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In Vitro Investigation of the Effect of Camel Milkproteins and Its Fractions on Insulin Receptor Function

Arshida Ashraf

United Arab Emirates University

College of Science

Department of Biology

IN VITRO INVESTIGATION OF THE EFFECT OF CAMEL MILK PROTEINS AND ITS FRACTIONS ON INSULIN RECEPTOR FUNCTION

Arshida Ashraf

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Dr. Mohammed Akli Ayoub

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Declaration of Original Work

I, Arshida Ashraf, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*In Vitro Investigation of the Effect of Camel Milk Proteins and its Fractions on Insulin Receptor Function*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Mohammed Akli Ayoub, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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This Master Thesis is approved by the following Examining Committee Members:

1) **Advisor (Committee Chair): Dr. Mohammed Akli Ayoub**
   Title: Assistant Professor
   Department of Biology
   College of Science
   Signature _____ Date 14/11/2019

2) **Member (Internal Examiner): Dr. Yusra Al-Dhaheri**
   Title: Assistant Professor
   Department of Biology
   College of Science
   Signature _____ Date 14/11/19

3) **Member (External Examiner): Dr. Amar Mansour Hamrouni**
   Title: Assistant Professor
   College of Pharmacy
   Institution: Al Ain University, U.A.E.
   Signature _____ Date 14/11/2019

This Master Thesis is accepted by:

Dean of the College of Science: Professor Ahmed Murad

Signature _____ Date 30/01/2020

Dean of the College of Graduate Studies: Professor Ali Al-Marzouqi

Signature Ali Hassan Date 2/2/2020

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Abstract

Camel milk (CM) has been reported to have anti-diabetic properties in many *in vitro* and *in vivo* studies but the molecular basis of such beneficial properties are still elusive. Recently, camel milk whey proteins (CWPs) have been shown to positively affect the activity of the human insulin receptor (hIR) in cell lines. In this study, we profiled crude CWPs and their hydrolysates as well as camel milk lactoferrin (CMLF) for their pharmacological and functional effects on hIR activity and its downstream signaling in both human embryonic kidney (HEK293) and hepatocarcinoma (HepG2) cell lines. For this, bioluminescence resonance energy transfer (BRET) technology was used to assess hIR activity in live cells and the phosphorylation status of the downstream protein kinase B (Akt) and the extracellular signal-regulated kinases (ERK1/2) was also analyzed in parallel. Moreover, glucose uptake was examined in order to link our data to more integrated cell response and to the hypoglycemic effects of camel milk. Our data clearly demonstrate the biological activity of CWPs, their hydrolysates, and CMLF, by promoting Akt and ERK1/2 phosphorylation in both HEK293 and HepG2 cells. In addition, our BRET assay confirmed the positive pharmacological action of CWPs and their hydrolysates on hIR activity in a dose-dependent manner. More interestingly, the combination of CWPs and their hydrolysates with insulin revealed an allosteric modulation of hIR that was drastically abolished by the competitive hIR-selective peptide antagonist S961. This clearly demonstrates the implication of hIR activation in the effects of CWPs and their hydrolysates. Finally, such effects on BRET data and kinase phosphorylation were clearly correlated with an increase in glucose uptake in HepG2 cells. Our data reveal the pharmacological effects of camel milk proteins on hIR activity and function. This provides for the first time the molecular basis of the anti-diabetic properties of camel milk that was unknown until now.

Keywords: Camel milk, Diabetes, Insulin receptor, Insulin, Glucose, BRET.

Title and Abstract (in Arabic)

دراسة تأثير بروتينات حليب الإبل على وظيفة مستقبلات هرمون الإنسولين

الملخص

أظهرت عدة دراسات أن حليب الإبل له تأثير على هرمون الإنسولين(hIR) كمضاد للسكري، لكن هذا التأثير لحليب الإبل غير واضح حتى الآن على مستوى البيولوجيا الجزيئية (المسارات الموجودة داخل الخلية). حديثاً، وجدت دراسة أن بروتينات حليب الإبل مثل بروتين مصل اللبن(CWPs) وأجزاءه (CWPs hydrolysates)بالإضافة إلى بروتين لا كتوفرين (CMLF)، لها تأثير إيجابي على زيادة نشاط مستقبلات الأنسولين في خلايا الإنسان. خلال دراستنا هذه، تمت تنقية البروتينات التي ذُكرت سابقاً من حليب الإبل ومعاينة كيفية تأثيرها على وظيفة ونشاط مستقبلات الإنسولين، بالإضافة إلى دراسة تأثيرها على المستوى الجزيئي للخلايا باستخدام نوعين من الخلايا (خلايا سرطان الكبد HepG2 و خلايا الكلى الجنينية (HEK293). لإتمام هذه التجارب، استَخدمت تقنية BRET لتقييم وظيفة مستقبلات الأنسولين في الخلايا الحيّة،إضافة إلى تقييم تفعيل نشاط كل من بروتين Akt و ERK1/2 عن طريق ما يسمى بالفَسفَتة (أي ارتباطهم بفوسفات). أيضاً قياس مستوى أخذ واستيعاب الخلايا للسكر للحصول على صورة أوضح لربطها مع تأثير حليب الإبل على خفض السكري. دراستنا أثبتت أن بروتينات مصل اللبن ولاكتوفرين بحليب الإبل، تقوم على تفعيل نشاط كل من Akt و ERK1/2 عن طريق ما يسمى بالفسفتة. بالإضافة إلى هذه النتائج، باستخدام تقنية BRET تم تأكيد التأثير الإيجابي لبروتينات مصل اللبن ولاكتوفرين على وظيفة ونشاط مستقبلات الأنسولين اعتماداً على تراكيز معينة. علاوةً على ذلك،عند إضافة بروتينات مصل اللبن وأجزاءها بذات الوقت مع هرمون الإنسولين، أظهرت زيادة أكبر لنشاط مستقبلات هرمون الإنسولين. وتم التأكد من صحة هذه النتائج باستخدام مضادات (antagonists) للإنسولين. حيث أن هذه المضادات (S961) تتنافس مع الإنسولين للإرتباط بدلاً منها بمستقبلات الإنسولين.أخيراً، النتائج التي حصلنا عليها باستخدام BRET و عمليات الفسفتة، توافقت بشكل رائع مع نتائج مستوى أخذ واستيعاب خلايا سرطان الكبد للسكر. وأثبتت كيفية تأثير حليب الإبل على وظيفة مستقبلات الإنسولين، والذي بدروه يمثّل أول دراسة تقوم بإظهار تأثير حليب الإبل على خفض السكر على المستوى الجزيئي للمسارات الداخلية للخلايا.

مفاهيم البحث الرئيسية: حليب الإبل، داء السكري، مستقبلات هرمون الأنسولين، الأنسولين، جلوكوز.

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Dedication

To my beloved husband, parents and siblings

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List of Abbreviations

Akt/PKB	Protein kinase B
BRET	Bioluminescence resonance energy transfer
BWP	Bovine milk whey protein
CM	Camel milk
CMLF	Camel milk lactoferrin
CWP	Camel milk whey protein
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DPP4	Dipeptidyl peptidase-4
EC50	Effective concentration 50
Emax	Maximum efficacy
ERK	Extracellular signal-regulated kinases
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon like peptide-1
GLUT4	Glucose transporter 4
HEK293	Human embryonic kidney cells
HepG2	Hepatocarcinoma cells
hIR	Human insulin receptor
HLA	Human leukocyte antigen
IGFR	Insulin like growth factor receptor
IL-	Interleukin-
IR	Insulin receptor

IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LF	Lactoferrin
MAPK	Mitogen-activated protein kinase
MODY	Maturity onset diabetes of the young
PDK1	Protein dependent kinase 1
PI3K	Phosphoinositide 3-kinase
PTB	Phosphotyrosine binding
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TNF-α	Tumor necrosis factor-α

Chapter 1: Introduction

1.1 Diabetes and the insulin receptor

1.1.1 Definition and characteristics

Diabetes mellitus (DM) is now recognized as the world's fastest growing chronic condition, and the most common one. With urbanization, easy access to fast food, inactive lifestyles and increasing incidence of obesity, its global prevalence has increased to 8.8% with 425 million adults (20-79 years) living with diabetes in 2017 (Figure 1) [1]. The International Diabetes Federation predicts that, following the current trend in increase, these numbers are expected to rise to 629 million in 2045. In the United Arab Emirates, the prevalence was recorded to be 17.3% in 2017, i.e., almost twice the current global average, and is expected to hike up to 23.4% in 2045 (Figure 2) [1, 2].

Figure 1: Estimated age-adjusted prevalence of diabetes in adults in the world in 2017 (20-79 years) [1].

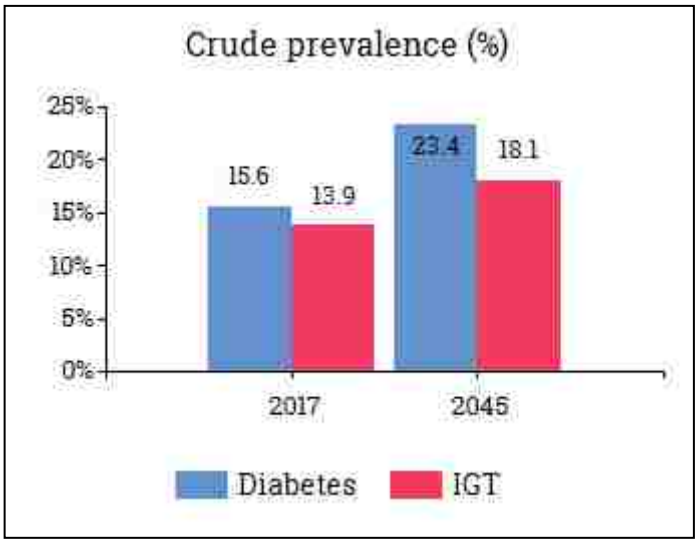

Figure 2: Prevalence of diabetes and impaired glucose tolerance (IGT) in the UAE in 2017 compared to the expected increase in 2045 [2].

Diabetes is mainly characterized by high blood glucose levels defined as >126 mg/dl while fasting and >200 mg/dl 2 hours after ingestion of glucose (by the American Diabetes Association) [3]. Currently, diabetes has been classified into two major types (Figure 3). Type 1 DM (T1DM) is defined as an autoimmune disorder where the body attacks its own insulin producing pancreatic β-cells. Type 2 DM (T2DM) develops in the later stages of life and is characterized by insulin resistance and deficiency in insulin secretion due to progressive loss of β-cell function [3]. It is still unclear which event precedes the other, but a popular opinion is that insulin resistance develops early in the course of type 2 DM. Pancreatic β-cells then begin and continue to overproduce insulin to compensate for this resistance. As the disease progresses over the years, there is a gradual impairment of these β-cells and consequently, in insulin production, hence, reducing the body's ability to compensate and resulting in hyperglycemia and diabetes [4, 5]. It is difficult to pinpoint an exact primary cause of DM, but adiposity, inflammation and genetic predisposition are a few key factors that contribute to the development of insulin resistance. The third common

type, gestational diabetes, usually develops in the initial stages of pregnancy and normally rectifies itself by delivery [3].

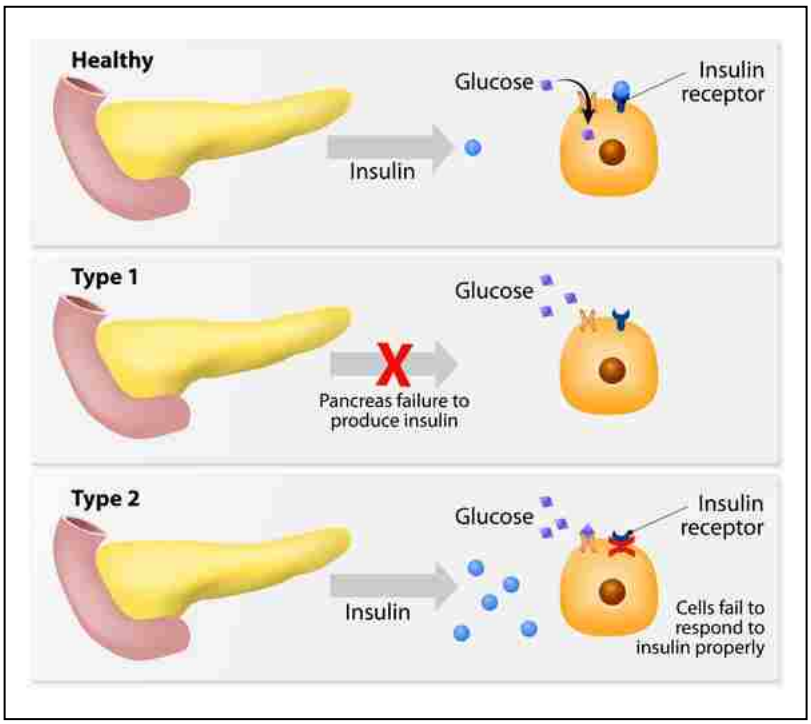

Figure 3: Differences in pancreatic insulin secretion and insulin receptor activity in healthy individuals versus T1DM and T2DM patients [6].

Hyperglycemia does not have any immediate harmful effects, but over the years, persistent high blood sugar levels can have deteriorating effects on blood vessels and organs like kidneys, eyes and heart. The pathophysiology of diabetes is complex and involves many biological processes. For majority of diabetics, in addition to insulin therapy, more than one drug is often used to combat different aspects of the disease and maintain glucose homeostasis. Apart from maintaining a healthy lifestyle and diet, effective clinical management of diabetes involves the careful selection of drugs that complement each other and minimize the risk of negative side effects such as hypoglycemia. Consistent control of blood glucose levels in patients with insulin dependent diabetes effectively delays and slows down the development of

complications like diabetic retinopathy and nephropathy [7]. As research uncovers more and more about the pathogenic complexity of diabetes, there is an ensuing increase in the search for better and easier treatment options.

1.1.2 Insulin receptor and its signaling pathways

Insulin is a 51 amino acid long anabolic hormone secreted by β-cells in the Islets of Langerhans of our pancreas in response to rises in blood sugar levels. Discovered almost a hundred years ago, it is most famously known for being the key player in glucose and lipid homeostasis in the body [8]. In broad terms, insulin promotes the uptake and storage of fuel as glycogen and fats, and prevents breakdown of stored fuel [9]. Insulin is initially synthesized in the pancreas as a 110 amino acid polypeptide called pre-proinsulin. Almost immediately it is transferred to the endoplasmic reticulum where it undergoes cleavage and loses its signal peptide to form proinsulin [10]. Finally, a middle fragment of proinsulin is removed by proteolytic cleavage leaving us with the two chains of mature insulin (A and B) held together by disulphide bridges [11]. Insulin achieves its effects through its human receptor (hIR) present in the plasma membranes of its target cells – the most prominent ones being hepatocytes, adipocytes, skeletal muscle cells, pancreatic β-cells and neurons [12].

The hIR belongs to the tyrosine kinase family of receptors (RTK). Together along with insulin-like growth factor receptors (IGFR) and the orphan receptor, they form the IR subfamily. All receptors in the IR family are synthesized as one protomer protein which then is proteolytically cleaved to form α and β polypeptide chains. For the hIR, these chains are finally assembled into disulfide linked homodimers where each monomer is the αβ heterodimer. This αβ monomer is coded for by a gene with 22 exons. After translation, the pre-mRNA undergoes alternative splicing of exon 11 to

create two variants of the hIR without and with the exon; IR-A and IR-B respectively. The α subunit of hIR is entirely extracellular and contains the ligand binding domains of the receptor. Meanwhile, the β subunit spans the plasma membrane once and then constitutes the cytoplasmic subunit, possessing its characteristic tyrosine kinase domain [13].

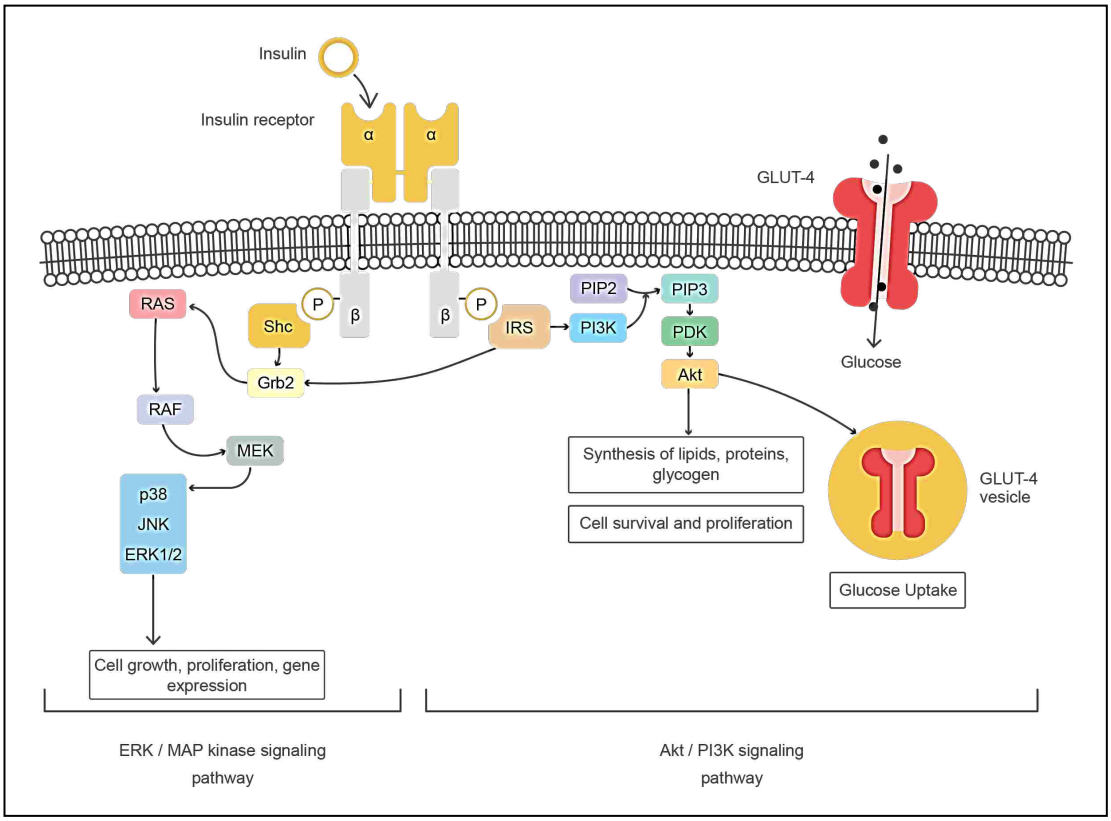

Figure 4: Schematic representation of the Akt/PI3K and MAPK/ERK pathways initiated by the insulin induced activation of the hIR. Adapted from [4].

When insulin binds to α subunit, it causes a conformational change in the receptor bringing the two β subunits together in the plasma membrane. This consequently allows autophosphorylation of hIR at specific tyrosine residues and activation of its kinase activity. We know now that the downstream signaling pathways initiated by insulin binding to the hIR cannot be explained as a single line of events

one preceding the other, but as a complex and integrated network involving branching out at several steps and crosstalk between pathways arising from hIR as well as other receptors. The major two pathways by which the hIR controls its metabolic and mitogenic effects are Akt/protein kinase B (PKB) pathway and ERK/mitogen-activated protein kinase (MAPK) pathway respectively (Figure 4). These pathways begin with the recruitment and subsequent activation of the scaffolding proteins, insulin receptor substrate protein (IRS) and Shc to the hIR via their phosphotyrosine binding (PTB) domains. In the PKB/Akt pathway, phosphoinositide 3-kinase (PI3K) bind to IRS, via its SH2 domain [14, 15]. Here, the IRSs do not contain any intrinsic kinase activity, but act as scaffolds to bring PI3K close and allow their phosphorylation by hIR. PI3K then activates protein dependent kinase 1 (PDK1) which in turn activates PKB/Akt. Activation of PKB is responsible for the mediation of major metabolic effects of insulin such as increase in glucose uptake by translocation of glucose transporter 4 (GLUT4) from cytoplasmic vesicles to the plasma membrane, and also activation of glycogenesis [9, 16]. Glucose transport proteins like GLUT4 help in uptake of extracellular glucose from the blood to inside the cell via an ATP independent mechanism [15].

Another signal transduction protein, Grb2 interacts with IRS to initiate the ERK/MAPK pathway. Grb2 then phosphorylates Ras which in turn initiates the Ras/Raf/MEK cascade leading to the activation of MAP kinases such as p38, c-Jun N-terminal kinase (JNK) and ERK1/2 [14]. The MAPK/ERK pathway is the mitogenic arm of IR signaling and is responsible for gene expression related to cell growth, proliferation and differentiation. Similar to IRS, Shc is another scaffolding protein that binds to hIR and is seen to initiate the ERK/MAPK pathway in an IRS independent mechanism [14].

1.1.3 IR, insulin resistance and diabetes

Insulin resistance is the body's inability to effectively use physiologically normal concentrations of insulin to maintain glucose homeostasis. Although insulin is a pleiotropic hormone, 'insulin resistance' usually refers to its actions on blood glucose levels. Everybody with insulin resistance is not diabetic and some can maintain glucose homeostasis by compensatory increase in insulin secretion, while most patients with both types of diabetes exhibit some level of insulin resistance [17, 18]. Moreover in T2DM,together along with obesity, is one of the major risk factors and is seen in pre-diabetic patients more than 10 years before the onset of the disease [18, 19].

The contributing factors for diabetes involve complex interplay between environmental and genetic parts. Obesity, inactive lifestyles, stress and excessive nutrition are some of the most common environmental triggers. The genetic contribution to the development of diabetes is made clear by its heritability. First degree relatives of diabetic patients have a much higher risk of developing the diseases as compared to the general population [20]. On a genetic basis, diabetes can be monogenic, i.e. arise as a result of just one defective gene, or polygenic, where effects of several altered genes (mainly involved in insulin signaling and β-cell growth and proliferation) add up to create the diabetic phenotype [21].

Genome wide association studies have identified more than 50 genetic loci associated with T1DM , the most studied of these being mutations in HLA genes [22]. When it comes to T2DM, these studies have exposed at least 75 different genetic loci. However, the reasons for the effects of these mutations are still unknown [23]. There are rare monogenic forms of T2DM such as maturity onset diabetes of the young

(MODY), but majority of them are polygenic involving mutations at multiple genetic loci [23].

Being the key pathway when it comes to maintenance of glucose homeostasis by the hIR, defects related to proteins involved in the metabolic PI3K/Akt signaling cascade obviously play an important role in the pathogenesis of insulin resistance. The first critical node in this pathway is the hIR itself [15]. More than 60 different mutations affecting the hIR have been identified, most of them resulting in diseases involving insulin resistance like the Rabson-Mendelhall syndrome, leprechaunism and type-A insulin resistance. Some of these variants have been associated with higher risk of developing T2DM [24]. Multiple *in vitro* studies on skeletal muscle cells isolated from diabetic patients reveal faults with IR including markedly reduced autophosphorylation in response to insulin, lower expression levels and defective kinase activity [25–28]. The IRS scaffolding proteins fall next in line in the pathway. Decreased tyrosine phosphorylation of IRS1 and IRS2 has been observed in diabetic and severely obese patients [25, 28]. It is interesting to note that in mice knockout studies with disruption of IRS2, the mice exhibited diabetic phenotype with insulin resistance and impaired beta cell secretion of insulin. However, with the disruption of IRS1, mice only developed insulin resistance suggesting compensatory increase in insulin levels by the pancreatic beta cells [5]. Reduction in tyrosine phosphorylation of PI3K is another factor that contributes to insulin resistance in diabetic and prediabetic individuals [25, 28, 29]. The next important protein in this signaling pathway is Akt. It is now known that mutations in the kinase domain of Akt2 are associated with severe insulin resistance and diabetes [15]. Consequent effects of defective IRS, PI3K and Akt activation is also seen significant attenuation of GLUT4 translocation and glucose uptake and utilization by insulin sensitive peripheral

tissues[18, 25, 30]. Another cause of ineffective control of glucose levels in the body are missense mutations in the insulin hormone gene. It results in the productions of structurally abnormal insulin leading to problems in its biological activity and receptor binding abilities [31].

1.1.4 Therapies targeting the hIR

Effective maintenance of glucose homeostasis over the years is the most effective way to decrease the adverse effects of hyperglycemia on organs such as eyes, heart and kidneys. Currently the best method of treatment for this is by administering exogenous or insulin analogs to help cope with insufficiency of biologically active insulin or impaired activity of the hIR. However, these treatments are not without negative side effects. Excessive activation of the insulin induced mitogenic MAPK/ERK pathway can cause weight gain and increased risk of cancer development. Occasional hyperinsulinemia can lead to hypoglycemic episodes [4, 32]. Additionally, these high levels of insulin can lead to an unwanted increase in the activation of other cellular processes like increased androgen production by the ovaries [17]. Recent research has been focusing on alternative treatments that target the hIR and activate only its glucose lowering metabolic pathways without the mentioned side effects [32]. A few examples have been discussed below.

Qiang *et al.* (2014) reported the discovery of 4548-G05, a metabolite of the fungi *Chaetomium gracile*, and a non-peptidyl insulin mimetic which specifically activates the insulin receptor by binding to its extracellular region in the absence of insulin [33]. 4548-G05 activates both Akt and ERK signaling pathways downstream of the hIR while also increasing glucose uptake. In mouse models of T1DM and T2DM, it reduces blood glucose back to normal healthy levels [33]. Another potential

candidate is the synthetic insulin mimetic peptide S597 which showed high hIR binding affinity and activation of its kinase activity. It partially phosphorylated the hIR and exhibited biased agonism by activating the metabolic pathway almost to the level of insulin with negligible stimulation of the mitogenic pathway. Even high concentrations of S597 only partially stimulated the MAPK/ERK pathway. Accordingly, this peptide did not show much effect on cell proliferation [34].

Bhaskar *et al.* (2012) identified a promising candidate, XMetA – a fully human monoclonal antibody which is a positive allosteric modulator highly specific to the hIR [35]. XMetA binds to hIR with an affinity higher than insulin but causes only partially activation of the metabolic Akt pathway (about 40% of what is activated by insulin) and does not potentiate the mitogenic MAPK/ERK pathway [35]. It's interesting to note that XMetA does not compete with insulin since it binds to completely different site giving it the advantage of being used in conjunction with insulin and other current diabetic drugs. Moreover, it takes away the risks associated with complete inhibition of natural hormonal mechanisms [36]. *In vitro* experiments in 3T3 cells confirmed consequent glucose uptake after activation of Akt, while *in vivo* experiments in STZ induced diabetic rats , cynomolgus monkeys and rhesus monkeys showed that XMetA normalized both fasting and non-fasting blood glucose levels, along with other metabolic indices of diabetes [35, 37–39]. Even in insulin resistant diet induced obese mice, XMetA improved fasting glucose as well as insulin tolerance [38]. This character offers XMetA the potential of therapeutic use in patients where activation of IR by insulin is defective [37]. More excitingly, these positive effects come without side effects such as hypoglycemia and weight gain [38]. Another studied monoclonal antibody is IRAB-A, identified by Hinke *et al.*by phage screening assays with the IR extracellular domain. Similar to XMetA, *in vitro* assays using IRAB-A

identified it as an allosteric agonist with specificity to the insulin receptor. It was also observed to stabilize ligand binding and also increased sensitivity of the hIR to insulin coupled with higher glucose uptake. The same group also identified an hIR antagonist, IRAB-B. IRAB-B treated mice showed rapid development of hyperglycemia, sever insulin resistance, decreased phosphorylation of Akt, IRS1, glucose uptake as well as body weight [40]. As compared to the current methods, IRAB-B shows the potential to be a more cost effective and faster way to induce insulin resistance in mammalian models to study the pathophysiology of impaired insulin receptors [40].

Along with XMetA, Bhaskar *et al*.(2014,2014) also identified two other antibodies that interact with the hIR – XmetD and XMetS [41, 42]. XMetD is a highly specific allosteric insulin receptor antagonist. *In vitro* experiments showed that XMetD markedly decreased insulin affinity, IR autophosphorylation as well as downstream events including Akt phosphorylation and glucose transport. What's more is that it did not show any effects in the absence of insulin [42]. Most importantly, in mice induced with hyperinsulinemic hypoglycemia XMetD treatment returned blood glucose to normal levels [42]. XMetS, on the other hand, enhanced the sensitivity of hIR to insulin by stabilizing the insulin – receptor conformation by decreasing their dissociation rate. Similar to XMetA, XMetS showed biased activity, enhancing the activation of only the metabolic or Akt pathway without much effect on the ERK pathway [41].

The modifications that these treatments cause to hIR signaling patterns are hypothesized to be the result of structural modulations to the IR brought about by binding to non-orthosteric sites [43]. While the research involving these treatments are still in the preliminary stages and not without the possibility of unidentified long term

effects, studying the structural mechanisms could bring us closer to understanding the working of the IR and also to the development of new therapeutics for diabetes [32].

1.2 Camel Milk

For centuries, in communities that inhabit the drier areas of Asia and Africa, CM has been more than just the traditional source of nutrition. Throughout history, the therapeutic nature of CM has been reported against multiple diseases like tuberculosis, asthma, jaundice and leishmania [44, 45]. Rightfully called 'the white gold of the desert', CM has come into the research spotlight in the recent years, with scientists in different parts of the world uncovering its various medicinal properties including antimicrobial, anti-carcinogenic, anti-hypertensive, anti-oxidant and anti-diabetic properties [46].

1.2.1 Chemical composition of CM

In general, CM is composed of about roughly 80-90% water, 2-4% fat, 3-4% protein, 4-5% lactose, along with vitamins and minerals [47]. However, the specific percentages of each of the constituents vary based on influence by factors such as lactation stage, age of the camel, geographic and seasonal conditions [48]. Compared to bovine milk, CM has lower cholesterol and fat content. Moreover, this fat is mainly polyunsaturated fatty acids. Its fat globules are also the smallest in size when compared to cow, buffalo and goat milk. The smaller size implies a larger surface to volume ratio contributing to its easier digestibility [49, 50]. Milk proteins are broadly classified into caseins and whey proteins. In CM, caseins constitute about 75% of all protein while whey proteins make up the other 25%. The major protein fractions in camel whey are immunoglobulins, camel serum albumin, α-lactalbumin, lactophorin A, and lactoferrin. In contrast to bovine milk where β-lactoglobulin is the most abundant whey

protein, α-lactalbumin is the most abundant camel whey protein. β-lactoglobulin is one of primary culprits when it comes to milk allergies and lactose intolerance. Moreover, camel whey actually lacks β-lactoglobulins, making it safe even for such patients [51, 52]. Camel whey also contains high concentrations of antimicrobial proteins and immunoprotective bioactive compounds like lactoferrins, immunoglobulins IgG and IgM and lysozyme [53]. To add to this exceptional list of benefits, CM is also rich in vitamin C, A, D and riboflavin. Additionally, its mineral profile is similar to that of human milk with high concentrations of iron, copper, zinc and potassium [48].

1.2.2 Antidiabetic properties of CM

Many previous studies using animal models and humans have shown beneficial effects of CM in diabetes by reducing blood sugar, improving pancreatic β-cell function, decreasing insulin resistance and improving lipid profiles [54–60]. It has been suggested as an adjunct to insulin therapy because it can effectively reduce the amount of insulin required by diabetics. One study contended a significant reduction (46.15%) in insulin doses required by Type 1 diabetic patients who consumed raw CM for 2 years (Figure 5) [61]. In another study conducted in the camel breeding and rearing Raika tribe of India, the incidence risk of diabetes was very low in communities that regularly consumed camel versus those that did not [62]. It is also known to alleviate other pathophysiological effects associated with diabetes such as obesity, inflammation, wound healing, diabetic nephropathy and oxidative damage [63–66].

Figure 5: Graphical representation of the reduction in mean doses of insulin required in T1DM patients consuming CM over the course of 2 years [61].

While we cannot pinpoint an exact reason for these antidiabetic effects, recent studies have identified a few contributing factors. CM contains approximately 3 times more insulin like peptide (52 micro unit/L) as compared to bovine milk. Interestingly, camel and bovine milk insulin is only one amino acid different from each other, but no hypoglycemic activity has been reported yet for bovine milk. Hence, it is more plausible that it is not the insulin itself but its cooperation with the other characteristics of CM that helps with glucose homeostasis [67]. An important factor here is that CM does not coagulate in our stomach's acidic environment, probably making it easier for this insulin-like peptide to be readily absorbed and passed into the blood stream [61]. Moreover, certain CM proteins have amino acid sequences that are rich in half cystine residues, much like the insulin family of proteins. Another explanation could be the unique property of these peptides in CM to be encapsulated by lipid nanoparticles, effectively protecting them from proteolysis in the stomach. The fact that CM is rich

in zinc could be another contributing factor, since zinc enhances and stabilizes insulin-IR interaction [68, 69].

The antidiabetic nature of CM is the most famous amongst all its medicinal values and has been continuously verified by in-vivo studies on both animal models and diabetic patients [54, 58, 61, 68]. However, when it comes to the cellular and molecular mechanisms behind this effect, we've only scratched the surface. Our research explores the effects of CWPs and its fractions on one of the primary targets in diabetes: the hIR. We have also looked into the subsequent activation of its signaling pathways and glucose uptake into cells.

1.2.3 Molecular and cellular mechanisms of the antidiabetic effects of CM

While many *in vivo* studies have proven the hypoglycemic effects of CM, we are still in the early stages of defining the responsible molecular mechanisms. These anti-diabetic effects cannot be attributed to one CM component. It is rather the result of harmonious action of multiple components on different targets in the body. The studies discussed below have proven that these targets can not only be those directly associated with diabetes such as the IR, pancreatic β-cells and glucose transporters, but also factors that contribute to the pathogenesis of diabetes, such as obesity, inflammation and oxidative stress (Figure 6) [55, 66, 70–76].

One study discovered potent dipeptidyl peptidase-4 (DPP4) inhibitory peptides in tryptic hydrolysates of CM [70]. DPP4 is a protease that inactivates incretins, i.e., glucagon like peptide-1(GLP-1) and gastric inhibitory polypeptide (GIP). Incretins are gut hormones released when food containing sugars and lipids travels into the small intestine. They signal the pancreas to produce insulin in accordance to the glucose

ingested and inhibit the release of glucagon. This is known as the incretin effect. Inhibiting DPP4 slows down incretin degradation allowing their effects to last longer, consequently increasing insulin secretion [70].

Additionally, treatment with CM reduced diabetic oxidative stress by significantly decreasing free radicals and production of pro-inflammatory cytokines like interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) [71]. Diabetes (both T1DM and T2DM) is well known to be a chronic inflammatory disease where high levels of inflammatory cytokines and continued oxidative stress contribute largely to the pathogenesis and also development of complications in diabetes [72, 73]. Conversely, CM increased their anti-inflammatory counterparts IL-2 and IL-4 in diabetic mice. This could be due to the direct action of CM proteins, like lactoferrin and β-casein, which exhibit anti-inflammatory and anti-oxidant properties. However, it is also possible that the lowered glycemic levels indirectly diminishes the existing inflammation [60, 75]. Additionally, peptic hydrolysis of colostral CM produces bioactive peptides with increased antioxidative properties as compared to the milk before hydrolysis [74]. ATF-3 expression and phosphorylated protein kinase B (Akt) are two other targets studied. High ATF-3 levels are indicative of increased pro-apoptotic genes, while decreased Akt phosphorylation influences the development of diabetes. Treatment with CWPs brings both these factors back to normal levels in diabetic mice [71]. It is possible that DPP4 inhibition along with anti-inflammatory, anti-apoptotic and anti-oxidant properties is the basis for increased pancreatic insulin secretion observed in diabetic patients and animals [55, 75, 76]. After all, inflammation and apoptosis of insulin-secreting pancreatic β-cells certainly is a prime event in both types of diabetes.

A recent *in vitro* study by A.O. Abdulrahman *et al.* suggests that CM directly interacts with hIR [77]. It weakly activated hIR but significantly increased its sensitivity to insulin, suggesting an allosteric mode of action. The study also narrowed down the putative agent to a peptide/protein component in the milk whey fraction [77]. In the light of this study, our main objective for this thesis was to test the effect of CWPs and its fractions on hIR function, and subsequent glucose uptake, *in vitro*. Direct interaction of CM with the hIR opens up the possibilities of positive allosteric modulation of other associated receptors such as those specific for GLP1 , GIP and the IGFR, or even negative allosteric modulation of the glucagon receptor [78].

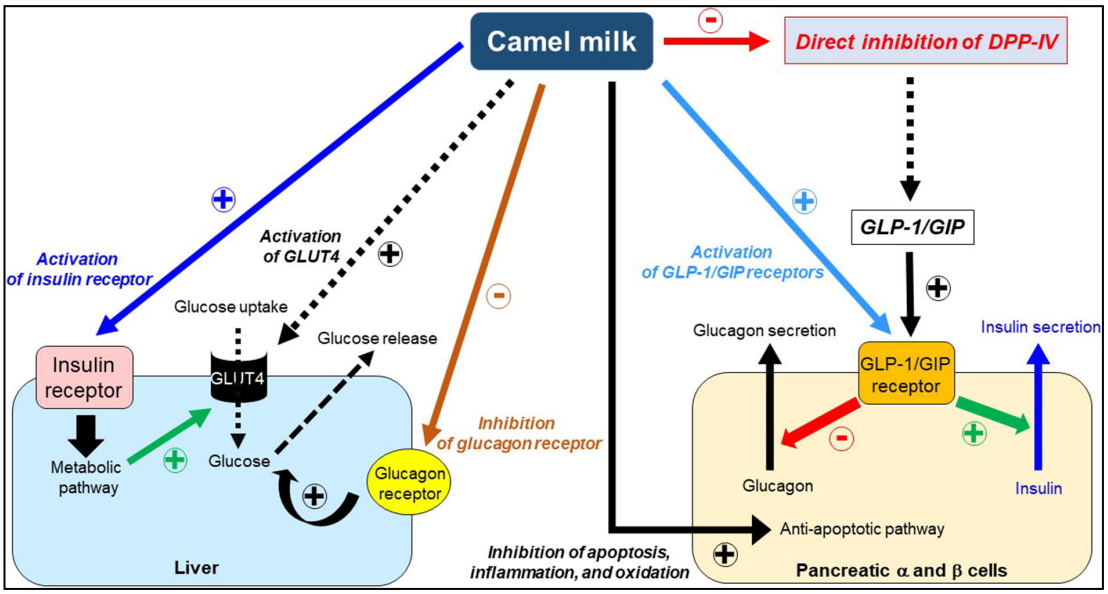

Figure 6: Probable molecular targets of camel milk components in the cell that could help explain its antidiabetic properties [78].

Among the constituents of CM, lactoferrin (LF) stands out as a potential player when it comes to its antidiabetic effects. Associations between LF and glucose homeostasis were initially made by Moreno Navarette *et al.* when they discovered that levels of plasma LF are significantly reduced in T2 diabetics with increased insulin resistance [79]. They highlighted that LF effects the insulin signaling pathway by

potentiating IR-mediated Akt phosphorylation and increasing GLUT4 and IR expression on adipocytes [79, 80]. Moreover, literature shows that increased plasma LF is associated with decreased expression of inflammatory markers and overall adiposity [81, 82]. Both of which are significant risk factors in the pathogenesis of diabetes. Plasma LF levels are also inversely related to abdominal as well as overall adiposity while consistent oral consumption decreased abdominal visceral fat in men [81, 83, 84]. Lastly, it even inhibits the production of several proinflammatory cytokines including TNF-α, IL6 and IL 1β [85].

Another possible candidate is adiponectin, a metabolic adipokine secreted by the white adipose tissue and present in high concentrations in CM. Higher circulating adiponectin levels have been correlated with increased insulin sensitivity while reduced levels of plasma adiponectin have been commonly observed along with insulin resistance, T2DM, obesity and cardiovascular diseases [86, 87].

Lastly, as mentioned in some studies above, CM peptides add one more category to its potential antidiabetic components. Gastrointestinal digestion of food proteins releases and activates encrypted peptides within it. These peptides have, more often than not, higher biological activity when compared to the parent protein. In general, milk is one of the major sources of food derived bioactive peptides. The peptides present in CM hydrolysates, generated by lysis by digestive enzymes, show significant DPP4 inhibition, wound healing and anti-obesity properties in the context of diabetes [57, 70, 88].

Chapter 2: Hypothesis, Objectives and Approach

We hypothesize that the hypoglycemic properties of CM observed *in vivo* may involve the action of CWPs, its hydrolysates and LF on the intracellular signaling pathways mediated by insulin.

In order to better understand how CWP affects the hIR at the molecular level, our objectives were to study these effects on different aspects of IR function *in vitro* as given below:

1. Pharmocological effect of CWPs, its hydrolysates and LF on the intracellular signaling pathways mediated by hIR by studying the phosphorylation status of intracellular kinases ERK1/2 and Akt as well as IR itself.
2. Physical association of IR with the substrate protein IRS1 on treatment with CWPs, its hydrolysates and LF.
3. Effect of CWPs, its hydrolysates and LF on the function of glucose uptake into the cells via translocation of glucose transporters (e.g. GLUT4) from their cytoplasmic vesicles to the plasma membrane.

Table 1**:** Objectives and their respective approaches used in this thesis

Objectives	Approaches
Phosphorylation status of IR and related intracellular kinases Akt and ERK1/2	By SDS-PAGE and western blotting techniques
Physical association of IR with IRS1	By BRET techniques
Glucose uptake into the cells	Luminiscence based assay

Chapter 3: Materials and Methods

3.1 Chemicals, reagents and plasmids

Human insulin and bovine LF was purchased from Sigma Aldrich. The hIR antagonist peptide, S961, was a gift from Dr. Lauge Schäffer (Novo Nordisk, Copenhagen, Denmark). Camel LF was generously provided by Dr. Elrashdy M. Redwan (King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia).The plasmids coding for *Renilla* luciferase-fused hIR (hIR-Rluc) and YFP-tagged IRS1 (IRS1-YFP) used for transient transfections in HEK293 cells were kindly provided by Dr. Rasmus Jorgensen (Hagedorn Research Institute, Novo Nordisk, Gentofte, Denmark), Dr. Tarik Issad (Cochin Institute, Paris, France), respectively. Correct expression of the yellow fluorescent proteins (YFP) tagged proteins was verified visually by using fluorescence microscopy and by reading light emission at 540 nm using the Tristar 2 plate reader. The same instrument was used to verify expression of hIR-Rluc by measuring light emissions at 480 nm upon the addition of Rluc substrate.

3.2 Bacterial transformation and plasmid extraction

The hIR-Rluc and IRS1-YFP plasmids were first dissolved in 50 µL nuclease free water. Transformation was carried out using NEB® 5-alpha competent E. coli cells (New England BioLabs Inc.) The bacteria and plasmids were mixed together as per manufacturer's instructions. Briefly, after gentle mixing, the plasmid-bacteria mixture was incubated on ice for 10 minutes followed by a heat shock for 42 seconds at 45°C on the heating block, and then returned to ice for 5 more minutes. Next, 500 µL of SOC Outgrowth Medium (New England BioLabs Inc.) was added to each tube and incubated for 1 hour at 37°C in the shaking incubator. This was followed by centrifugation at 5000 rpm for 5 minutes, after which the supernatant was discarded,

and the pellet resuspended in 50-100 µL of SOC medium. This mixture was spread evenly onto agar plates containing the antibiotic ampicillin (Sigma) for colony selection and incubated at 37°C for 24-36 hours.

One colony from the transformed plates for each plasmid was picked and dropped into 200 mL of LB media supplemented with ampicillin. The resuspended colony was grown in liquid culture overnight at 37°C overnight in the shaking incubator. The transformed bacteria were pelleted from the suspension culture the next day by centrifugation at 6000 rpm for 15 minutes. The pellets were stored at -20°C before being used for plasmid extraction.

Plasmids were extracted from the bacterial pellets using the Qiagen® Plasmid Maxi kit following manufacturer's protocols. Extracted DNA was finally resuspended in nuclease-free water and plasmid concentrations were measured using the NanoDrop 2000 (Thermo Scientific). Plasmid integrity was checked using agarose gel electrophoresis.

3.3 CM collection, fractionation and hydrolysis

Milk of *Camelus dromedarius* was kindly provided by Ms. Aysha, (PhD student in the College of Science) fresh every week from her local farm (Al Ain, UAE). To obtain the whey proteins for cell treatment, whole milk was first skimmed by centrifugation at 5000 rpm for 30 minutes at 4°C to remove fat. Caseins were then separated out by acid precipitation by bringing the pH down to 4.6 using 1M HCl followed by incubation at 37°C for 30 minutes. The sample was then centrifuged again for 5000 rpm at 30 minutes at 4°C to precipitate caseins. Clear whey proteins were collected (supernatant) and pH adjusted to 7.4 (physiological pH) prior to cell

treatment. All CWP hydrolysates were kindly provided by Dr. Sajid Al Maqsood from the College of Food and Agriculture, UAEU.

3.4 Cell culture and transfection

Both HEK293 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplied with 10% fetal bovine serum and 100 IU/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich) at 37°C in 5% CO_2.

Transient transfections were carried out when HEK293 cells were approximately 70% confluent in T75 flasks using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. Briefly for each flask, plasmids and lipofectamine were separately mixed with 1mL Opti-MEM (Gibco) each and incubated for 5 minutes at room temperature. The two mixes were then combined and incubated for another 20 minutes before it was added to the cells in T75 flasks along with DMEM. For BRET experiments, each flask was transfected with 7.5 µg hIR-Rluc2.5 µg IRS1-YFP, while for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting, each flask was transfected with 5 µg of hIR-Rluc.

Twenty four hours after transfection, cells were harvested using Trypsin-EDTA (Gibco) and counted with the improved Neubauer chamber. Cells were then seeded into 96-well plates at a density of 10^5 cells per well for BRET, or into 6-well plates at a density of 10^6 cells per well for western blotting experiments. All experiments were carried out a total of 48 hours after transfection.

HepG2 cells endogenously expressing hIR were seeded into 6-well plates at a density of 10^6 cells/well 48 hours prior to the experiment.

3.5 Cell treatment and lysis

HEK293 cells and HepG2 cells transiently and endogenously expressing hIR, respectively, were starved in serum-free DMEM overnight in their 6-well plates. Two protocols were followed for the treatment: 1) Cells were treated or not with insulin, camel and bovine whey proteins, hydrolysates of CWPs and LF (bovine and camel), for 5 minutes to observe ERK1/2 and hIR phosphorylation and 10 minutes for Akt phosphorylation, at 37°C. The timings for cell treatment were chosen based on the kinetics for these two pathways when activated by insulin [77]. 10 minutes was the optimum time for Akt accumulation in the cells after treatment with insulin, while the peak for ERK1/2 accumulation was at 5 minutes [77]. The same method was also used to study the effects of co-treatment of the mentioned treatments with insulin. 2) Cells were pre-treated with whole CWPs or its hydrolysates for 60 minutes before the addition or not (control) of insulin. In experiments involving the hIR-selective antagonist (S961), 1 µM of the antagonist was added along with the initial treatments.

After treatment, the cells were washed in ice cold PBS followed by homogenization, for 90 min at 4°C, with 200 µL/well of ice-cold RIPA lysis buffer (Merck Millipore) supplemented with phenylmethylsulfonyl fluoride (PMSF) (Roche) and protease inhibitors (Sigma-Aldrich). Cell lysates that were then scraped and collected were centrifuged for 15 min at 15000 g (4°C) to remove cell debris. The supernatants were stored at -20°C until further use.

3.6 Protein quantification by BCA assay

Protein concentrations of the lysates were determined by the BCA assay using the BCA Protein Assay Kit (Pierce, Thermo Scientific).

3.7 SDS-PAGE and Western blotting

20 µg of protein from each cell lysates sample was mixed with Laemmli buffer (Bio-rad) and heated for 5 minutes at 95°C. The samples were then separated by SDS-PAGE (225 V for 75 minutes) and transferred to polyvinylidene fluoride (PVDF) (Bio-Rad) membranes by conventional wet-transfer technique (100V for 90 minutes). Transfer was confirmed by staining with Ponceau (Sigma-Aldrich). The membranes were then blocked in 5% skimmed milk prepared in PBS (Gibco) containing 0.1% Tween 20 (Bio-Rad) (PBST) for 1 hour before moving on to immunoblotting. The membranes were incubated overnight in primary antibodies against Akt, pAkt, ERK1/2, pERK1/2, IR-β, and pIR-β (Table 2). Anti- rabbit IgG or anti- mouse IgG conjugated to horseradish peroxidase (HRP) were used as secondary antibodies as per the source of the primary antibodies. Membranes were treated with secondary antibodies for 45 minutes before treatment with the chemiluminescent substrate for detection (Pierce ECL and SuperSignal West Femto, Thermo Scientific). HRP activity was detected by chemiluminescence using the LI-COR C-digit Blot Scanner (LI-COR Biosciences.). The whole protocol in also described in Figure 7. All antibodies were purchased from Cell Signaling Technology, except the anti-pIR (Tyr1334) (Thermo Fischer).

Table 2: List of antibodies used and their working dilutions

Antibody	**Catalog no.**	**Dilution used**
Akt	9272	1:1000
pAkt (Ser473)	9271	1:800
ERK1/2	4695	1:1000
pERK1/2 (Thr202/Tyr204)	9106	1:2000
IR-β	3020	1:1000
pIR-β (Tyr1345)	3026	1:1000
pIR (Tyr 1334)	44-809G	1:1000
Anti-mouse IgG –HRP	7076	1:3000
Anti-rabbit IgG –HRP	7074	1:3000

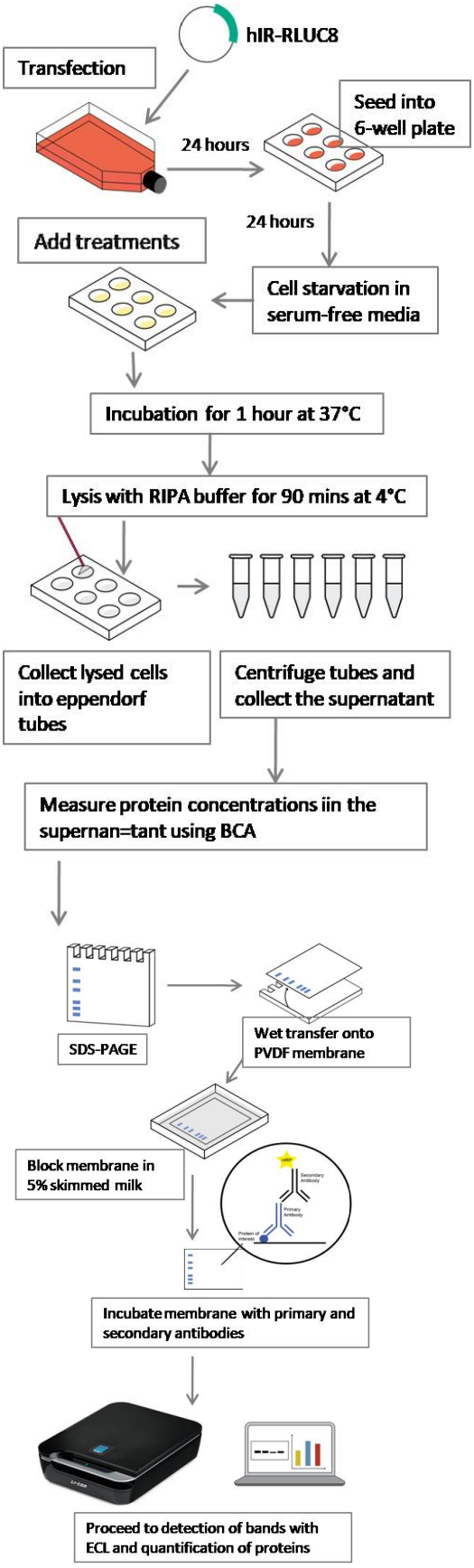

Figure 7: Schematic representation of cell treatment and western blotting.

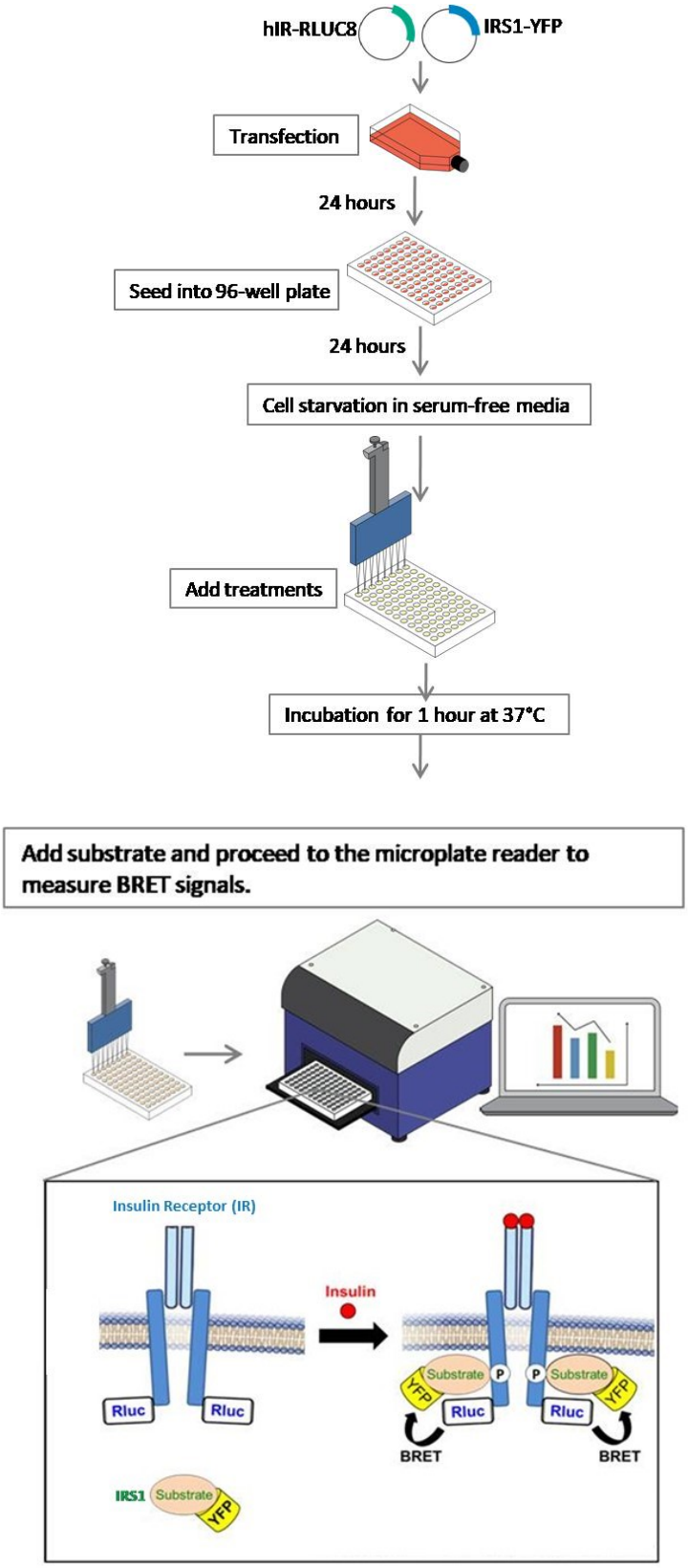

Figure 8: Schematic representation of cell treatment for BRET assays.

3.8 BRET assays

The BRET assay is a technique that can be used to protein-protein interactions in live cells and in real-time (Figure 8). It operates on the principle of resonance energy transfer from a bioluminescent donor to fluorescent acceptor based on their proximity. When the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor, transfer of energy occurs from the former to the latter. Thus, when the two proteins are in close proximity (<10 nm), we are able to observe an increase in the BRET signal (Figure 9). Transfected HEK293 cells co-expressing hIR-Rluc (BRET-donor) and IRS1-YFP (BRET–acceptor) proteins were starved overnight in serum-free DMEM. Cells were treated with 50 µL/well of respective treatments or PBS (control) and incubated at 37°C for 60 minutes. When required, S961 was added along with the treatments at this stage. This was followed by removal of treatment and addition of 40 µL of 2.5 µM coelenterazine h (Promega) prepared in PBS. BRET measurements of emitted light were immediately carried out using the Tristar 2 multilabel plate reader (Berthold, Germany) at 480 n and 540 nm.

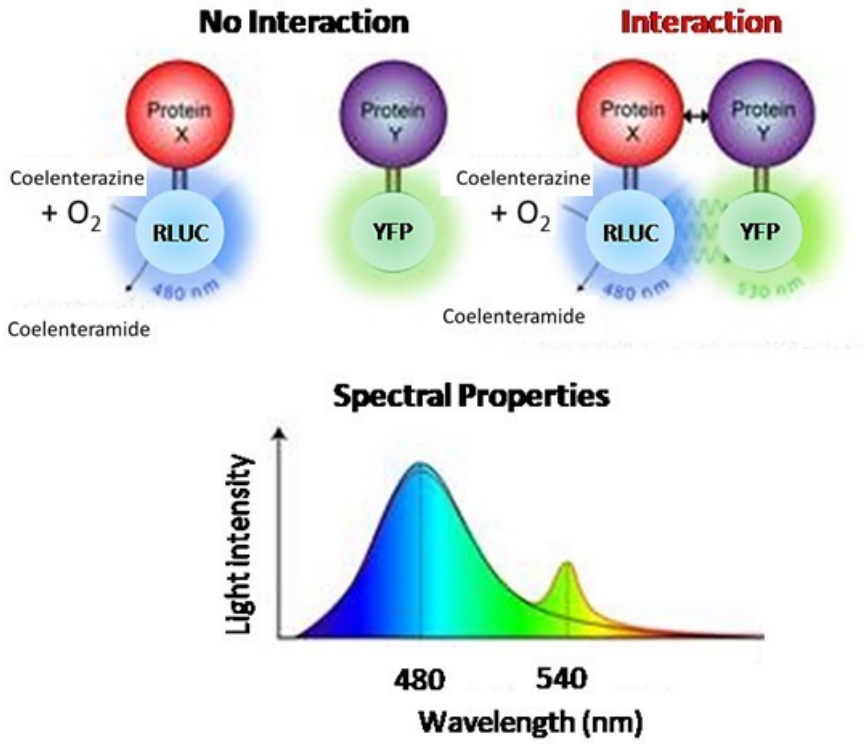

Figure 9 : Principle of the BRET assay (adapted from [89]).

3.9 Glucose uptake assay

Glucose uptake into non-transfected HepG2 cells was measured using the Glucose Uptake-Glo Assay (Promega). Briefly, HepG2 cells that were seeded into 96 well plates at a density of 10^5 cells/well were washed with PBS and starved in glucose and serum free medium on the day before the experiment. The experiment was started by removing the media and adding respective treatments to the wells followed by incubation for 60 minutes at 37°C. The stimulants were then removed and 25 µL of freshly prepared 1mM 2-deoxyglucose was added to each well. After 10-15 minutes at room temperature the reaction was stopped, and cells lysed using provided buffers. 50 µL/well of freshly prepared 2-deoxyglucose-6-phosphate detection reagent

containing Ultra-Glo™ Recombinant Luciferase was added before incubating the plate for 15-60 minutes at room temperature. The luciferase activity was measured at 15-minute intervals using the Glo-Max® Discover Microplate reader.

3.10 Data and statistical analysis

All BRET, western blotting and glucose uptake experiments were carried out in triplicates. Bands visualized after western blotting were quantified by taking the ratio of phosphorylated protein over the respective total protein to compare the rates of phosphorylation among samples. Quantification of bands and graph generation were carried out using Image Studio software, Version 5.2.BRET ratios were calculated as light emissions at 540 nm over 480 nm. From this, the ligand induced BRET was obtained by subtracting the control (only PBS treated cells) values. Next, all the values were normalized by taking the insulin induced positive control as 100%. Graphs were plotted using GraphPad Prism software. Statistical analyses were performed with two-way ANOVA and Tukey's multiple comparisons test to determine statistically significance between the different conditions. ****p-value < 0.0001, ***p-value < 0.001, ** p-value < 0.01, * p-value < 0.05, and ns p-value > 0.05.

Chapter 4: Results

4.1 Successful CM whey proteins (CWPs) fractionation and hydrolysis

After removing the fat and casein fractions from fresh CM, we ran them on an SDS-PAGE gel to confirm the presence of required fractions as well the integrity of whey proteins. Coomassie staining of the gel revealed clear single bands at around 80 kDa in the lanes for camel and bovine LF signifying the presence of unadultered and intact LF. Camel and cow whey proteins showed in intact bands as well (Figure10).

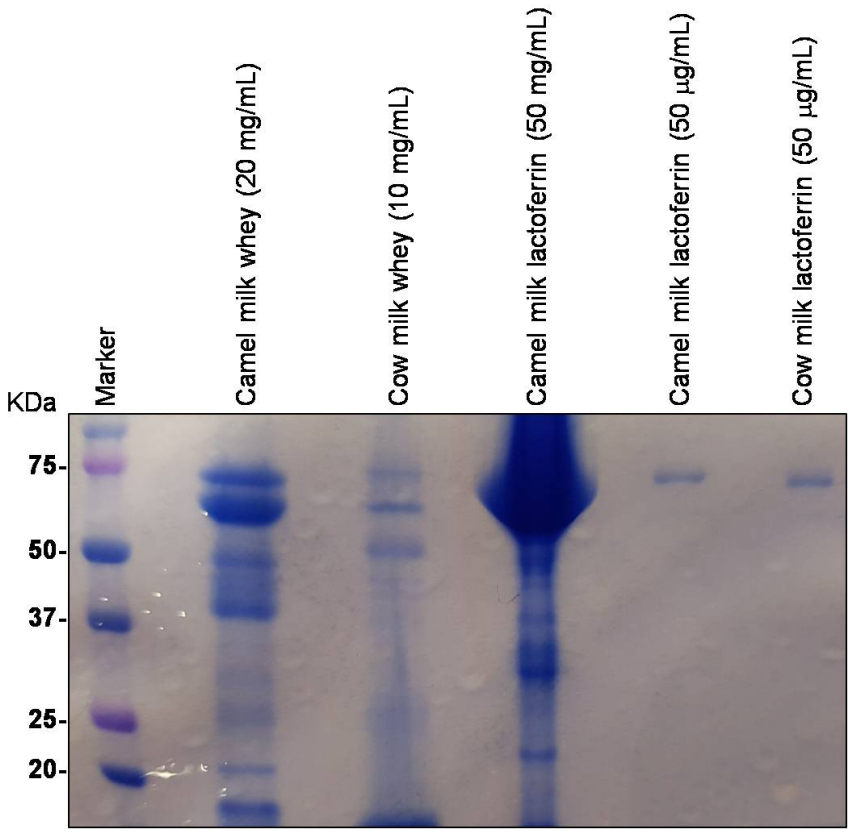

Figure 10: Coomassie blue staining of the gel confirming the presence of CWPs and LFs in the samples.

4.2 CWPs activate Akt and ERK1/2 pathways in HEK293 and HepG2 cells

As stated above, CM has been reported to have significant anti-diabetic effects in many *in vivo* studies. Therefore, we attempted to link such interesting properties of CM with more integrated cell responses and molecular pathways using two different cells models, HepG2 cells endogenously expressing hIR and HEK293 cells transiently expressing or not hIR. For this, we investigated the functional activity of CWPs on the two major intracellular signaling pathways, Akt and ERK1/2 phosphorylation, known to be the key hIR-mediated downstream signaling involved in glucose uptake and homeostasis. As shown in Figure 11A, insulin (1 μM) used as a positive control significantly increased Akt and ERK1/2 phosphorylation in both HEK293 and HepG2 cells indicating the activation of hIR and validating the experiment. Interestingly, CWPs (1 mg/ml) also strongly induced Akt and ERK1/2 phosphorylation in HEK293 and HepG2 cells similarly to insulin action (Figure 11A). The overexpression of hIR in HEK293 seems to increase both insulin- and CWPs-mediated response compared to mock HEK293 cells (Figure 11A). These data demonstrate the functional activity of camel milk proteins on Akt and ERK1/2 signaling pathways that might explain the hypoglycemic effect of camel milk.

Next, we examined the biological activity of CWPs upon *in vitro* proteolysis into heterogeneous peptide fractions. The rationale behind this was to mimic the obvious gastric proteolysis of CM proteins after its consumption. For this, we subjected CWPs to enzymatic digestions using the key gastric and pancreatic proteolytic enzymes, trypsin, chymotrypsin and pepsin and we then tested the effect of their respective hydrolysates at 1 mg/ml on Akt phosphorylation in HEK293 cells. As shown in Figure 11D, while trypsin hydrolysate fully lost its activity the

chymotrypsin and the pepsin ones were still functional like insulin and CWPs. This indicates that CWPs hydrolysis by chymotrypsin and the pepsin did not impair the biological activity of the proteins suggesting the existence of biologically active peptides contained in such fractions.

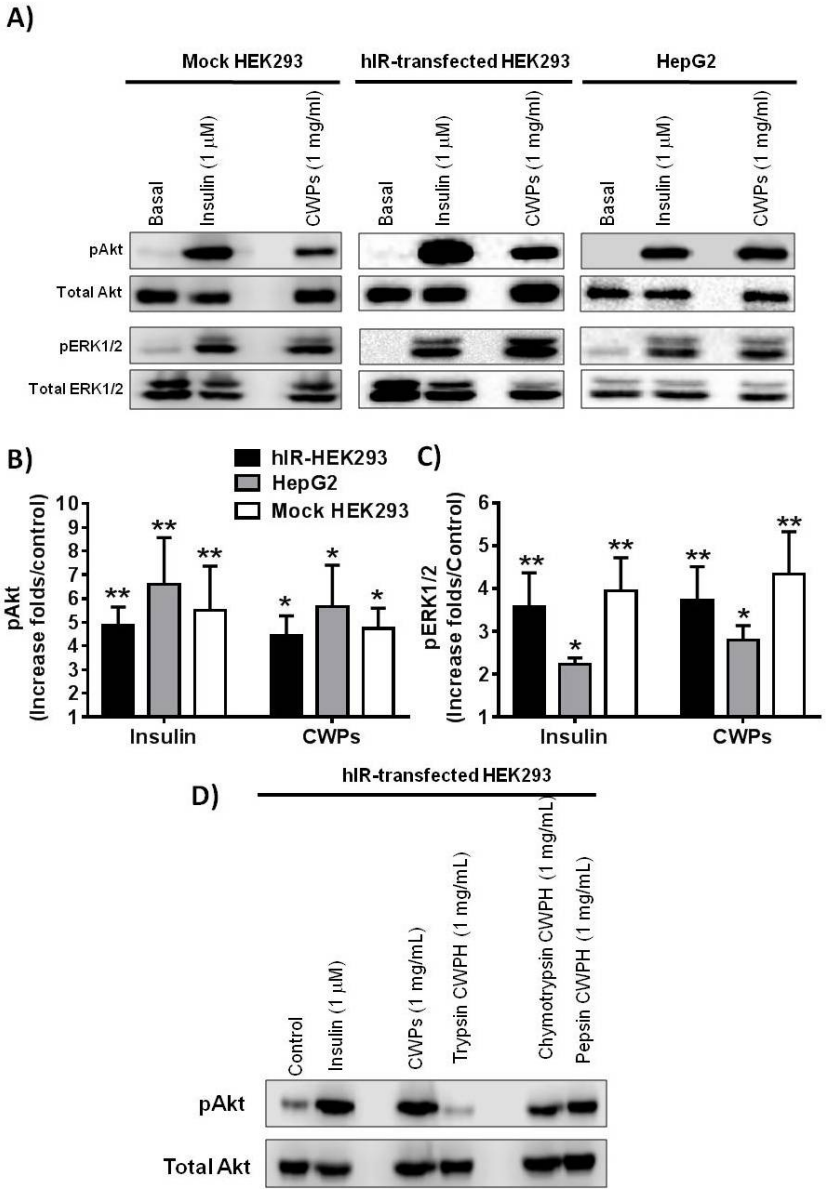

Figure 11: CWPs activate Akt and ERK1/2 pathways in HEK293 and HepG2 cells. HEK293 cells transiently over-expressing hIR or not, and HepG2 cells endogenously expressing hIR showing phosphorylation of Akt and ERk1/2 on treatment with insulin or CWPs. A) pAkt, Akt, pERK and ERK bands visualized after western blotting. B) and C) The fold increase in phosphorylation of Akt and ERK1/2 (respectively) over the control.

4.3 Effects of LF on Akt and ERK1/2 pathways in HEK293 and HepG2 cells

Following this, we investigated the effects of two potential candidates in CWPs: LF and adiponectin, on the Akt and ERK pathways. Both camel and bovine LF potentiated an increase in the phosphorylation of ERK1/2 that seemed to increase with over expression of hIR in HEK293 cells. However, the increase was more pronounced by bovine LF (Figure 12). Moreover, bovine LF also induced the phosphorylation of Akt, which was not evident when both HEK293 and HepG2 cells were treated CMLF (Figure 12). In HepG2 cells, however, CMLF potentiated ERK phosphorylation more than bovine LF. The results with bovine LF are consistent with previously published research that showed that LF increases insulin induced Akt phosphorylation [79, 80]. Adiponectin did not show any activation of both pathways and was not continued with for further investigations (data not shown).

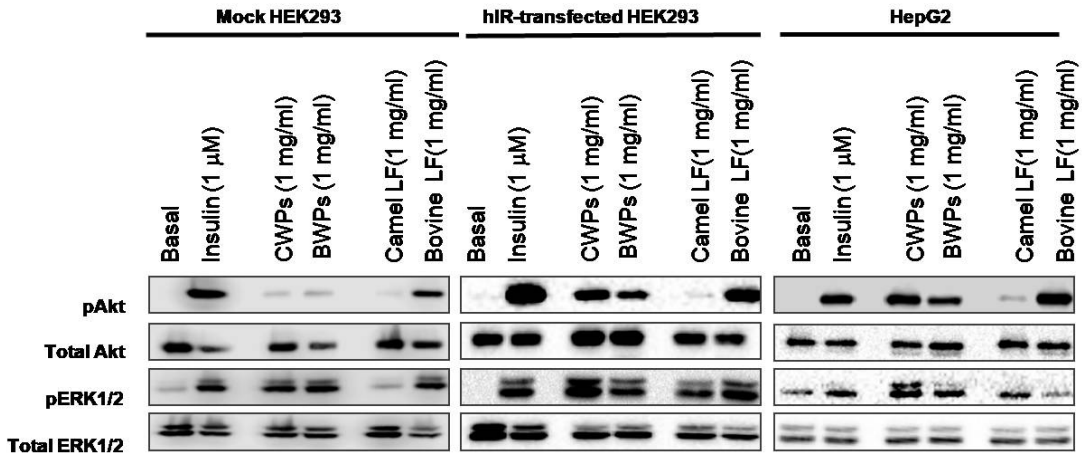

Figure 12: Effects of LF on Akt and ERK1/2 pathways in HEK293 and HepG2 cells. Western blotting data of cells lysates from HEK293 cells transiently over expressing hIR or not, and HepG2 cells endogenously expressing hIR showed significant phosphorylation of Akt and ERK1/2 when treated with CWPs, bovine milk whey proteins (BWPs) and bovine LF. Camel LF on the other hand, showed significant activation of ERK1/2 pathway and only very slight phosphorylation of Akt.

4.4 Positive effects of CWP hydrolysates on Akt and ERK1/2 pathways in HEK293 and HepG2 cells

Next, we profiled the most potent peptides obtained from pepsin hydrolysis of the raw CWPs for their biological activity on Akt and ERK1/2 pathways. These peptides are designated as P3:1, P5:1, P5:2, and P6:2 and used at 1 mg/ml along with the original CWPs (1 mg/ml). This dose was selected based on dose response studies of CWP and its hydrolysates on cells, showing maximum phosphorylation at 1mg/ml (data not shown). Among these peptides, P3:1, P5:1, and P5:2 showed to different extents, a stronger response on both Akt and ERK1/2, while P6:2 only induced a weak response with a relative stronger action of the peptide fractions on ERK1/2 compared to Akt pathway (Figure 13). Akt and ERK phosphorylation was observed in mock HEK293 cells as well as HepG2 cells with a few differences in the effect of the different peptide fractions in the two cell types. HEK cells overexpressing hIR however, stimulated only ERK phosphorylation and did not show any significant phosphorylation of Akt on treatment with the peptides (Figure 13). Together, these observations demonstrate the biological activity of peptide fractions obtained from pepsin hydrolysis of the whole CWPs and further confirm the existence of bioactive peptides in CWPs.

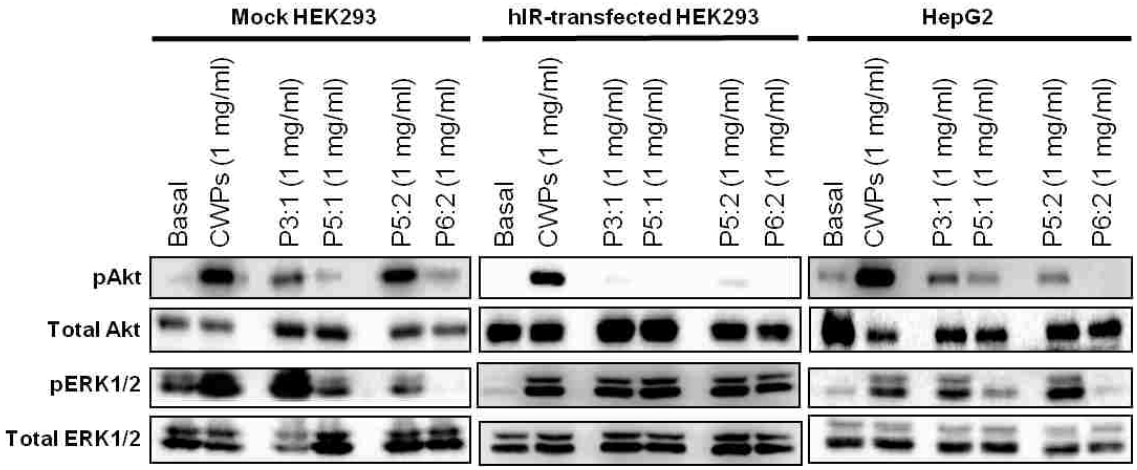

Figure 13: Positive effects of CWP hydrolysates on Akt and ERK1/2 pathways in HEK293 and HepG2 cells. These western blots show the successful phosphorylation of Akt and ERK1/2 by 3 out of the 4 peptic hydrolysates of CWP tested on mock HEK293 cells and HepG2 cells endogenously expressing hIR. HEK293 cells over expressing hIR showed only ERK phosphorylation.

4.5 Positive effects of CWPs and their hydrolysates on hIR activity studied by BRET in HEK293 cells

Next, we wanted to link our data on Akt and ERK1/2 with the putative effects of the raw CWPs and their peptide fractions on the activation of the hIR transiently expressed in HEK293 cells. For this, we used BRET technology as previously described. The assay measures in live cells the physical recruitment of IRS1-YFP protein to hIR-Rluc upon its activation by insulin and camel milk fractions as described in the methods sections (Figure 9). As a validation of the BRET assay, insulin promoted a nice dose-dependent increase in the BRET signal between hIR-Rluc and IRS1-YFP with the expected potency (EC50 value of 386.52± 32.34 nM, n=5) (Figure 14A). Next, we examined the effect of a single stimulation on BRET signals in cells upon their incubation 60 minutes at 37ºC with either insulin (1 µM), CWPs (1 mg/ml), or the different peptide fractions (1 mg/ml). As shown in Figure 14B, CWPs and their peptide fractions clearly increased the BRET signal between hIR-Rluc and IRS1-YFP and this to different extent (50 - 75%, $p<0.001$, n=5-8) compared to insulin used as a

positive control. These observations suggest a positive pharmacological effect of camel milk fractions on hIR activity. To further confirm these observations, we performed dose-response analysis showing a significant dose-dependent BRET increase with CWPs (Figure 14C) as well as the different peptide fractions (Figure 14D). Together these results reveal positive effects of CWPs and their peptide fractions on hIR activity suggesting either a direct or an indirect on hIR in HEK293 cells that may explain our data on Akt and ERK1/2 pathways shown in Figure 11 and 13.

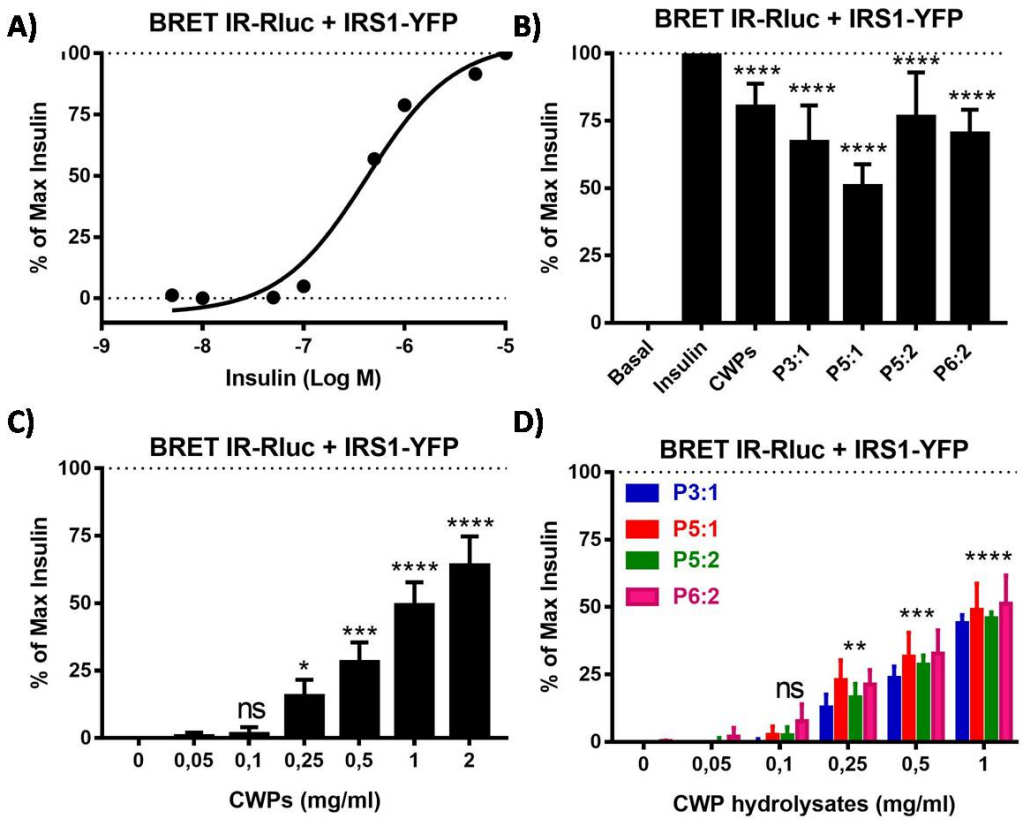

Figure 14: Positive effects of single treatments of CWPs and their hydrolysates on hIR activity studied by BRET in HEK293 cells. BRET experiments carried out in transfected HEK293 cells showing positive stimulation of the IR and subsequent recruitment of IRS1. A) Dose response of insulin, B) BRET response after 1 hour treatment of single dose of insulin (1uM), CWPs or its fractions (1mg/ml), C) Dose response of CWPs, D) Dose response of peptic hydrolysates of CWPs.

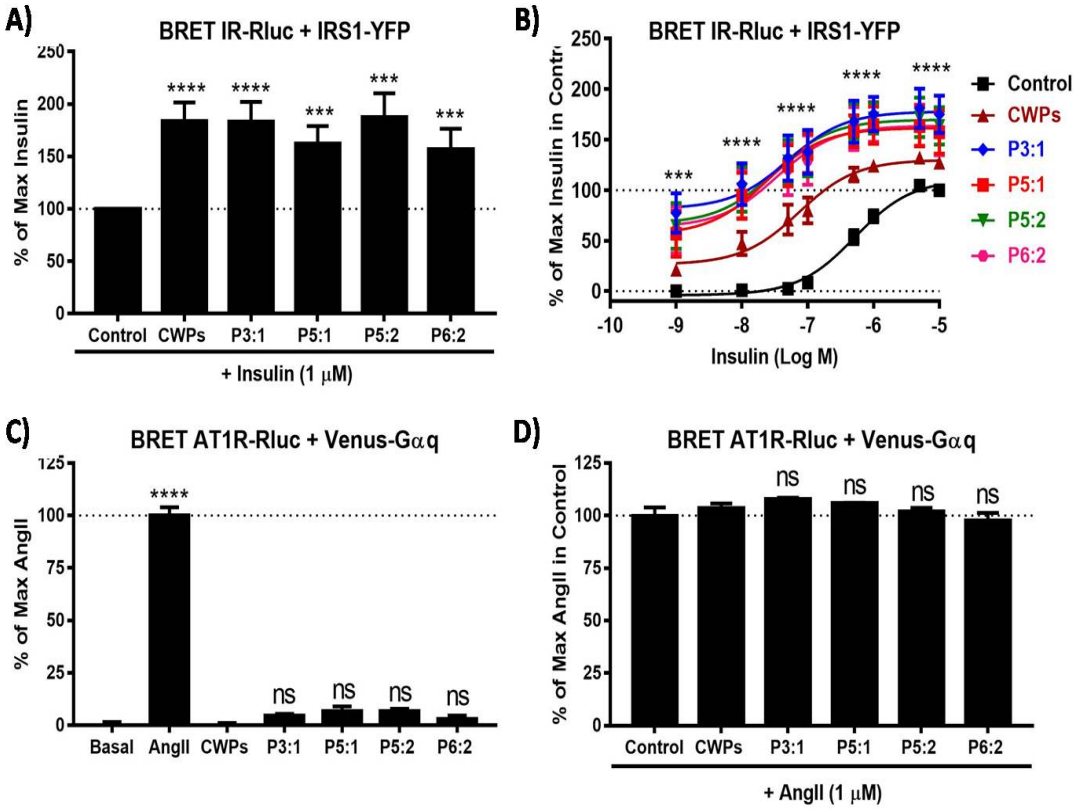

Figure 15: Positive effects of co-treatment of insulin with CWPs or their hydrolysates on hIR activity studied by BRET in HEK293 cells. BRET experiments carried out in transfected HEK293 cells showing significant increase in insulin-induced stimulation of the IR and subsequent recruitment of IRS1. A) Co-treatment of cells with CWPs or its hydrolysates with single dose of insulin (1μM), B) Dose response of insulin on cells pre-treated with CWPs or its fractions, C) Dose response of CWPs, D) Dose response of peptic hydrolysates of CWPs.

We also tested the effect of the combined stimulation of the cells co-expressing hIR-Rluc and IRS1-YFP with insulin (1 μM) in the presence of 1 mg/ml of either CWPs or their peptide fractions in the aim to reveal any allosteric effects on hIR. This was based on the previous study reporting a positive allosteric action of camel milk on hIR activity [77]. As shown in Figure 15A, the co-treatments with CWPs and their peptide fractions significantly potentiated, to different extent (60 - 100%, $p<0.001$, $n=5-8$), the insulin-mediated BRET signals. Such a positive effect was also confirmed on insulin dose-response showing a significant increase in both the efficacy (Emax,

$p<0.001$, n=5-8) and the potency (given in Log EC50, $p<0.001$, n=5) of insulin (Figure 15B) (Table 3) revealing a positive allosteric action of CWPs and their peptide fractions on hIR. Additionally, we used the angiotensin receptor (AT1R) conjugated to Rluc as BRET donor and its downstream signaling protein, Gαq conjugated to Venus (a variant of YFP) as a negative control for this experiment. As shown in Figure 15C and 15D, single treatment of CWPs and its hydrolysates as well as their co-treatment with Angiotensin II (Ang II) did not significantly increase the BRET signal compared to the control. This confirms that CWPs will not randomly potentiate any receptor in the cell.

To demonstrate this positive allosteric action on hIR, we also examined the effect of the co-treatment on ERK1/2 response in cells pre-treated or not with either CWPs (1 mg/ml) or the different peptide fractions (1 mg/ml) for 60 minutes at 37ºC before their stimulation with insulin (1 μM) for 5 minutes at 37ºC. As shown in Figure 16, the co-treatment with CWPs and the different peptide fractions strongly potentiated the insulin-mediated ERK1/2 phosphorylation and this was consistent with our BRET data shown in Figure 15. Together these results also reveal a positive allosteric action of CWPs and their peptide fractions on hIR activity in addition to their insulin-independent effects.

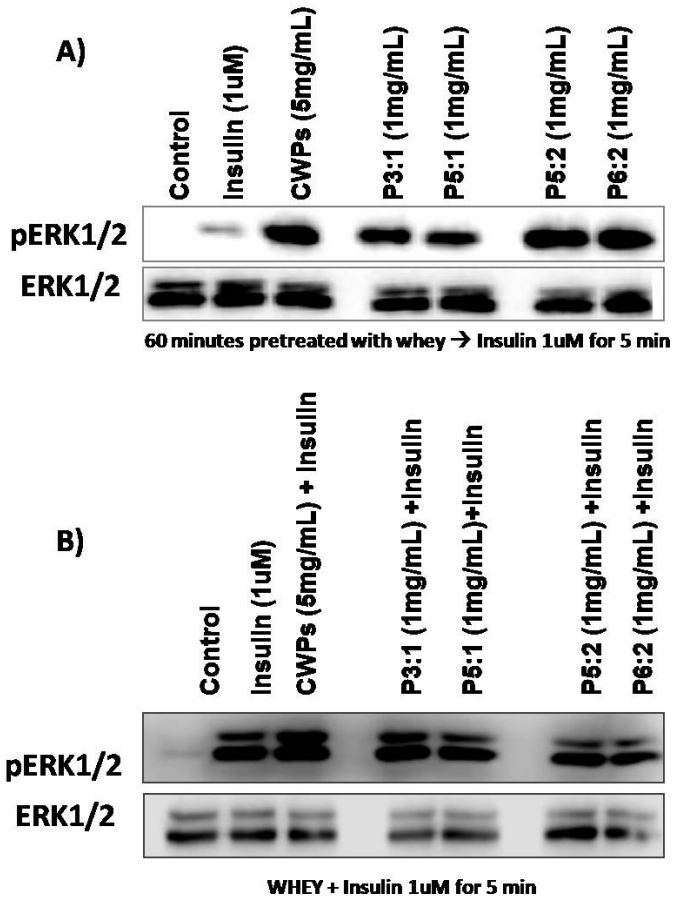

Figure 16: Positive effects of co-treatment of insulin with CWPs or their hydrolysates on ERK1/2 phosphorylation in HEK293 cells. In HEK293 cells transiently over expressing hIR A) pre-treatment of cells with CWPs and their hydrolysates for 60 minutes followed by 5 minutes with insulin and B) Co-treatment of cells with insulin and CWPs and their hydrolysates for 5 minutes, showed increase in ERK1/2 phosphorylation to almost the levels of insulin.

4.6 The positive allosteric effect of CWPs and their peptide fractions on hIR depends on its activation by insulin

In order to characterize the positive allosteric action of CWPs and their peptide fractions on hIR activity, we examined the effect of the hIR-selective peptide antagonist, S961, as previously reported [90]. For this, BRET measurements were performed in HEK293 cells transiently co-expressing hIR-Rluc and IRS1-YFP and treated or not with either insulin (1 μM), CWPs (1 mg/ml), or the different peptide fractions (1 mg/ml), in the absence (control) or presence of 1 μM of S961. As shown

in Figure 17A, the different treatments significantly elicited BRET increase between hIR-Rluc and IRS1-YFP ($p<0.001$, n=3) further confirming the positive action of CWPs and their peptide fractions on hIR activity. In addition, S961 treatment drastically inhibited insulin-mediated BRET by~80 % compared to the control ($p<0.0001$, n=3) indicating that the increase in the BRET signal indeed reflects hIR activation by insulin. However, S961 had no significant effects on the BRET signals promoted by CWPs and the different peptide fractions (Figure 17A). Next, to confirm our positive BRET data indicating the potentiation of IR, we analysed the phosphorylation status of the IR. As we expected, IR was phosphorylated by the whey proteins as well all the hydrolysates (Figure 18A) further supporting the suggestion that CWPs interact directly with the hIR in some way.

In the co-treatment protocol where cells were treated with insulin in the presence of either CWPs or the different peptide fractions, a very strong potentiation in the BRET signals was observed compared to the control ($p<0.0001$, n=3) further confirming the positive allosteric action on hIR (Figure 17B). More interestingly, the treatment with S961 drastically abolished the potentiation of the BRET signals ($p<0.0001$, n=3) (Figure 17B) and the remaining partial response reflects the direct effects of CWPs and different peptide fractions on hIR similarly to what we obtained in Figure 17A. Adding to this result, we also observed that co-treatment of CWPs with insulin increased phosphorylation of hIR (Figure 18B). More importantly, this phosphorylation was completely blocked in the presence of S961, further proving that the increase in sensitivity to insulin observed in BRET is through the insulin receptor.

Moreover, we initially used two different pIR antibodies recognizing two different phosphorylated tyrosines in the hIR. Interestingly, although insulin

phosphorylated both these tyrosine residues, CWPs phosphorylated only one (i.e., Tyr 1345) indicating differential phosphorylation of the receptor by CWPs (Figure 18C). This could be an explanation for the increase in IR potentiation when cells were pre-treated with CWPs before challenging them with insulin. For all further experiments with the hydrolysates, anti-pIR (Tyr 1345) was used.

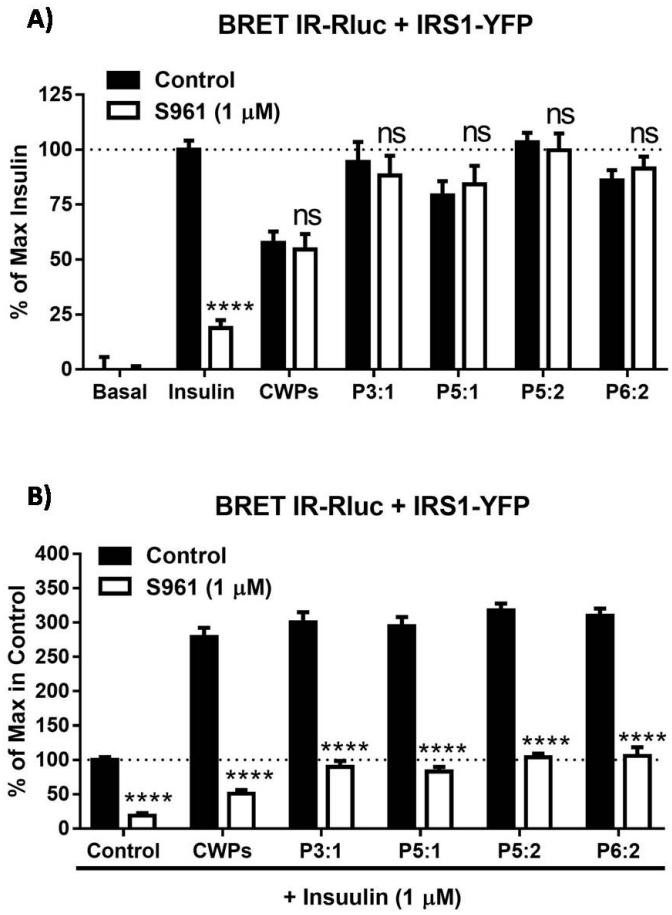

Figure 17: The positive allosteric effect of CWPs and their peptide fractions on hIR depends on its activation by insulin. These graphs show the differences in BRET potentiation with A) single treatment of CWPs and its hydrolysates with and without the hIR antagonist S961 B) co-treatment of CWPs and its hydrolysates with insulin in the presence and absence of S961.

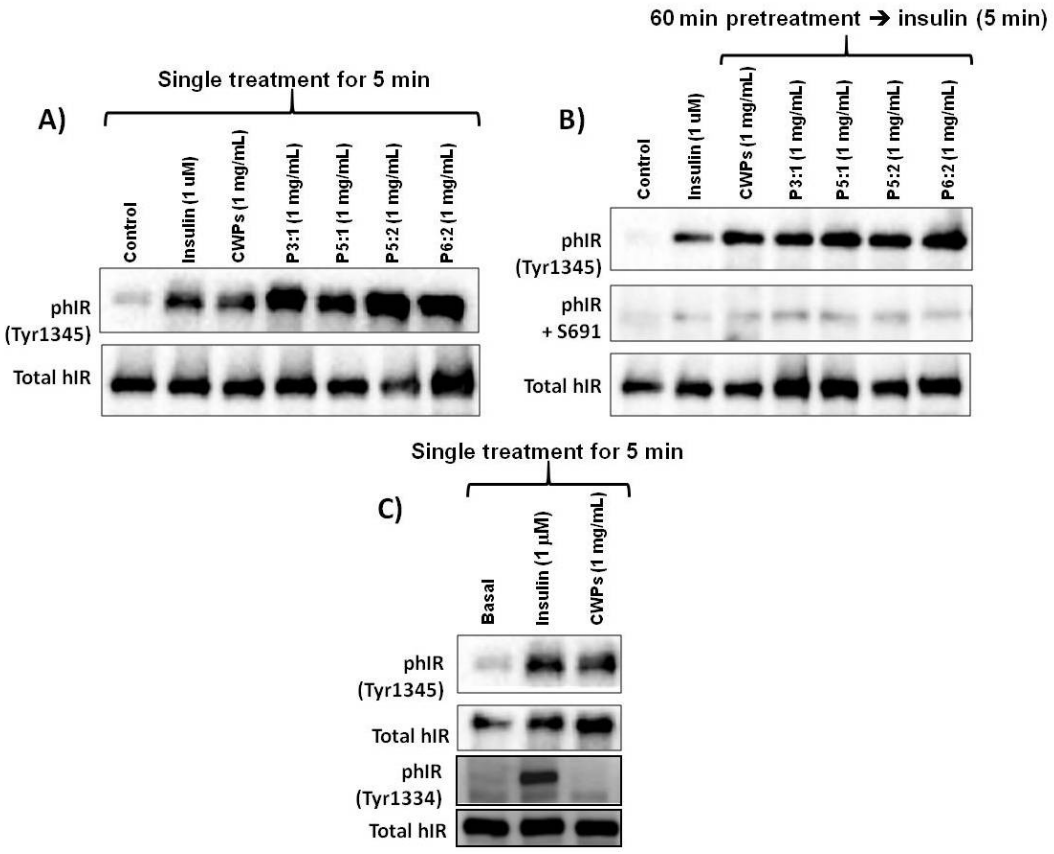

Figure 18: The phosphorylation of IR showing that the positive allosteric effect of CWPs and their peptide fractions depends on its activaion by insulin. In HEK293 cells overexpressing hIR: A) Single treatment by insulin, CWPs or its hydrolysates results in phosphorylation of IR. B) Pretreatment with CWPs or its hydrolysates followed by treatment with insulin (1 uM) leads to increase in phosphorylation of IR and this is blocked by S961. C) Differential phosphorylation of the insulin receptor by CWPs.

4.7 Positive effects of CWPs and their hydrolysates on glucose uptake in HepG2 cells

In order to translate our BRET and kinase (Akt and ERK1/2) phosphorylation data into more integrated cell response and to make a link with the hypoglycemic properties of camel milk demonstrated in many *in vivo* studies, we also examined the effects of CWPs and different peptide fractions on glucose uptake in native/non-transfected HepG2 cells. As shown in Figure 19, unexpected in contrast to insulin all the other treatments significantly ($p<0.0001$, $n=5$) and to different extent promoted glucose uptake in HepG2 cells. HepG2 cells being liver cells predominantly express

glucose transporter 2 (GLUT2), while insulin induces glucose uptake through GLUT4 [91, 92]. Glucose uptake through GLUT2 is insulin independent. Together, this could explain the low levels of glucose uptake into insulin treated HepG2 cells.

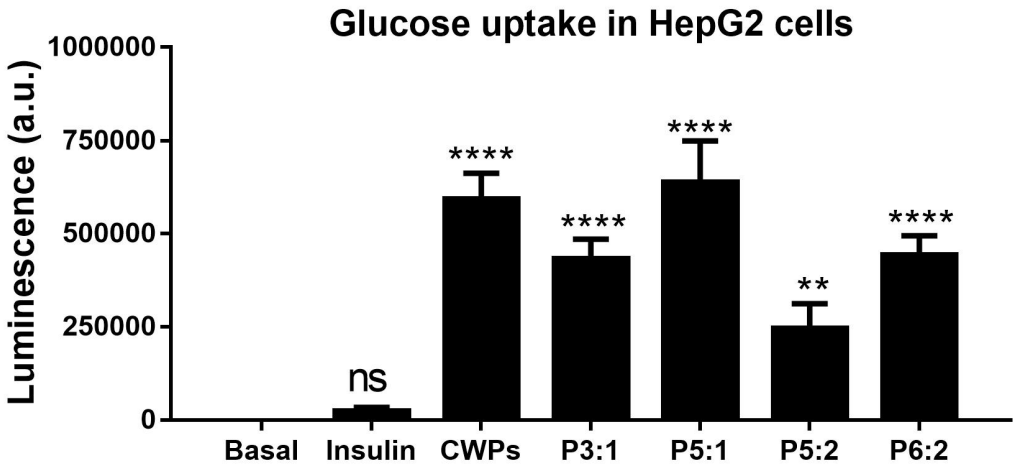

Figure 19: Positive effects of CWPs and their hydrolysates on glucose uptake in HepG2 cells. CWPs and its hydrolysates stimulated glucose uptake into HepG2 cells endogenously expressing the hIR.

Table 3: Emax and Log EC50 values determined from the different single and combined treatments with insulin, CWPs, and the different peptide fractions (as shown in Figure 14C and Figure 15B).

Treatment	E_{max} (%) in single treatment	E_{max} (%) in combined treatment	Log EC_{50} of insulin in combined treatment
Insulin (Control)	**100**	**100**	**-6.28 ± 0.07 (n=5)**
CWPs	**80 ± 8 (n=8)**	**184± 18 (n=8)**	**-7.13 ± 0.14 (n=5)**
P3:1	**67 ± 13 (n=5)**	**183± 19 (n=5)**	**-7.29 ± 0.34 (n=5)**
P5:1	**51 ± 8 (n=8)**	**162 ± 17 (n=8)**	**-7.63 ± 0.35 (n=5)**
P5:2	**76 ± 16 (n=5)**	**188± 23 (n=5)**	**-7.50 ± 0.34 (n=5)**
P6:2	**70 ± 9 (n=8)**	**157± 19 (n=8)**	**-7.46 ± 0.39 (n=5)**

Chapter 5: Discussion and Conclusion

The antidiabetic nature of CM has been proved time and again by multiple *in vivo* studies in both animal models of the disease and diabetic humans. As mentioned earlier, a recent study revealed that raw CM significantly increased the recruitment of substrate proteins involved in insulin- induced downstream signaling pathways, to the hIR [77]. This potentiation was narrowed down to be the action of a component in the whey fraction of CM. Due to its obviously major role in glucose homeostasis, we hypothesized that this effect was linked to the activation of hIR by CWPs. To answer our question, we looked into effect of CWPs on induction of different mechanisms that are normally carried out by insulin for glucose homeostasis via the hIR: phosphorylation Akt and ERK1/2, physical association of IRS1 to hIR and increase in glucose uptake. The *in vitro* effects of CWPs, its hydrolysates, LF and adiponectin were studied on these three parameters. CWPs and its hydrolysates clearly potentiated Akt and ERK phosphorylation to levels that were comparable to insulin. These two proteins play key roles in two major pathways activated by the insulin receptor, Akt in the metabolic pathway and ERK1/2 in the mitogenic pathway. This was true even for the peptic and chymomtryptic hydrolysates of CWP as well as the different peptide groups obtained after peptic hydrolysis and bovine LF. CMLF however, was only seen to activate the ERK1/2 signaling pathway, and to a lower extent than bovine LF. It is possible that the higher activity of bovine LF when compared to CMLF is due to the fact that bovine LF used here was commercially purchased, while CMLF was isolated in the university labs. However, we can also speculate that CMLF when given to cells as pure treatment does not elicit the same response as when it enters the body as part of whole CM. As mentioned before, a number of factors like the pH and stability of

CM along with the encapsulation of milk proteins by lipid nanoparticles, all contribute to the better bioavailability of CM proteins once ingested [61, 67].

It's important to note here that since the phosphorylation of Akt and ERK are not events that are exclusive to hIR activation and is possible through other receptors, we cannot use these results to confirm activation of hIR or the binding of CWPs and its hydrolysates to the hIR.

Stepping one step closer to hIR activation, CWPs, its hydrolysates and LF also showed increased BRET signals (upto 75% that of insulin) signifying the physical association of hIR with IRS1. IRS1 is an important scaffolding protein involved in both the metabolic Akt and mitogenic ERK pathways induced by insulin through the hIR. It is the first intracellular protein that interacts with the hIR upon activation[14, 15]. The consequent increase in glucose uptake into HepG2 cells confirms the intracellular translation of these signals into responses.

Excitingly, when we co-treated hIR expressing cells with insulin along with CWPs or their hydrolysates, we witnessed a 60-100% jump in insulin induced -BRET signals. This is consistent with the observation from the previously mentioned study by A.O. Abdulrahman and colleagues which suggested a positive allosteric action of CM on hIR [77]. Comparing dose response studies of insulin with and without CWPs or its hydrolysates, we can clearly see that these proteins and peptides increase both the potency and efficacy of insulin. Fractions of CWP increased insulin induced recruitment of IRS1 to hIR even at saturating doses of insulin. Additionally, these signals were higher than those achieved at saturating single doses of CWPs or their hydrolysates. Moreover, this view was strengthened further when blocking the hIR with the antagonist S961 significantly reduced insulin–induced BRET but had almost

no effect on that induced by any of the CWP fractions. Blocking of IR in cells treated with both insulin and CWPs remarkably diminished the insulin effect in the treatment and brought them back to the single treatment levels. All of these points suggest an allosteric mode of action of action of CWPs and their peptide fractions on hIR. The binding of insulin to the extracellular domain of the receptor, changes and stabilizes the conformation of the receptor in such a way that enables its intracellular kinase domains to undergo successful autophosphorylation and initiate signaling. We know now that the different ligands can exist for a single receptor and they can stabilize and potentiate the receptor to different extents and/or to be selective to certain downstream pathways [93]. In this scenario, it is possible that the allosteric binding of CWPs to the hIR stabilizes the insulin-hIR complex in a conformation that enables better interaction with downstream proteins, higher affinity to insulin and/or a lower dissociation rate of the ligand-receptor complex (Figure 20). A similar model of activation of the hIR is seen in XMetS, an allosteric antibody to the hIR [41]. These results also support the finding by Agrawal *et al*. (2011) where the amount of insulin required by diabetics considerably reduced over the course of 2 years if they were consuming camel milk [61]. Increased potency with co-treatment of insulin with CWPs or its fractions implies that, in the presence of CWP, a smaller quantity of insulin is required to achieve the same effect.

It is important to note here that this allosteric effect of CWPs and its hydrolysates is in addition to the insulin-independent effects observed initially. While this study indeed postulates some degree of direct activation of the insulin receptor by CWPs, we cannot rule out the possibility that CWPs also have some indirect effects on the hIR and its related pathways. It is possible that it is potentiating downstream signaling targets in insulin induced hIR signaling pathways via activation of other

receptors. Another possibility is that CWPs affect completely different pathways that contribute to glycemic control via the activation of other receptors. Receptors like IGFR and cell surface receptors for glucagon, incretins and cytokines are some potential receptor candidates for this cause [78] (Figure 20).

Another point to note is that in the previously mentioned study by A.O. Abdulrahman *et al*. (2016), CM proteins only increased the recruitment of Grb2 to hIR and not IRS1 [77]. On the contrary, our study shows significant potentiation through hIR-IRS1 interaction. These differences can perhaps be reconciled by the fact that differences in storage of the milk, lactation stage, breed and living environment of the camels can affect the concentration of proteins in the milk [46]. Throughout the course of this thesis work, CWPs were fractionated from fresh CM every week. Though the source of CM was always the same farm, minimal differences were observed in Akt and ERK phosphorylation levels and also in the IR activation levels seen in BRET experiments with different preparations of CWPs. These differences can be attributed to the reported changes in CM protein levels that come along with changes in seasons and changes in lactation periods [46]. The final values and results are averages of three or more distinct experiments.

Increasing the sensitivity of hIR via allosteric binding is an exciting possibility that comes with the potential to provide a new door for drug development strategies for diabetes. Studying the structural and physical properties of these interactions will help us understand the molecular interactions associated with hIR signaling better. Since they potentially bind to a site completely different spatially and structurally to that of insulin, allosteric modulator' actions can be more specific to the receptor than that of the orthosteric ligand. This is because sequence similarity in these sites is more

unlikely across receptors from the same family (for example: IR and IGFR) [36]. Therapies that increase the sensitivity of the hIR can reduce the requirement for exogenous insulin and hence, also be less likely to induce hyperinsulinemia. When therapies like this are started at early stages of diabetes, they may even postpone or nullify the need for exogenous insulin by preserving β cell function in the pancreas [41].

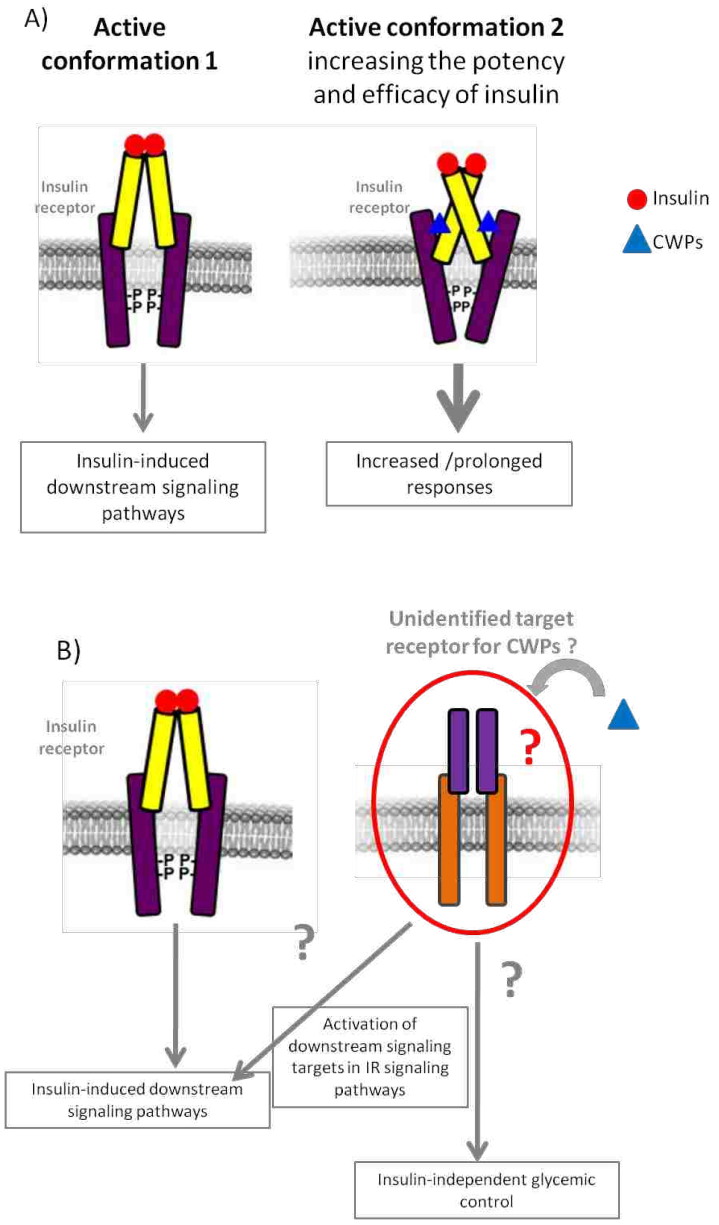

Figure 20 : Putative model of the action of CWPs on the IR.
A) Allosteric mode of action by direct interaction with the IR, B) Indirect effects on IR activated pathways via another receptor (adapted from [77]).

Chapter 6: Future Directions

This research definitely takes us one step closer to understanding the molecular mechanisms that are responsible for the hypoglycemic effects of CM observed *in vivo*. Although, it will be beneficial to investigate the action of CWPs and its hydrolysates on other receptors involved in glucose homeostasis, such as the IGFR, glucagon and incretin receptors. Another interesting target would be the IR-IGFR hybrid receptors, since they respond to insulin and IGF in ways that are different to their homodimeric counterparts. Additionally, our study prioritized only a subset of the hydrolysates produced by CWPs on gastric digestion. Further investigations are required to assess the effects of hydrolysates generated by different enzymes/ combinations of enzymes in different conditions (e.g. time, pH) to bring us closer to actual stomach conditions.

It is also essential to confirm the *in vitro* effects of CWP hydrolysates in *in vivo* systems such as diabetic mice models. *In vitro* studies rule out the physiological effects of persistent high blood glucose levels in the environment. The cancerous characteristics of established cell lines like HepG2 cells can lead to altered results [94]. These effects may very well contribute to differences in the hypoglycemic activity of camel milk *in vivo*.

Finally, sequencing and studying the peptides obtained after peptic hydrolysis of CWP is another possibility that could help us move further. Protein modeling and binding studies of these hydrolysates can provide us with better insights into the working and activation of the IR. Above all, it can give us a new perspective on modulation of IR signaling.

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List of Publications

1- M. A. Ayoub, A. R. Palakkott, A. Ashraf, and R. Iratni, “The molecular basis of the anti-diabetic properties of camel milk,” Diabetes Research and Clinical Practice, vol. 146, pp. 305–312, Dec. 2018.

2- A. Ali, A. R. Palakkot, A. Ashraf, I. Zamel, B. Baby, R. Vijayan, M. A. Ayoub, “Positive Modulation of Angiotensin II Type 1 Receptor-Mediated Signalling by LVV-Hemorphin-7,” Frontiers in Pharmacology, vol. 10, pp. 1258, Oct. 2019.