Camel and bovine milk lactoferrins activate insulin receptor and its related AKT and ERK1/2 pathways

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ABSTRACT

Lactoferrin (LF) is a milk protein that may be an interesting candidate for the antidiabetic properties of milk due to its well-documented bioactivity and implication in diabetes. Here, we investigated the functional action of LF purified from camel and bovine milk (cLF, bLF) on insulin receptors (IR) and their pharmacology and signaling in hepatocarcinoma (HepG2) and human embryonic kidney (HEK293) cells. For this, we examined IR activation by bioluminescence resonance energy transfer (BRET) technology and the phosphorylation of its key downstream signaling kinases by western blot. The purified cLF and bLF induced phosphorylation of IR, AKT, and ERK1/2 in HepG2 and HEK293 cells. The BRET assays in HEK293 cells confirm the pharmacological action of cLF and bLF on IR, with a possible allosteric mode of action. This reveals for the first time the bioactivity of LF toward IR function, indicating it as a potential bioactive protein behind the antidiabetic properties of camel milk.

Key words: camel milk, lactoferrin, insulin receptor, diabetes, bioluminescence resonance energy transfer (BRET)

INTRODUCTION

Camel milk (CM) is traditionally thought to have many nutritional and medicinal virtues, including antidiabetic effects, in certain tribes and countries. However, little is known about the chemical properties of CM and its mode of action. Recent advances have led to get more insight into the chemical composition and charac-

teristics of CM (Mihic et al., 2016; Singh et al., 2017; Alhaj et al., 2020; Swelum et al., 2021). The antidiabetic effects of CM have been demonstrated in many in vitro and in vivo studies using various animal models as well as diabetic patients (Shori, 2015; Ayoub et al., 2018). However, such a beneficial action of CM in diabetes remained for a long time elusive at the molecular and cellular levels because the antidiabetic agent or agents and the molecular mechanisms involved have not been appropriately studied or fully dissected. Recent studies have investigated this aspect, revealing interesting CM whey proteins and their derived hydrolysates and peptides with bioactivity against different components of glucose homeostasis and insulin and glucagon-like peptide-1 (GLP-1) secretion pathways, including insulin and insulin receptors (IR), α -amylase, α -glucosidase, and dipeptidyl peptidase IV (DPP-IV; Abdulrahman et al., 2016; Mudgil et al., 2018; Nongonierma et al., 2018, 2019; Ashraf et al., 2021; Baba et al., 2021; Kilari et al., 2021). Indeed, crude hydrolysates obtained from in vitro hydrolysis of raw CM whey proteins with various proteases have been shown to inhibit DPP-IV, which is responsible for GLP-1 degradation and, thereby, the decrease of insulin secretion by the β pancreatic cells. Interestingly, we recently demonstrated that raw CM whey proteins and their derived hydrolysates, using a recombinant gastric pepsin endopeptidase, are able to activate IR and its downstream signaling pathways, which was associated with an increase in glucose uptake in both human embryonic kidney (HEK293 cell line) and hepatocarcinoma cells (HepG2 cell line; Abdulrahman et al., 2016; Ashraf et al., 2021). Moreover, a positive synergistic action of CM whey proteins and their derived hydrolysates on insulin-mediated IR activation was observed, indicating a possible allosteric mode of action of CM on IR (Ashraf et al., 2021). Together, these studies clearly revealed the existence of bioactive

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proteins and peptides in CM whey fractions, targeting the key pathways of glucose homeostasis and regulation that may provide a molecular basis for the antidiabetic effects of CM (Ayoub et al., 2018). However, all these *in vitro* data have yet to be proven *in vivo* and in more integrated systems, and further studies are required to demonstrate whether the CM products of degradation resulting from digestion have similar mechanisms. Moreover, it is not clear which protein really confers to CM such bioactivity and pharmacological actions toward IR, DPP-1V, and other pathways relevant to the antidiabetic action. Some studies suggested that insulin or insulin-like peptides, or both, could be the antidiabetic agents of CM (Agrawal et al., 2005). Lactoferrin (LF) may also be an interesting candidate due to the facts that (1) it represents one of the most abundant proteins in milk whey fractions (Konuspayeva et al., 2009, 2007; Mohamed et al., 2021), and (2) it has diverse biological activities (antiviral, antibacterial, antiproliferative, and antidiabetic; Moreno-Navarrete et al., 2009a,b; El-Fakharany et al., 2013; Mayeur et al., 2016; Zapata et al., 2017; Mohamed and Schaalan, 2018; Moreno-Expósito et al., 2018; Cutone et al., 2020). In addition, the possible use of intact LF in different dietetic or clinical situations has already been evidenced (Cutone et al., 2020). For instance, in infants, LF has been shown to survive to gastric digestion and has the ability to interact with its specific receptor in the small intestine. In human adults, LF from cow milk appears to be more resistant to proteolytic degradation than LF from human milk. Some studies have also speculated about the possible encapsulation of CM proteins in particles that protect them from digestion in the gut, but no experimental proof of this is available as of yet (Malik et al., 2012). Finally, LF has been classified as a “generally recognized as safe” substance by the US Food and Drug Administration, and several tests in both animals and humans have proven its safety and tolerability (Cutone et al., 2020). However, the biological activity of LF can also be mediated by bioactive peptides released upon its digestion during its passage and hydrolysis in the gut. Nevertheless, such a case needs to be tested experimentally, and any clinical application of LF should also consider intraperitoneal or intravenous injection of the bioactive LF-derived peptides.

Milk protein content is characterized by 2 major fractions, caseins and whey proteins, which represent 75 to 80% and 20 to 25% of total milk proteins, respectively (Farah, 1993; Konuspayeva et al., 2009; Al haj and Al Kanhal, 2010; Benmeziiane-Derradji, 2021). The whey protein fraction is principally composed of several bioactive proteins, including immunoglobulins, β -lactoglobulin, α -lactalbumin, lactoperoxidase, lysozyme, lactophorin, and lactoferrin, with various nutri-

tional and medicinal benefits (Krissansen, 2007; Badr et al., 2017). These proteins are present at different concentrations in human milk, bovine milk (BM), and CM, and the absence of β -lactoglobulin, known as an allergenic milk protein in human milk and CM, constitutes one of the major differences with BM. Lactoferrin constitutes one of the proteins found in the whey fraction of milk, with different concentrations and percentages reported, varying from 1.33% to 7.28% depending on the study, the breed, the type of milk (human, cow, camel), and the lactation stage (Abd El Gawad et al., 1996; Zhang et al., 2005; El-Hatmi et al., 2006; Konuspayeva et al., 2007; Cutone et al., 2020). Moreover, the concentrations of LF in camel and cow milk has been reported to vary from ~0.2 mg/mL to ~5 mg/mL, with higher levels in camel than in cow milk (Abd El Gawad et al., 1996; Zhang et al., 2005; El-Hatmi et al., 2006; Konuspayeva et al., 2007; Cutone et al., 2020). Lactoferrin is principally known for its role in innate immunity, with well-established antibacterial and antiviral properties, as well as anti-proliferative effects in cancer cells (Redwan and Tabll, 2007; El-Fakharany et al., 2013; Redwan et al., 2014; Moreno-Expósito et al., 2018; Niaz et al., 2019; Soboleva et al., 2019; Li et al., 2021). Lactoferrin is an iron-binding globular glycoprotein of approximately 70 to 80 kDa, belonging to the transferrin family, that is found not only in mammalian milk but also in other secretory fluids, including, saliva, tears, and nasal secretions (Baker and Baker, 2005). In terms of identity between different LF species, bovine LF has been shown to have 70% and 75% identity with human and camel LF, respectively, whereas camel LF shares 74% identity with human LF (Schanbacher et al., 1993; Kappeler et al., 1999).

By contrast, several studies have reported a potential link between LF and diabetes and its complications, such as insulin resistance, inflammation, and obesity (Moreno-Navarrete et al., 2009a,b; Mayeur et al., 2016; Zapata et al., 2017; Mohamed and Schaalan, 2018; Moreno-Expósito et al., 2018). Indeed, circulating LF in diabetic patients was found to be negatively associated with hyperglycemia and positively associated with insulin sensitivity and anti-inflammatory responses (Moreno-Navarrete et al., 2009a). Lactoferrin has been shown to significantly improve the baseline levels of HbA1c, body mass index, and lipid profiles of type 2 pediatric diabetic patients via the TLR4-NF κ B-SIRT-1 signaling cascade (Mohamed and Schaalan, 2018). Moreover, LF has been found to increase insulin-mediated AKT phosphorylation in hepatocytes and adipocytes, suggesting an action on IR (Moreno-Navarrete et al., 2009b). This may support the implication of LF in glucose transport and uptake, as activation of the downstream AKT pathway of IR controls the expression and function of

glucose transporter 4 (GLUT4), mediating glucose uptake in the major insulin-sensitive tissues (Haeusler et al., 2018). Finally, studies have found that CM-derived LF can significantly improve insulin sensitivity in type 2 diabetes patients, and had anti-inflammatory and immune-regulating effects (Badr et al., 2017).

In this study, we hypothesized that LF from CM and BM may directly activate IR and its related downstream signaling pathways, and this may constitute a new molecular basis for the potential implication of LF in the antidiabetic properties of CM. For this, we used LF purified from CM (**cLF**) as previously reported (Redwan and Tabll, 2007; Redwan et al., 2014; Almelhdar et al., 2019), as well as commercial LF purified from BM (**bLF**). The study consists of profiling cLF and bLF for the first time at the molecular and cellular levels for their pharmacological effects on IR activity and function in both HepG2 and HEK293 cells, endogenously and transiently expressing IR, respectively. The phosphorylation status of IR and the 2 key kinases, AKT and ERK1/2, was investigated as evidence for the activation of IR and its downstream signaling pathways by LF. Moreover, bioluminescence resonance energy transfer (**BRET**) technology was used to demonstrate the activation of IR by LF at the molecular level in live cells, as previously reported (Abdulrahman et al., 2016; Ashraf et al., 2021).

MATERIALS AND METHODS

Chemical and Reagents

The mammalian expression plasmids coding for the human IR-Rluc and its signaling protein substrate, IRS1-YFP, were generously provided by Tarik Issad (Cochin Institute, Paris, France). Fresh CM was supplied by local farmers at Al Ain, the United Arab Emirates. Human insulin and bovine LF (purity: >90% by protein Biuret assay, and >85% by SDS-PAGE) were purchased from Sigma Aldrich. The antibodies recognizing IR, AKT, ERK1/2, and GAPDH (both native and phosphorylated forms) were from Cell Signaling Technology; coelenterazine h for BRET assays was purchased from Promega Corp.; the bicinchoninic acid protein assay and Lipofectamine 2000 for cell transfection were purchased from Thermo Fisher Scientific. Ninety-six-well white plates were from Greiner Bio-One. The cLF was purified as previously reported, with purity >90% as estimated by HPLC and SDS-PAGE analysis (Redwan and Tabll, 2007; Redwan et al., 2014; Almelhdar et al., 2019) and generously provided by Elrashdy M. Redwan (King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia). The competitive IR antagonist, S961 peptide (GSLDESFYDWFER-

QLGGGSGGSSLEEEWAQIQCEVWGRGCPSY), was a kind gift from Lauge Schäffer (Novo Nordisk, Copenhagen, Denmark).

Preparation of Whey Protein Fractions from Milk

To obtain whey proteins from camel and cow milks, we used the acid precipitation method, followed by ultracentrifugation. For this, commercial BM was purchased from local shops, and raw CM was obtained from healthy camels (*Camelus dromedarius*) at local camel farms in Al Ain, the United Arab Emirates. Milk samples were immediately transferred to the laboratory under ice storage conditions. Milk was skimmed twice by centrifugation at $4,255 \times g$ for 20 min at 10°C. The skim milk was then subjected to acid precipitation to pH 4.0 by addition of 1 N HCl, and milk samples were allowed to settle for 1 h, followed by centrifugation thrice at $10,000 \times g$ for 10 min at 4°C, to separate whey from the casein fractions. This step was repeated 2 times to separate the whey fraction efficiently. Protein content of the obtained milk whey was estimated using the bicinchoninic acid protein assay. The final samples were first analyzed by SDS-PAGE, followed by Coomassie blue staining, and then samples were used fresh or frozen at -20°C for further experiments.

Cell Culture and Transfection

The HepG2 and HEK293 cells were maintained at 37°C, 5% CO₂ in complete RPMI and Dulbecco's modified Eagle's medium, respectively, containing 0.3 mg/mL glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin supplemented with 10% fetal calf serum (Gibco). The HepG2 cells were used for endogenously expressing IR, and HEK293 cells were used for transient expression of IR-Rluc with IRS1-YFP. The transient transfection was carried out in 75-cm² flasks containing 10⁷ cells, using Lipofectamine 2000 (ThermoFisher Scientific), 5 µg of IR-Rluc, and 5 µg of IRS1-YFP (for BRET and phosphorylation experiments), as previously described (Abdulrahman et al., 2016; Ashraf et al., 2021). All assays were carried out 48 h post-transfection.

Protein Phosphorylation Assay

The HepG2 and transfected HEK293 cells were seeded onto 6-well plates at 10⁶ cells/well overnight in complete media, and then treated with either insulin (1 µM), CM whey protein fraction (**cWP**) and BM whey protein fraction (**bWP**), or their respective purified LF (cLF and bLF; 1 mg/mL or increasing doses for cLF) for 10 min at 37°C, respectively. Following treatment,

cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer and thereafter centrifuged at $12,000 \times g$ for 30 min at 4°C. Thereafter, the extracted cell lysate proteins were quantified using the bicinchoninic acid protein assay as previously reported (Ali et al., 2019). To examine the phosphorylation status of IR, AKT, and ERK1/2, 30 to 40 µg of proteins were loaded on 10% SDS-PAGE gel, followed by western blotting using the following antibodies: mouse monoclonal anti-phospho-IR (pIR-Tyr1334, 1:1,000 dilution), mouse monoclonal anti-phospho-AKT (pAKT-S473, 1:800 dilution), and mouse anti-phospho-p44/42 (pERK1/2, 1:2,000 dilution), as previously described (Ashraf et al., 2021). The corresponding total proteins loaded were also analyzed in parallel, using the specific antibodies against the native forms of the different proteins, including GAPDH as a control. Densitometry analysis of the different western blots was performed using Image Studio Version 5.2 software from LI-COR Biosciences.

BRET Assay

We used BRET technology as previously described for hIR-IRS1 interaction (Ashraf et al., 2021). Cells coexpressing hIR-Rluc and IRS1-YFP were first seeded in white, 96-well plates, washed with 50 µL/well of PBS, and treated for 60 min at 37°C with 40 µL of PBS with or, as control, without the indicated doses of insulin, the different CM and BM whey fractions (cWP, cLF, bWP, bLF), or the combination of each of these CM or BM fractions with insulin, as indicated. For the BRET experiments with the competitive IR antagonist S961, cells were first pretreated with 10 µM of S961 for 20 min at 37°C, followed by treatments with the indicated doses of insulin or CM or BM fractions. Following treatments, 10 µL of coelenterazine h (the substrate of Rluc in IR-Rluc fusion protein required for the luminescence emission at 480 nm during the BRET assay) was added to a final concentration of 5 µM, and BRET measurements were carried out using a Tristar 2 multi-label plate reader (Berthold Technologies GmbH & Co.), allowing luminescence recording and signal integration at 480 and 540 nm.

Data Presentation and Statistical Analysis

For BRET, the data were first converted to ligand-induced BRET signals by subtracting the 540 nm/480 nm ratio obtained from vehicle-treated cells from the same ratio obtained from cells treated with insulin or CM fractions. Then the data were normalized by taking as 100% the maximal insulin-induced BRET signal in the control (absence of any treatment with CM fractions). All the data were analyzed and fitted using

GraphPad Prism software version 7. Statistical analyses were performed with 2-way ANOVA and Bonferroni test to determine statistical significance between the different treatments and conditions, as indicated in the figures and their legends. For this, the different treatments with insulin, whey fractions, or purified cLF and bLF, combined or not, were analyzed for their statistical difference and significance relative to either control or basal condition in each experiment. The statistics are shown in letters, where the same letters indicate no significant difference ($P > 0.05$), whereas different letters indicate statistically significant effects, between the different treatments compared with each other, including the control condition (untreated cells; $P < 0.05$). However, in dose-response graphs, statistical differences are marked with asterisks: **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$.

RESULTS

LF Purified from CM and BM Promoted IR Phosphorylation in HepG2 Cells

In this study, we investigated the pharmacological and functional actions of LF purified from CM (cLF) as previously reported (Redwan and Tabll, 2007; Redwan et al., 2014; Almeshdar et al., 2019) and commercial LF purified from BM (bLF) on IR activity and its related downstream signaling pathways. For this, we used HepG2 cells endogenously expressing IR for all phosphorylation assays and HEK293 cells transiently transfected with the plasmids coding for IR-Rluc and its substrate protein, IRS1-YFP, for both phosphorylation and BRET assays.

Importantly, a previous study showed no cytotoxic effect of purified LF on HepG2 cells (El-Fakharany et al., 2013). Moreover, we previously verified that CM whey fractions had no effect on the viability of HepG2 and HEK293 cells within the short time range of our experimental conditions (data not shown). Indeed, all our BRET and western blot experiments were carried out upon treatments of cells for 1 h (for BRET) or 10 min (for SDS-PAGE and western blot) with the different CM and BM fractions. Thus, within this time of treatment no variations would be expected in terms of cell cycle regulation and cell proliferation.

First, we verified the integrity of the cLF and bLF proteins, as well as cWP and bWP, using SDS-PAGE followed by Coomassie blue staining. As shown in Figure 1A, both cLF and bLF showed intact protein bands with the expected molecular weight (~70–80 kDa) and correct electrophoretic profile.

Next, we profiled cLF, bLF, and their respective whey protein fractions (cWP and bWP) for their ef-

fects on the activation of IR endogenously expressed in HepG2 cells. For this, IR phosphorylation was examined as a readout of its activation upon cell treatment with 1 mg/mL of the different milk fractions using a subsaturating dose of insulin (1 μ M) as the positive control. As shown in Figure 1B, both cWP and bWP and their respective LF significantly promoted IR phosphorylation to similar levels compared with insulin. The quantification of 4 independent western blot experiments and their statistical analysis relative to the control condition (untreated cells; $P < 0.05$) confirm such a positive effect on IR phosphorylation (pIR) with no significant difference between the different treatments compared with each other (Figure 1C). These observations demonstrate the activation of IR upon treatment of cells with cWP and bWP fractions as well as their purified LF.

LF Purified from CM and BM Promoted AKT and ERK1/2 Phosphorylation in HepG2 Cells

Next, we wanted to link our data on pIR with its downstream signaling pathways in HepG2 cells by in-

vestigating the effects on the phosphorylation of AKT and ERK1/2, known as the 2 major canonical IR signaling pathways (Haeusler et al., 2018). As was the case for pIR, the cWP and bWP fractions and their respective LF (cLF and bLF) significantly promoted AKT and ERK1/2 phosphorylation to similar levels compared with insulin (Figure 2A). This was confirmed through the quantification of 4 independent western blot experiments showing statistically significant effects of the different treatments relative to the control condition (untreated cells; $P < 0.05$), with no significant difference between the different treatments compared with each other (Figure 2B and C).

To further confirm the bioactivity of cLF on IR, AKT, and ERK1/2 pathways, we carried out dose-response analysis using various doses of cLF (0, 0.05, 0.1, 0.5, 1, and 2 mg/mL). As shown in Figure 3, cLF promoted the phosphorylation of IR, AKT, and ERK1/2 in a dose-dependent manner. Moreover, taken along with the BRET dose-response shown in Figure 4B, these data indicate that the dose of 1 mg/mL used in the whole study can be considered a saturating dose of LF (Figure 3).

Figure 1. Insulin receptor (IR) phosphorylation induced by camel milk (CM) and bovine milk (BM) fractions in hepatocarcinoma (HepG2) cells. (A) Whey protein fractions of CM and BM (cWP, bWP) samples (10 mg/mL) as well as their respective purified lactoferrins (LF: cLF and bLF, 0.5 mg/mL) were first subjected to 10% SDS-PAGE followed by Coomassie blue staining for protein purity and integrity. (B) HepG2 cells were treated as indicated, and IR phosphorylation (pIR) was analyzed as described in Materials and Methods. (C) Quantification of western blot data for IR phosphorylation using densitometry analysis; data are presented as means $\pm$ SEM of 4 independent experiments. Different lowercase letters (a, b) indicate statistically significant effects of the different treatments relative to the control condition (untreated cells; $P < 0.05$).

Figure 2. Phosphorylation of AKT and ERK1/2 induced by camel milk (CM) and bovine milk (BM) fractions in hepatocarcinoma (HepG2) cells. (A) HepG2 cells were treated as indicated, and AKT and ERK1/2 phosphorylation (pAKT and pERK1/2) was analyzed as described in Materials and Methods. (B) and (C): Quantification of western blot data for AKT and ERK1/2 phosphorylation using densitometry analysis; data are presented as means $\pm$ SEM of 4 independent experiments. cWP, bWP = whey protein fractions of CM and BM; cLF, bLF = purified lactoferrin of CM and BM. Different lowercase letters indicate statistically significant effects of the different treatments relative to the control condition (untreated cells; $P < 0.05$).

LF Purified from CM and BM Promoted IR Activation in HEK293 Cells as Assessed by BRET

With the aim of demonstrating the direct effects of cWP and bWP and their respective LF on IR activation at the molecular level of IR protein, we used BRET technology in HEK293 cells, as previously reported (Abdulrahman et al., 2016; Ashraf et al., 2021). In live cells, this proximity-based assay measures the change in the distance and the interaction dynamics between proteins upon cell treatment. Here, we measured the physical translocation of the insulin receptor substrate 1 (IRS1) fused with the yellow fluorescent protein (IRS1-YFP as a BRET acceptor) to the activated IR fused to *Renilla* luciferase (IR-Rluc as a BRET donor) upon its activation with CM and BM fractions. Thus, upon cell treatment, any increase in the BRET signal between IR-Rluc and IRS1-YFP reflects the proximity between them, which is indicative of IR activation and phosphorylation, resulting in IRS1 recruitment. For this, HEK293 cells were first transfected with the mammalian expression plasmids coding for IR-Rluc and IRS1-YFP and then treated with either 1 mg/mL of cWP and bWP fractions and their respective LF or with insulin (1 μ M) used as a positive control for IR activation. As shown in Figure 4A, the different CM and BM fractions significantly promoted the increase in the BRET signals between IR-Rluc and IRS1-YFP, which occurred at similar levels compared with insulin. The statistical analysis confirms the significant effects of the different treatments relative to the control condition (untreated cells; $P < 0.05$), with no significant difference between the different treatments compared with each other (Figure 4A). This BRET result is con-

sistent with our data on IR phosphorylation, shown in Figure 1, and further demonstrates the activation of IR by cWP and bWP and, more importantly, by the purified cLF on IR activation assessed by BRET, we also carried out dose-response analysis with cLF (0, 0.01, 0.05, 0.1, 0.5, 1, and 2 mg/mL). As shown in Figure 4B, cLF promoted increases in the BRET signals between IR-Rluc and IRS1-YFP in a dose-dependent manner, with a maximal response observed at 1 mg/mL of cLF. This confirms that the dose of 1 mg/mL is a saturating dose of LF, similar to our data on the phosphorylation of IR, AKT, and ERK1/2 (Figure 3).

Synergistic Effects of LF Purified from CM and BM on Insulin-Mediated IR Activation in HEK293 Cells as Assessed by BRET

Next, we examined the effects of combining cLF or bLF with insulin on IR activation using BRET assay in HEK293 cells transfected with the plasmids coding for IR-Rluc and IRS1-YFP. For this, we performed dose-response analysis by stimulating cells with increasing doses of insulin (0, 0.05, 0.1, 0.5, 1, 5, and 10 μ M) in the absence and presence of a saturating dose (1 mg/mL) of either cLF or bLF. As shown in Figure 4C and D, insulin induced BRET increase in a dose-dependent manner, with expected potency compared with our previous study (Abdulrahman et al., 2016; Ashraf et al., 2021). Interestingly, in the presence of either cLF (Figure 4C) or bLF (Figure 4D), a synergistic effect was observed on the insulin dose curve, characterized by a potentiation of the maximal insulin response ($E_{\max}$) by $\sim 145\%$ and $\sim 130\%$, respectively, compared with the

condition with insulin alone (Figure 4 and Table 1). Moreover, a left-shift of the dose curve of insulin was observed in the presence of cLF (Figure 4C) but not bLF (Figure 4D), resulting in a decrease in the half-maximal effective concentration (EC_{50}) value of insulin from 466 nM (insulin alone) to 193 nM (cLF; Table 1). These effects on insulin E_{max} and its EC_{50} suggest that cLF increased both insulin efficacy and its potency for IR activation.

Pharmacological Blockade of IR Had No Effect on CLF/BLF-Mediated BRET Increase but Reduced Their Potentiating Action

To further investigate the effect of cWP, bWP, and their respective LF on IR activation, we used the competitive IR antagonist S961 (Schäffer et al., 2008), as previously (Ashraf et al., 2021). For this, we used a BRET assay in HEK293 cells transfected with the plasmids coding for IR-Rluc and IRS1-YFP, similar to the experiments represented in Figure 4. As expected, the different treatments (black histograms) significantly, and to different extents, increased the BRET signals ($P < 0.05$) between IR-Rluc and IRS1-YFP (Figure 5A and B). In addition, pretreatment of cells with S961 (10 μ M) drastically reduced insulin-promoted BRET (red histograms), indicating the blockade of IR (Figure 5A and B). However, S961 had no significant effect on the BRET responses mediated by cWP, bWP, and their respective LF ($P > 0.05$; Figure 5A), consistent with our previous study on cWP and their hydrolysates (Ashraf et al., 2021). Moreover, the combined treatment (insulin + cLF or bLF) led to a significant potentiation of BRET response to $137.5 \pm 11.5\%$ with cLF and $129 \pm 9.7\%$ with bLF compared with insulin alone ($P < 0.05$; Figure 5B). This further confirms the positive effect of cLF and bLF on insulin-mediated BRET response, as revealed in the dose-response analysis shown in Figure 4C and D. Interestingly, S961 treatment only partially inhibited the BRET responses mediated by the combination of insulin with cLF and bLF (Figure 5B). Again, this observation is in agreement with our previous study on the effects of S961 on IR activity mediated by cWP and their hydrolysates (Ashraf et al., 2021).

LF Purified from CM and BM Promoted IR, AKT, and ERK1/2 Phosphorylation in HEK293 Cells

To correlate our BRET data in the transfected HEK293 cells with IR phosphorylation and its downstream signaling pathways, we also examined the effects of cWP and bWP fractions and their respective LF (cLF and bLF) on the phosphorylation status of IR, AKT, and ERK1/2 in transfected HEK293 cells in parallel with BRET experiments. As shown in Figure

6A, both cLF and bLF promoted, to different extents, the phosphorylation of IR, AKT, and ERK1/2, similar to cWP and bWP as well as insulin. The statistical analysis of the quantification of 3 (pIR; Figure 6B) and 4 (pAKT and pERK1/2; Figure 6C and D) independent western blot experiments confirms the significant effects of the different treatments relative to the control condition (untreated cells; $P < 0.05$), with no significant difference between the different treatments compared with each other. This is consistent with our BRET data shown in Figure 4A and Figure 5A and further confirms the bioactivity of the different milk fractions on IR function and its downstream signaling pathways in transfected HEK293 cells, as demonstrated in HepG2 cells endogenously expressing IR (Figure 1 and Figure 2).

DISCUSSION

In this study, we investigated for the first time the bioactivity of one of the major CM and BM protein

Figure 3. Dose-response of camel milk lactoferrin (cLF)-promoted phosphorylation of insulin receptor (IR), AKT, and ERK1/2 in hepatocarcinoma (HepG2) cells. HepG2 cells were treated with increasing doses of cLF (0, 0.05, 0.1, 0.5, 1, and 2 mg/mL), and the phosphorylation status of IR, AKT, and ERK1/2 phosphorylation (p) was analyzed by western blot using the appropriate antibodies as described in Materials and Methods. The figure is representative of 3 different independent experiments.

Figure 4. Insulin receptor (IR) activation promoted by camel milk (CM) and bovine milk (BM) fractions and assessed by bioluminescence resonance energy transfer (BRET) technology in human embryonic kidney (HEK293) cells. HEK293 cells were first transfected with the plasmids coding for IR-Rluc (BRET donor) and IRS1-YFP (BRET acceptor). Thereafter, the cells were treated as indicated by single treatments (A and B) or by a combination of increasing doses of insulin (0, 0.05, 0.1, 0.5, 1, 5, and 10 μ M) in the absence or presence of 1 mg/mL purified lactoferrin of either CM or BM (cLF, C; bLF, D). BRET signals were measured as described in Materials and Methods. All BRET responses were normalized by taking the insulin-mediated BRET signal as 100%. Data are presented as means $\pm$ SEM of 3 to 5 independent experiments. For the single treatments (A), different lowercase letters indicate statistically significant effects of the different treatments relative to the control condition (untreated cells; $P < 0.05$). For the dose-response in (B), statistical analysis indicates significance when comparing the different doses of cLF with the condition in the absence of cLF (0 mg/mL). For the dose-response data in (C) and (D), statistical analyses indicate significant differences when comparing the combined treatment condition (Insulin + cLF/bLF, blue and red curves) with insulin alone (black curves). **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, and ns $P > 0.05$. cWP, bWP = whey protein fractions of CM and BM.

components, LF, at the molecular and cellular levels in the context of the well-documented antidiabetic properties of the milks (Shori, 2015; Ayoub et al., 2018). For this, we used LF purified from CM and BM, either in-home (cLF) or commercially (bLF). The novelty of the study resides in the demonstration of the bioactivity of the purified cLF and bLF toward activity of IR

and its downstream signaling in HepG2 and HEK293 cells endogenously and transiently expressing IR, respectively. Indeed, SDS-PAGE followed by western blot for protein phosphorylation demonstrated that both cLF and bLF promoted IR phosphorylation in a dose-dependent manner, indicating its activation. Moreover, LF-induced IR phosphorylation was associated with

Table 1. Different pharmacological parameters of the positive effects of camel and bovine milk lactoferrin (cLF and bLF) on insulin dose-response, as shown in Figure 4¹

Item	Insulin	Insulin + bLF (1 mg/mL)	Insulin + cLF (1 mg/mL)
Basal BRET (%)	1.15 $\pm$ 5.19 ^a	25.46 $\pm$ 7.05 ^b	28.98 $\pm$ 15.13 ^b
Maximal BRET (%)	100 ^a	129.8 $\pm$ 12.5 ^b	144.4 $\pm$ 15.6 ^b
LogEC ₅₀	-6.332 $\pm$ 0.130	-6.377 $\pm$ 0.155	-6.714 $\pm$ 0.255
EC ₅₀ (nM)	465.5 ^a	419.7 ^a	193.0 ^b

^{a,b}Different lowercase letters indicate statistically significant effects of the different treatments relative to the condition where cells were treated with insulin alone ($P < 0.05$).

¹Statistical analysis was performed by comparing the condition with insulin alone to the combined treatments (Insulin + bLF or cLF). BRET = bioluminescence resonance energy transfer; EC₅₀ = half-maximal effective concentration. All values indicated in the table are means $\pm$ SEM.

Figure 5. Effect of insulin receptor (IR) blockade on camel milk (CM) and bovine milk (BM) fraction-mediated bioluminescence resonance energy transfer (BRET) responses in human embryonic kidney (HEK293) cells. HEK293 cells were first transfected with the plasmids coding for IR-Rluc (BRET donor) and IRS1-YFP (BRET acceptor) and pretreated (red histograms) or not (black histograms) with the competitive IR antagonist S961 (10 μ M). Thereafter, cells were stimulated with insulin (1 μ M) alone (A and B) or with 1 mg/mL of either whey protein fractions of CM and BM (cWP, bWP), purified lactoferrin of CM or BM (cLF and bLF) (A), or the combination of insulin with cLF and bLF (B), as indicated and described in Materials and Methods. All the BRET responses were normalized by taking the insulin-mediated BRET signal as 100%. Data presented as means $\pm$ SEM of 3 independent experiments. Different lowercase letters (a–d) indicate statistically significant effects when the different treatments were compared ($P < 0.05$).

the activation of its 2 canonical kinase pathways, represented by AKT and ERK1/2, known for their implication in the control of glucose transport and metabolism in the major insulin-sensitive tissues (Haeusler et al., 2018). Such biochemical evidence of the bioactivity of cLF and bLF was also confirmed by BRET technology, demonstrating the pharmacological targeting of IR and its activation in live HEK293 cells. Indeed, cLF and bLF significantly increased BRET signals between IR-Rluc and IRS1-YFP, indicative of IRS1 recruitment to the activated IR. Both phosphorylation and BRET data showed that the effects of cLF and bLF occurred to similar levels as that of the insulin used as a positive control and their respective crude whey fractions, further evidencing the specificity of LF effects. Moreover, our data suggest that both CM and BM components exert similar effects on IR and its activation in HepG2 and HEK293 cells.

We previously reported the positive pharmacological and functional effect of crude cWP and their hydrolysates on IR activation and its downstream signaling pathways at the cellular level (Abdulrahman et al., 2016; Ashraf et al., 2021). In addition, we emphasized that the cWP fraction contains bioactive components, either proteins or peptides, which may explain the beneficial effects of CM in diabetes, as reported in vitro and in vivo (Shori, 2015; Ayoub et al., 2018). Thus, the positive effect of LF on IR and its downstream signaling is very consistent with our previous finding on CM whey proteins and hydrolysates, suggesting that

LF is one of the potential antidiabetic agents of CM. This may constitute a substantial advance toward the identification and isolation of the antidiabetic agent contained in CM. Furthermore, such a novel finding on LF as one of the potential antidiabetic candidates contained in CM is credible when considering all the previous studies reporting the link between LF and diabetes and its pathology. Indeed, LF has been reported to be involved in insulin resistance and related disorders, such as obesity and inflammation (Moreno-Navarrete et al., 2009a,b; Mayeur et al., 2016; Zapata et al., 2017; Mohamed and Schaalán, 2018; Moreno-Expósito et al., 2018). Also, circulating LF has been negatively associated with hyperglycemia and positively associated with insulin resistance (Mayeur et al., 2016). On the other hand, our data also support the conclusion that BM and its LF may also be considered for antidiabetic applications, as they also showed a positive action on IR and its downstream signaling pathways.

At the molecular level, we demonstrated for the first time that LF positively modulates IR activity and function, resulting in the activation of its 2 canonical kinase pathways, AKT and ERK1/2. This finding can be considered seminal in the sense that we revealed that LF-induced IR, AKT, and ERK activation occurred in an insulin-independent manner, demonstrating that LF was able to activate such a signaling axis in both HepG2 and HEK293 cells even in the absence of insulin treatment. Moreover, our BRET data in HEK293 cells, using the treatment of insulin in combination with cLF

or bLF, are of great importance, as we also observed a positive effect of both cLF and bLF on insulin-mediated response. This is in agreement with our previous studies on cWP and their hydrolysates (Abdulrahman et al., 2016; Ashraf et al., 2021), suggesting a synergistic or allosteric action of cWP or bWP and cLF or bLF on IR activity and signaling. Such a synergistic action is consistent with a previous study that reported a positive effect of human LF on insulin-dependent response by increasing insulin-mediated AKT phosphorylation in HepG2 and adipocyte 3T3 cells (Moreno-Navarrete et al., 2009b). Moreover, *in vivo* studies have showed that CM led to a need for lower insulin doses injected in diabetic rats and also increased insulin secretion by the β pancreatic cells (Agrawal et al., 2011, 2007; Korish, 2014).

The quantitative analysis of the BRET data shown in Figure 4 and Figure 5 indicates that cLF- and bLF-mediated potentiation of BRET responses occurred in more a synergistic rather than an additive manner. Indeed, the level of the responses mediated by the combined treatments (insulin + cLF or bLF) was not consistent with the sum of the individual responses mediated by either insulin or cLF or bLF alone. Moreover, cLF seems to increase both insulin efficacy ($E_{\max}$ increased) and its potency (EC_{50} decreased) for its receptor (Table 1). From a pharmacological point of view, the changes of these 2 key parameters are more

in agreement with a synergistic and positive allosteric effect of LF on IR. Indeed, the dose-response curve with cLF is typically consistent with the effect of a positive allosteric modulator, since cLF significantly left-shifted the insulin dose curve and increased its potency (reduction of insulin EC_{50} value, as shown in Table 1). However, the potentiation of the maximal insulin-mediated BRET response ($E_{\max}$) can be considered somewhat intriguing. Indeed, at the concentration 5 μM and 10 μM of insulin, the BRET response was saturated (Figure 4C and D), indicating that all IR molecules were occupied by insulin and the system was fully activated. Accordingly, any further increase of the BRET signals by cLF or bLF at these saturating doses of insulin cannot be explained by further engagement of IR molecules in BRET process. This may be indicative of the engagement of other LF receptors cross-talking with IR, as speculated in our model shown in Figure 7. In addition, one would speculate that cLF and bLF induce or stabilize specific conformational changes within IR, leading to higher BRET efficiency between IR-Rluc and IRS1-YFP. This point requires further investigation using appropriate tools and models.

Regarding the possible binding of cLF and bLF to the orthosteric and allosteric binding site of IR, our data are in agreement with the implication of an allosteric binding site. In fact, the combination of insulin with cLF or bLF did not result in a competition be-

Figure 6. Phosphorylation (p) of insulin receptor (IR), AKT, and ERK1/2 induced by camel milk (CM) and bovine milk (BM) fractions in human embryonic kidney (HEK293) cells. HEK293 cells were first transfected with the plasmids coding for IR-Rluc and IRS1-YFP as for bioluminescence resonance energy transfer (BRET) analysis. Cells were thereafter treated as indicated, and the phosphorylation status of IR, AKT, and ERK1/2 was analyzed by western blot using the appropriate antibodies as described in Materials and Methods. Panel (A) is representative of 3 (IR) and 4 (AKT and ERK1/2) independent experiments. Quantification of western blot data using densitometry analysis is presented as means $\pm$ SEM of 3 (for IR; B) or 4 (for AKT and ERK1/2; C and D) independent experiments. Different lowercase letters (a, b) indicate statistically significant effects of the different treatments relative to the control condition (untreated cells; $P < 0.05$). cWP, bWP = whey protein fractions of CM and BM; cLF, bLF = purified lactoferrin of CM and BM; hIR = human insulin receptor.

Figure 7. Speculative model illustrating the action of camel milk (CM) lactoferrin (cLF) and bovine milk (BM) lactoferrin (bLF) on insulin receptor (IR) activity and its downstream signaling pathways. Overall, our data demonstrate the positive action of LF purified from CM and BM on IR activity and its downstream signaling pathways. This may be mediated either through a direct binding of LF on IR, most likely via an allosteric binding site, leading to the activation of IR and its related AKT and ERK1/2 pathways (1), or through the binding of LF to another unknown receptor (2) that synergistically cross-activates IR (3) or promotes AKT and ERK1/2 activation independently on IR (4). P = phosphorylated tyrosine residues in IR upon its activation.

tween them at IR, which would have resulted in the inhibition of IR activation and BRET responses. This excludes any possibility for cLF and bLF to bind in the orthosteric site of IR. Furthermore, our data with the competitive IR antagonist S961 are consistent with our previous study on cWP and their hydrolysates (Ashraf et al., 2021), revealing an interesting mode of pharmacological action of cWP and bWP and their respective LF. Indeed, S961 did not block the pharmacological effects of cWP or bWP and their respective LF on IR, whereas it specifically abolished insulin-mediated IR activation. Because S961 is an antagonist competing with insulin binding and action on IR, this indicates that cWP and bWP and cLF and bLF do not bind to the orthosteric (insulin) binding site on IR. This implies either the existence of a positive allosteric binding site for cWP and bWP and cLF and bLF in IR or their indirect effect via binding to another cell surface receptor that cross-talks with IR, as stated previously and as illustrated in Figure 7. Interestingly, S961 only partially reduced the BRET response mediated by the

combined treatment with insulin and cLF or bLF to the levels corresponding to the effects of cLF and bLF alone (Figure 5B). This should be interpreted in the context of S961 blocking the orthosteric (insulin) binding site on IR as well as in line with our speculation on the existence of either an allosteric binding site on IR or another LF receptor that allosterically interacts with IR. The positive effect of cLF and bLF on IR when they were individually applied suggests that binding cLF or bLF to such a putative allosteric site in IR may be enough to induce conformational changes within IR that leads to its activation. However, this was not affected by the competitive IR antagonist (S961), suggesting a different mode of activation via allosteric binding rather than the orthosteric binding of insulin. However, the synergistic effects of cLF and bLF indeed require the binding of insulin in its orthosteric binding site on IR, which further supports the positive allosteric modes of action of cLF and bLF. All together, these data and arguments imply the possible existence of such an allosteric site in the extracellular domain of IR.

This can be supported by previous studies reporting the allosteric modulation of IR using selective antibodies (Bhaskar et al., 2012; Issafras et al., 2014).

Together, these observations converge toward the speculation that LF plausibly represents one of the bioactive proteins behind the pharmacological and functional action of cWP on IR previously reported (Abdulrahman et al., 2016; Ashraf et al., 2021). When comparing the effective doses of the whole whey fraction and LF, our data showed that 1 mg/mL of both quantitatively led to similar responses in terms of IR activity (BRET and phosphorylation). This is somewhat intriguing, considering 1 mg/mL of LF does not correspond with the real concentrations of LF in 1 mg/mL of the whey fraction, which are most likely much less. One plausible explanation of our results is that LF may not be the only bioactive protein or peptide in the whey fraction activating IR. Otherwise, how to explain similar levels of response and signal between the whey fraction and the purified LF, admitting that the real concentration of LF in 1 mg/mL of whey fraction would probably be lower than 1 mg/mL used for the purified one? This may be due differences in the pharmacological properties (potency, efficacy, synergistic interaction, different binding sites on IR or other receptors) of each bioactive component contained in the whey fraction. Indeed, 1 mg/mL of whey fraction may contain other bioactive components (including LF) that also positively modulate IR and its signaling. Thus, the final response and signal (BRET and phosphorylation) would represent the sum of the effects mediated by these bioactive whey components and their pharmacological interactions, each with its specific and intrinsic potency and efficacy on IR. This may also indicate different binding properties of the different bioactive whey components, with all of them being involved in a positive cooperative or allosteric interaction, leading to high activation of IR and its signaling. One would expect that individually these bioactive components at their real concentrations in CM would have only weaker effect on IR activation and signaling compared with the whey fraction and insulin. Along this line, 1 mg/mL of purified LF may constitute a very high dose, which is probably beyond its real concentration in our whey fractions. This may explain why, at the end, the purified LF led to effects (quantitatively) similar to the sum of all the bioactive whey proteins or peptides. Of course, we cannot exclude the implication of other nonprotein components present in the whey fractions.

Accordingly, our speculative model shown in Figure 7 describes the different mechanisms and possibilities to reconcile all of our data with the effects of cLF and bLF on IR and its downstream signaling pathways in HepG2 and HEK293 cells. Of course, further studies are

required, to demonstrate the exact mode of action of LF on IR by investigating the possibility of LF binding on IR or not. The physiological relevance of our finding and its plausible clinical application may be not obvious based on our *in vitro* study using intact LF, raising the question of its digestion and hydrolysis in the gut. However, LF, as a protein, is known as an interesting CM component with diverse biological activities demonstrated in many *in vitro* and *in vivo* studies (antiviral, antibacterial, antiproliferative, antidiabetic, and more; El-Fakharany et al., 2013; Cutone et al., 2020). Therefore, with the aim of using LF in humans and whole animals, efforts have made to address the question of LF bioavailability and absorption for plausible applications in therapy and clinically. As result, evidence has accumulated in favor of the possible use of intact LF in different dietetic or clinical situations. For instance, in infants, LF seems to survive to gastric digestion and has the ability to interact with its specific receptor in the small intestine. In contrast, studies on gastrointestinal digestion kinetic of LF in adults have highlighted that recombinant human LF (rhLF) from transgenic cows is completely digested, whereas about 60% of bLF was found undigested. In human adults, bLF appears to be more resistant than human LF against proteolytic degradation. Some studies have also speculated about the possible encapsulation of CM proteins in particles that protect them from digestion in the gut, but no experimental proof of it has yet been found (Malik et al., 2012). Finally, LF has been classified as a “generally recognized as safe” substance by the US Food and Drug Administration, and several tests in both animals and humans have proven its safety and tolerability, even at high dosages. All these aspects of LF are described by Cutone et al. (2020). Nevertheless, if LF really contributes to the antidiabetic effects of CM, its biological activity may simply be due to its hydrolysis by the gastric proteases, and its derived peptides would be the bioactive agents. Thus, it would be interesting to examine the effects of LF hydrolysates and derived peptides on IR activity and glucose transport inside the cells. Furthermore, as stated earlier, studies have reported a link between LF and diabetes. Indeed, circulating LF has been negatively associated with hyperglycemia and positively associated with insulin resistance, respectively (Mayeur et al., 2016). This suggests that LF may be an important component in glucose homeostasis that gives a solid rationale for investigating LF in the context of the antidiabetic effects of CM. Again, our *in vitro* study does not show any direct link between LF and the antidiabetic effects of CM and BM. The targeting of IR and IR signaling pathways should be considered interesting and promising for further investigations. At more integrated levels and systems, it is

obvious that our data in HepG2 and HEK293 cells need confirmation in vivo, using diabetic animal models in which the purified intact or hydrolyzed cLF and bLF would be tested for their beneficial effects toward various parameters of diabetes pathology such as glycemia, lipid levels, and insulin secretion and response.

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