Cytokine 69 (2014) 39–46

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

The critical role of macrophage migration inhibitory factor in insulin activity

Milica Vujicic [a,1], Lidija Senerovic [b,1], Ivana Nikolic [a], Tamara Saksida [a], Stanislava Stosic-Grujicic [a], Ivana Stojanovic [a,*]

[a] Department of Immunology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Belgrade, Serbia
[b] Institute of Molecular Genetics and Genetical Engineering, University of Belgrade, Belgrade, Serbia

ARTICLE INFO

Article history:
Received 2 December 2013
Received in revised form 22 April 2014
Accepted 12 May 2014
Available online 4 June 2014

Keywords:
Macrophage migration inhibitory factor
Insulin
Insulin synthesis
Insulin activity

ABSTRACT

Macrophage migration inhibitory factor (MIF) is a molecule with plethora of functions such as regulation of immune response, hormone-like, enzymatic and chaperone-like activity. Further, MIF is a major participant in glucose homeostasis since it is an autocrine stimulator of insulin secretion. MIF absence in male knockout mice (MIF-KO) results in development of glucose intolerance, while sensitivity to insulin is fully preserved. Since our results confirm that beta cells from MIF-KO mice express, produce and secrete insulin similarly to beta cells of their wild type (WT) counterparts C57BL/6 mice, we hypothesize that MIF-KO-derived insulin is less active. Indeed, insulin from MIF-KO islets is unable to significantly induce glucose uptake into hepatocytes and to efficiently promote insulin-triggered Akt phosphorylation determined by immunoblot. However, MIF's tautomerase function is not crucial for insulin biosynthesis since MIF inhibitors had no impact on WT insulin activity. Importantly, MIF recognition by anti-MIF antibody (ELISA) after *in vitro* co-incubation with purified insulin was significantly lower suggesting that insulin covers MIF immunodominant epitope. In addition, MIF binds insulin within beta cell as confirmed by co-immunoprecipitation. WT and MIF-KO-derived insulin exhibited different cleavage patterns suggesting different protein conformations. Finally, pre-incubation of recombinant MIF with insulin promotes formation of insulin hexamers. These results imply that MIF probably enables proper insulin folding what results in insulin full activity. This newly discovered feature of the cytokine MIF could be potentially important for commercially produced insulin, for increasing its stability and/or bioavailability.

1. Introduction

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine first identified as a soluble product of activated T lymphocytes that inhibits macrophage migration *in vitro* [1]. MIF is expressed in a variety of cell types including epithelial, endothelial and immune cells [2]. In addition, MIF is highly expressed in several tissues of the endocrine system including hypothalamus, pituitary, adrenal glands and islets of pancreas [3]. Unlike other cytokines, MIF is constitutively expressed and stored in intracellular pools and does not require de novo protein synthesis before secretion [4]. In addition to well established effects in modulation of immune response, MIF possesses properties of an enzyme [5], endocrine molecule [6] and a chaperon-like protein [7].

MIF has been implicated in pathogenesis of many inflammatory and/or autoimmune diseases including type 1 diabetes (T1D) and type 2 diabetes (T2D) [8,9]. Increased MIF serum level is associated with development of T1D, obesity and insulin resistance [10–12]. In animal models of diabetes, MIF inhibition or deletion prevents spontaneous or chemically-induced autoimmune diabetes [13,14]. Furthermore, MIF absence protects pancreatic islets from detrimental influence of cytokines and high level of nutrients [15,16]. Although MIF's role in diabetes pathogenesis is usually examined from the immunological point of view, current knowledge indicates that MIF directly modulates insulin secretion

Abbreviations: MIF, macrophage migration inhibitory factor; MIF-KO, MIF-knockout mice; WT, wild type; ELISA, enzyme-linked immunoadsorbent assay; T1D, type 1 diabetes; T2D, type 2 diabetes; FCS, fetal calf serum.

* Corresponding author. Address: Department of Immunology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Bulevar despota Stefana 142, 11108 Belgrade, Serbia. Tel.: +381 11 2078 390; fax: +381 11 2761 433.
E-mail address: ivana@ibiss.bg.ac.rs (I. Stojanovic).

[1] Authors equally contributed.

http://dx.doi.org/10.1016/j.cyto.2014.05.013
1043-4666/

and glucose metabolism and that treatment of pancreatic islets with anti-MIF antibody reduces glucose-stimulated insulin release [17]. Moreover, MIF is secreted together with insulin and it acts as an autocrine factor to stimulate insulin release [17]. The absence of MIF in MIF-knockout mice (MIF-KO) results in the development of obesity, glucose intolerance at 6 months of age and consequent hyperglicemia [18]. Our results support these findings, although our colony of MIF-KO mice develops intolerance to glucose around 7 weeks of age [19]. On the other hand, these mice are sensitive to administration of exogenous insulin indicating that receptor for insulin is fully functional as well as insulin-triggered signaling pathway in target cells [18]. Therefore, the explanation for the observed condition of MIF-KO mice may reside either in the function of beta cells or insulin activity. This study identifies MIF as a crucial contributor in proper insulin biosynthesis and that it is responsible for insulin full bioactivity.

2. Material and methods

2.1. Animals

Homozygous male *Mif* gene deficient mice (MIF-KO) were bred onto a C57BL/6 background [20] and kept under standard conditions (nonspecific pathogen free) along with male C57BL/6 mice (WT) in the Animal Facility at the Institute for Biological Research "Sinisa Stankovic", University of Belgrade. All animal procedures were in compliance with the EEC Directive (86/609/EEC) on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Sinisa Stankovic", University of Belgrade (No: 5/2013).

2.2. Insulin production during intraperitoneal glucose tolerance test

Tolerance to glucose was estimated by intraperitoneal injection of D-glucose (Sigma–Aldrich, St. Louis, MO, USA) (2 mg/g body weight) to 2–3 months old WT and MIF-KO mice. Blood from orbital plexus was collected before and 30 min after glucose administration for measurement of insulin concentration.

2.3. Isolation of pancreatic islets

Pancreatic islets from pancreas of 2–3 months old WT and MIF-KO mice were isolated by collagenase V (Sigma–Aldrich) digestion technique followed by handpicking [21]. Before performing experiments, islets were cultured overnight in RPMI 1640 medium containing 10% vol/vol FCS (PAA Chemicals, Pasching, Austria), 10 mmol/l HEPES, 5 μmol/l β-mercaptoethanol, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate (all from Sigma–Aldrich) and 1X PenStrep (PAA Chemicals) in a humidified (5% CO_2, 95% air) atmosphere at 37 °C.

2.4. Functional assays for pancreatic islets

WT and MIF-KO islets (groups of 10) were placed in 96-well non-adhesive plate (Sardstedt, Numbrecht, Germany) and subjected to *in vitro* test of glucose-induced insulin release as described [21]. Insulin release was measured by ELISA (Mercodia, Uppsala, Sweden) following manufacturer's instructions. Islet insulin content was measured from ultrasonically (Sonics & Materials Inc., Vibra Cell, Danbury, CT, USA) disrupted islets by ELISA and normalized by DNA concentration (measured using Eppendorf Biophotometer, Eppendorf Austria GmbH, Wien, Austria) to exclude variation between samples due to difference in islet cell number.

2.5. Preparation of conditioned media for in vitro tests

For preparation of conditioned media containing insulin, 24 h after isolation WT and MIF-KO islets were placed 96-well non-adhesive plates in Krebs–Ringer buffer (Sigma–Aldrich) supplemented with 10 mmol/l HEPES (Sigma–Aldrich) and 2 mg/ml BSA (AppliChem GmbH, Darmstadt Germany) (KRBH) and containing 11 mmol/l glucose for stimulation of insulin secretion. After 2 h at 37 °C, conditioned media was collected and insulin concentration was determined by ELISA. For inhibition of MIF's tautomerase activity ISO-1 ((S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester) or Vitamin E were used [22,23]. WT islets were cultured in the presence of ISO-1 (50 μg/ml) or Vitamin E (10 μmol/l) for 24 h (both chemicals purchased from Sigma–Aldrich). Islets were then placed in KRBH with 11 mmol/l glucose for 2 h and insulin concentration was estimated from conditioned media using ELISA.

2.6. Measurement of glucose uptake

To determine the activity of WT and MIF-KO derived insulin, the insulin-responsive cells of hepatocellular carcinoma – Hep G2 (ATCC® HB-8065™) was utilized. Hep G2 cells were cultured in 96-well plates (4×10^4 cells/well) in DMEM (PAA Chemicals) supplemented with 4 mmol/l L-glutamine, 1X PenStrep (PAA Chemicals) and 10% vol/vol FCS. After 24 h, cells were stimulated by 2 ng/ml of human recombinant insulin (Sigma–Aldrich), WT or MIF-KO insulin, or mouse recombinant MIF (R&D Systems, Minneapolis, MN, USA; 2 ng/ml) or pre-incubated recombinant MIF + recombinant insulin (2 ng/ml each), or insulin from either ISO-1 or Vitamin E-treated pancreatic islets. Glucose uptake was measured indirectly by determination of glucose concentration by glucometer (Sensimac, IMACO GmbH, Lüdersdorf, Germany) in the supernatants of treated Hep G2 cells after specific time intervals. The results are shown as reduction in glucose concentration after specific intervals of incubation with different insulin preparations compared to the value of glucose concentration at the beginning of incubation.

2.7. Viability

Viability of treated Hep G2 cells (4×10^4) was assessed by MTT assay as previously described [16]. Colorimetric reaction was measured using automated microplate reader (LKB 5060-006, LKB, Vienna, Austria).

2.8. Western blot

Hep G2 cell (10^6) treated either with WT or MIF-KO-conditioned media containing insulin or conditioned media with insulin derived from ISO-1-treated islets (all 2 ng/ml) for 6 h were disrupted in the lysis buffer containing 62.5 mmol/l Tris–HCl (pH 6.8 at 25 °C), 2% w/v SDS, 10% vol/vol glycerol, 50 mmol/l DTT, 0.01% w/v bromophenol blue (all from Sigma–Aldrich). The concentration of proteins was measured using Lowry method. All samples (50 μg of each) were subjected to electrophoresis on 15% vol/vol SDS–polyacrylamide gel (SDS–PAGE). After electro-transferring to polyvinylidene difluoride membranes at 5 mA/cm^2, using semi-dry blotting system (Semi-Dry Transfer Unit, GE Healthcare, Buckinghamshire, England), the blots were blocked with 5% w/v BSA in PBST buffer (80 mmol/l Na_2HPO_4; 20 mmol/l NaH_2PO_4; 100 mmol/l NaCl; 0.1% vol/vol Tween-20) (all obtained from Sigma–Aldrich) and probed with specific rabbit antibodies for Akt and phosphorylated Akt on Ser473 (pAkt) (Cell Signaling Technology, Boston, MA, USA) at 1/1000, mouse-anti mouse insulin (Abcam, Cambridge, UK) at 1/1000, rabbit-anti mouse glucagon,