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16α-Bromoepiandrosterone as a new candidate for experimental diabetes-tuberculosis comorbidity treatment

Short title: Glucocorticoid govern T2D/TB immunopathology

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Abbreviations:

Tuberculosis (TB), Type 2 diabetes mellitus (T2D), Glucocorticoids (GCs), 11-β-hydroxysteroid dehydrogenase type 1 (11-βHSD1), 11-β-hydroxysteroid dehydrogenase type 2 (11-βHSD2), Dehydroepiandrosterone (DHEA), 16α-bromoepiandrosterone (BEA), Diabetic mice with Tuberculosis (T2D/TB), Mycobacterium tuberculosis (Mtb), Glucocorticoid receptors (GR), Type 1 T helper (Th1), Type 2 T helper (Th2), Hypothalamus-Pituitary-Adrenal (HPA), High-fat diet (HFD), Streptozotocin (STZ), Colony forming units (CFU), Central nervous system (CNS).

Abstract

Tuberculosis (TB) is the leading cause of death from a single bacterial infectious agent and is one of the most relevant issues of public health. Another pandemic disease is type 2 diabetes mellitus (T2D) that is estimated to affect half-billion people in the world. T2D is directly associated with obesity and a sedentary lifestyle and frequently associated with immunosuppression. Immune dysfunction induced by hyperglycemia increases infection frequency and severity. Thus, in developing countries the T2D/TB comorbidity is frequent and represents one of the most significant challenges for the healthcare systems.

During the chronic phase of both diseases, several immunoendocrine abnormalities are occurring, such as high extra-adrenal production of active glucocorticoids (GCs) by the activity of 11-β-hydroxysteroid dehydrogenase type 1 (11-βHSD1). 11-βHSD1 catalyzes the conversion of inactive cortisone to active cortisol or corticosterone in lungs and liver, while 11-β-hydroxysteroid dehydrogenase type 2 (11-βHSD2) has the opposite effect. Active GCs have been related to insulin resistance and suppression of Th1 responses, which are deleterious factors in both T2D and TB. The anabolic adrenal hormone dehydroepiandrosterone (DHEA) exert antagonistic effects on GCs signaling in immune cells and metabolic tissues, however its anabolic effects prohibit its use to treat immunoendocrine diseases. 16α-bromoepiandrosterone (BEA) is a water miscible synthetic sterol related to DHEA that lacks an anabolic effect while amplifying the immune and metabolic properties with important potential therapeutic uses.

In this work, we compared the expression of 11-βHSD1 and the therapeutic efficacy of BEA in diabetic mice infected with TB (T2D/TB) with respect to non-diabetic TB-infected mice (TB). T2D was induced by feeding mice with a high-fat diet and administering a single low-dose of streptozotocin. After four weeks of T2D establishment, mice were infected intra-tracheally with a high-dose of Mycobacterium tuberculosis strain H37Rv. Then, mice were treated with BEA three times a week by subcutaneous and intra-tracheal routes.

Infection with TB increased the expression of 11-βHSD1 and corticosterone in the lungs and liver of both T2D/TB and TB mice, however T2D/TB mice developed a more severe lung disease than TB mice. In comparison with untreated animals, BEA decreased GC and 11-βHSD1 expression while increasing 11-βHSD2 expression. These molecular effects of BEA were associated with a reduction in hyperglycemia and liver steatosis, lower lung bacillary loads and pneumonia. These results uphold BEA as a promising effective therapy for the T2D/TB comorbidity.

Keywords: Diabetes-tuberculosis comorbidity, active glucocorticoids, 11-βHSD1, BEA, immunotherapy.

Introduction

Type 2 diabetes (T2D) is a metabolic disease with high incidence and prevalence in low- and middle-income countries and tightly associated with immune dysfunction. Hyperglycemia impairs overall immunity, increasing infection frequency and severity. Therefore, T2D is a critical susceptibility factor for tuberculosis (TB) (1). In immunocompetent individuals, Mycobacterium tuberculosis (Mtb) infection is controlled by immune mechanisms that contribute to granuloma development and control of bacterial growth (2). However, in subjects with T2D/TB comorbidity the innate and adaptive immunity are impaired and is associated with poor prognosis (3).

A type 1 T helper (Th1) cell response mediates the protective immune response to TB through macrophages activation by IFN-γ and TNFα (4), while type 2 T helper (Th2) immune response has been shown that is a susceptibility factor, due that the Th2 cytokines IL-4 and IL-13 favor microbial growth by reducing TNF-α and iNOS (5). T2D patients show an impaired response to Mtb infection (6), consisting in alterations in bacterial recognition, phagocytosis, and a deficient immune cellular response with low cytokine and chemokine production (7). These immune response dysfunctions in T2D individuals results in severe TB manifestations (8). The advance in the understanding of the metabolic factors and neuro-immune-endocrine regulation in T2D has been an important aspect for clarifying the higher susceptibility of T2D to develop TB.

Glucocorticoids (GCs) are steroid hormones with critical homeostatic functions. GCs exert their effects by binding to glucocorticoid receptors (GR), endocrine nuclear receptors present in all cell types. GR signaling plays an essential role in the modulation of many biological functions in metabolic organs including liver, lung, adipose tissue and muscle, and in immune tissues and cells such as thymus, macrophages and lymphocytes (9). Cortisol suppresses the expression of pro-inflammatory and adhesion molecules, thus preventing the extravasation of neutrophils to the site of inflammation (9). Chronic exposure induces an anti-inflammatory gene expression profile of resident macrophages and decreases their phagocytic activity. Furthermore, active GCs suppress Th1 cytokine production and induce cell death (10), promoting a Th2 environment that impairs

granuloma formation (2). GCs inhibit the proliferation of effector T cells and induce apoptosis of neutrophils, basophils, and eosinophils resulting in reduced inflammation (11). In homeostatic conditions and under acute stress, the principal source of GCs are the adrenal glands, which are regulated by the negative feedback loop of the Hypothalamus-Pituitary-Adrenal (HPA) axis(12). However, diverse environmental insults and metabolic or endocrine alterations prompt GCs overproduction leading to negative effects on metabolism and the immune response (13).

GCs have an important participation in the physiopathology of TB. It has been reported in an experimental model of progressive TB that at the time of maximal protective activity mediated by IFN-γ, TNF-α and NO production, the HPA axis is activated, thereby stimulating the adrenal glands to secrete GCs (corticosterone) with the apparent aim of avoiding tissue damage produced by excessive lung inflammation. However, the excess of corticosterone also inhibits the activity of Th1 lymphocytes and induces differentiation of Th-2 lymphocytes, favoring bacterial survival and proliferation, ultimately causing death (10).

GCs also play a pivotal role in metabolic diseases and is determinant in the progression of obesity and metabolic syndrome to T2D. Although circulating GCs in T2D individuals are not different than in healthy subjects (14), there is a local conversion of inactive to active GCs in metabolic organs such as the liver and adipose tissue (15). Non-adrenal glucocorticoids synthesis is mediated by 11-βHSD1, an oxo-reductase enzyme that uses NADP(H) as a cofactor. This enzyme converts inactive cortisone to active cortisol in humans or corticosterone in rodents (16). Interestingly, the isozyme 11-βHSD2 exert the opposite effect. This NAD-dependent dehydrogenase converts active cortisol or active corticosterone to inactive cortisone (17). The higher activity of 11-βHSD1 in organs such as lung, liver and adipose tissues in T2D results in increased local GC production with deleterious consequences. The increased conversion of active glucocorticoids in visceral adipose tissue increases lipolysis and circulating free fatty acid content (18). Experimental studies with transgenic mice overexpressing 11-βHSD1 selectively in adipose tissue demonstrated that an increase in local levels of corticosterone induces visceral obesity, insulin resistance, diabetes and hyperlipidemia (19).

Moreover, upregulation of 11-βHSD1 expression in liver of T2D patients increases local synthesis of GCs leading to augmented glycogenolysis. Hepatic GCs also stimulates glucose output by activating phosphoenolpyruvate carboxykinase (PEPCK) gene expression (20), resulting in unrestrained glucose release. Sustained hyperglycemia induces glucotoxic damage and immune dysfunction, deteriorating the condition of diabetic patients (21). Since the increased activity of 11-βHSD1 in liver and adipose tissues is implicated in the development of T2D (22) is plausible that the same mechanism occurs in the lung during TB. However, as far as we know, there are no published studies about the pulmonary production of active GCs mediated by 11-βHSD1 in TB. Accordingly, novel therapeutic approaches for T2D or TB should be aimed to prevent abnormal GCs signaling and 11-βHSD1 expression in non-adrenal organs.

DHEA is an anabolic hormone of the adrenal cortex involved in the conversion of sexual steroids. This hormone displays antagonistic activity to cortisol, counteracting the deleterious effect of cortisol on immune response (23) and glucose metabolism. The cortisol-DHEA ratio is modified in T2D/TB comorbidity and represents a deleterious factor for both diseases (24). However, the anabolic effects of DHEA prevents its pharmacologic use in the treatment of these diseases. 16α-bromoepiandrosterone (BEA) is a synthetic analog of DHEA that modulates immune and metabolic responses [25, 26]. Compared to DHEA, BEA does not display anabolic activities, which makes it a feasible candidate drug for T2D and TB.

Here, we studied the expression of 11-βHSD1 and 11-βHS2 in the lung and liver, as well as the concentrations of GCs in a murine model of T2D/TB comorbidity and evaluate the therapeutic effect of BEA administration during experimental late TB and T2D/TB.

Materials and Methods

Ethics Statements

All the animal work was performed according to the guidelines of the Mexican law NOM 061-Z00-1999 and approved by the Internal Committee for the Care and Use of Laboratory Animals (CICUAL) of the National Institute of Medical Sciences and Nutrition in México.

Experimental Model of Type 2 Diabetes and Progressive Pulmonary TB in BALB/c Mice

To induce T2D, we used a non-genetic mouse model of diet-induced insulin resistance and pancreatic beta cell insufficiency as previously reported [27, 28]. Briefly, male BALB/c mice three weeks old were fed with a high-fat diet (HFD) containing 45% of calories from fat during the whole course of the experiment. Control animals were fed with rodent chow. After 5 weeks, animals received a single low dose (100 mg/kg) of streptozotocin (STZ) (ChemCruz, Santa Cruz Biotechnology, Dallas, TX) by intraperitoneal injection (i.p). This low-dose of STZ protocol does not cause diabetes in chow-fed mice (27). We assessed the combined effect of HFD and STZ in fasting blood glucose levels before and one month after STZ administration with glucose test strips (Accu-chek active, Roche diabetes care Mannheim, Germany) in blood obtained from the tail vein. Serum cholesterol and triglycerides concentration were measured in blood obtained from the tail vein with test strips (Accutrend plus system, Roche) one month after STZ administration.

After one month of STZ administration, animals were treated with either metformin (250 mg/kg) or glibenclamide (15 mg/kg) intragastrically and fasting glucose was determined 8 and 24 hours after administration. Animals confirmed with T2D, were infected by the intra-tracheal route with a high dose of the Mtb reference strain H37Rv (ATCC No. 25618), in order to induce progressive pulmonary TB. Briefly, Mtb strain H37Rv was grown in Middlebrook 7H9 broth (Difco, Detroit, MI, USA) supplemented with 0.2% glycerol, 10% OADC enrichment and 0.02% Tween-80 and maintained at 37◦C in agitation. Mid log-phase cultures were used for infection. Mycobacteria were counted and stored at −80◦C until use. Bacterial aliquots were thawed and pulse-sonicated to remove clumps. For the infection protocol, groups of T2D and control animals were anesthetized in a gas chamber using Sevoflurane and infected intra-tracheally with 2.5 Å~ 10^5 live bacilli using a cannula in a biosafety level III cabinet and maintained in vertical position until spontaneous recovery. Mice were

maintained in groups of five throughout the study in cages fitted with micro-isolators connected to negative pressure in an animal biosafety level III facility.

In order to compare the evolution of TB in T2D and control mice, groups of 5 mice were euthanized by exsanguination under pentobarbital anesthesia after 14, 21, 28 and 120 days after infection. Left lungs were immediately excised, cleared from hilar lymph nodes and thymic tissues, frozen by immersion in liquid nitrogen and kept to −80∘C for bacillary loads determination by colony forming unit's quantification and TNFα expression determined by RT-PCR, as described below. Right lungs were intra-tracheally perfused with absolute ethanol and after 24hr the tissue was paraffin embedded for histology and automated morphometry studies. Ten animals per group were left untouched and their survival was recorded to construct survival curves.

Expression and cellular source of 11-6HSD1, 11-6HSD2 and glucocorticoids in the lungs and liver during late active pulmonary TB

Groups of five mice with T2D, TB and T2D/TB were euthanized by exsanguination under pentobarbital anesthesia after 60 and 120 days after infection, in two independent experiments. In this model, the late active disease is well established by this time, as characterized by extensive pneumonia, high bacillary loads and low expression of IFNγ and TNFα. The lungs were processed as described above. Left lungs were used for total RNA isolation and mRNA determination of 11-βHSD1 and 11-βHSD2 by RT-PCR, while right lungs were used for histology and immunohistochemistry studies, including the determination of the cellular source of corticosterone and cortisone and their concentrations by digital pathology. Tissue fragments from the liver were processed in the same way.

BEA treatment in infected animals with and without T2D

Two months after infection, groups of mice with TB or T2D/TB were administered each other day with 0.02 mg/kg BEA either by intra-tracheal route to evaluate its direct effect on the infected lung or by subcutaneous route to determine the effect of BEA in the liver. After 30 and 60 days of

treatment, groups of six animals were euthanized by exsanguination under anesthesia with 210 mg/kg of i.p pentobarbital, inside in a cabinet of biosecurity level III. Three left lungs per time were perfused with absolute ethanol, fixed, and prepared for histopathological studies as mentioned above. Five right lungs were frozen for bacilli load quantification and three lungs were used to determine mRNA abundance by RT-PCR. Two independent experiments were performed. Animals were monitored daily and humanely euthanized if exhibited respiratory insufficiency or substantial weight loss.

Determination of colony-forming units in lung

Left lungs and spleen from four mice at each time-point, in two different experiments, were used for bacillary load determination by colony forming units (CFU) quantification. Lungs were homogenized in 1mL of 0.05% Tween-80 in a sample homogenizer (FastPrep-24, MP Biomedicals, Santa Ana, California, USA). Four consecutive logarithmic dilutions were made from this homogenate. Ten microliters of each dilution were plated in duplicate on Bacto Middlebrook 7H10 agar (Difco) enriched with OADC. Plates were incubated at 37ºC and 5% CO_2 during 21 days for CFU quantification.

Preparation of lung tissue for histological and immunohistochemistry analysis

Right lungs of mice were perfused with absolute ethanol by endotracheal route and fixed for 24 h and then embedded in paraffin blocks. Sections of 4µm width were mounted on glass slides, deparaffinized and stained with hematoxylin and eosin. For quantification and morphometric analysis for determining the surface area affected by pneumonia, three different mouse lungs per time point in two independent experiments were evaluated.

For immunohistochemistry, lung tissue was sectioned and mounted on charged glass slides and then deparaffinized. Slides were first blocked for unspecific peroxidase activity with 3% methanol peroxide for 1 h. For tissue 11-βHSD1 and 11-βHSD2 abundance detection, rabbit anti-mouse monoclonal antibodies (Biorbyt, Cambridge, UK) were used at a concentration of 1:250, incubated

overnight in agitation, followed by incubation with secondary anti-rabbit IgG labeled with peroxidase. For cortisone and corticosterone immunostaining, rabbit antimouse monoclonal antibodies (LsBio, Seattle, Washington, USA) were used at a final concentration of 1:250. In both cases, bound antibodies were detected with diaminobenzidine and counterstained with hematoxylin.

Immunostained sections were analyzed by digital pathology. Briefly, stained sections were digitalized at 200 x magnification using an Aperio ScanScope CS (Aperio, Sausalito, CA, USA), with a spatial resolution of 0.45 μm/pixels. The images were analyzed using ImageScope software (Aperio). All the lung tissue sections were circumscribed and sent for automated image analysis using the Spectrum Software V11.1.2.752 (Aperio). For immunostaining GCs intensity quantification, an algorithm was developed to determine the total lung expression of cortisone and corticosterone in the whole tissue section (29,30),(31).

Gene expression determination of 11-6HSD1, 11-6HSD2 and cytokines in lung and liver by RT-PCR

The right lungs of three mice per time point were rapidly excised, collected in 1.5-ml cryotubes, immediately frozen in liquid nitrogen and maintained at −80∘C until processing. For homogenization, lungs were slowly defrosted and homogenized in the FastPrepR-24 (MP Biomedicals) in the presence of zirconium flint beads. Total RNA extraction was performed with the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Total RNA was quantified by spectrophotometry (A260/280) and 100 ng of RNA from each lung was used for the production of complementary DNA (cDNA) by retro-transcription using a Omniscript kit (Qiagen), following the manufacturer's instructions. An endpoint PCR was then run to amplify the constitutive housekeeping gene RPLP0 (Ribosomal protein large P0, Gen ID:11837, GenBank, NCBI) and its integrity was analyzed by running at 2% agarose gel stained with SYBR green.

 The cDNA obtained from each sample was analyzed by real-time PCR (qPCR) using the Real-Time PCR system 7500 (Applied Biosystems, Foster City, CA, USA) and the Quantitech SYBR Green

Mastermix kit (Qiagen) with specific primers designed with the first BLAST (ncbi.nlm.nih.gov) for 11-βHSD1, 11-βHSD2, IFN-γ, TNF-α. Cycling conditions used were as follows: initial denaturation at 95∘C for 15min, followed by 40 cycles at 95∘C for 20 s, 60∘C for 20 s, and 72∘C for 34 s. Relative 11-βHSD1, 11-βHSD2 mRNA abundance was calculated by the $2-(\triangle\triangle Ct)$ method (32). For absolute quantification, of IFN-γ, TNF-α and 11-βHSD1, 11-βHSD2 mRNA in liver, the number of copies of each target gene was normalized to 1 million amplicons of the housekeeping gene RPLP0 mRNA, including the standard curves and a negative control.

Statistical Analysis

All the statistical analyses were performed using GraphPad Prism Software (version 6.0, La Jolla, USA). The data was analyzed using paired t-test, one- and two-tailed ANOVA with Bonferroni correction for multiple comparisons. *P*-values <0.05 were considered significant.

Results

Biochemical characterization of the T2D mice

To compare the progression and severity of TB in mice with T2D with respect to non-diabetic mice, we induced diet-induced insulin resistance and pancreatic beta cell insufficiency in mice by feeding them for 5 weeks with a high-fat diet containing 45% of calories from fat. Afterwards, animals received a single low dose (100 mg/kg) of STZ via i.p (Fig. 1A). Mice fed with the high-fat diet showed significant weight gain with respect to control mice fed rodent chow (Fig. 1B). Two months after SZT administration, T2D mice showed significant higher serum glucose, triglycerides, cholesterol and insulin concentration (Fig. 1C-F), confirming that these mice developed the metabolic abnormalities of T2D. Moreover, treatment of these mice with the hypoglycemic drugs metformin or glibenclamide reduced blood glucose to control levels (Fig. 1G), indicating that this model of diabetes is responsive to pharmacological antihyperglycemic treatments. In accordance with the biochemical

characterization, the histological analysis of the liver showed extensive lipid cytoplasmic vacuolization of hepatocytes (steatosis) in T2D mice (Fig. 1H).

Comparative evolution of pulmonary disease in TB and T2D/TB mice

In comparison with chow-fed mice, T2D mice showed significant higher pulmonary bacilli burdens after 28 or 120 days after infection with Mtb (Fig. 2A). T2D/TB mice also showed a decreased survival rate and lower expression of TNFα in lungs at 120 days after infection than TB mice (Fig. 2B, C). Thus, T2D/TB mice developed a more severe disease than TB mice.

Comparative expression of GCs and GCs converting enzymes in the late phase of TB & T2D/TB lungs

To determine the role of locally synthesized GCs in the lung in the progression of lung damage, we evaluated corticosterone (active GC) and cortisone (inactive GC) content in the lungs of non-infected controls and T2D mice, as well as TB and T2D/TB mice at day 120 of infection by immunohistochemistry. As show in Fig 3A, the lungs of control non-infected mice showed scarce bronchial epithelial cells with corticosterone positive immunostaining, while numerous bronchial cells are strongly positive in T2D mice. In the TB and T2D/TB groups the bronchial epithelium was negative for corticosterone. However, the pneumonic areas showed intense corticosterone immunostaining in some lymphocytes and numerous macrophages, being many of them vacuolated or foamy macrophages. Gene expression of the GC converting enzyme 11-βHSD1 was significantly higher in T2D mice in comparison with the control non-infected group (Fig. 3B). TB and T2D/TB groups also presented a significant increase in 11-βHSD1 mRNA content with respect to the control group. Immunostaining revealed that in T2D mice, the 11-βHSD1 was limited to bronchial epithelial cells, whereas in TB and T2D/TB this enzyme displayed strong immunostaining in foamy vacuolated macrophages of the pneumonic areas.

Immunohistochemical detection of the inactive GC cortisone revealed positive immunostaining in alveolar epithelium of control mice, while bronchial cells are negative in all the conditions. TB mice showed strong immunostaining in alveolar epithelium and in some inflammatory cells, while T2D and T2D/TB were negative to this hormone (Fig. 3C). The immunohistochemical results are well correlated with the 11-βHSD2 mRNA abundance, which showed significant higher expression in non-infected lungs, while it was lower in all other groups, where the T2D/TB group showed the lowest content (Fig. 3D). These results revealed that at a late stage of the disease there is a high expression of 11-βHSD1 and corticosterone production in the inflammatory cells of pulmonary TB, which is even higher in mice with T2D/TB. Conversely, in TB and T2D/TB mice the 11-βHSD2 that converts active cortisol or active corticosterone to inactive cortisone is repressed, increasing TB severity.

Effect of BEA in lung and spleen bacillary burdens, pulmonary cytokine expression and tissue damage in TB and T2D/TB mice

To evaluate the therapeutic effect of BEA on Mtb-infected mice, TB and T2D/TB mice were administered with BEA or vehicle to evaluate bacillary loads (CFUs) in the lung and spleen, extent of tissue damage (percentage of lung surface affected by pneumonia), and gene expression of the protective cytokine's TNF-α and IFN-γ by RT-PCR. In comparison with mice receiving vehicle, both TB and T2D/TB groups showed significant reduction of pulmonary bacillary burdens after 30 or 60 days of treatment with BEA (Fig. 4A). BEA administration reduced the percentage of lung affected by pneumonia in the T2D/TB mice at 60 days (Fig. 3B). The administration of BEA increased IFNγ and TNFα mRNA content in both groups after 30 days of treatment, and IFNγ in TB group after 60 days of treatment (Fig. 4C); as well as reduction of pneumonia. Interestingly, 60 days of BEA treatment induced significant reduction of CFUs, and weight increase in spleen of the T2D/TB group (Fig. 4E, F). These results indicates that BEA administration to T2D/TB mice allows spleen hyperplasia leading to a better control of bacillary dissemination in this experimental group.

Effect of BEA on the expression of 11-βHSD1 and 11-βHSD2, corticosterone and cortisone in the lungs and liver.

In the lung, treatment of TB and T2D/TB groups with BEA reduced the expression of 11-βHSD1 after 30 or 60 days of treatment when compared with the control group (Fig. 5A). This effect was even more evident in the liver of T2D/TB group, which also showed complete remission of steatosis and hyperglycemia (Fig. 6).

The effects of BEA on 11-βHSD1 gene expression in the infected lung were in concordance with the corticosterone immunostaining, and its quantitative evaluation by digital pathology (Fig. 5A-C). BEA significantly decreased corticosterone content and 11-βHSD1 mRNA abundance in TB and TB/T2D mice, being more pronounced in the TB group (Fig 5B, C). In contrast, BEA administration increased cortisone content and 11-βHSD2 expression in TB or T2D/TB mice after 30 and 60 days (Fig 5D-F).

In the liver, treatment of TB and T2D/TB groups with BEA also reduced the expression of 11-βHSD1 and increased that of 11-βHSD2 when compared with the control group (Fig. 6A, B). This effect was even more evident in the liver of T2D/TB group, which also showed complete remission of steatosis and hyperglycemia (Fig. 6C, D).

These results indicate that BEA act as a regulator of 11-βHSD1 and 11-βHSD2 transcription in the lung and liver, repressing 11-βHSD1 and upregulating 11-βHSD2 gene expression. In consequence treatment of Mtb-infected mice with BEA induced a lower production of active corticosterone and a higher production of inactive cortisone, which in turn contribute to the increased expression of the pro-inflammatory cytokine's TNF-α and IFN-γ, decreasing the pulmonary bacillary loads. BEA administration improved lipid and glucose metabolism in the liver and normalized liver histology and glucose blood concentrations.

Thus, BEA administration improved the immune response and metabolism in T2D/TB comorbidity, suggesting that it could potentially be used as a novel immune therapeutic agent with beneficial immune, endocrine and metabolic activities.

Discussion

T2D/TB comorbidity is an emerging area of interest due to its alarming increase in incidence worldwide. T2D is a metabolic and endocrine disease characterized by a progressive whole-body glucose intolerance that leads to chronic hyperglycemia, affecting multiple organs. T2D compromises host immune responses and impairs host ability to control microbial infections (6,7). Indeed, T2D impairs the immune response against Mtb, worsening the course of the disease requiring longer courses of chemotherapy (8,9). Our results confirm that T2D/TB comorbidity leads to a more aggressive course of TB in a murine model of T2D/TB; and for the first time, demonstrates that one significant pathogenic factor for increased TB severity is the higher expression of 11-βHSD1 and corticosterone production during late advanced disease in the lungs and liver. Moreover, treatment with BEA efficiently decreases the expression of 11-βHSD1 reducing local corticosterone synthesis. This effect of BEA significantly reduced bacillary burdens and hyperglycemia. These results suggest that this synthetic hormone could be considered a feasible candidate drug for T2D and TB co-morbidity.

Mtb affects mainly the lungs producing chronic and excessive inflammation in which innate and adaptive immunity are severely affected (3,4). Mtb infection initiates by inhalation of saliva droplets with Mtb that are engulfed by alveolar macrophages. Macrophages are key cells in bacilli elimination, and together with dendritic cells process Mtb antigens that are presented to T lymphocytes in regional lymph nodes. Lymphocytes migrate to the lung and along with macrophages and other cells, form containment structures known as granulomas which are the histopathological hallmark of TB (6). In early stages of active infection, Th1 cellular immune responses are protective, as IFNγ, TNFα and other cytokines such as IL-12 induce macrophage activation, allowing containment of bacterial growth. Nevertheless, during late active disease, extensive inflammation produces a shift toward anti-inflammatory responses, such as Th-2 cytokine pattern in which IL-4, IL-13 and other anti-inflammatory cytokines such as IL-10 and TGF-β. This shift in immune response induces a local immunosuppressive/anti-inflammatory effect, resulting in poor containment of infection and progression of tissue damage (33). Moreover, besides the derangement in the

immunologic response, there is a heightened neuroendocrine response involving a complex network of cytokines, hormones, and neurotransmitters that exacerbates TB pathogenesis (10,34). These neuroendocrine responses include the cytokines released during the immune response to Mtb (pro-inflammatory mediators), abnormal production of several hormones and central nervous system (CNS) responses, prompting the activation of the two major stress systems, the HPA axis (35) and the sympathetic nervous system (SNS) (7,36).

HPA and GCs have an important contribution to the physiopathology of TB (37)(38). It was reported in the experimental model of progressive TB in BALB/c mice that at the time of maximal protective activity mediated by IFN-γ, TNF-α, IL-1β and NO production (day 21 after infection), pro-inflammatory cytokines such as TNF-α and IL-1β strongly activate the HPA axis, producing high expression of CRF in the hypothalamus and adrenal hyperplasia with high serum concentrations of corticosterone (35). Then, during the chronic or late phase after 28 days of infection, there is progressive adrenal atrophy and a decrease in circulating corticosterone associated with extensive pneumonia (35). Thus, there is high GCs adrenal production apparently with the aim to avoid tissue damage produced by excessive lung inflammation. During the late phase of the disease when adrenal glands atrophy, peripheral macrophages become an alternative. It seems that there is another source of corticosterone available by local production in unidentified tissues or cells.

Our results show for the first time, that this extra-adrenal source of GCs are macrophages, particularly foamy cells which strongly express the enzyme 11-βHSD type 1 and serve as an alternative source of corticosterone in the inflamed tuberculous lung. This local source of corticosteroids acts in an autocrine and paracrine manner. However, local excess of corticosterone also inhibits Th1 lymphocyte activity and induces differentiation of Th-2 lymphocytes that favors bacterial survival and proliferation causing animals death (15). Thus, this local anti-inflammatory activity mediated by high production of corticosterone in affected organs during active late TB has deleterious effect. Antagonistic hormones such as DHEA can restore balance to the system.

DHEA is the most abundant product of the human adrenal gland after puberty with maximum levels occurring in the late 20s. Its production then falls steadily with advanced age. DHEA has glucocorticoid counterregulatory effects in several systems. For example, in obesity DHEA shows opposite effects to GCs on enzyme expression in the liver (18) and in the immune system (39). In a previous study, treatment with DHEA starting on day 60 after Mtb infection in BALB/c mice reduced the pulmonary bacillary burden and decreased tissue damage by reactivating the Th1 cytokine response (40). In humans, a deficit in DHEA relative to cortisol can be striking in severe TB (12). However, DHEA is suboptimal for human use, partly because it is metabolized into sex steroids.

16a-Bromoepiandrosterone (BEA; 16a-bromo-5a-androstan-3b-ol-17-one) is a synthetic adrenal steroid derivative that does not enter sex steroid pathways and has been demonstrated in tuberculous BALB/c mice treated with BEA, every other day beginning on day 60, that it produces a significant inhibition of bacterial proliferation and higher expression of protective cytokines (TNF-α, IFN-γ). Moreover, when given as an adjunct to conventional chemotherapy, BEA enhances bacterial clearance (41). Interestingly, this synthetic hormone showed therapeutic benefit in patients with TB, Malaria (26,42) malaria and in the Acquired Immune Deficiency Syndrome (AIDS) (43).

In the present study we used a new water-miscible formulation of BEA. Its ability to form a stable suspension in water is derived from a new method of formulating the molecule. Water miscibility avoids the problems associated with the organic solvents required to administer the former, lipid soluble formulation. Mice treated with this new BEA formulation showed similar results as the previous DHEA synthetic analog, producing significant increase of IFNγ and TNFα. This effect was associated with pulmonary bacillary loads reduction and increase survival (41). In the present study, we demonstrated that BEA administration also reduced pulmonary expression of 11-βHSD1 and corticosterone. Conversely, BEA increased 11-βHSD2 which shifts the GCs equilibrium to inactive cortisone. Thus, BEA can induce CD4 Th1 cells and macrophages activation by direct activity, and as shown in the present work, by the suppression of the local production of corticosterone in the lungs.

Obesity is commonly associated with T2D and other manifestations of the metabolic syndrome (44). The metabolic syndrome is characterized by insulin resistance in adipose tissue, skeletal muscle and liver leading to hyperlipidemia, hypertension, hepatic steatohepatitis and T2D. GCs activity antagonizes insulin action and are important causative factors in the development of metabolic syndrome and T2D. GC impair glucose uptake, enhance lipolysis and hepatic gluconeogenesis and promote proteolysis. Moreover, GCs directly inhibit pancreatic secretion of insulin, enhance glucose secretion by inhibiting gluconeogenesis in the liver and oppose other metabolic actions, such as insulin signaling and glucose uptake by inhibiting the translocation of the glucose transporter GLUT4 to the plasma membrane (22).

Our results show that in comparison with healthy control animals, mice with T2D without Mtb infection exhibits higher 11-βHSD1 expression and strong corticosterone immunostaining in bronchial epithelial cells and in hepatocytes. BEA treatment decreased the expression of both enzyme and hormone in lung and liver and corrected glucose concentration in blood. Thus, BEA significantly improved metabolic abnormalities by down-regulating 11-βHSD1 gene expression as one of its therapeutic mechanisms. These results are in agreement with previous publications and contribute new information such as the increase of corticosterone production in the airways epithelium of T2D individuals which could be a factor for higher severity of respiratory infections, such as TB.

Our results demonstrates that T2D/TB mice presents a higher pulmonary bacillary load than TB mice without T2D, as well as lower expression of IFNγ and TNFα. These alterations were associated with higher expression of 11-βHSD1 and strong immunostaining to corticosterone in the pulmonary inflammatory intra-alveolar infiltrate, particularly in foamy macrophages. Thus, it seems that the higher pulmonary production of corticosterone mediated by 11-βHSD1 is a factor that worsens the course of TB in T2D and thus, regulation of 11-βHSD1 expression in the lung could be a new therapeutic target.

In this regard, our results suggest that BEA can block 11-βHSD1 in the lung and in the liver, making it an effective novel treatment for this co-morbidity. Trials for the use of BEA in the treatment of human TB, T2D and the co-morbidity T2D/TB are now justified.

Conclusion

The study of T2D/TB comorbidity from the immunoendocrine point of view is of utmost importance to understand the background of this binomial, as well as to develop novel strategies for the control of this comorbidity. Active GCs represent a negative factor in the development of both entities, with a negative effect on glucose metabolism and the development of a non-protective immune response. Treatment with BEA represents a novel strategy as an adjuvant in the treatment of TB alone and comorbidity with T2D. For these reasons, it is of utmost importance to continue with the study of the BEA through its escalation to clinical studies to be used in patients.

Conflict of interest

We declare not conflict of interest during the course of this work.

Author contributions

LT-MO, AC and HP-R contributed to the background work and conceived the experiments. LT-MO and RE-O performed, organized, and analyzed the results. LT-MO, B-E, EB and MC-B experiments design and execution. BP-J performed animal infections and drug administration. ME-D performed the culture and preparation of Mycobacterium tuberculosis H37Rv strain for infection and CFU experiments, IT-V, NT, AT designed the hypercaloric diet. C-W, G-Y prepare and supply BEA for the animal treatment. LT-MO, IT-V and HP-R wrote the paper.

Acknowledgments

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Figures

Figure 1.- Metabolic abnormalities and liver pathology of mice with type 2 diabetes mellitus in comparison with non-diabetic animals (sham). A) General description of the experimental groups. B) mice were weighted weekly for weight gain monitoring. Data are presented as a mean and standard derivation of 5 mice per group for 16 weeks. C) Blood glucose levels were assessed in two and four weeks after administration of STZ intraperitoneally in animals fed with a high-fat diet (HFD) and in control animals not treated with STZ and fed with rodent chow. Serum concentrations of D) triglycerides, E) cholesterol, and F) insulin in control (white bars) and T2D (black bars) mice, after four months of HFD-STZ treatment. G) Effect of hypoglycemic drugs on serum glucose levels in T2D animals, before and after 27 hours of oral administration of metformin (Met) or glibenclamide (Gly). Asterisk represents statistical significance (one-way ANOVA, P <0.05, and two-way ANOVA for graph 1 B, 1 C, and 1 G). H) Representative micrograph of liver tissue from a control mouse (left) and T2D mouse (right). Extensive cytoplasmic vacuolization of hepatocytes corresponding to steatosis is show in the liver of T2D mouse.

Figure. 2. Comparative course of pulmonary tuberculosis in diabetic mice infected with Mtb (T2D/TB) and non-diabetic mice infected with Mtb (TB). A) Five mice per group were euthanized at the indicated time points post-infection and the right lungs were used to determine the bacterial loads by counting colony-forming units (CFU). A significant increase in the bacillary load of the T2D/TB group was observed after one and four months of infection in comparison with control group.

Figure 3.- Gene expression and cellular localization of glucocorticoids converting enzymes and glucocorticoid content in late TB (day 120). A) Representative micrographs of immunohistochemical

staining of corticosterone in the epithelium of the lung airways and the alveolar epithelium. B) Comparative gene expression of 11-βHSD1 determined by qPCR in the different experimental groups at day 120 (Top panel). Immunohistochemical detection of 11-βHSD1 (Bottom panel). C) Representative micrographs of immunohistochemical detection of cortisone in bronchial epithelial cells and alveoli. D) Comparative 11-βHSD2 gene expression and immunohistochemical staining. Asterisks represent statistical significance (**** p< 0.0001, two- way ANOVA and n.s. no significance).

Figure 4.- Therapeutic effect of BEA in TB and T2D/TB mice. Groups of TB and T2D/TB mice after 60 days of intratracheal infection with high dose Mtb strain H37Rv were treated during one or two months with BEA, while the control TB or T2D/TB groups received only the vehicle. Lungs were processed for the indicated determination. In comparison with the control TB or T2D/TB mice, BEA induced significant decrease of pulmonary bacillary loads and tissue damage (pneumonia) in both TB and T2D/TB groups, particularly after 60 days of treatment. After one month of treatment, BEA induced higher expression of the protective cytokines IFNγ and TNFα. In the spleen, treatment with BEA did not produced significant changes in the weight and CFUs in TB mice, while induced significant decrease of bacillary burdens and higher weight in T2D/TB mice after two months of treatment. Asterisks represent statistical significance (**** p< 0.0001, two- way ANOVA and n.s. no significance).

Figure 5.- Effect of BEA on lung GCs production and gene expression of their converting enzymes in TB and T2D/TB mice. A) Representative low power and mild power micrographs of immunohistochemistry detection of corticosterone, in both groups after one and two months of BEA treatment. B) In comparison with the TB or T2D/TB groups, the digital pathology analysis showed significant lower immunostaining of corticosterone induced by BEA treatment after one month and two months in TB mice and T2D/TB mice. C) BEA treatment induced lower 11β-HSD1 gene expression in TB and T2D/TB in comparison with TB or T2D/TB mice treated with vehicle. D) Representative micrographs of cortisone detection by immunohistochemistry in the lungs of TB and T2D mice treated with BEA at one and two months of infection. E) The digital pathology study shows higher expression of cortisone after one month of

treatment in the TB and T2D/TB groups. F) Gene expression of 11b-HSD2 corticosterone converting enzyme after BEA treatment in TB and T2D/TB animals. Asterisk represents statistical significance (p<0.05).

Figure 6.- Effect of BEA in the liver of T2D and T2D/TB mice. Liver samples from groups of five T2D and T2D/TB mice treated with BEA for one month and their respective control mice that received only the vehicle, were used to isolate total RNA and 11-βHSD1 (A) and 11 -βHSD2 (B) mRNA determination by RT-PCR. C) Treatment with BEA corrected hyperglycemia in T2D and T2D/TB mice. Asterisks represent statistical significance (*** p <0.0001, **** p <0.0001, two-way ANOVA, n.s. without significance). D) Liver tissue sections stained with H&E reveals steatosis in T2D and T2D/TB mice, which was reverted by treatment with BEA.
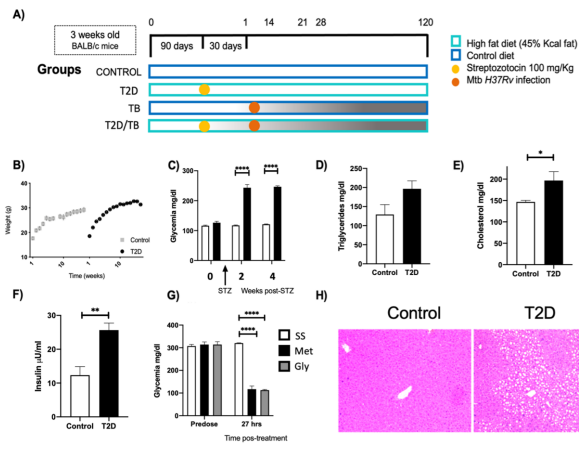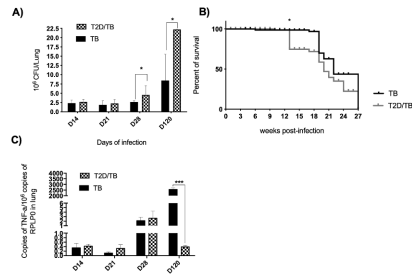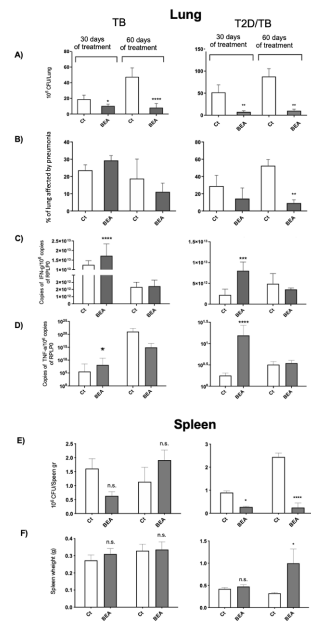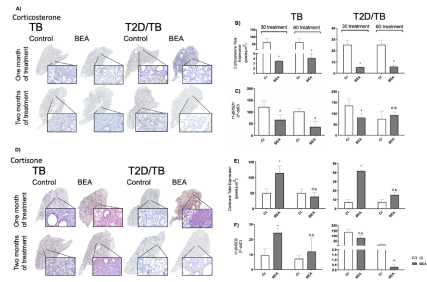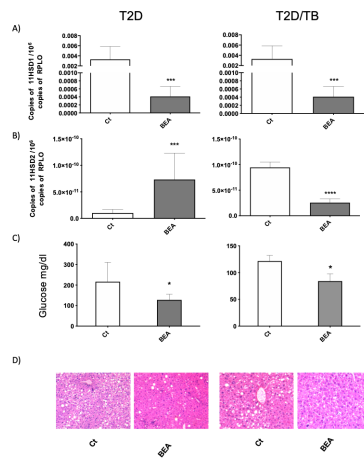
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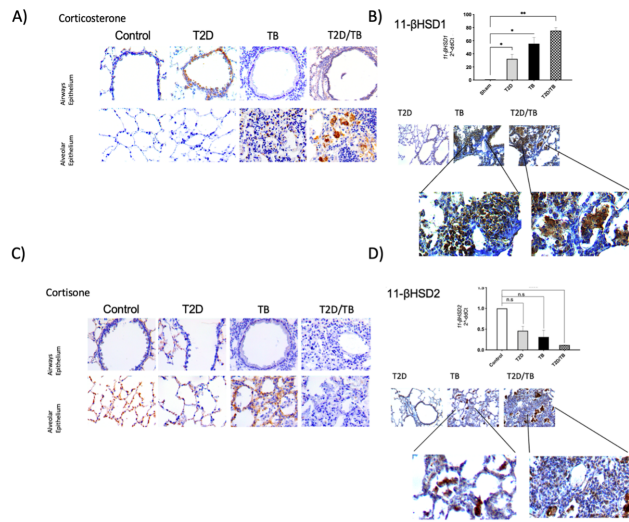
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