Briefs on Insulin and Innate Immune Response

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Key Words

Insulin • Diabetes Mellitus • Alveolar macrophages • Innate immune system • Leukotrienes • Sepsis • Phagocytosis • Cytokines • NF- κ B • MAPK • Akt • PKC

Abstract

Insulin is a pivotal regulator of glucose metabolism and exerts an important anabolic function throughout the body. Insulin commands the glucose uptake by the cells and might control the processes in which there is need for energy such as mitogenesis and gene transcription. In certain conditions, diabetes mellitus for example, when insulin is diminished, the homeostasis of many tissues and organs are broken what can lead to a higher mortality due to an enhanced susceptibility to infections. This vulnerability to infections can partially be explained by a change in response to inflammation. In fact, diabetic animals and patients show a deficient inflammatory response. Many animal models have shown that neutrophils chemotaxis and recruitment are dampened and macrophages from diabetic patients have low phagocytic and microbicidal activities. In most cases, once insulin therapy is introduced, clinical symptoms and signs can be reverted. In addition, there are a

number of studies trying to demystify pathways under insulin command. Researchers are also trying to understand how insulin is able to keep inflammatory response under control, restores innate immune cells ability to fight against pathogens and harmlessly activates adaptive immunity response. This review provides an overview on how inflammatory response is driven in the absence of insulin in diabetes and discusses recent findings on the influence of insulin on innate immune response. At the end, some signaling pathways are also highlighted and important enzymes and proteins that control DNA transcription are presented.

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Diabetes and Inflammation

Diabetes mellitus (DM) is a complex syndrome characterized by the loss of the homeostasis of glucose metabolism. It is associated to high mortality and morbidity rates, partially attributed to an enhanced susceptibility to secondary infections, when compared to non-diabetic patients [1]. Gram-positive bacteria are responsible for

most cases of superficial infections that affect the extremities of the limbs. Severity of lesions can be attributed to a failure in the clearance of pathogens by what may lead to amputation of a foot or leg [2]. Diabetic women are more frequently affected by urinary infections. It has been shown that almost two thirds of the women with pyelonephritis are diabetic and had three times higher amounts of bacteriuria when compared to non-diabetic ones. Moreover, upper urinary tract infection, which can lead to sepsis and death, is also more common among diabetic patients [3]. Pneumonia caused by Gram-negative bacteria and *Mycobacterium tuberculosis* are more frequent in diabetic patients, despite higher mortality and morbidity rates are related to those caused by *Streptococcus pneumoniae* and influenza virus. In some other cases, DM is considered a predisposition factor for bacteremia [2-4].

Many type I diabetes animal models were launched and we would like to emphasize those that use diabetogenic drugs such as alloxan and streptozotocin. These drugs are cytotoxic and mediate enhanced production of reactive oxygen species (ROS) that accumulate in the pancreatic islets causing an irreversible lesion of the cells responsible for insulin synthesis, the β cells [5]. Alloxan is an unstable hydrophilic compound, with half-life of 90 seconds. This is also enough time to allow it to destroy the β cells when administered intravenously [6]. In higher doses, these drugs may have adverse effects: alloxan can cause necrosis of the renal tubular cells [5] and streptozotocin can be toxic to lymphoid cells of the spleen and thymus [7].

Anjos-Valota et al. [8] showed that rats with alloxan-induced DM present deficient cell adhesion and migration and lower expression of intercellular adhesion molecule (ICAM)-1 in the internal spermatic fascia after stimulation by tumor necrosis factor (TNF)- α and that these parameters are reverted by insulin treatment. In the same model of DM, it was found that intratracheal instillation of lipopolysaccharide (LPS) induces lower migration of neutrophils and lower levels of TNF- α , interleukin (IL)-1 β , IL-6 and IL-10 to the bronchoalveolar space when compared to non-diabetic rats. [9, 10].

Phagocytes from diabetic patients present low phagocytic and microbicidal activities, which can partially explain their higher susceptibility to infection [11]. Using the experimental model of alloxan-induced DM, it was shown that neutrophils from diabetic rats have a diminished capacity to phagocytose *Candida albicans* [11]. Phorbol myristate acetate (PMA)-induced phagocytosis of zymosan and production of hydrogen

peroxide by neutrophils and macrophages from diabetic rats are also lower when compared to cells from healthy rats [12]. The deficient function of phagocytes in diabetic patients is due to lower synthesis of pro-inflammatory mediators what contribute to their higher susceptibility to infection and worse prognosis [11, 12]. However, comprehensible mechanisms responsible for the deficient response of these cells are not entirely elucidated.

Other important acute inflammatory mediators, the leukotrienes (LTs), are also hindered in diabetes [13-15]. Many essential functions are attributed to LTs, which are synthesized at early stages of infection, being crucial stimulators of phagocytosis and inducers of microbicidal activity. LTs have been reported to increase resistance to *Leishmania amazonensis* [13], *Trypanosoma cruzi* [14] and HIV [15]. They are generated from the metabolism of the arachidonic acid (AA) which is abundant in different tissues such as liver, muscles and brain [16]. Free AA is oxygenated at its C-5 position by 5-lipoxygenase (LO) in concert with 5-LO-activating protein (FLAP) to generate the epoxide intermediate LTA₄, which can subsequently be hydrolysed to form LTB₄ or conjugated with reduced glutathione to form cysteinyl-LTs (cys-LTs), LTC₄, LTD₄, and LTE₄ [16]. LTs act on different types of G-protein-coupled receptors. LTB₄ can act on high (BLT1) or low affinity (BLT2) receptors and the cys-LTs on cys-LT1 or cys-LT2 receptors with distinct affinities [16, 17].

It has been demonstrated that LTs are capable of potentiate phagocytic and microbicidal activity of alveolar macrophages (AMs), vital line of defense in the lungs against infection [16]. Mancuso et al. [18] showed that the phagocytic capacity of AMs in rats is significantly increased by LTs production during phagocytosis of IgG-opsonised targets and that inhibition of LT synthesis reduces the phagocytic ability of these cells. LTs also increase phagocytosis and killing of IgG-opsonised *Klebsiella pneumoniae* [19].

Since LTs stimulate phagocytosis and killing activity of AMs, and insulin deficiency is associated with a reduction of phagocytic function, Ferracini et al. [20] investigated the role of LTs on phagocytosis using AMs from diabetic rats. AMs from diabetic and non-diabetic rats were compared for phagocytosis of IgG-opsonised targets and intracellular signaling elicited by Fc γ R engagement. The effects of LT synthesis inhibitors or receptor antagonists, and insulin addition to AMs *in vitro* were also investigated [20]. In this study it has been shown that the phagocytic capacity of AMs from diabetic rats is significantly impaired compared to AMs from non-dia-

betics. The inhibition of LT synthesis in AMs from non-diabetic rats decreases the phagocytic capacity of these cells, whereas the phagocytic capacity of AMs from diabetic rats is not affected by LT synthesis inhibition [20], suggesting that AMs from diabetic rats lack this LT-dependent potentiating effect, which is not related to a deficient production of LTs or to the capacity to respond to LTs. However, lung homogenates from diabetic rats have been shown to produce less cys-LTs when stimulated with calcium ionophore than lungs from non-diabetic rats [21]. AMs from diabetic rats produce similar amounts of LTB₄ and LTD₄ as AMs from non-diabetics upon the engagement of FcγR [20].

The interaction of an IgG-opsonised particle with FcγR that is linked to immunoreceptor tyrosine-based activation motif (ITAM) sequences activates a variety of signaling pathways [22] that promote phagocytosis, trigger microbicidal mechanisms [23] and induce the release of cytokines and lipid mediators, such as LTs [22, 23]. Campos et al. [24] showed that among the molecules activated in rat AMs during phagocytosis via FcγR, protein kinase B (Akt), which is a downstream target of phosphatidylinositol 3'-kinase (PI3K), protein kinase C (PKC) and mitogen-activated protein kinases (MAPK) are upregulated by LTs. However, in AMs from diabetic rats, upregulation of the FcγR signaling by LTs seems not to occur, because similar levels of LTs are produced by non-diabetic and diabetic AMs upon FcγR engagement [20]. In addition, LTs inhibition by zileuton does not affect the phosphorylation of signaling molecules, which strengthens the hypothesis that, in AMs from diabetic rats, the coupling of LTs to FcγR signaling does not occur [20]. At which level this coupling occurs is a matter of speculation, but Serezani et al. [25] showed that in AMs a proportion of FcγR localizes within lipid raft membrane microdomains and upon engagement of this receptor, there is a redistribution of these receptors and triggering of signaling cascade that culminates with PKC activation. It has been suggested that in AMs from diabetic rats the dynamics of cell membrane could be altered in such a way that these receptors redistribution might not occur [20, 25]. Moreover, the addition of insulin to AMs culture increases phagocytosis in both non-diabetic and diabetic cells without changes in the levels of LTs in cultures of AMs with IgG opsonised sheep red blood cells (IgG-SRBC) [20]. At the molecular level, insulin increases the phosphorylation of PKC-δ in both groups, which might explain the upregulation of phagocytosis caused by insulin addition [20]. In conclusion, these abnormalities in the course of the inflammatory response in DM might con-

tribute to increased susceptibility and severity of infections in the diabetic host. In agreement with these findings, critical evaluations of the topic show that infection can be more serious and possibly more difficult to eradicate in the diabetic host [26-28].

Another important advance in understanding the pathogenesis of phagocyte dysfunction and inflammatory disorders in diabetes is the observation that glucose or its analogues interacts with proteins or lipids. Since neutrophil function requires energy, metabolic changes (i.e., glycolytic and glutaminolytic pathways) may be involved in the reduction of neutrophil function observed in diabetic states [28]. Metabolic routes by which hyperglycemia is linked to neutrophil dysfunction include the advanced protein glycosylation reaction, the polyol pathway, oxygen free radical formation, the nitric oxide-cyclic guanosine-3'-5'-monophosphate pathway, and the glycolytic and glutaminolytic pathways [28]. Neutrophils from diabetic rats present impaired metabolism of glucose and glutamine. On the other hand, increased fatty acid oxidation may compensate for the reduction in glucose and glutamine utilization to maintain the ATP supply for these cells [28].

Insulin and the Inflammatory Response

Insulin through direct or indirect effects regulates leukocyte behavior in inflammation (Fig. 1) [28]. For example, intensive insulin therapy reduces morbidity and mortality among critically ill patients, even among those who are not diabetic [29]. The authors showed in a study conducted in an intensive care unit (ICU) that strict control of blood glucose levels with insulin reduces morbidity and mortality. Nevertheless, in contrast to the results of this study, others have established that the benefit of insulin therapy is inconsistent and may increase the risk of hypoglycemic episodes [30-31]. Thus the role of intensive insulin therapy in critically ill patients is uncertain as well as whether the beneficial effects are due to insulin per se or to tight glycemic control. This issue has been extensively discussed in the Editorial of The New England Journal of Medicine [32].

Insulin regulates many cellular processes, such as glucose transport [33], glycogen synthesis [34], mitogenesis [35], and gene transcription [36]. Throughout different signaling pathways insulin exerts a negative effect on the transcription of a subset of genes, and for other genes the effect is positive [36]. Downregulation of insulin-like growth factor binding protein (IGFBP)-1

Fig. 1. Insulin and Neutrophils - in normal individuals (controls), when a pathogen, represented by lipopolysaccharide (LPS), enters the tissue neutrophils, the first line of defense, through adhesion molecules expression, such as intercellular adhesion molecule (ICAM)-1, reach the inflammatory site. During inflammation, polymorphonuclear phagocyte pathogens, produce leucotrienes (LTs) and reactive oxygen species (ROS). Neutrophils also produce interleukin (IL)-8 and tumor necrosis factor (TNF)- α , which recruit other cells to the site of injury. Matrix metalloproteinases (MMPs) are also important mediators of inflammation produced by these cells from innate immunity. When an individual becomes diabetic, neutrophils fail to exert their function. Phagocytosis is dampened and immune cells produce lower amounts of ROS and LTs. All these factors added to lower production of cytokines and MMPs making the diabetic individual prone to opportunistic infections. When diabetic individuals are treated with insulin, all hindered functions are recovered.

gene [37], insulin receptor substrate (IRS)-2 gene [38] and LPS-induced signaling pathways in AMs [39, 40], and upregulation of insulin-like growth factor (IGF)-1 gene [41] and insulin receptor (IR) gene [42] are some examples of insulin action on gene transcription. In fact, Martins et al [10] demonstrated that alloxan-induced diabetic rats exhibit reduced IL-1 β and TNF- α mRNA levels in the lungs and mesenteric lymph nodes after instillation with LPS, when compared to non-diabetic rats. The expression of these cytokine genes is normalized after treatment of diabetic rats with insulin. So, the presence of IL-1 β and TNF- α in the BAL fluid is associated with increased expression of their transcripts in lung and mesenteric lymph nodes [9, 10]. These early-response cytokines amplify the inflammatory response by stimulating the release of chemoattractant factors by AMs and lung epithelial cells, and the expression of adhesion

molecules by leukocytes and the endothelium [43].

Survival depends on the ability of the host to respond appropriately to pathogenic challenges. A dysregulation of the mechanisms that trigger the innate immune response against bacterial pathogens contributes to the pathogenesis of bacterial sepsis [26]. It has long been recognized that certain infections occur almost exclusively in diabetic patients, and many of these patients have a worse prognosis once infection occurs [26-28]. Adequate concentrations of insulin are essential for normal function of endothelial cells and neutrophils during the course of the inflammatory process [8-10].

PKC plays a central role in signal transduction and participates in diverse biological and biochemical functions [44-46]. For example, PKC may participate in glycogen metabolism, release of neurotransmitters, and protein transactivation by phosphorylation [44-47]. Akt is

implicated on PI3K-mediated regulation of nuclear factor- κ B (NF- κ B) [48] an important transcription factor for pro-inflammatory mediators. Previous studies from our group have established a protocol for alloxan-induced diabetic rats treatment, in which the dose of insulin was chosen based on its ability to reverse inflammatory parameters that were reduced in diabetic rats [9, 10, 49-51]. Although this dose of insulin only partially reduces hyperglycemia, it maintains blood insulin levels elevated during the time of the experiment [9, 10, 49-51]. It is plausible that the observed effects would be primarily due to the increased levels of insulin rather than to the reduction of glycemia. Using this protocol, we showed that relative to LPS-treated control rats, LPS-treated diabetics exhibit reduced phosphorylation of the extracellular signal-regulated kinase (ERK), p38, Akt, PKC- α and PKC- δ [50] and that treatment of diabetic rats with insulin completely or partially restores all parameters [50]. In addition, insulin regulates MAPK, PI3K, PKC and NF- κ B pathways in the allergic lung inflammation in diabetic rats [51], which suggests that insulin is required for optimal transduction of the intracellular signals that follow inflammatory processes.

Protective Effect of Insulin

Hyperglycemia and insulin resistance are common in severe illness and are associated with adverse outcomes [52-54]. It has been discussed that in critically ill patients a single drug is unlikely to be of significant benefit. However, in some particular situations or, in some patients, insulin is beneficial. Das [55] suggested that a combination of naturally occurring endogenous anti-inflammatory molecules is one additional tool that can be used in the management of patients with sepsis, a condition that any adequate therapy is not available at present.

Another important event that triggers sepsis/septic shock is the nuclear translocation of NF- κ B and induction of NF- κ B-dependent effector genes [56]. Böhler et al. [57] observed that all patients with septic shock show increased NF- κ B binding activity in peripheral blood mononuclear cells (PBMC), but mortality is higher when the binding activity exceeds 200% of the initial values. They also showed that somatic gene transfer with an expression of plasmid coding for I- κ B α reduces LPS-mediated NF- κ B activation and increases mice survival after LPS administration [57]. This suggests that NF- κ B mediates mortality in animal models of sepsis. They also

reported that gene transfer with I- κ B α is not effective when given simultaneously with or after LPS, suggesting that gene transfer has to be done before the cells are stimulated to release crucial mediators for the pathophysiology of sepsis [57]. The central role of NF- κ B in mediating inflammatory processes is evident from both the importance of its target genes and from the phenotypes of mice lacking the NF- κ B p65 subunit [58, 59]. Therefore, compounds that inhibit NF- κ B are of great interest for the development of therapeutic agents for the treatment of acute and chronic inflammation purposes. In other study, LPS activation of AMs induces alterations in the activation of NF- κ B p65 subunit and I- κ B α phosphorylation. These alterations induced by LPS are reversed by insulin [40]. Indeed, insulin might have a potent acute anti-inflammatory effect including a reduction in the intranuclear NF- κ B and an increase in I- κ B in mononuclear cells from obese subjects [60].

It has been shown that insulin inhibits LPS-induced ERK 1/2, p38, Akt, PKC α and PKC δ phosphorylation and TNF release in AMs *in vitro* [39]. This inhibitory effect of insulin on signaling molecules corroborates previous findings *in vivo* showing a protective effect of insulin on systemic inflammation related to sepsis [61]. Taken together, these studies suggest that the protective effect of insulin in sepsis could be, at least partially, attributed to inhibition of signaling pathways and synthesis of mediators released by the lungs during this condition.

In addition, insulin exerts an anti-inflammatory effect at the cellular and molecular levels *in vitro* and *in vivo* [39, 40, 60-62]. A low dose infusion of insulin (2.5 IU/h) reduces ROS generation by PBMCs, suppresses NADPH oxidase expression and intranuclear NF- κ B binding, induces I- κ B expression and suppresses plasma ICAM-1 levels, and monocyte chemotactic protein (MCP)-1 concentrations [60, 63]. Finally, insulin suppresses the proinflammatory transcription factor early growth response gene (Egr)-1, plasminogen activator inhibitor (PAI)-1 and MCP-1 concentrations [60, 63].

Conclusion

Insulin has a pivotal importance on controlling the inflammatory response in diabetic patients [9, 10, 28, 49-51]. It is true that much effort is still necessary to determine the ideal doses and concentration to treat diabetic patients mainly when hyperglycemia is out of control due to infection or other adverse conditions such as stress and surgery. Being regulator of many cellular

processes and capable of activating gene transcription, it is crucial to demystify clear pathways that are able to keep inflammatory response under control, restore innate immune cells ability to fight against pathogens and harmlessly activate adaptive immunity response. Although insulin treatment was introduced almost 100 years ago, there is still a lot to improve and to find out. It has been shown that insulin can restore neutrophils migration in a LPS-induced inflammation environment [9, 10, 28]. However the shortcoming here is that most of the work was settled based on a single dose of insulin what differs from a type I diabetic patient, who needs insulin at least once a day. Interestingly, it seems that AMs do not show impaired phagocytic capacities when blockage of LTs was induced what could be an important pathway to target, once LTs seem to be an important acute inflammatory mediator [20].

AKT and PKC are crucial molecules on lymphocyte survival and proliferation cascades [47, 48, 64]. In the other words, would it be possible to extrapolate and use the anabolic effect of insulin to revert apoptotic processes inducing immune cells to last for longer periods and control this pathway in order to improve diabetic responses to inflammation? Another important point to check out is

the set of the genes that are up or down regulated by insulin. Would the upregulation of IR gene also occur in type II patients treated with insulin? Is insulin treatment for some periods beneficial and able to partially restore insulin resistance developed by these patients?

Far from being cleared, the mist that hovers over the topic insulin and inflammation is dense and sometimes impenetrable. Hopefully with new insights and better understanding of molecular pathways on how insulin exerts its function, new perspectives will be brought up in favor of many patients fencing complications and improving life expectancy.

Acknowledgements

We apologize to the many researchers whose work we have not been able to discuss in this limited review. The authors are supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Projeto Universal), and Pró-reitoria de Pesquisa da Universidade de São Paulo (Projeto I and Novos Docentes), Brazil.

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