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Toll-like receptor 4 (TLR4) as a possible pathological mechanism in hyperglycemia-associated testicular dysfunction

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A B S T R A C T

Hyperglycemia is a chief factor in diabetes, a complex disease associated with reproductive disorders, mainly testicular dysfunction, which contributes to male infertility. Leydig cells are the predominant cell population in the testis interstitium and, when stimulated, they are capable of initiating immune responses playing crucial roles in the mechanisms related to testis' homeostasis. These cells express TLR4, an innate immune receptor, which is known to be modulated by hyperglycemia in other cell populations and tissue types. Still, whether TLR4 contributes to hyperglycemia-associated testicular dysfunction remains elusive. Activation of TLR4 in response to high glucose levels involves redox imbalance and stimulation of transcriptional factors, especially nuclear factor (NF)-κB, which could be a potential pathological mechanism compromising the testis integrity. Additionally, emerging evidence shows crosstalk between TLR4 and Nrf2, an anti-inflammatory mediator that was previously shown to be reduced in diabetic testis. Therefore, we hypothesize that hyperglycemia-mediated TLR4 activation in testicular cells, especially Leydig cells might be a crucial event triggering oxidative stress and inflammation, which in turn, drives testicular dysfunction.

Introduction

Hyperglycemia is the main characteristic of diabetes mellitus, a multifactorial disease that has been on the rise in the past decades [1]. Currently, this disease affects more than 400 million people and is reported to be the 9th leading cause of death worldwide [1,2]. Diabetes is known to negatively impact female and male fertility [3] and has been shown to abnormally affect cells of reproductive organs [4,5], which might have severe implications on the patient's quality of life. Its association with reproductive problems in both genders has made this disease even more important to rectify [3].

One of the target reproductive male organs that are most susceptible to hyperglycemia-induced dysfunction is the testis [6,7]. Testicular dysfunction includes reduced sperm count and motility [8–10], damaged seminiferous tubules [7,10] and increased apoptosis of germ cells [11]. Additionally, it was previously demonstrated that diabetic rats have decreased testes' weight [7,12,13], abnormal histoarchitecture of seminiferous epithelium, vacuolization of Sertoli cells, and disruption of the blood-testis barrier [7,14], a physical structure important for maintaining the testicular immune privilege environment.

The mammalian testes belong to one of the few organs where systemic immunological responses are prominently reduced [15] and the effective protection of testicular tissues from infection relies on the local cell-initiated innate immune responses. The two main somatic cell populations in the testis are the Sertoli and Leydig cells. Both are equipped with the innate immune machinery contributing to testicular defense. The latter cell type (Leydig cells) is the first line of testicular resistance against invading microbial pathogens, as well as the major cell population in the testicular interstitium [16]. It has been demonstrated that Leydig cells have anti-viral capability and can directly regulate testicular macrophage numbers [17–19]. Following immune system activation in the testis, a massive number of macrophages are involved leading to the secretion of many pro-inflammatory mediators [20,21], which can also be produced by most testicular cells, including Leydig cells. It has been previously speculated that pattern recognition receptors (PRR) could be mediators of innate immune responses in testicular cells [16]. Among these receptors, TLR4 is the most investigated in the pathways associated with hyperglycemia, and several studies have identified its expression in the testis of different species such as rat [22], mice [23], and, even human [24]. Likewise, many different testicular cell types, inside and outside of the blood-testis barrier, such as dendritic cells, macrophages, Sertoli cells, and Leydig cells express this receptor. In mouse Leydig cells, TLR4 is shown at a relatively higher level compared with other TLR members, and its activation suppresses steroidogenesis [23], which further correlates with reduced testosterone levels and testicular dysfunction [7]. Notably, experimental and clinical research have identified diminished testosterone production in diabetic animals [7,12] as well as in diabetic men [25].

The mechanisms by which steroidogenesis is declined in diabetes is

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not very well understood, but it is known to involve hyperglycemia-induced metabolic damage of the hypothalamic-pituitary-gonadal axis and dysregulation of hormonal signaling [25]. Thus, studies described in the literature stated a decreased production of the luteinizing hormone (LH) [4,6] in the pituitary gland as well as reduced expression of gonadotropin [26] and androgen receptors in Leydig cells [6]. Oxidative stress is an essential component in the pathophysiology of diabetes and contributes to reduced steroidogenesis by inhibiting steroidogenic enzymes and cholesterol transport into the mitochondria [27]. Altogether, these factors justify decreased testosterone production and diminished steroidogenic capacity in Leydig cells of diabetic testis. Recently, it was also suggested that testosterone contributes to immune system regulation through a crosstalk with TLR4 [28]. However, it is still not clear how these conditions are linked to diabetes and, accordingly, this interesting topic is still being extensively investigated.

Hyperglycemia triggers diabetes-induced tissue damage in different organs. Additionally, this condition is the primary component in the generation of reactive oxygen species (ROS) through an array of mechanisms mainly including mitochondrial processes (oxidative phosphorylation and steroidogenesis) [27,29,30], elevated activity of MAP kinases [31], and stimulation of inflammatory factors *via* the nuclear factor (NF)-κB pathway [32]. This nuclear factor promotes cell proliferation and differentiation, potentially causing severe damage in tissues, including the testis [32]. Studies suggest that the inflammatory factors involved in the NF-κB pathway, play an inhibitory role in steroidogenesis [33]. Taken into account that TLR4 can initiate the testicular immune response in Leydig cells [23], and that activation of this receptor in response to hyperglycemia in other cells is associated with oxidative stress and inflammation *via* the NF-κB pathway, one could say that TLR4 stimulation in Leydig cells contributes to testicular dysfunction during diabetes.

Hypothesis

Immunoprotection is highly important in the testes and an intricate network of redox imbalance strictly drives testicular dysfunction, increases production of pro-inflammatory markers, and diminishes steroidogenic capacity. All these events can be affected by immune responses, such as that mediated by activation of TLR4. Taken into our consideration, in this work, we hypothesized that TLR4 contributes to hyperglycemia-associated testicular dysfunction by triggering ROS and inflammation (Fig. 1) and by impairing the anti-oxidant defense, potentially through Nrf2.

Evaluation of the hypothesis

The testis comprises two compartments: the seminiferous tubules and the interstitial space. Spermatogenesis occurs within the seminiferous tubules and steroidogenesis is achieved by Leydig cells, which are located outside of the blood-testis barrier. These two processes comprise the dual function of the testis. Moreover, this organ holds a unique immunological environment with an extraordinary immune privilege and effective local innate immunity. The mechanisms underlying testicular innate immunity are emerging based on the investigation of PRR-mediated innate immune responses in testicular cells, especially Sertoli and Leydig cells. TLR4 expression was identified in whole testes as well as in different testicular cell populations [22–24,34,35]. Activation of this receptor in different organs and cells during diabetes is associated with tissue damage by increasing ROS production and inflammation *via* the cytokine profile [36,37]. In mouse Leydig cells, TLR4 activation initiates testicular immune response [23], and in diabetic rats this cell primes testicular alterations [38].

Sustained hyperglycemia results in testicular dysfunction and reduced fertility [13,39]. During diabetes, oxidative stress impairs sperm nuclear DNA quality, the survival of germ cells, and spermatogenesis; all of which contribute to male infertility [40]. Furthermore, in diabetic

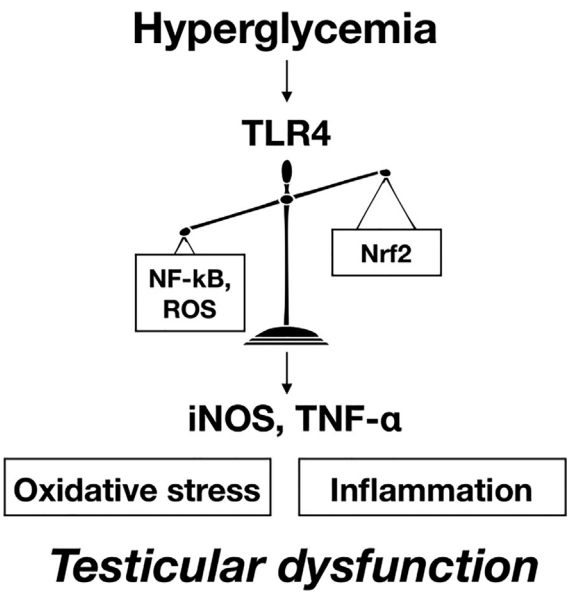

Fig. 1. The hypothetical view that TLR4 mediates hyperglycemia-associated testicular dysfunction. Hyperglycemia modulates TLR4 in Leydig cells triggering the production of ROS, the stimulation of NF-κB as well as down-regulation of Nrf2. This ultimately leads to the release of iNOS and TNF-α, two factors contributing to tissue damage in the testis through inflammation and oxidative stress.

conditions, hyperglycemia affects the microcirculation and hemodynamics of the testis resulting in oxidative stress [41]. It has also been suggested that interstitial edema with inflammatory infiltration and microvascular disorders are present in the testicular tissue of diabetic mice, and inflammatory factors have an inhibitory influence on testosterone-producing Leydig cells [42].

We have previously demonstrated the beneficial effects of the TLR4 blockade in a model of diabetes-associated erectile dysfunction (ED) [43] as well as in diabetic bladder dysfunction [44]. Additionally, hyperglycemia was shown to modulate this receptor in mesangial cells [45], endothelial cells [46], and in H9c2 cardiac cells [47], which suggests the versatile expression pattern of this receptor. Overall, TLR4 activation in response to hyperglycemia culminates in a significant increase in NF-κB activation along with enhanced secretion of pro-inflammatory cytokines such as TNF-α. This is notably so, as TNF-α is a known negative regulator of steroidogenesis [33] and was previously demonstrated to be upregulated in a model of diabetes [48]. Additionally, this receptor mediates ROS production as well as stimulation of the inducible nitric oxide synthase (iNOS). It is clear that the functionality of Leydig cells is affected by inflammation [33], but the pathways triggered by these cells during diabetes needs further investigation.

In pathological conditions, rat Leydig cells showed elevated ROS levels with higher phosphorylation rates of mitogen-activated protein kinases (MAPKs) cascades and overall diminished testosterone synthesis [27,31,49,50]. Activation of MAPK pathways with increased phosphorylation of JNK, p38, and ERK1/2 kinases in testicular tissues were identified in diabetic-induced rodent models [31,51]. Phosphorylation of these kinases is also observed in response to activation of TLR4, leading to the secretion of inflammatory molecules [52]. In Leydig cells, mitochondrial-derived ROS plays a critical role in phosphorylation of ERK1/2 [50]. ERK is an essential mediator of TLR4 cascades and it was recently suggested that fluctuations in TLR4/ERK cascades are associated with testosterone deficiency in specific immune cells [28]. Although this mechanism is not entirely clear, there is a link between testosterone levels and TLR4 signaling regulation, which may contribute to hyperglycemia-induced testicular dysfunction.

There is a growing body of evidence supporting the immunomodulation effects of testosterone. Lipopolysaccharide (LPS) is the most studied ligand for TLR4. LPS-mediated production of pro-inflammatory cytokines exhibits an inhibitory role in Leydig cell function through the generation of ROS, and consequently, disruption of mitochondrial membrane permeability [53,54]. Additionally, LPS-induced inflammation causes oxidative stress and apoptosis in Leydig cells, which may be a major influential factor involved in reduced steroidogenesis and testicular dysfunction under pathological conditions [55].

Another component contributing to Leydig cells dysfunction is nitric oxide (NO). In a LPS-treated inflammation model, Leydig cells are inhibited by NO leading to decreased testosterone production. Although an increase in NO is not the major component associated with dysfunctionality in Leydig cells, upregulation of iNOS may contribute to damage in the seminiferous epithelium [56]. Regarding male infertility, NOS is implicated in interaction with oxidative stress, which may be associated with overstimulation of NF-κB. Interestingly, this transcriptional factor is known to regulate the transcriptional pattern of iNOS and has the potential to influence the apoptotic status of testicular cell populations [57].

It is well accepted that disruption of redox homeostasis contributes to the development of many diseases. Thus, it is not surprising that the dysregulation of the antioxidant status plays a crucial role in testicular dysfunction. The transcriptional factor Nuclear Factor-Erythroid 2-Related Factor (Nrf2) is expressed in all tissues of the human body and is a significant regulator of the endogenous antioxidant system. This transcriptional factor provides cells the ability to adapt to oxidative stress by mediating the induction of the cytoprotective genes and maintaining cellular redox status, especially during diabetes [58,59]. Decreased Nrf2 was detected in diabetic humans and mice, contributing to increased oxidative stress, endothelial dysfunction, nephropathy, and cardiac insult [58,60]. Under high glucose conditions, cells from Nrf2 knockout mice showed higher levels of ROS compared to wild-type cells [61]. Furthermore, in cardiac cells, diabetic downregulation of Nrf2 activity contributes to increased levels of ROS *via* ERK pathway [60]. Lack of Nrf2 has been associated with male infertility by causing testicular and epididymal cell apoptosis accompanied by augmented ROS and spermatogenesis disruption [62]. Importantly, over time, increases in oxidative stress contribute to, or cause, reduction in testosterone production by aging Leydig cells and Nrf2 plays a critical role to delay the age-related oxidative damage in these cells [63].

Nrf2 regulates several anti-inflammatory cytokines by triggering its downstream targets genes [64]. Many Nrf2 activators have shown to preserve pancreatic β-cell functions against oxidative and inflammatory stress-induced apoptosis, and its potential to decrease pro-inflammatory cytokine toxicity during hyperglycemia [65], and attenuate testicular apoptosis in diabetes [66]. In cardiomyocytes, ERK-mediated suppression of Nrf2 activity leads to oxidative stress-induced insulin resistance [67]. An activator for Nrf2, Rosiglitazone, is suggested to regulate TLR4 activity and preserve Nrf2 expression in macrophages during inflammatory conditions [68]. Additionally, it was proposed that stimulation of Nrf2 decreases inflammation through activation of anti-oxidant substances, which associates with downregulation of TLR4 [69]. In a model of lung injury and inflammation, Nrf2 showed a protective effect on lung cells by modulating TLR4 and Akt signaling, which regulates cell survival and protects against oxidative stress, thus, suggesting a role for this axis (Nfr2/TLR4/Akt) in lung tissue damage [70].

All this information together, provide insights into the possible involvement of TLR4 in testicular dysfunction under hyperglycemic conditions. Although it is well accepted that oxidative stress and inflammation contribute to damage in many tissue types during diabetes, a precise mechanism leading to testicular dysfunction in this condition is not clarified yet. Over the last decade, TLR4 has been suggested to participate in the pathophysiological mechanisms of hyperglycemia, as its activation in persistent high glucose associates with increased MAPKs phosphorylation and ROS generation, which contributes to tissue injury. Therefore, it would be unsurprisingly that the release of pro-inflammatory cytokines such as TNF-α, upregulation of iNOS, and downregulation of the antioxidant capacity *via* Nrf2, would be triggered in the testis following stimulation of TLR4 by hyperglycemia.

Conflict of interest

None declared.

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