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Insulin Promotes Wound Healing by Inactivating NFkβ$^{P50/P65}$ and Activating Protein and Lipid Biosynthesis and alternating Pro/Anti-inflammatory Cytokines Dynamics.

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Abstract: Four hundred and twenty-two million people have diabetes due to excess free body glucose in their body fluids. Diabetes leads to various problems including retinopathy, neuropathy, arthritis, damage blood vessels etc; it also causes a delay in wound healing. Insufficiency of insulin is the main reason for diabetes-I and systemic insulin treatment is a remedy. The perspective of the potential use of insulin/insulin based drugs to treat chronic wounds in diabetic conditions is focused on in this review. At the site of the wound, TNF-α, IFN-Y, IL-1β and IL-6 pro-inflammatory cytokines cause the generation of free radicals, leading to inflammation which becomes persistent in diabetes. Insulin induces expression of IL-4/IL-13, IL-10 anti-inflammatory cytokines etc which further down-regulates NFkβ$^{P50/P65}$ assembly. Insulin shifts the equilibrium towards NFkβ$^{P50/P50}$ which leads to down-regulation of inflammatory cytokines such as IL-6, IL-10 etc through STAT6, STAT3 and c-Maf activation causing nullification of an inflammatory condition. Insulin also promotes protein and lipid biosynthesis which indeed promotes wound recovery. Here, in this article, the contributions of insulin in controlling wound tissue microenvironments and remodulation of tissue have been summarised, which may be helpful to develop novel insulin-based formulation(s) for effective treatment of wounds in diabetic conditions.

Keywords: Insulin; Inflammation; Wound Healing; Diabetes; NFkβ.

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List of abbreviations

Akt, protein kinase B; bFGF, basal fibroblast growth factor; 4EBP-1, 4E binding protein; eIF4B, eukaryotic initiation factor 4B; ERK, extracellular signal–regulated kinase; eNOS, endothelial nitric oxide synthase; FOXO, Fork head box protein O1; HIF PHD, hypoxia inducible factor prolylhydroxylase; JAK, Janus kinase; JNK, jun N-terminal kinase; IAgNP, Insulin coated silver nanoparticles; IDF, international diabetes foundation; IFN, Interferon-alpha; IGF, insulin like growth factor; IGF-1R, insulin like growth factor 1 receptor; IL, Interleukin; IP, induced protein; IL-IR, Interleukin insulin receptor; IRS-1, insulin receptor substrate; MAPK, mitogen-activated protein kinase; MEK, MAPK ERK kinase MIF, migration inhibitory factor; MIP, macrophage inflammatory proteins; MMP- 2,4, matrix metalloproteinase; mTOR, mechanistic target of rapamycin; NADH, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NFkβ, nuclear factor kappa beta; NK, natural killer; NO, nitric oxide; NOX, NADP oxidase; PDH, pyruvate dehydrogenase; PFK-1, 6-phosphofructo-1- kinase; PI3K, phosphoinositide-3-kinase; PKC, protein kinase C; PPAR-Y, Peroxisome Proliferator Activated Receptor Gamma; RAF, rapidly accelerated fibro sarcoma; ROS, reactive oxygen species; rpS6, ribosomal protein; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; TLR, toll like receptors; TNF, tumour necrosis factor; TSC ½, tuberous sclerosis protein ½; VEGF, vascular endothelial growth factor; WHO, world health organisation.

Introduction

Over the last 25 years, there has been fourfold increase in cases of diabetes mellitus, commonly known as diabetes [1]. In the year 2016, worldwide 422 million people have

been reported to have diabetes. According to the World health Organisation (WHO), diabetes is the 8th leading cause of death and was directly associated with 1.5 million deaths worldwide in the year 2012 [2]. Diabetes is characterized by the presence of a chronic high free glucose level in the body fluid, including blood, urine, sweat etc [3]. Among one of the main reasons for the occurrence of the diseases is the failure of hormone-mediated metabolic regulation. Hormones such as insulin and glucagon play the most important role in maintaining the sugar balance in the blood [4]. Maintaining a steady balance of sugar in the blood is very critical for the normal functioning of the body [5]. The presence of excess glucose in body fluids leads to various pathological conditions such as susceptibility to infection, inducing the onset of various diseases like cataract, retinopathy, neuropathy, arthritis, hypertension, cardiovascular problems, kidney damage, damaged blood vesicles, and a delay in wound healing etc [5, 6]. Due to the association of these diseases with diabetes, the International Diabetes Foundation (IDF) estimated the loss of 4.9 million lives, ~1.25% of all diabetic patients in 2014, as being directly or indirectly caused due to diabetes [7]. These diseases are associated and affect various organs of the body with different pathologies however; all these pathological conditions are associated with tissue inflammation [8]. Diabetes induces low grade systemic inflammation and promotes disease conditions such as hypertension, arthritis, retinopathy etc [9].

One of the important aspects of diabetes to inflammation is its association with delayed wound healing [10]. The chronic diabetic wound is characterized by the persistent increment of pro-inflammatory cytokines and the absence of growth signal in damaged tissues [11]. Various diabetes treatments are helpful in controlling blood glucose level and thereby can also delay progression of the diseases associated with diabetes, such as hypertension, arthritis, retinopathy, cataract, neuropathy etc, but very little is known about their effect on the recovery of diabetic wounds [12]. Wounding induces tissue inflammation and induces the release of various pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-12, IL-18, interferon gamma (IFN-γ), and tumour necrosis factors (TNFs) [13,14]. Pro-inflammatory cytokines, such as IL-1β, IL-6, secreted from monocytes and macrophages in wound tissue triggers pain responses by stimulating neuronal signalling [15]. IFN-γ, TNF-α, and IL-1β induce tissue apoptosis and pyroptosis mediated by oxidative stress and by activating innate immunity [16]. IFN-γ is a potent activator of tissue macrophages by modulating STAT1 expression to generate a foreign pathogen defence state in the affected area [17]. IL-12 stimulates the production of IFN-γ and TNF-α and reduces IL-4, anti-inflammatory cytokine mediated suppression of IFN-γ, IL-4 inhibits IFN- γ through STAT3 signalling [18]. IL-18 activates Natural killer (NK) cells and T cells thereby promoting the release of IFN-γ in wound conditions for pathogen defence [19]. However, the persistence of prolonged inflammatory conditions is detrimental and causes tissue damage which eventually delays the repair process. IL-1 is a potent activator of TNFs and therefore induces cell damage. Overproduction of IL-1β is especially involved in neuronal inflammation and causes the damage of neuromuscular junctions, which delays the wound healing procedure. The FDA has approved the use of IL-1 blocker as an effective therapeutic approach to fight against rheumatoid arthritis [20]. Macrophages, in response to foreign pathogens, secrete IL-6 which infers Toll-like receptor (TLR), especially the TLR-9 response-mediated defence against the foreign pathogen by killing it. The TLR-9 pathway is activated by unmethylated DNA which is present in prokaryotes, and kills the pathogen. But in the case of a wound, spillage of mitochondrial DNA takes place, which is essentially unmethylated DNA, and triggers a similar response in the injured tissue [21]. IL-12 inhibits the formation of new blood vessels in the tissue by IFN-γ-mediated over expression of Interferon gamma-induced protein 10 (IP-10 or CXCL-10) [22]. IL-18 suppresses the expression of vascular endothelial growth factor (VEGF) which is essential for cell blood vessels development of the wound tissue and it is therefore essential for growth and repair [23].

Overcoming the inflammatory response is important to initiate and control the repair mechanism. In the normal course, anti-inflammatory cytokines such as IL4, IL-10, IL-11, IL-13, Interferon-alpha (IFN-α) and transforming growth factor-beta (TGF-β) play crucial roles in the wound recovery process [24]. TLR-9 response at an early phase causes a rapid production of pro-inflammatory signals such as TNF-α via rapid activation of p38/ mitogen-activated protein kinase (MAPK) and the c-Jun N-terminal kinase (JNK) pathway [25]. In normal conditions, the prolonged activation of MAPK results in activation of MAPK phosphatase which works as a feedback inhibitor of p38/MAPK and JNK pathways, resulting in the down regulation of TNF-α production. Dephosphorylated p38/ MAPK results in the production of anti-inflammatory cytokines IL-10, homodimeric (35 kDa each) cytokine, produced by monocytes, macrophages and induces TGF-β signalling which promotes cell division [26]. Like IL-10, IL-4 and IL-13 also stimulate the synthesis of fibrinogen and the extracellular matrix, especially collagens. IL-4, a 20 kDa cytokine, is secreted from inflamed T cells, mast

cells, and macrophages and activates Janus kinase/signal transducer and activator of transcription-6 (Jak/STAT6) pathway promoting wound repair [27]. IL-4 helps in the synthesis of the extracellular matrix, especially collagen which provides the physical support for wound recovery [28]. Another cytokine, L-1RA, is secreted by cells including immune cells, epithelial cells, and adipocytes, and shows inhibition of the IL1β pro-inflammatory effect by binding itself to the interleukin-1 receptor (IL-1R). On the other hand, deregulation of IL-1β and TNF-α prolongs the inflammatory phase and delays healing [29]. IL-11, a 23 kDa protein released by bone marrow cells shows its anti-inflammatory activity. IL-11 inhibits IL-1 and TNF-α synthesis through $NFk\beta^{P50/P65}$ inhibition by up-regulating inhibitory $NFk\beta^{P50/P50}$ synthesis in monocytes/macrophages cells [30]. The dynamics between pro-inflammatory and anti-inflammatory cytokines is critically maintained in normal physiological conditions for effective wound recovery process, but in diabetic conditions, the normal dynamics of the cytokines get impaired.

The first line of diabetes treatment for insulin dependent, type-I diabetes is the administration of insulin systematically. Insulin, a peptide hormone produced by beta cells of the islets of Langerhans of the pancreas. The precursor of insulin is proinsulin, a single polypeptide encoded by the INS gene in humans, after processing 2 secretory proteins are generated, with one consisting of two chains namely A and B (21 and 30 amino acids respectively) that generates mature insulin and the second is the C-chain of 31 amino acids, commonly known C-peptide [31, 32]. The "A" chain is fairly compact and has 2 small alpha helical regions whereas the B chain has only one of such kind. Two disulfide linkages between A7-B7 and A20-B19 hold the A and B chains together, other than the internal disulfide bridge of the A chain (A7-A11). In presence of Zn^{2+} and a favorable pH of ~6.0,insulin folds into a hexameric form and get stored in the pancreas. Upon diffusion into the blood, due to alteration of pH, hexameric insulin converts into the monomeric form and binds to its receptor [33, 34]. Receptor binding depends upon several regions present in insulin monomers. These regions are mainly present at the surface; mutations in these regions reduce the affinity of binding of insulin [35, 36]. The regions are located at GlyA1, IleA2, ValA3, GluA4 on the N terminus and TyrA19, CysA20, AsnA21 on the C-terminus of the "A" chain, and at GlyB23, PheB24, PheB25, and TyrB26 at C-terminus of the "B" chain [37].

Not only present in humans, insulin-like peptides are also found in invertebrates such as molluscs and insects. The growth-related function of insulin-like peptides shows that insulin is not only involved in glucose metabolism [38]. Drugs which can stabilize the balance between pro- and anti-inflammatory cytokines can be useful to treat insulin-dependent or independent diabetes and its associated disease conditions. Although very few studies have been performed involving insulin as a wound healing agent, S.E. Greenway from Harbor-UCLA Medical Center, Torrance, California in 1999 showed almost 25% faster recovery of the wound in the case of diabetic patients. They found that the wounds of the five diabetic subjects healed in 6.6 ± 1.7 days with insulin treatment and 8.8 ± 1.6 days with saline, a difference of 2.2 ± 0.6 days. They also found that insulin has wound healing activity even in non-diabetic patients. They found that the wounds of the six nondiabetic subjects healed in 4.8 ± 0.4 days with insulin treatment and 7.3 ± 0.7 days with saline, a difference of 2.5 ± 0.5 days, which is almost 35% faster than the placebo [39]. But in spite of the promising result, not much follow-up has been done in this respect. In another study in 2012 it was shown that the application of insulin topically inhibits the infiltration of neutrophils in wound tissue in diabetic mice [40]. A few other discrete works in 2017 showed that topical insulin is beneficial in burn wound healing in diabetic rats [41]. In one of our recent studies, we have shown that both insulin and insulin-protected silver nanoparticles promote wound recovery in both diabetic and non-diabetic conditions [42]. In this work, we have shown that insulin promotes wound recovery in both in vitro and in vivo both in diabetic as well as normoglycemic conditions. On the 5th day of treatment 20% and 12% faster recovery of the wound was observed in IAgNPs for diabetic and normoglycemic rats in comparison with the control. Whereas free insulin also showed faster recovery with lesser efficiency in comparison with IAgNPs with an increased rate of 4.67% and 7.27% respectively for the diabetic and normoglycemic rate in comparison with placebo-treated animals. On the 11th day, the percentage was 73.33% and 60.0% with IAgNPs and 40% and 33.33% with free insulin in diabetic as well as nondiabetic models respectively in comparison to respective controls. Further serum quantification showed an increase in the percentage of anti-inflammatory cytokines and a decrease in inflammatory cytokines in both diabetic and non-diabetic animals after treatment with IAgNPs and insulin in comparison to respective controls. In diabetic rats on the 5th day the concentration of IL-6 was 25% and TNF-α in 2 fold higher concentration in comparison to the non-diabetic control. After treatment with IAgNPs, there was 50% inhibition of this cytokine expression in both groups which is higher than free insulin. Expression of IL-6 and TNF-α on the 11th day was 30% and 50% respectively in control in comparison to a non-diabetic model which

reduces to 45% in both sets diabetic as well as non-diabetic upon treatment with IAgNPs and with free insulin the inhibition was around 40% in IL-6 and 30% in TNF-α. In contrast to the expression of inflammatory cytokines, the percentage of anti-inflammatory cytokines (IL-10) increases after treatment with IAgNPs and free insulin. On the 5th day, the concentration of IL-10 increased 70% in normal and 50% in diabetic rats with IAgNPs treatment and free insulin-treated groups showed 45% and 30% increment in IL-10 concentration in normal and diabetic models respectively in comparison to control. On 11th the concentration of anti-inflammatory cytokines further increases by 65% and 50% with IAgNPs and slightly less with free insulin in non-diabetic and diabetic models respectively. Histological evaluations on the 5th and 11th day showed a significant decrease in the level of leukocyte infiltration, faster deposition of collagens and rapid re-epithelization was observed with IAgNPs and insulin in comparison with other sub-groups [42]. In spite of a few discrete studies, the underlying mechanism of insulin function as a wound healing agent, the proper evaluation of insulin as an antioxidant, anti-inflammatory and as wound recovery agent is yet to be done. The objective of this review is to explore the wound healing mechanism of insulin. The authors revealed that insulin induces wound tissue regeneration and healing by controlling glucose metabolism, protein biosynthesis, lipid biosynthesis and alternating pro/anti-inflammatory dynamics by driving NFkβ equilibrium from p50/p65 to p50/p50.

Anti-inflammatory cytokine activation and increase in cell differentiation by insulin signalling

Insulin is a peptide hormone, which can also act as an anti-inflammatory agent by activating cytokines which can reduce inflammation and help to recover the wound. Also, through metabolism and synthesis activities, it plays an important role in cell differentiation and survival. Insulin promotes up regulation of NF-kβ$^{P50/P50}$ by suppression of p65 expression and TNF-α. Suppression of NF-kβ$^{P50/P65}$ decreases the expression of IL-6, IL-1β, IL-12 and TNF-α cytokines in the wound site [43, 44-46]. Inhibition of proinflammatory cytokines drives the equilibrium toward the expression of anti-inflammatory cytokines, such as IL-10, IL-4, VEGF etc, which inhibits cellular apoptosis and induces cell proliferation like IGF [47-49]. In the following section the regulation of the dynamics of cytokines under the influence of insulin is discussed under the following sections: a) Insulin inactivated NFkβ$^{p50/p65}$ to decrease inflammation by inducing glucose uptake, b) Insulin induces fatty acid biosynthesis and thereby inactivates the TNFα mediated inflammatory pathway, c) Insulin induces cell growth and differentiation by protein synthesis and inhibits proteolysis through FOXO inactivation to promote cell survival, d) Insulin behaves as an IGF growth factor and can activate the same signalling pathway to reduce inflammation, and e) Insulin modulates inflammation through the reduction of proinflammatory cytokines and inducing anti-inflammatory cytokines.

a) Insulin inactivated NFkβ$^{p50/p65}$ to decrease inflammation by inducing glucose uptake

Presence of excess glucose at the site of a wound allows microbial growth, leading to activation of inflammatory pathways. Insulin is mainly recognized for its anti-glycemic activity. With the help of insulin, glucose molecules get internalized by the cells via glucose transporters and get stored as glycogen. In muscle tissue, stored glycogen works as an energy source and gets utilized aerobically [50]. Wounds which result from microcirculatory damage mainly in peripheral nerves, the retina and the renal cortex and, due to increases in oxygen consumption by inflammatory cells, leads to promotion of glycolytic aerobic to anaerobic switch [51]. The direct consequence of this is the release of lactic acid as an end product of glycolysis. Additional sources of anaerobic glycolysis are the proliferating cells in wound tissues, performing anaerobic respiration in muscles [51, 52]. Lactic acid present in the blood gets reused to synthesize glucose in liver.

Lactate dissociates into nicotinamide adenine dinucleotide (NADH) and pyruvate; the NADH formed during this acts as substrate for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, results in the production of lactate-induced reactive oxygen species (ROS) [53]. Due to the synthesis of more NADPH, the ratio of NADPH to NAD^+ decreases, leading to the activation of VEGF, and facilitation of angiogenesis and pyruvate both together are responsible for the synthesis of collagen and angiogenesis by inactivation of prolylhydroxylase hypoxia inducible factor (HIF PHD) [54, 55].

HIF may cause tissue damage and inflammation at the wound site [56]. Peripheral blood vessel damaged responsive hypoxia causes oxidative stress through activation of NADP oxidase (NOX) at wound site which is a key regulating factor in the wound healing process and leads to the over expression of intercellular ROS [57, 58] . High level of ROS in turn induces the oxidation of protein and lipid peroxidation leads to cellular apoptosis [57-60]. Further production of ROS induces accumulation

of $NFk\beta^{P50/P65}$, which inhibits the expression of HIF1-α and mTOR. Other than inhibition of HIF1 and mTOR, NFkβp50/p65 induces expression of resistin which is responsible for intercellular insulin resistance [61, 62]. Resistin activates a vicious cycle by inducing over expression of p65 [63]. Activation of p65 drives the equilibrium of NFκβ from p50/p50 to p50/p65 which results in development of insulin resistance [61-63]. Insulin on the other hand induces HIF1 and mTOR through activation of AKT pathway and inhibits TNFα [64]. Activation of HIF1 drives back the NFκβ equilibrium from p50/p65 to p50/p50. The normalization of blood glucose level with proper functioning of insulin also effectively reduces the $NFk\beta^{P50/P65}$ expression level [65]. Activation of $NFk\beta^{P50/P50}$ inhibits expression of proinflammatory cytokines like IL-6, IL1β [66, 67] and induces over expression of anti-inflammatory cytokines which results in decline in inflammatory conditions and promotes tissue repair. [66-69].

NADPH and pyruvate inactivate HIF PHDs, through oxidation of Fe (II) and ascorbic acid. HIF PHDs are dioxygenase, dependent on Fe (II) and 2-oxoglutarate, requiring ascorbic acid. Elsewhere in the presence of lactate, Fe (II) and ascorbic acid are oxidised. As a result of this, lactate acts as an inhibitor of tissue damage and promotes the release of basal fibroblast growth factor (bFGF), IL-8 and activation of NF-$k\beta^{P50/P50}$ [70]. The upregulation of NF-$k\beta^{P50/P50}$ by lactate through suppression of the generation of NF-$k\beta^{P50/P65}$ ultimately decreases the release of IL-6, IL-1β, IL-12 and TNF-α cytokines. This signalling ultimately leads to increase in cell viability [43]. In addition, ROS-dependent inhibition of IkBα and expression of VEGF receptors are responsible for collagen synthesis and angiogenesis [71]. IkBα is responsible for the translocation of NFkβ to the nucleus and expression of the p65 gene which in turn induces inflammation [72, 73]. In addition to this, suppression of expression of NF-$k\beta^{P50/P65}$ takes place by the phosphorylation of ERK through insulin signalling [74]. In contrast to the popular belief on the role of lactate in wound healing, Cheol *et al*. suggested that lactate, mainly produced in skeletal muscles, either inhibits glucose metabolism by impairing insulin signalling or decreases uptake of glucose by decreasing transmembrane glucose gradient [75]. The insulin signalling for glucose metabolism takes place through 6-phosphofructo-1-kinase (PFK-1), which is produced by fructose-2, 6-biphosphate and pyruvate dehydrogenase (PDH) used to convert pyruvate to oxaloacetate. Lactate inhibits this insulin signalling by increasing citrate level and decreasing fructose-2, 6-biphosphate which inhibit and promote PFK-1 respectively. Inhibition of PDH by increasing NADH to NAD ratio ultimately inhibits transformation of pyruvate to oxaloacetate [75, 76]. This shows that lactate acts as inhibitor of glycolysis resulting in increase of glucose concentration in blood [77]. A high concentration of glucose in blood, in turn, leads to prolonged inflammation at the site of wound.

b) Insulin induces fatty acid biosynthesis and thereby inactivates the TNFα mediated inflammatory pathway

Insulin also has several other functions. It stimulates lipogenesis and protein synthesis, as well as cell growth and differentiation [78]. Lipogenesis is a process of synthesising fatty acid from acetyl-CoA that eventually gets converted to triglycerides [79]. Insulin stimulates lipogenesis by activating two types of enzymes, PDH (pyruvate dehydrogenase) which allow the conversion of pyruvate to acetyl CoA and acteyl CoA carboxylase which convertsacetyl CoA to malonyl CoA. Malonyl CoA provides the 2-carbon building blocks that are used to synthesise the larger fatty acids in the cytoplasm [80]. The transportation of acetyl CoA from the mitochondria to the cytoplasm takes place using the enzyme tricarboxylate translocase, after its conversion to citrate by the reaction with oxaloacetate. Glucose plays an important role in the stimulation of the release of both insulin and citrate [81, 82].

Fatty acids, mainly polysaturated ones, play a role in the production of the cell membrane. The composition of the membrane of the cell affects the absorption of the enzymes responsible for the functioning of the cell's phosphatidylinositol 4-kinase (PI4K), a membrane-associated phosphatidylinositol kinase, which plays a central role in cell signalling [83-85]. The products of fat metabolism activate PI4K which regulates the functioning of Protein Kinase C (PKC) which controls the signalling of TNF-α the proinflammatory cytokines [86]. PKC induces inflammation by increasing the expression of p38MAPK and NFkβ. In the presence of PI4K, the activity of PKC inhibited ultimately reduces the release of proinflammatory cytokine TNF-α [87]. The free fatty acid components thus play the role in wound healing.

c) Insulin induces cell growth and differentiation by protein synthesis and inhibits proteolysis through FOXO inactivation to promote cell survival

The role of insulin in protein synthesis is not very clear. Insulin can stimulate protein synthesis in many types of cells and tissues in various animals including humans. In muscle tissue insulin induces changes in blood flow and induces increased delivery and uptake of amino acids by muscle tissues which help in muscle anabolism [88, 89]. Though, many times it has been found that patient with diabetes-I undergoing systemic insulin uptake loses

muscle volume which happens mainly as systemic insulin infusion results in a decrease in the concentration of free amino acids in the blood, which are essential for muscle anabolism [90]. This phenomenon can be overcome by applying exogenous amino acids systematically [91]. Insulin stimulates essential protein synthesis in tissues by increasing the RNA contents and translocation of mRNA mainly through the phosphoinositide-3-kinase (PI3K) pathway of the insulin signalling pathway [92]. In the PI3K pathway, Akt inhibits tuberous sclerosis protein ½ (TSC1/2) that acts as an inhibitor of mechanistic target of rapamycin (mTOR) which ultimately activates eukaryotic initiation factor (eIF4B) through the phosphorylation of 4E Binding protein (4EBP1). eIF4B binds with the eukaryotic secondary structured mRNA 5'end. During protein synthesis, eIF4B binds to eIF4G and eIF4A, which are further linked with the 40S ribosome and has RNA helicase activity respectively. If insulin is insufficient or in the case of diabetic condition, phosphorylation of 4EBP1 is limited or absent resulting in impaired protein synthesis. Also, the activation of mTOR inhibits proteolysis through MAPox activation [93-95]. Hyperinsulinemia in the muscle also inhibits degradation of protein which results in expansion of the muscle tissue [96, 97]. Insulin decreases the concentration of free amino acids in the blood due to inhibition of overall protein degradation in the body [98]. These roles of insulin in the regulation of amino acid metabolism clearly suggest that insulin can play a very important role in wound recovery in case of diabetic condition where patients are undergoing systemic insulin treatment.

d) Insulin behaves as an IGF growth factor and can activate the same signalling pathway to reduce inflammation

Insulin-like growth factors (IGFs) are proteins comprised of IGF ligand (IGF-I and IGF-II) that regulate growth and development during embryogenesis, differentiation in adult tissues and has an anti-inflammatory effect. Insulin shows an anti-inflammatory effect via stimulating the release of IL-4/13 and IL-10 (more significantly 100-150%) chemokines and decreasing the release of IFN-Ɣ proinflammatory cytokine [47, 99]. IGFs bind to the IGF-1 receptor, insulin receptor (IR), insulin related receptor, IGF-2 receptor and other receptors. Most functions of both IGF-I and IGF-II are mediated through IGF-Insulin receptor (IGF-IR) [100]. IGF-I is an important growth factor produced by fibroblast cells, keratinocytes, macrophages and platelets. It promotes the migration of endothelial cells into the wound. It also induces the proliferation or mitosis of fibroblast cells for the formation of extracellular matrix and angiogenesis by activating the protein kinase B signalling pathway. In addition, IGF also induces protein synthesis and blocks muscle atrophy in order to catalyze skeletal hypertrophy [101].

Upon receptor binding, IGF-I activates insulin receptor substrate 1 (IRS1) which phosphorylate protein kinase B (Akt) via phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K). Phosphorylated Akt then activates mTOR, PI3K related kinase which controls cellular proliferation [102]. Again IGF-I promotes cellular growth by activating extracellular signal–regulated kinase/mitogen-activated protein kinase / (ERK/MAPK pathway via phosphorylation of RAS/RAF kinase [103]. In addition, receptor binding of IGF-I leads to secretion of anti-inflammatory cytokine interleukin-10 (IL-10) which can again activates Akt through AMPK signalling [44]. Similarly, like IL-10, IL-4 also can bind to Akt and helps in the infiltration of M2 macrophages at wound site [45, 46].

e) Insulin modulates inflammation through reduction of proinflammatory cytokines and inducing anti-inflammatory cytokines

Decreased insulin action, either due to insulin resistance or insufficient release of insulin, leads to diabetes. Insulin decreases either due to loss of functions of β-cells, malfunctioning of insulin receptors or disease in the kidney [104]. Systemic insulin treatment is taken regularly by 6 million Americans to control hyperglycemic conditions. Hyperglycemia can lead to damage of tissue through oxidative stress by increasing the flux of glucose and other sugars through the polyol pathway, it increases the expression of advanced glycation end products and its activating ligand receptor, the over activation of hexosamine pathway and activation of protein kinase. These mechanisms mainly take place through mitochondrial ROS overproduction [105]. In the polyol pathway, more redox stress appears as NADPH consumption in glucose transport remains insufficient to form ROS scavengers i.e. reduced GSH. Formation of the precursors of advanced glycinated product modifies the plasma proteins that bind to the advanced glycination product receptors present on vascular endothelial cells, macrophages and smooth cells. This activates transcription factor NFkβ, which activates HIF-α to lead into the production of hypoxia stimulated chemokines through the production of ROS [106]. Hyperactivity of protein kinase, in the presence of high glucose, stimulates the eNOS expression in cells of smooth muscles and leads to tissue destruction. Increased ROS production shows the activation of a number of proinflammatory pathways and generates epigenetic changes that lead to persistent expression of proinflammatory genes during wounds. Excessive production of matrix metalloproteinase

(MMP-2, 4) impairs wound healing leading to breakdown of extracellular matrix proteins like fibronectin and vitronectin [105-107].

In a normal wound, the healing process relies on activation of a cascade of physiological events such as inflammation, proliferation, epithelisation, vascularisation, maturation and remodelling at the scar site [108-110]. Macrophages play an important role throughout the whole process. In the early wound healing phase, macrophages function through the release of cytokines and activating leucocytes in order to produce inflammatory response [111].

Macrophage infiltration takes place into the wound site due to chemotaxis induced by factors such as PDGF, LPS (Lipopolysaccharide), PAMP (Pathogen-associated molecular patterns), Toll-like receptor (TLR) ligand and IFN-gamma (IFN-Y) [112, 113]. M1 are responsible for the secretion of high levels of IFN-β/TNF-α and STAT1. Insulin via PI3/Akt pathway activates STAT3 which inhibits STAT1 synthesis and induces class switching of M1 to M2 macrophages repair macrophages that functions in the constructive process like in tissue repair and wound healing. M2 macrophages also produce polyamines and ornithine through the arginase pathway and anti-inflammatory IL-4, IL-10 and IL-13 cytokines [114, 115]. Insulin together with M2 macrophages induced anti-inflammatory activates IP3K/Akt pathway to induce protein and fatty acid biosynthesis, cell division, cell migration and angiogenesis to promote wound recovery [113, 48, 49, 114]. In diabetes with insulin resistance there are consistent elevated levels of TNFα and IL-6, the proinflammatory cytokines have been shown. In normal glycemic conditions, the adipocytes produce cytokines, like IL-13, that promotes the activation of alternative or M2 macrophages. M2 or alternatively activated macrophages are responsible for the secretion of anti-inflammatory cytokines like IL-10, and may secrete insulin-sensitizing factors, PPAR-Y (Peroxisome Proliferator Activated Receptor Gamma), which generates a vicious circle for insulin activity [110, 115, 116]. PPAR-Y can also activate the anti-inflammatory cytokine IL-10 [117].

In the diabetic condition, there is prolonged expression of the pro-inflammatory macrophage phenotype sustained by IL-1β and TNF-α and wound healing gets impaired. Over expression of IL-1β, TNFα, or IL-17 cytokines decreases the expression of inflammatory cytokines, upregulates wound healing related genes and accelerates healing of wounds [117-121]. Furthermore, in adipose tissue and the blood have elevated TNFα cytokines, and TNFα neutralization improves sensitivity of insulin in the animals. Diabetes induces changes in gene expression and metabolism in adipocytes and results in increased lipolysis and production of free fatty acids (FFAs) and pro-inflammatory factors that recruit and induce activation of macrophages, such as monocytes chemotactic protein-1 (MCP-1) and tumour necrosis factor α (TNFα). Activation of M1 macrophages produces a huge concentration of inflammatory cytokines, like IL-1β, resistin and TNFα that acts on adipocyte cells to make them insulin resistant. This signalling forms a kind of feedback loop that increases the inflammation and resistance to insulin [110, 122].

TNF-α, inflammatory cytokine, plays an important role in the normal healing process, but its activation for long period of time leads to an increase in protease activity. In non-healing wounds of humans, MMPs were detected at very high concentrations. In chronic or inflamed wounds, there is an imbalance in pro-inflammatory cytokines and its inhibitors, proteases and their anti-proteases expression [123-125].

The transition of macrophages gets delayed in the hyperglycemic condition due to oxidative stress of IL-6, IL-1β, MMPs and ROS etc cytokines (Figure 1). This delay in transition is responsible for prolonged inflammatory phase leading to delay in wound healing [126]. The role of insulin in transition from inflammatory to anti-inflammatory state is shown in Figure 2. M1 and M2 macrophages and their transition are depicted in Figure 3 [127, 128].

Similar role of C-peptide

C-peptide is a short 31 amino acid peptide, has glycine rich regions and acts as a linker between the A and B peptides of proinsulin [129]. C-peptide shows angiogenesis through the ERK1/2 and Akt phosphorylation pathway. This signalling pathway of angiogenesis is similar to the VEGF pathway and ultimately results in the production of NO through eNOS activation. It plays an important role in mitogenesis like insulin, through the same signalling pathway as insulin [130]. C-peptide can bind to the insulin receptor, resulting in the phosphorylation of the intracellular substrate in the Ras/MAPK and the PI3K/Akt signalling pathways leading to cell division or mitogenesis. In addition to these two functions, C-peptide also shows an anti-inflammatory effect. It shows this activity through the inhibition of the expression of IL-6, IL-8, MIP-1α and MIP-1β proinflammatory cytokines [131]. C-peptide, like insulin, also prevents complications related to diabetes, like neuropathy, nephropathy, and vascular inflammation, in case of diabetes especially type 1 diabetes mellitus [132].

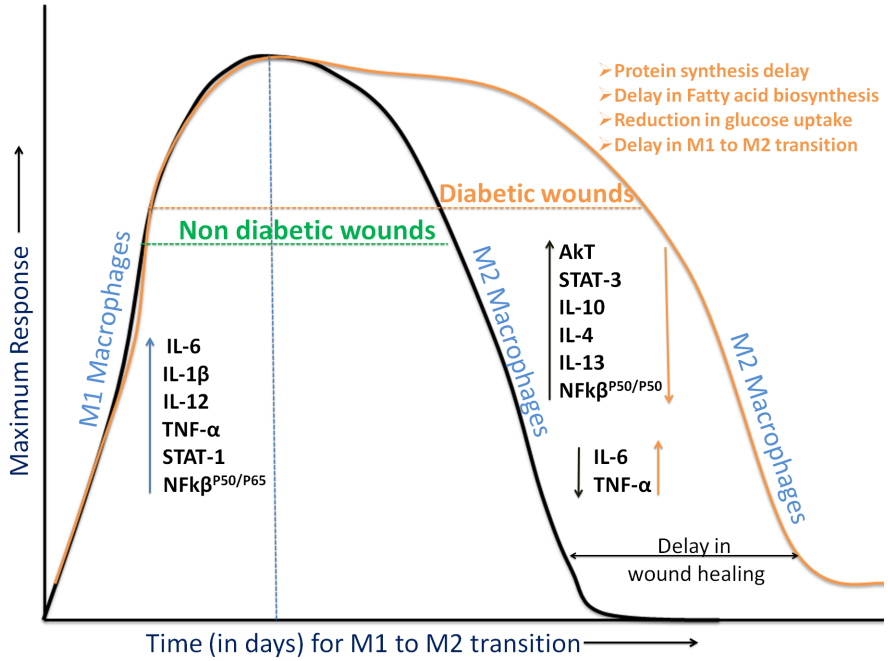

Figure 1: M1 (classically activated) macrophages, like IL-10, IL-1β, IL-12, TNF-α, STAT1 and NFkβ$^{P50/P65}$, are responsible for inflammation at the wound site. M2 macrophages, like PKC, HIF-α, STAT3, NFkβ$^{P50/P50}$ etc, help in wound recovery by reducing the inflammation. Patients having diabetic wounds show persistent expression of M1 macrophages as compared to normal wounds that result in a delay of transition of the M1 to M2 macrophages phenotype.

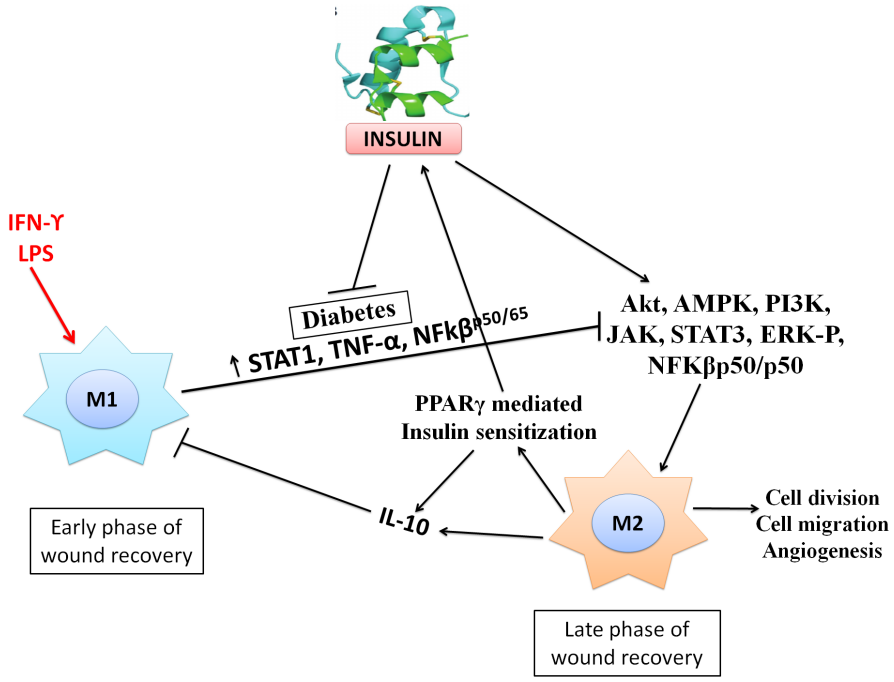

Figure 2: Effect of insulin in transition of M1 to M2 macrophages. In the presence of insulin the expression of M2 macrophages Akt, PI3K, AMPK, PKC, HIF-α, STAT3, NFkβ$^{P50/P50}$, and ERK increases leads to anti-inflammatory response and help in wound recovery.

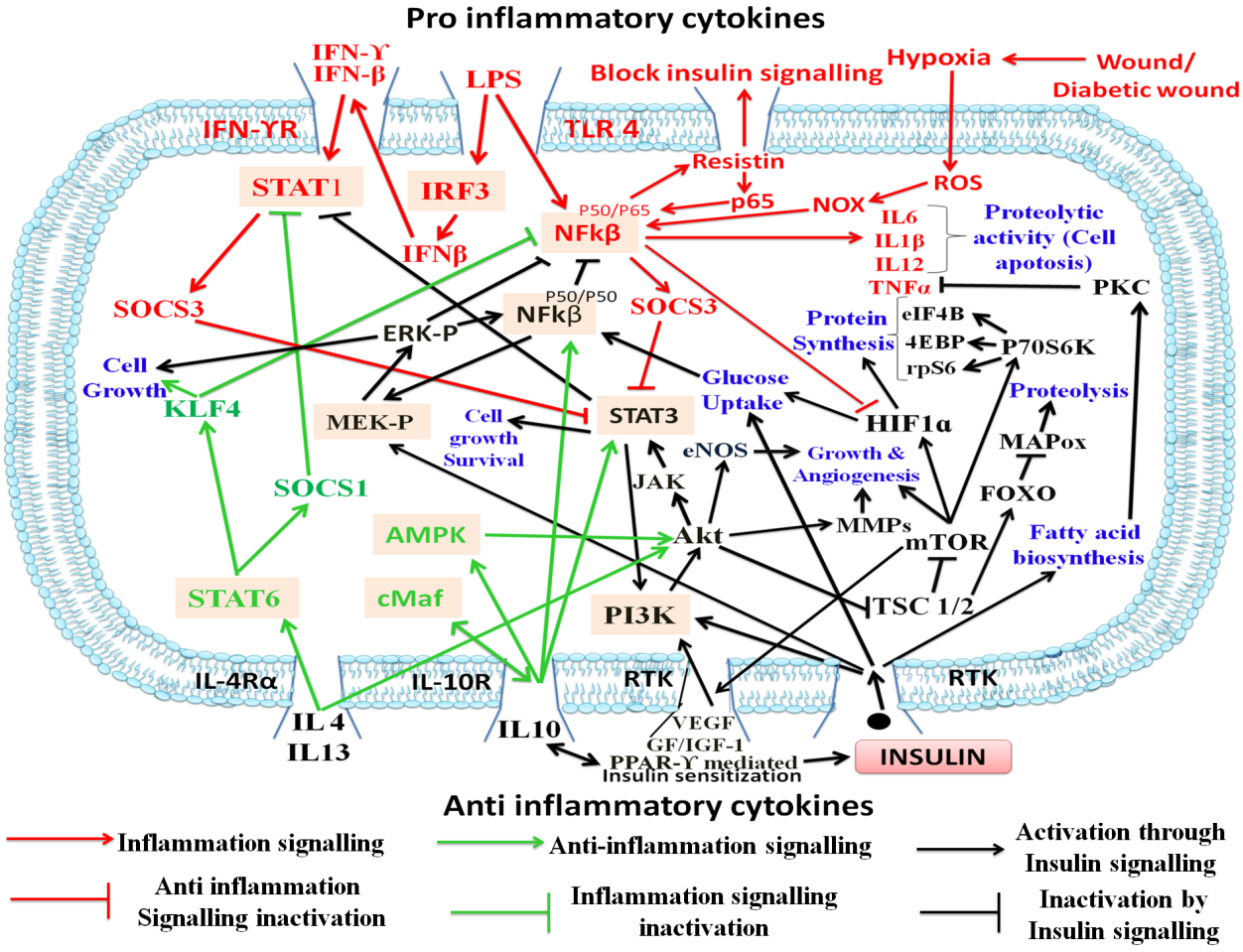

Figure 3: Molecular pathway for transition of macrophages i.e. from the M1 to M2 phase. IFN Y and TNF α generation in wounds activates NFk β, IRF-3, STAT1 which helps in secretion of IL-10, IL-1β, IL-12, TNF-α, STAT1 and NFkβ$^{P50/P65}$, responsible for inflammation. The transition of M1 to M2 macrophages is necessary for wound healing. IL-4, IL-13, IL-10, IGF, VEGF and insulin can activate PKC, HIF-α, STAT3, NFkβ$^{P50/P50}$ etc cytokines in order to produce an anti-inflammatory effect.

The blood level of C-peptide also increases during type 2 diabetes, which is due to insulin resistance [133]. During this, endothelial dysfunction gets initiated followed by deposition of C-peptide in the intima of the vessel wall. The deposition of C-peptide leads to increased inflammation in vessels of aortic arch and atherosclerotic lesions. C-peptide shows this inflammation effect due to its chemotactic behaviour towards inflammatory macrophages. Macrophages/T-lymphocytes/monocytes migrate through the vessel wall and then release proinflammatory cytokines, IL-6, TNF-α, MIF etc, chemokines and nitric oxide, and activate the intracellular signalling pathway [134].

Conclusions

Insulin is a peptide hormone which plays multiple functions in our body such as the control of inflammation, increase in cell differentiation, lipid and protein biosynthesis etc, in addition to controlling glucose levels in the blood through glucose metabolism. During glucose metabolism, IL-8 and NFkβP50/P50 get activated causing inactivation of the pro-inflammatory cytokines TNF-α, IFN- Y, IL-1β, IL-6 NFkβP50/P65, NOX, and resistin. Similarly in case of fat metabolism insulin inactivates proinflammatory cytokines by inactivating TNF-α mediated inflammatory pathway. Protein synthesis also gets induced by insulin through the PI3K, Akt pathway which helps in cell survival through the formation of 4EBPI, Ribosomal protein S6 (rpS6). This shows that along

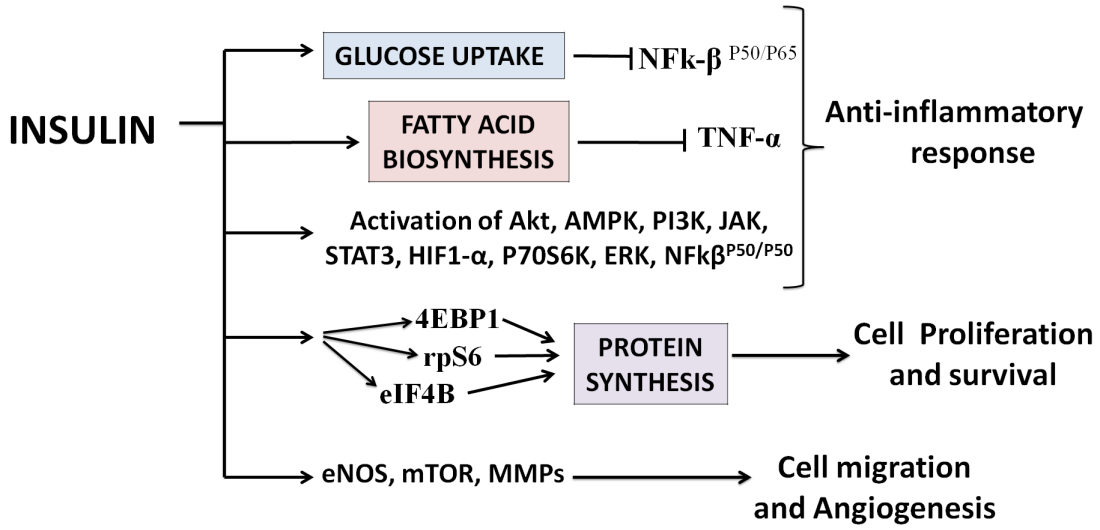

Figure 4: Insulin plays a role in anti-inflammation and cell survival through metabolic and synthesis pathways. Metabolism of glucose and fats leads to the activation of TNF-α and NFk-β respectively which inactivates the inflammatory signalling. With this signalling it helps in cell survival and protein synthesis. In addition insulin can activate the Akt pathway and to increase the expression of eNOS, MMPs, mTOR leading to angiogenesis in addition to the anti-inflammatory response. Insulin can also reduce the expression of NFkβ$^{P50/P65}$ through the MEK, ERK pathways such as the glucose uptake pathway.

with antiglycemic activity insulin also exhibits an anti-inflammatory action, although the mechanistic aspects of the anti-inflammatory role of insulin remain to be well understood and elucidated. Other than metabolism and biosynthesis pathways, as insulin has structural similarities with IGF-I, it can bind to the IGF receptor and can show anti-inflammatory activity through PI3K, Akt etc signalling pathways, which ultimately activates pro-inflammatory cytokines like STAT-3 which can activate Akt again and promote angiogenesis and growth by increasing the production of eNOS. Similarly, due to structural similarity insulin can bind the IGF receptors and activate the same pathway as GF/IGF-I, necessitating further studies on insulin, IGFs and their role in anti-inflammatory responses (Figure 4]. About 5 % of the world population is diabetic and are therefore at risk of slow recovery/non recoverable wound formation. Insulin can promote wound recovery by modulating inflammatory dynamics, therefore novel formulations based on insulin or insulin-like inflammatory modulators (such as IGF) would have a huge potential for the various types of clinical application including diabetic care and should be explored for beneficiary purposes.

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