

EFFECT OF INSULIN ON LPS-INDUCED INFLAMMATORY MARKERS IN MOUSE COLON SMOOTH MUSCLE CELLS *IN VITRO*

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ABSTRACT

Objective: The aim of the research is to determine the effect of supraphysiological doses of insulin on mouse colon smooth muscle cells (CSMCs) expression and secretion of pro-inflammatory cytokines interleukin 1 alpha (IL-1 α) and tumor necrosis factor alpha (TNF- α) *in vitro*.

Methods: Freshly isolated CSMCs from BALBc mice were cultured in Dulbecco's Modified Eagle Medium and treated with various doses of insulin (0, 1, 5, 10, and 20 nM) for 48 h, with/without lipopolysaccharides (LPS; 1 μ g/mL) to induce inflammation. The levels of IL-1 α and TNF- α in the cell homogenates and conditioned media were measured using ELISA.

Results: Insulin alone (1, 5, 10, and 20 nM) did not elicit a significant change in the expression or secretion of IL-1 α or TNF- α from CSMCs; however, insulin (10 and 20 nM) significantly ($p < 0.05$) increased the expression and secretion (~1.3–1.6-fold) of both IL-1 α and TNF- α from CSMCs in the presence of inflammatory stimulus LPS when compared to LPS alone.

Conclusion: This study highlights the role of hyperinsulinemia on CSMC inflammation and its potential role in the pathogenesis of inflammatory bowel disease (IBD) during obesity. Measures that prevent obesity may protect against the development of IBD since the worldwide incidence of both obesity and IBD is increasing in a parallel fashion.

Keywords: Insulin, Smooth muscle, Inflammation, Inflammatory bowel disease.

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INTRODUCTION

Despite the important role of insulin in glucose homeostasis, lipid metabolism, growth, and energy metabolism, clinical and experimental evidence suggests that chronic hyperinsulinemia is major risk factor for the development of multiple diseases such as ischemic heart disease, stroke, atherosclerosis, cancers, Alzheimer's disease, and non-alcoholic fatty liver disease [1].

Hyperinsulinemia is usually observed in patients with obesity, type 2 diabetes mellitus, metabolic syndrome, and inflammatory bowel disease (IBD) [2-5]. The role of hyperinsulinemia in development of certain conditions such as cardiovascular diseases, stroke, and cancer is well established [1]; however, its role in the development of IBD is not extensively investigated. The occurrence of IBD in obese patients was considered uncommon in the past [6,7]; however, recent epidemiological evidence suggests that prevalence of both obesity and IBD is increasing worldwide in a parallel fashion in the developed nations, and obesity as well as insulin resistance are relatively prevalent in IBD patients [2,3,8-11], suggesting a possible link between the altered metabolic state accompanying obesity, and the development of inflammation in the bowel. Obesity is considered a state of low-grade systemic inflammation and has been shown to be associated with altered endocrine profile, hyperinsulinemia, increased plasma leptin, decreased plasma adiponectin, elevated plasma cytokines, and immune dysregulation [12]. Obesity in IBD patients may increase the disease activity, impair drug actions, and cause post-operative surgical complications, especially in the presence of hypertrophied mesenteric adipose tissue in Crohn's disease (creeping fat) [2,9].

Conflicting data about whether insulin exerts pro-inflammatory or anti-inflammatory effects on different tissues exist [13]. Several studies

showed that small doses of insulin may exert anti-inflammatory effects during euglycemic or moderately hyperglycemic conditions in the myocardium and vascular endothelial cells through the phosphoinositide 3-kinase-protein kinase B (PI3K-AKT) pathway and suppression of P-selectin and intercellular adhesion molecule 1 expression [14,15]. On the other hand, chronically elevated insulin levels during hyperglycemic conditions may exert pro-inflammatory effects through activation of mitogen-activated protein kinase (MAPK)-NF- κ B signaling pathway in macrophages, hepatocytes, and vascular smooth muscle cells (SMCs), which result in stimulation of inflammatory cytokine expression [16-18].

The pathogenesis of IBD is complex and can involve multiple cytokines where they control several aspects of the inflammatory response and development of multiple extraintestinal manifestations such as arthritis, stomatitis, cholelithiasis, and hepatotoxicity [19,20]. Clinical and experimental evidence have shown that blockade of certain cytokines such as tumor necrosis factor (TNF- α) and interleukin-1 α (IL-1 α) by specific antibodies is effective in suppression of IBD symptoms [21-23]. The major source of cytokines in IBD is the infiltrating inflammatory cells and epithelial cells [20]; however, several studies suggest that intestinal SMCs are also greatly involved in inflammatory conditions of the bowel [24]. Clinical and experimental evidence shows that intestinal SMCs are affected structurally and functionally in IBD leading to phenotypic switching, altered contractility, and growth properties [24-26]. SMCs are capable of expression and secretion of multiple inflammatory mediators during different pathological conditions and can actively participate in IBD pathogenesis through recruitment of inflammatory cells and increased proliferation [27].

The aim of this research is to explore the effect of supraphysiological doses of insulin on the expression of inflammatory markers IL-1 α and

TNF- α from mouse colon SMCs after exposure to inflammatory stimulus lipopolysaccharide (LPS). We hypothesize that elevated insulin levels which accompany obesity can stimulate the intestinal SMCs to produce different inflammatory mediators, thereby playing an important role in IBD pathogenesis. Measures that control obesity and prevent hyperinsulinemia can be effective in the prevention of IBD.

MATERIALS AND METHODS

Materials

Smooth muscle buffer (SMB) was prepared in-house and contained 120 mM NaCl, 4 mM KCl, 2.6 mM KH₂PO₄, 2 mM CaCl₂, 25 mM HEPES (N-2-hydroxyethylpiperazine -N' 2- ethanesulfonic acid), 14 mM glucose, and 2.1% essential amino acid mixture (pH was adjusted to 7.4). The digestion solution contained 0.5 mg collagenase and 0.33 mg trypsin inhibitor per ml of SMB. All of these chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Cell culture media used during the incubation process were prepared by adding 10% fetal bovine serum (FBS) (GE Healthcare, Little Chalfont, UK), 100 U/ml of penicillin, 100 μ g/ml of streptomycin, and 2.5 μ g/ml of amphotericin B to Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine (Capricorn Scientific GmbH, Ebsdorfergrund, Germany). The remaining reagents were purchased from Euroclone S.P.A. (Pero, Italy). LPS was purchased from Sigma-Aldrich Inc. Human recombinant insulin was purchased from Sigma. Protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Specific ELISA kits for mouse TNF- α and IL-1 α were purchased from Sigma. 500 μ m Nitex mesh was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Animals

Twenty young mature male BALB/c mice (~12 weeks of age, 26.5–30 g) were provided by the animal house of Jordan University of Science and Technology. Mice were euthanized by inhalation of CO₂ and the colon was rapidly excised. The colon was cut into small pieces (2–3 cm) in length, then placed in cold SMB with the composition described previously. All procedures were performed according to the guidelines of the Animal Care and Use Committee at Jordan University of Science and Technology.

Preparation of dispersed SMCs

Colonic SMCs were isolated by sequential enzymatic digestion of muscle strips, filtration, and centrifugation. The mucosa was scraped off with fine scissors, then the tissues were cut into thin slices (2 mm long; 2 mm thin), and incubated for 20 min in SMB containing 0.5 mg/ml collagenase and 0.33 mg/ml soybean trypsin inhibitor in a shaking water bath at 31°C. The tissues were continuously gassed with 100% oxygen during the entire isolation procedure. The partly digested tissue was washed twice with 50 ml of collagenase-free SMB, and the muscle cells were allowed to disperse spontaneously for 10 min in collagenase-free medium. To eliminate broken cells and other organelles, cells were harvested by filtration through 500 μ m Nitex mesh and centrifuged twice at 350 g for 10 min, and the process was repeated 4–5 times. The cells were counted in a hemocytometer, and it is estimated that 95% are viable using trypan blue exclusion assay.

Colon SMCs (CSMCs) proliferation assay

Freshly isolated CSMCs were cultured in 96-well plates at 1×10^5 cells/well. Cells were serum deprived for 24 h before stimulation with either vehicle, insulin (1 nM), insulin (5 nM), insulin (10 nM), insulin (20 nM), LPS (1 μ g/ml), LPS (1 μ g/ml) + insulin (1 nM), LPS (1 μ g/ml) + insulin (5 nM), LPS (1 μ g/ml) + insulin (10 nM), or LPS (1 μ g/ml) + insulin (20 nM). Cell proliferation was assessed after 48 h using MTT assay (Intron Biotechnology, Korea) according to manufacturer's instructions. Briefly, CSMCs were incubated with MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h at 37°C. MTT reagent was converted to an insoluble formazan and the concentration determined by optical density at 570 nm.

Evaluation of inflammatory cytokines expression using ELISA

Ten thousand dispersed mouse colonic SMCs (n=3/group) were seeded in 6-well plate in DMEM containing 10% FBS, 100 U/ml of penicillin,

100 μ g/ml streptomycin, and 2.5 μ g/ml amphotericin B. The cells were incubated in a humidified incubator with 95% air and 5% CO₂ at 37°C for 48 h and treated with either vehicle, insulin (1 nM), insulin (5 nM), insulin (10 nM), insulin (20 nM), LPS (1 μ g/ml), LPS (1 μ g/ml) + insulin (1 nM), LPS (1 μ g/ml) + insulin (5 nM), LPS (1 μ g/ml) + insulin (10 nM), or LPS (1 μ g/ml) + insulin (20 nM). After completion of the incubation period (48 h), the treated samples were centrifuged at 1000 rpm for 5 min at 4°C. The conditioned media were stored in -20°C for further analysis, and the cells pellets were immediately lysed.

Cell lysates were prepared using BashingBeads Lysis tubes (Zymo Research, Irvine, CA, USA) and the cell lysis buffer containing protease inhibitor cocktail provided by the kit according to manufacturer's instructions. Then, lysates were centrifuged for 10 min at 14000 rpm at 4°C and the supernatant collected for further analysis. Total protein concentration of the supernatants was measured using the DC protein assay kit. The protein concentration was then adjusted to 100 μ g/mL in all samples.

To evaluate differential cytokine expression in the treated and control samples, specific ELISA kits for IL-1 α and TNF- α were used to measure the levels in the cell lysates and the conditioned media in the control and treated samples according to the manufacturer's protocol.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). One-way analysis of variance followed by Fisher's *post hoc* analysis was used to examine significant differences between groups. A $p < 0.05$ was considered statistically significant. All data are shown as a mean \pm standard error of the mean. Values shown are representative of three independent experiments performed in triplicates.

RESULTS

Colonic SMCs identification

Freshly isolated mouse cells were viewed at $\times 20$ objective of inverted Nikon TMS-f microscope, and they appeared spindle-shaped with varying lengths (Fig. 1a).

CSMCs viability assay

To ensure that CSMCs viability is not affected by LPS or insulin treatment, we performed an MTT assay on the treated cells. Our results indicate that cell viability is comparable between all treatment groups indicating that neither LPS nor insulin is affecting the growth of CSMCs within 48 h period (Fig. 1b).

Assessment of cytokines expression and secretion using ELISA

To evaluate the levels of differentially expressed cytokines in the treated samples and control, specific ELISA assays were used. Data showed that insulin alone (1, 5, 10, and 20 nM) did not elicit any change in the expression or secretion of IL-1 α or TNF- α from CSMCs when compared to control group. LPS treatment caused a marked increase in the expression of both IL-1 α and TNF- α (~2.6-fold increase, $p < 0.01$) when compared to control. Interestingly, insulin (10 and 20 nM) significantly ($p < 0.05$) increased the expression (~1.3–1.7-fold) of both IL-1 α and TNF- α from CSMCs in the presence of inflammatory stimulus LPS when compared to LPS alone (Fig. 2a and b). To further evaluate whether altered cytokine expression may affect their secretion into the media, levels of IL-1 α and TNF- α were measured in the conditioned media of treated groups. Data showed that levels of both IL-1 α and TNF- α did not change with insulin treatment (1, 5, 10, and 20 nM) alone when compared to control; however, they are significantly increased with LPS treatment (~3-fold, $p < 0.05$). Furthermore, insulin (10 and 20 nM) significantly ($p < 0.05$) increased the secretion (1.3–1.7 fold) of both IL-1 α and TNF- α from CSMCs in the presence of inflammatory stimulus LPS when compared to LPS alone (Fig. 3a and b). This data suggest that inflammatory stimuli can alter the behavior of gastrointestinal SMCs by enhancing expression of inflammatory cytokines. On the other hand, high doses of insulin can augment cytokine production in the

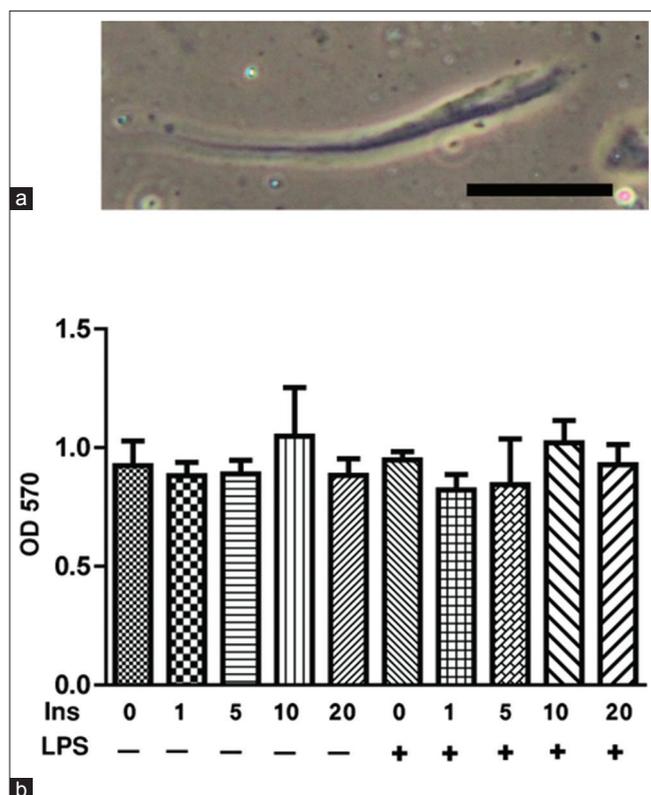


Fig. 1: (a) Isolation and identification of single mouse colon smooth muscle cells where they appeared in spindle shape under phase contrast microscopy. Cells were viewed through inverted Nikon TMS-f microscope, $\times 20$ magnification, and image captured with Canon digital camera. (b) cell viability assay for CSMCs treated with insulin (Ins) in various nanomolar doses with/without (Lipopolysaccharide) LPS at $1 \mu\text{g}/\text{mL}$ concentration

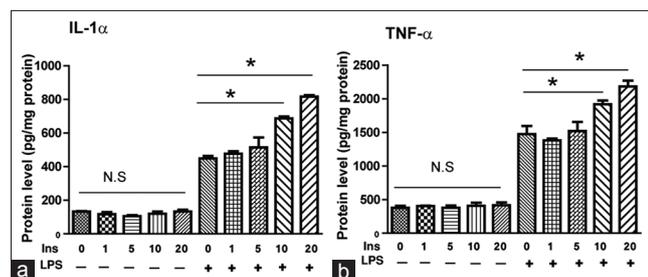


Fig. 2: Effect of insulin (Ins) treatment on the expression of inflammatory cytokines (a) interleukin and (b) tumor necrosis factor by mouse colon smooth muscle cells using ELISA in the presence or absence of inflammatory stimulus lipopolysaccharide. Cytokine levels were measured in the cellular lysate according to the manufacturer's protocol. * indicates a significant difference between groups where $p < 0.05$. Bars represent mean \pm standard error of the mean

presence of inflammatory stimulus, suggesting a possible link between hyperinsulinemia and IBD pathogenesis in obese subjects.

DISCUSSION

Substantial body of evidence suggests that intestinal SMCs are affected functionally and structurally by most inflammatory conditions involving the intestine, especially IBD, leading to altered contractility, morphology, and secretion of various inflammatory mediators, thereby contributing to the pathogenesis of IBD [24,27,28]. IBD is a chronic disease with

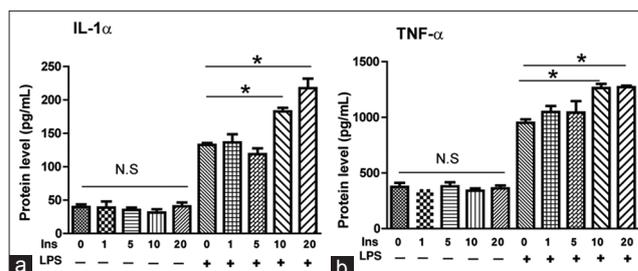


Fig. 3: Effect of insulin (Ins) treatment on the secretion of inflammatory cytokines (a) interleukin -1 α and (b) tumor necrosis factor- α by mouse colon smooth muscle cells using ELISA in the presence or absence of inflammatory stimulus lipopolysaccharide. Cytokine levels were measured in the conditioned media according to the manufacturer's protocol. *Indicates a significant difference between groups where $p < 0.05$. Bars represent a mean \pm standard error of the mean

multifactorial etiologies characterized by intestinal inflammation, extraintestinal symptoms, and elevated serum cytokine levels [29]. Several studies reported an increase in the prevalence of IBD in obese hyperinsulinemic patients [2,10]; however, the effect of insulin on development of inflammation in the bowel is not investigated. Here, we report that supraphysiological doses of insulin augment LPS-induced cytokine expression and secretion from CSMCs.

Clinical and experimental evidence suggests that hyperinsulinemia can contribute to the development of several diseases such as atherosclerosis, colon cancer, non-alcoholic steatohepatitis, and stroke [1]. However, contrasting information from different experiments show that insulin can exert both pro-inflammatory and anti-inflammatory effects in different tissues [13]. Animal experiments showed that diet rich in fructose markedly increased serum insulin levels and consequently increased reactive oxygen species, decreased nitric oxide production, and resulted in the activation of the transcription factor NF- κ B and expression of downstream inflammatory genes in the cardiovascular tissues [30]. Moreover, insulin has been shown to directly induce production of different cytokines such as IL-1 β , IL-8, CCL2, and OSM in U937 macrophages by an insulin-dependent activation of NF- κ B [18]. Furthermore, insulin activated pro-inflammatory gene expression through activation of PI3K independently from NF- κ B in vascular SMCs [17]. *In vitro* studies also showed that insulin amplified LPS-induced cytokines production in THP-1 monocytes [16].

On the other hand, insulin has been reported to exert anti-inflammatory effects in multiple tissues such as cardiomyocytes, hepatocytes, and vascular endothelial and SMCs in an NF- κ B-dependent manner in euglycemic or moderately hyperglycemic conditions [31]. Iwasaki *et al.* found that insulin exerts short-term anti-inflammatory effects mediated by PI3 Kinase-NF- κ B pathway and long-term pro-inflammatory effect through activation of MAPK-NF- κ B pathway in hepatocytes [13].

Our data suggest that high doses of insulin mimicking hyperinsulinemic state exerted a significant pro-inflammatory effect on mouse CSMCs *in vitro* in the presence of LPS and increased the expression and secretion of IL-1 α and TNF- α , suggesting a pathogenic role of hyperinsulinemia in IBD development.

IL-1 α has a central role in innate immunity and inflammation [32]; it plays a key role in the early phases of inflammation in experimental colitis and its tissue levels correlate with the severity of the disease [33]. High levels of IL-1 α have been reported in colonic tissues taken from IBD patients, where it is thought to enhance recruitment of neutrophils, stimulation of IL-6 production by macrophages, and promotion of intestinal tumorigenesis [34]. Moreover, specific blockade of IL-1 α receptor reduces the inflammatory responses associated with experimental colitis, suggesting an essential role of IL-1 α in IBD

pathogenesis [23]. Some of the effects of TNF- α are mediated through IL-1 α . IL-1 α expression is greatly enhanced during inflammatory conditions where it initiates a cascade of event to increase inflammatory cytokines and chemokines, hence accounting for the early phases of sterile inflammation. Increased expression and secretion of both IL-1 α and TNF- α together is considered an alarm cytokine signal that aggravates inflammatory responses by inducing a cascade of other pro-inflammatory genes [20].

TNF- α is potent pro-inflammatory cytokine produced as a transmembrane protein that is released as soluble cytokine by proteolytic cleavage by the metalloproteinase TNF-converting enzyme [35]. TNF- α is primarily secreted from activated macrophages as well as many other cell types. Several human diseases are associated with dysregulation of TNF- α production, and it has been reported that expression of both membrane-bound and soluble TNF- α by submucosal inflammatory cells is significantly increased in patients with IBD [35]. Expression of TNF- α in IBD stimulates different pro-inflammatory actions, such as initiation of systemic inflammatory response, increased angiogenesis, Paneth cell death, production of matrix metalloproteinase, and damage of intestinal epithelial cells [20]. Anti-TNF- α therapeutic antibodies such as infliximab and adalimumab are being used in the management of IBD to lessen the disease symptoms and suppress its progression, highlighting the role of TNF- α in the pathogenesis of IBD [21]. In our study, 10 or 20 mM insulin enhanced production of TNF- α from CSMCs in the presence of inflammatory stimulus LPS, suggesting an important role for hyperinsulinemia on CSMCs-driven TNF- α expression in the pathogenesis of gastrointestinal inflammatory diseases possibly through recruitment and activation of different inflammatory cells.

CONCLUSION

Although the CSMCs are important in the pathogenesis of IBD, their role is rarely explored compared to the role of inflammatory and epithelial cells. Their secretory functions are also overlooked since most studies focus on the morphological changes and altered contractility that occurs during colonic inflammation. Therefore, drugs and lifestyle changes that prevent obesity and hyperinsulinemia may confer great benefits in preventing IBD, through inhibition of intestinal smooth muscle's expression and secretion of inflammatory mediators.

AUTHOR CONTRIBUTIONS STATEMENT

ANA: Conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the manuscript, revising the manuscript critically for important intellectual content. OAA, MAA, AGM, and MA: Analysis and interpretation of data, revising the manuscript critically for important intellectual content.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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