS (a and c) or containing 0.3 mM water-soluble cholesterol (b and d) and incubated for 2 h. Each well was washed with a cholesterol-free medium, given the experimental medium supplemented with free FCS containing either TFE-α-LA (a and b) or TFE-β-LG (c and d), and cultured for 24 h at 37°C in 5% CO_2. Control cells were cultured without FCS. Cell growth was measured using WST-1 assays. Each bar is expressed as mean ± SD ($n = 4$). Asterisks indicate significant differences as determined by one-way ANOVA followed by Dunnett's multiple comparison test: ****$P < 0.0001$, ***$P < 0.001$, and *$P < 0.05$.

proliferation in a medium containing 10% FCS, and its proliferation activity gradually decreased with increasing exposure time.

Effects of membrane cholesterol levels on cell growth

The impairment of cell viability by misfolded proteins is significantly related to the cholesterol content in the cell membrane (Cecchi *et al.*, 2005). We evaluated the influence of cell membrane conditions on the cell response to TFE-treated whey proteins. Because of the low cholesterol levels in undifferentiated cell membranes (Karnell *et al.*, 2005), water-soluble cholesterol was added 2 h before the cell experiments. The cells were then exposed to TFE-treated proteins for 24 h.

TFE-α-LA exhibited potent cytotoxic effects, irrespective of cholesterol pre-treatment (Fig. 3a, b). Similar to Fig. 1b, without cholesterol pre-treatment, TFE-β-LG exhibited opposite effects on cell proliferation depending on concentration: stimulation at 1.0 mg/ml but inhibition at 5.0 mg/ml (Fig. 3c). However, cholesterol pre-treatment resulted in a marked reduction in both cytotoxicity and cell proliferation after TFE-β-LG treatment (Fig. 3d).

Probing the signal transduction pathway involved in cell proliferation

Signal transduction regulates cell survival and death. We probed the signal transduction pathways involved in the cellular response to whey proteins using several inhibitors. We first investigated the signal transduction pathways involved in apoptosis. The intrinsic apoptotic pathway regulates the activity of the Bcl-2 family of proteins, which controls the integrity of the mitochondrial membrane (Cagnol and Chambard, 2010). BIP-V5 is a cell-permeable pentapeptide (Yoshida *et al.*, 2004), inhibits Bax activation, which plays a role in mitochondria-related apoptosis (Suzuki *et al.*, 2017; Peña-Blanco and García-Sáez, 2018). BIP-V5 significantly suppressed the cytotoxic effect of TFE-α-LA but not that of TFE-β-LG (Fig. 4a, b). Likewise, the cytotoxicity of TFE-α-LA

disappeared when PFT-α was used, which inhibits p53-mediated apoptosis (Komarova and Gudkov, 2000), whereas that of TFE-β-LG was unaffected (Fig. 4c, d).

Next, we examined signal transduction pathways involved in cell proliferation. Protein tyrosine kinases (PTK) play central roles in cell growth, division, and differentiation (Hubbard and Till, 2000). Genistein has been widely used to determine whether cell proliferation is mediated by PTK (Akiyama *et al.*, 1987; Akiyama and Ogawara, 1991). The cell proliferation effects of both native α-LA and β-LG were significantly suppressed by genistein treatment (Fig. 4e, f). We further investigated the activity using tyrphostin inhibitor AG490, which is a non-receptor tyrosine kinase and JAK 2/3 inhibitor (Meydan *et al.*, 1996). The activity of native α-LA was inhibited by AG490, whereas that of native β-LG was unaffected (Fig. 4g, h). The phosphorylation of tyrosine is essential for the cell proliferation effect of both native whey proteins; however, the details of the α-LA and β-LG signalling pathways appear to be different.

Discussion

Protein misfolding can cause the aggregation of molecules and is toxic to living systems. Neurodegenerative diseases such as Alzheimer's and Parkinson's disease are well-known debilitating misfolding disorders (Dobson, 2003). The ability to form aggregated structures is not an unusual feature of the small number of proteins associated with these diseases but is instead a general property of polypeptide chains (Stefani and Dobson, 2003).

In this study, as well as other previous studies, we obtained cytotoxic protein specimens by exposing native proteins to TFE and removing the co-solvent by dialysis. This procedure was described by Bucciantini *et al.* (2002), who showed that the SH3 domain from bovine phosphatidyl-inositol-3′-kinase and the amino-terminal domain of the *Escherichia coli* HypF protein, both irrelevant to diseases, underwent misfolding and cytotoxic transformation in such conditions. Before performing the
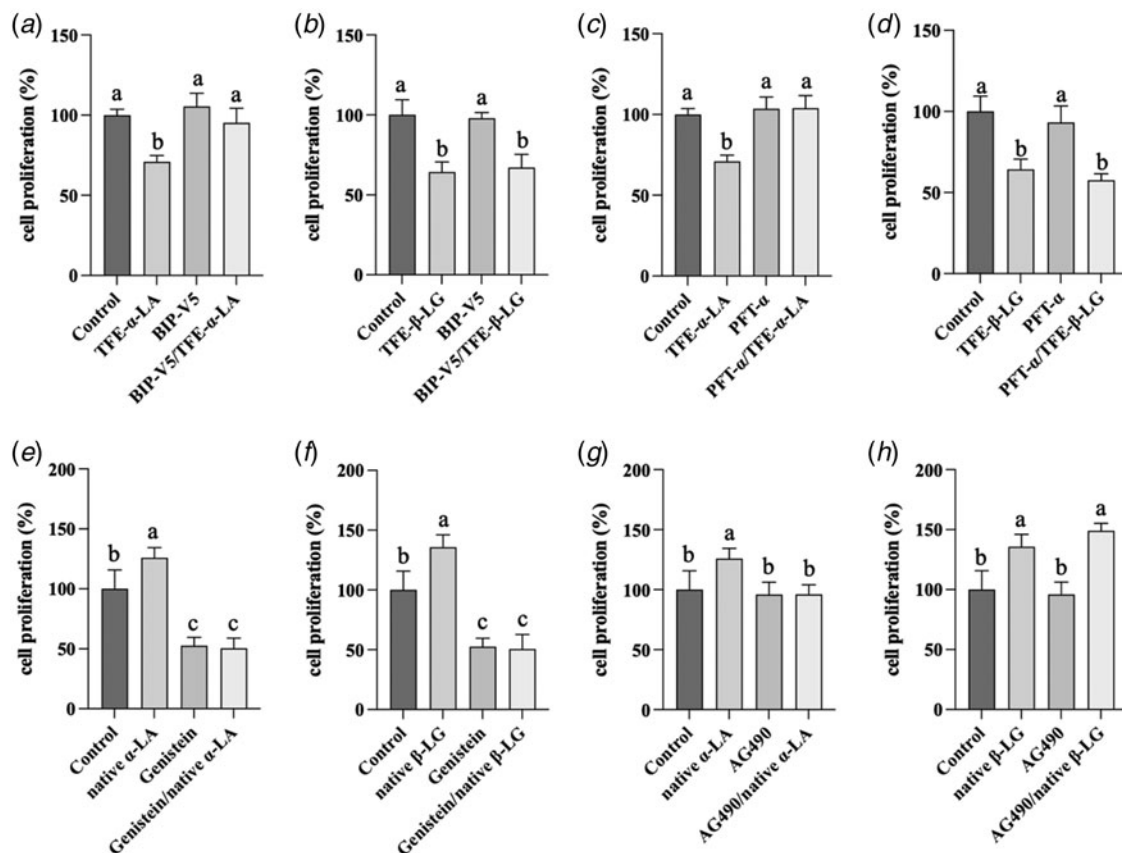
Figure 4. Effects of signal transduction pathway inhibitors on apoptosis (a–d) and cell proliferation (e–h) induced by native and TFE-treated whey proteins. Cells were seeded and incubated for 24 h. Then, the cells were given the DMEM medium without FCS containing the inhibitor [a and b, BIP-V5 (200 μM); c and d, PFT-α (30 μM); e and f, genistein (92.5 μM); g and h, AG490 (5 μM)] and the whey protein sample [a and c, TFE-α-LA at 1.0 mg/ml; b and d, TFE-β-LG at 2.5 mg/ml; e and g, native α-LA at 5.0 mg/ml; f and h, native β-LG at 5.0 mg/ml], then cultured for 24 h at 37°C in 5% CO_2. Cell growth was measured using WST-1 assays. Each bar is expressed as mean ± SD (n = 4). Means with different letters are significantly different ($P < 0.05$) as determined by one-way ANOVA followed by Tukey's multiple comparison test.

experiments using IEC-6 cells, we looked for evidence of misfolding. Comparative analysis of the conformational structure between the native and TFE-treated proteins using circular dichroism spectroscopy showed no significant structural difference between native and TFE-α-LA (Supplemental Fig. S1A and S1B) (Xijier et al., 2012; Inagaki et al., 2017). TFE-β-LG slightly altered its tertiary structure while maintaining its native secondary structure (Supplemental Fig. S1C and S1D). Possible misfolding-induced oligomerisation was indicated by gel filtration chromatography, which showed a fast-eluting peak before a major peak eluting at the same position as the native molecule: very small for TFE-α-LA and considerably large for TFE-β-LG (Supplemental Fig. S2) Xu et al. 2005b and Kobayashi et al. 2021. SDS-PAGE analysis indicated SDS-resistant oligomer formation in both TFE-treated whey proteins (data not shown) (Xu et al., 2005b and Kobayashi et al., 2021). According to Soto and Pritzkow (2018), smaller soluble misfolded oligomers, which are precursors of fibrillar aggregates, appear to be the real culprits of misfolding diseases. They reported that oligomeric species were highly dynamic and existed in equilibrium with monomers and fibrils. The large heterogeneity, rapid interconversion between species, and propensity to form higher-order aggregates make it difficult to obtain high-resolution structural information on misfolded oligomers. Because of their cytotoxic properties (Fig. 1) and oligomer formation (Supplemental

Fig. S2), we assumed that both TFE-α-LA and TFE-β-LG contained considerable amounts of misfolded molecules.

Both the denatured whey proteins were cytotoxic to undifferentiated IEC-6 cells; however, their effects differed considerably. The cytotoxicity of TFE-α-LA was concentration-dependent (Fig. 1a), rapid-acting, and very potent (Fig. 2a), which is consistent with the results of an initial study conducted in our laboratory (Xu et al., 2005a). Present observations using the signal transduction inhibitors BIP-V5 and PET-α strongly support the apoptotic action of TFE-α-LA (Fig. 4a, c). It acted on the intrinsic apoptotic pathway via BAX and p53. On the other hand, the action of TFE-β-LG seemed to be independent of this apoptotic pathway (Fig. 4b, d) and was variable: higher concentrations/longer exposure and lower concentration/shorter exposure induced cytotoxicity and cell growth stimulation, respectively (Fig. 1b, 2a). Even after 3 h of exposure to TFE-β-LG at high concentrations (5.0 mg/ml), cell proliferation was not affected (Fig. 2b). A similar biphasic cellular response has also been reported for unaggregated A-β(1-42) peptide in Neuro-2A cells (Dahlgren, et al., 2002). Its toxicity to neuronal viability was attributed to its molecular size, which was observed to be 40-fold less than that of smaller-sized oligomers.

Native α-LA and β-LG had similar effects on the overall cell response, but distinct effects were observed. Both native states showed cell proliferative activity in a concentration-dependent

manner (Fig. 1); however, the effects of β-LG were greater than those of α-LA. Tyrosine phosphorylation is necessary for the proliferation of both native whey proteins. However, α-LA only may also require activation of the selective epidermal growth factor receptor tyrosine kinase and JAK2/3 pathway (Fig. 4g). Native β-LG stimulated another signal transduction pathway, inducing robust stimulation of proliferation. Thus, native α-LA and β-LG induced similar yet discrete responses in undifferentiated intestinal cells.

Misfolded molecules cause aggregation of protein molecules, which is toxic to immature cells in living systems. Aggregation is a common consequence of the failure of a polypeptide chain to reach or maintain its functional three-dimensional structure. Such events may result from specific genetic mutations, misprocessing phenomena, aberrant interactions with metals and ions, changes in environmental conditions, such as pH or temperature, or chemical modifications, such as oxidation and proteolysis (Stefani and Dobson, 2003). Misfolding of globular milk proteins might occur spontaneously through various environmental changes, such as pH, enzymes in the digestive tract, and the biosynthesis of milk components in mammary gland cells. If neonates and premature cells in their digestive tracts require large amounts of maternal globular proteins, the risk of misfolded proteins must be avoided. One possible option is a low-risk protein such as β-LG: although it misfolds, its toxicity is much milder than that of a high-risk protein such as α-LA.

In Bovidae, the colostrum contains large amounts of β-LG. Its concentration is higher than 15 mg/ml, and it rapidly declines (Levieux and Ollier, 1999). The intestine of bovine neonates changes drastically within 24–36 h after birth, that is, gut closure, which refers to barrier formation against the transfer of macromolecules, such as maternal antibodies, into neonates. Our results also showed that cholesterol addition to IEC-6 cells dramatically abrogated the biphasic cell growth action of stimulation and inhibition by TFE-β-LG (Fig. 3d). Cholesterol limits excessive fluidity of cell membranes. The cellular responses induced by native and denatured β-LGs appeared to be weakened depending on the stability of the cell membrane. Additionally, β-LG, both native (Hashimoto *et al.*, 1995, 1998) and misfolded oligomers (Kobayashi *et al.*, 2021), stimulate the barrier function of tight junctions in differentiated Caco-2 cells. Based on our present observations and findings from previous reports on β-LG, we speculate that the possible involvement of bovine colostrum β-LG might be significant, if not essential, in the gut closure event immediately after birth.

In conclusion, β-LG at a high concentration induced a robust proliferative response in immature intestinal cells. Even if misfolding occurred, proteins could still stimulate cell proliferation. These findings may help our understanding of the function of β-LG in bovine colostrum.

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