

ORIGINAL ARTICLE

EFFECT OF VISFATIN ON TESTICULAR STEROIDOGENESIS IN PURIFIED LEYDIG CELLS

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Background: Lower testosterone levels have been reported in men suffering from diabetes mellitus. Men with insulin resistance states such as obesity and type 2 diabetes mellitus have significantly lower testosterone levels than age-matched normal weight and non-diabetic controls. Adipose tissue secretes variety of adipokines including adiponectin, resistin, visfatin, TNF- α . The study was designed to study the effect of visfatin on testicular steroidogenesis in purified Leydig cell *in vitro*. **Methods:** Leydig cells of Sprague Dawley rats were isolated and purified by Percoll. Cells were incubated for 3 hours with/without visfatin in the presence/absence of LH and intracellular signalling blockers including PKC blocker, PKA blocker and Raf1/Ras blocker. Cell culture extracts were stored at -80 °C before analysis for levels of testosterone hormone by ELISA. **Results:** Visfatin increased testosterone production ($p < 0.001$) from cultured Leydig cells. Raf1/Ras blocker decreased visfatin induced steroidogenesis ($p < 0.001$). **Conclusion:** Visfatin increased testicular steroidogenesis from Leydig cells *in vitro* models and operates through variety of enzymes, but especially through Ras/Raf1 kinase enzymes.

Keywords: Visfatin, adipocytokines, Leydig cells, testicular steroidogenesis

INTRODUCTION

An inverse correlation between serum testosterone and fasting insulin levels in men has been documented.¹ Men with type 1 and type 2 diabetes mellitus have significantly lower testosterone levels than age-matched normal weight and non-diabetic controls.² It has been suggested that the inverse relationship between testosterone and insulin is due to obesity.^{3,4}

Hormones, growth factors, and cytokines modulate Leydig cell steroidogenesis.⁵ Insulin receptors are present on Leydig cells⁶, and insulin stimulates testosterone production in Leydig cell cultures^{6,7}. Adipose tissue secretes a variety of adipokines, which include leptin and the newly discovered hormones adiponectin, resistin, visfatin, TNF- α . Leptin levels are inversely correlated with testosterone secretion.^{8,9}

Visfatin was first described in 2005 by Fukuhara *et al.*¹⁰ It has an insulin-mimetic action because it reduces blood glucose level. Visfatin binds to insulin receptors but does not compete with insulin, suggesting that the two proteins bind to different sites. Visfatin levels increase in plasma following a high-fat diet suggesting that it has an important role in diet or obesity-induced insulin resistance.¹¹ However the effect of visfatin on testicular steroidogenesis is yet to be evaluated.

MATERIAL AND METHODS

The project and relevant regulations governing the study were approved by the Committee for Postgraduate Studies, Army Medical College, Rawalpindi.

Rats were obtained from the National Institute of Health (NIH), Islamabad. They were kept in its

animal house facility where the temperature was maintained at 22 \pm 3 °C. Food and water was available *ad libitum*. The rooms were well ventilated and 12-hour light-dark cycle was maintained.

Medium 199 (M 199), LH, HEPES, Theophylline, Collagenase, Bovine Serum Albumin (BSA), Dulbecco's modified Eagles medium, Gentamycin, Percoll, Trypan blue, Nitro blue tetrazolium, Dehydroandrosterone, 25-hydroxy testosterone, β -NAD, Protein Kinase C inhibitor were purchased from Sigma Chemicals (St. Louis, MO). Phosphate buffer saline (PBS) (Anagen Technologies Inc, USA), Protein Kinase A inhibitor (Upstate Biotechnology), Ras/Raf1 Kinase Inhibitory Peptide and Visfatin, Soluble (rat) (recombinant) (His) from ALEXIS Biochemicals, AXXORA, USA were also used.

Two adult male Sprague-Dawley rats (90–120 days old) were obtained from NIH, Islamabad. After killing the rats by decapitation, testes were dissected out and decapsulated. Four decapsulated testes were treated with collagenase (0.25 mg/ml) to obtain dispersed cells. Briefly dispersed cells were diluted to a volume of 24 ml with M 199 containing 0.595 g/ml HEPES, 0.00009 g/ml Theophyllin, 1 μ l/ml Gentamycin and 0.1% BSA at pH 7.4. On the top of each preformed discontinuous Percoll gradient of 20 to 90% a 6 ml cell suspension was layered¹² and centrifuged for 25 min at 800 g at 27 °C. Third fraction was collected from the top of gradient, followed by dilution and centrifugation at 800 rpm at room temperature for 20 min. Pellet was re-suspended in M-199 with BSA. The purity of the Leydig cells was evaluated by 3- β -HSD staining method¹³ and obtained

at >80%. More than 90% viability was indicated by trypan blue exclusion test.¹⁴

Purified Leydig cells were incubated in M-199 with BSA at 37 °C for 3 hours along with LH, visfatin with/without Protein Kinase C inhibitor, Protein Kinase A inhibitor, and Ras/Raf1 Kinase Inhibitory Peptide. Number of Leydig cells/tube was 85,000. The samples were run in triplicate. Cell culture tubes were placed in a 60 °C water bath for 30 minutes to stop the incubation. Testosterone production was measured by EIA kit from Cayman Chemicals, USA.

Data were analysed using SPSS-13. Groups were compared using ANOVA. Tukey test was used for Post Hoc Comparison, and $p < 0.05$ was taken as statistical significant.

RESULTS

Visfatin resulted in significant increase of LH supported testosterone production by rat Leydig cells in a dose dependent fashion. Maximum stimulation was observed with 10^{-6} M visfatin. The Leydig cells were then incubated with 25-Hydroxy Cholesterol in the presence/absence of visfatin and LH. Visfatin was not able to increase 25-Hydroxy Cholesterol stimulated steroidogenesis ($p > 0.05$) significantly (Table-1). This implied that visfatin acted at a level before the StAR protein and cyt P450 scc enzyme.

In order to further investigate the intracellular mechanism of action of visfatin, Leydig cells were incubated with either Protein Kinase C inhibitor, Protein Kinase A inhibitor and Ras/Raf1 Kinase Inhibitory Peptide or combination of these intracellular enzyme blockers, in the presence/absence of visfatin and LH. (Table-1)

Table-1: Combined effect of LH and Visfatin on testosterone release by Leydig cells of rat

Sample	Mean Testosterone (pg/85,000 cells/3 hr)
Basal levels (without LH)	71.68±6.23
LH 1,000 ng	220.12±4.20
Visfatin 10^{-10} M with LH 1,000 ng	229.12±2.14
Visfatin 10^{-8} M with LH 1,000 ng	2.41±1.15
Visfatin 10^{-6} M with LH 1,000 ng	286.23±4.21*
25 Hydroxycholesterol 10 µg, LH 1,000 ng	310.12±1.21
25 Hydroxycholesterol 10 µg, LH 1,000 ng, Visfatin 10^{-8} M	312.21±1.08†

LH=Luteinizing Hormone, *LH 1,000 ng with Visfatin 10^{-6} M vs LH 1,000 ng ($p < 0.001$), †25 Hydroxycholesterol 10 µg, LH 1,000 ng with Visfatin 10^{-8} M vs LH 1,000 ng ($p > 0.05$)

Both, Protein Kinase C inhibitor and Protein Kinase A inhibitors reduced testosterone production from Leydig cells, individually and synergistically, in the presence of visfatin and LH. However, Ras/Raf1 Kinase Inhibitory Peptide produces most significant ($p < 0.001$) inhibition of visfatin and LH stimulated Leydig cell steroidogenesis (Table-2). This implied that

the site of stimulation of testosterone production by visfatin was before the level of StAR protein at the intracellular enzymes of Leydig cells, especially the Ras/Raf1 kinase enzyme system.

Table-2: Combined effect of LH, Visfatin and intracellular blockers on testosterone release by Leydig cells of rat

Sample	Mean Testosterone (pg/85,000 cells/3 hr)
LH 1,000 ng	220.12±4.20
Visfatin 10^{-6} M with LH 1,000 ng	286.23±4.21
Visfatin 10^{-6} M, LH 1,000 ng, PKC blocker 20 µM	241.12±1.05*
Visfatin 10^{-6} M, LH 1,000 ng, PKA blocker 20 µM	264.13±1.43**
Visfatin 10^{-6} M, LH 1,000 ng, Ras/Raf1 kinase blocker 20 µM	229.21±1.09†
Visfatin 10^{-6} M, LH 1,000 ng, PKC blocker and PKA blocker (20 µM each)	239.12±1.21**†
Visfatin 10^{-6} M, LH 1,000 ng, PKC, PKA and Ras/Raf1 kinase blocker (20 µM each)	196.13±1.31***†

LH=Luteinizing Hormone, * $p < 0.05$, ** $p < 0.05$, † $p < 0.001$, ** $p < 0.05$, *** $p < 0.001$ vs Visfatin 10^{-6} M with LH 1,000 ng

DISCUSSION

Visfatin was originally identified as pre β colony enhancing factor (PBEF), and was found to have a role in the maturation of B cell precursors.¹⁵ Later on visfatin was characterised as an adipokine with a high expression in visceral fat exhibiting insulin-like functions.¹⁰ These insulin mimetic actions of visfatin are mediated through binding to the insulin receptor at a site separate from that of insulin.¹⁶

Incubation of Leydig cells with varying concentrations of LH for three hours resulted in a significant rise in the testosterone release in a dose dependent fashion as compared to the basal release of testosterone. This is in accordance with literature^{5,17} as it is an established fact that LH/hCG stimulates Leydig cell steroidogenesis.

Our data presented here signifies that visfatin directly stimulated Leydig cell steroidogenesis and the site of steroidogenic stimulation caused by visfatin was prior to the formation of pregnenolone. These steroidogenic steps included the transport of cholesterol from outer to inner mitochondrial membrane via steroidogenic acute regulatory (StAR) protein, and the use of cholesterol by Cyt P450 scc enzyme. This was evident because visfatin was not able to increase 25-hydroxy cholesterol stimulated steroidogenesis. Various studies have demonstrated that insulin receptors are present on Leydig cells⁶, and insulin stimulates testosterone production in Leydig cell cultures⁷. Since visfatin acts on the insulin receptor^{10,18}, it is therefore plausible that visfatin had the same effect as that of insulin.

Our results suggest that visfatin alone caused a significant increase in testosterone production from

Leydig cells in the presence of LH 1,000 ng. However the intracellular mechanism of visfatin action remains complex. Various studies^{19,20} have implicated the importance of intracellular enzymatic pathways in testosterone synthesis by the Leydig cell. It seems that PKC and PKA blocker alone and synergistically cause a decrease in testosterone production. However the most significant decrease in testosterone production is seen when all three blockers are simultaneously used. It implies that most of the action of visfatin is via Ras/Raf1 kinase system and less through PKC and PKA system of enzymes.

Previous studies²¹ have demonstrated the inhibiting effect of leptin on testicular steroidogenesis *in vitro*. Thus it appears that adipocytokines may play a major role in decreased testosterone levels in diabetic patients.

It is pertinent to mention that in the present study experiments were performed on Percoll purified Leydig cell preparations which had only 82% purity. As such the presence of various paracrine factors which could be released from Sertoli and germ cells and may have mediated the effects of visfatin cannot be ruled out.

CONCLUSION

Visfatin increases serum testosterone levels by causing direct stimulation of Leydig cell steroidogenesis before the level of StAR protein. As such further studies may be carried out *in vivo* and in human subjects to give visfatin as an adjunct to insulin in diabetic patients to improve their testosterone secretion.

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of bioavailable, free, and total testosterone with insulin resistance:

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