Modulation of Cytokines, Adiponectin and Leptin Expression in Mature Bovine Adipocytes by Serotonin

Mohamed Mohamed Soliman, Zein Shabaan Ibrahim, Abeer Abdel Aleem Abdel Aal and Randa Saad Ismaiel

Abstract: Serotonin (5-HT) is an important neurotransmitter in the mammalian central nervous system (CNS) and synthesized from gastric and intestinal mucosa. It is involved in numerous physiological and behavioral disorders such as major depression, anxiety, schizophrenia, obesity and drug addiction. Here we tested the immuno-modulatory effects of serotonin on various adipokines expression in mature bovine adipocytes. Isolated mature adipocytes were incubated with either lipopolysaccharide LPS (1\mu g/ml), serotonin 1\mu M, or LPS for 2 h then with serotonin for 24 h. Using RT-PCR analysis, various adipokines that are secreted and expressed in adipocytes was examined. The results showed that treatment of cells by LPS stimulated the expression of IL-1\beta, IL-8, SOC3, adiponectin and leptin mRNA but has an inhibitory effect on mRNA expression of TNF-\alpha and PPAR-\gamma. Serotonin treatment has no effect on IL-1\beta, TNF-\alpha, IL-8 and SOC3 mRNA but stimulated adiponectin, leptin and LPL expression. Prior treatment of cells by LPS (2h) then serotonin for 24 h induced additive stimulatory effect on IL-1 and IL-8 expression but inhibited LPS induced adiponectin, leptin and LPL expression. The results indicate that serotonin has both immuno-modulatory effect during infection and inflammation, and an anti-obesity effect through its effect on adiponectin and leptin in normal state. Those effects probably induced at the transcriptional level of both PPAR-\gamma and LPL in bovine adipocytes.

Key words: adipokines expression, bovine adipocytes, LPS, serotonin

INTRODUCTION

Serotonin is a neurotransmitter synthesized from the amino acid tryptophan so named 5-hydroxytryptamin (5-HT) and is present in the brain and peripheral tissues. It has a variety of physiological functions including regulation of temperature and memory. It can affect the activities of the gastrointestinal tract and the cardiovascular system. Serotonin is released by blood platelets during inflammatory processes and may interact with circulating immune cells.

During inflammation, the activated blood platelets release serotonin and that leads to an increase in its local concentrations at the inflamed region (Benedict et al., 1986 and Medzhitov et al., 1997). In infectious diseases caused by Gram-negative bacteria, endotoxin (lipopolysaccharide, LPS) is released to activate the serotonin containing platelets by a direct mechanism (Timmons et al., 1986). Therefore, serotonin interacts with the inflamed tissue macrophages to secret various cytokines. The immunomodulatory role of serotonin on immune cells including B, T and NK cells, and monocytes/ macrophages has been documented (Iken et al., 1995). The presence of serotonin at the site of inflammation suggests its possible involvement in the control of this equilibrium between inflammatory and anti-inflammatory cytokines.
Results from several studies indicate that serotonin up-regulates the proliferation of T-cells in humans and plays a role in immune regulation (Cloez-Tayarani & Changeux 2007) in humans and rodents. It up-regulates the expression of interleukin (IL)-2 by human T-cells and differentially modulates the production of monocyte-derived cytokines such as tumor-necrosis-factor (TNF) and IL-1β in human peripheral blood mononuclear cells (PBMC) after induction by LPS (Cloez-Tayarani et al., 2003). Serotonin exerts its function through a well known membrane receptors which represents 14 distinct receptor subtypes in humans (Hoyer and Martin 1997). A number of 5-HT receptor subtypes reported in various cells (Khan & Poisson 1999 and Stefulj et al., 2000) which include 5-HT1A, 5-HT3, 5-HT1B, 5-HT1F, 5-HT2A, 5-HT2B, 5-HT3, 5-HT6 and 5-HT7 receptor subtypes have also been detected in humans and rats lymphocytes.

White adipose tissue is an endocrine organ that secretes leptin that acts as anti-obesity protein together with other secreted factors known as adipokines or adipocytokines. They are pharmacologically active, exhibiting both beneficial and pathologic effects on the target cells. Imbalanced expression of cytokines has been implicated in the progression of many diseases [Arend & Gab 2004]. Some established data in rodents and humans showed the importance of adipose tissue in regulation of inflammation and defensive mechanism in different diseases [Trayhurn & Beattie 2001]. Cytokines such as IL-1, TNF-α and IL-6 are proteins involved in lipid metabolism and in inflammation and stress response such as haptoglobin (Mohamed-Ali et al., 1998 and Trayhurn & Beattie 2001). Adipose tissue secretes proteins that have a direct relation with lipolysis and lipogenesis such as peroxisome proliferator activated receptor gamma (PPAR-γ) and lipoprotein lipase (LPL). Leptin regulates body weight and metabolism and may also act as a metabolic signal to the reproductive axis (Friedman & Halaas 1998). Recently it has been shown that leptin may act on serotonergic cells in the brain to mediate some of its effects on digestive behavior, metabolism, and reproduction (Finn et al., 2001). Serotonin increases with feeding just like leptin. It is believed to be a potent inhibitor of feeding behavior (Fletcher & Paterson 1989). Depletion of serotonin increases food intake and promotes obesity (Breisch et al., 1976) indicating that serotonin could be a mediator of leptin’s actions possibly by alteration in some adipokines. For our knowledge, the exact interaction between serotonin and adipokines secreted from adipocytes is not elucidated up till now. Therefore, this study was undertaken to test the relationship between serotonin and cytokines by testing its effect on the expression of TNF-α, IL-1β, IL-8, SOCS, LPL, PPAR-γ, adiponectin and leptin in bovine adipocytes alone and following LPS challenge.

MATERIALS AND METHODS

Materials:

Dulbecco’s modified Eagles’s medium (DMEM), bovine serum albumin (BSA), Hank’s balanced salt solution and serotonin were bought from Sigma–Aldrich Fine Chemical (St. Louis, MO, USA). Fetal calf serum (FCS) was from Trace Scientific Ltd. (Melbourne, Australia). LPS and collagenase were purchased from Wako Pure Chemicals Co. (Osaka, Japan). Serotonin was prepared as described in manufacture catalogue.

Isolation of Mature Adipocytes:

Subcutaneous adipose tissue was obtained from 3 non-pregnant, non-lactating healthy Holstein cows (5 years old) and dissected into small pieces in Hank’s balanced salt solution containing 2 mg/ml collagenase and 0.1% BSA in sterile 50 ml plastic tube. Following digestion at 37 °C for 90 min with gentle shaking, the solution was filtered through sterile nylon mesh with 80 μm pores. The filtrate was centrifuged at 1000×g for 5 min at room temperature. Mature adipocytes (supernatant cells) were washed twice with DMEM containing 10% FCS, 100 U /ml penicillin, and 100μg/ml streptomycin. The cells were counted by haemocytometer and the percentage of live to dead cells was encountered by trypan blue staining. 5X10⁶ cells were used for each ml media. The cells were cultured in DMEM (2.5 mM glucose) containing 10% FCS 6-well plates under different experimental conditions for 24 h.

Experimental Procedures and RT-PCR Analysis:

Cells were incubated with PBS as control or LPS (μg ml⁻¹) and/or with serotonin (1μM). LPS and serotonin were dissolved in PBS. Cells were incubated at 37 °C in 5% CO2 for 24 h. Total cellular RNA was isolated from cultured adipocytes by the guanidine-isothiocyanate method using TRizol reagent (Gibco BRL, Rockville, MD, USA). RNA (2μg) was treated at 72 °C for 5 min and reverse transcribed using 100 units of Moloney murine leukemia virus reverse transcriptase (Gibco), 50 pmol of poly (dT) primer and 20 nmol of dNTPs in a total volume of 10 μl at 37 °C for 1 h. After heating at 94 °C for 5 min, PCR amplification was performed with 2.5 units Taq polymerase (Perkin-Elmer, Foster City, CA, USA), 3 mM MgCl2 and 50 pmol
of forward and reverse primers specific for respective genes in a total volume of 50 μl. The PCR conditions for different tested genes are shown in Table 1. After electrophoresis in 1.5% agarose gel, the PCR products were stained with ethidium bromide and visualized under UV lamp. Intensities of PCR bands were analyzed densitometrically using NIH Image program (http://rsb.info.nih.gov/nih-image/).

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Treatments</th>
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<tr>
<td>G3PDH (452 bp)</td>
<td>5'-ACCACTGTCCACGCAATCAC-3'</td>
<td>5'-TCCACACCCCTTGTGCCTGA-3'</td>
<td>Annealing at 59 °C for 30 sec for 25 cycles</td>
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<tr>
<td>TNF-α (590 bp)</td>
<td>5'-TAACAAGGCCGATGCCCCAG</td>
<td>5'-GCAAGGGGCTCTTGATGGCAGA-3'</td>
<td>Annealing at 61 °C for 30 sec for 30 cycles</td>
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<tr>
<td>IL-1β (726 bp)</td>
<td>5'-ATGGCAACGTACCTGAAACCA-3'</td>
<td>5'-GCTGAAATGTCCCGAGA-3'</td>
<td>Annealing at 60 °C for 1 min for 28 cycles</td>
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<tr>
<td>Bovine leptin (286 bp)</td>
<td>5'-GCGACTGCTTCCCTCCTGACAG-3'</td>
<td>5'-TCGTTGGAGTAGAGGGAAGG-3'</td>
<td>Annealing at 61 °C for 70 sec for 33 cycles</td>
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<tr>
<td>Adiponectin (360 bp)</td>
<td>5'-GCCGCTTATGATGTCGCTCAG-3'</td>
<td>5'-TGCAATGACCCCATTGTGATT-3'</td>
<td>Annealing at 60 °C for 75 sec for 30 cycles</td>
</tr>
<tr>
<td>IL-8 (308 bp)</td>
<td>5'-ATGACTCCACCTCGGGCTGTCG-3'</td>
<td>5'-TCCTGGATCTTCTGCTAGGCT-3'</td>
<td>Annealing at 58 °C for 1 min for 30 cycles</td>
</tr>
<tr>
<td>PPAR-γ (214 bp)</td>
<td>5'-GCAGCCTGGAATTCATGACTGGACGC-3'</td>
<td>5'-CACAAATGCTGAGGCTACGTG-3'</td>
<td>Annealing at 55 °C for 60 s, 30 cycles</td>
</tr>
<tr>
<td>LPL (294 bp)</td>
<td>5'-GCAGAGGATGTCCTGGGCAATAA-3'</td>
<td>5'-CTTCACCAAGGTCCTCCACAT-3'</td>
<td>Annealing at 58 °C for 30 sec for 30 cycles</td>
</tr>
<tr>
<td>SOCS3 (360 bp)</td>
<td>5'-GCCTTACACTCCTACTGCGGG-30</td>
<td>5'-AAGCGGGGCCATCTGTTG-3'</td>
<td>Annealing at 60 °C for 30 sec for 30 cycles</td>
</tr>
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PCR cycle of respective genes are shown, while temperature and time of denaturation and elongation steps of each PCR cycle are 94 °C, 30 s and 72 °C, 60 s, respectively.

Statistical Analysis:
All data were expressed as means±SEM and analyzed by Dunnett's t-test for multiple comparisons with a single control group using specific program (StatView Version-5; SAS Institute, Inc USA) for Macintosh computers. Significance was reported as *P*< 0.05.

Results:
Effect of Serotonin on IL-1β, TNF-α, IL-8 and SOCS3 mRNA Expression:
In Fig.1 and 2, treatment of bovine adipocytes by LPS induced (*P*<0.05) mRNA expression of IL-1β, IL-8 and SOCS3 mRNA expression. During disease and infection, LPS is secreted by gram negative bacteria inducing inflammation. The different body cells are in turn stimulated to secret cytokines that induce anti-inflammatory mechanisms. As shown in figures 1 and 2 serotonin has no effect on the expression of cytokines in non inflammatory conditions. But pre-treatment of cells with LPS for 2 h then serotonin for 24 h induced significant (*P*<0.05) additive stimulatory effect on mRNA expression of IL-1β and IL-8 only but not on SOCS3 expression and TNF-α. Of note, LPS when incubated with cells alone, it inhibited TNF-α expression. Serotonin has no effect on LPS inhibited TNF-α expression and so no effect on LPS induced SOCS expression.

Effect of Serotonin on Adiponectin and Leptin mRNA Expression:
Interestingly, the results in this study show that serotonin has direct stimulatory effect on 2 major adipokines that play a crucial role in maintaining body homeostasis and insulin sensitivity. As seen in Fig.3, both LPS and serotonin individually up-regulated significantly leptin and adiponectin mRNA expression. But when the cells incubated with both LPS and serotonin this stimulatory effect was abolished as serotonin inhibited LPS induced leptin and adiponectin expression.

Effect of Serotonin on PPAR-γ and LPL mRNA Expression:
To test the role of serotonin in lipid metabolism, we examined the expression of PPAR-γ and LPL in bovine adipocytes. PPAR-γ stimulates lipogenesis while LPL inhibit lipogenesis and stimulate lipolysis and fatty acids oxidation. Fig.4 shows that LPS inhibited PPAR-γ expression without any alteration on LPL expression. On the same time serotonin alone has no effect on PPAR-γ but stimulated LPL expression and that support the idea that serotonin has anti-obesity function. When the cells treated with LPS then serotonin, LPL expression was abolished in presence of LPS as seen in Fig.4.
Fig. 1: Effect of serotonin and LPS on IL-1β and TNF-α expression in bovine adipocytes. Mature bovine adipocytes were cultured in 6-well plated at 5X10^6 per well with or without serotonin (1μM) in presence or absence of LPS (1μg/ml). 2 μg RNA was extracted and reverse transcribed using RT-PCR analysis for IL-1β and TNF-α expression. The bands of both genes were visualized using 1.5% gel stained with ethedium bromide. Intensity of expression was normalized with that of GAPDH (as internal standard) using NIH image for Densitometric analysis. Values are means±SEM obtained from 3 experiments. *P< 0.05 vs. control, and # p<0.05 vs. LPS.

Fig. 2: Effect of serotonin and LPS on IL-8 and SOCS3 expression in bovine adipocytes. Mature bovine adipocytes were cultured as described in Fig.1. 2 μg RNA was reverse transcribed and subjected to RT-PCR analysis using specific primers for IL-8, SOCS3 and GAPDH as internal standard. Values are means±SEM obtained from 3 experiments. *p<0.05 vs. control and # p<0.05 vs. LPS.

Discussion:
The present findings showed that serotonin has immuno-modulatory effect on LPS induced IL-1β and IL-8 expression but not TNF-α and SOCS. Serotonin act as potent inhibitors of TNF release but display opposite effects on IL-1β and IFN-γ productions (Cloez-Tayarani & Changeux 2007). When adipocytes treated by serotonin alone, it did not modulate IL-1β and IL-8 expression but during inflammation it stimulated their expression. (Figs.1 & 2). Most of antidepressants induced its action through inhibition of serotonin reuptake and consequently release some cytokines as IL-1β and IL-8 as reported in PBMC as well as adipocytes.
Fig. 3: Effect of serotonin and LPS on adiponectin and leptin expression in bovine adipocytes. Mature bovine adipocytes were cultured as described in Fig.1. 2 μg RNA was reverse transcribed and subjected to RT-PCR analysis using specific primers for leptin, adiponectin, and GAPDH as internal standard. Values are means±SEM obtained from 3 experiments. *p<0.05 vs. control, and # p<0.05 vs. LPS.

Fig. 4: Effect of serotonin and LPS on PPAR-γ and LPL expression in bovine adipocytes. Mature bovine adipocytes were cultured as described in Fig.1. 2 μg RNA was reverse transcribed and subjected to RT-PCR analysis using specific primers for PPAR-γ, LPL and GAPDH as internal standard. Values are means±SEM obtained from 3 experiments. *p<0.05 vs. control, and # p<0.05 Vs. serotonin.

Although cell variation was reported by various studies [Cloez-Tayarani et al., 2003; Heisler et al., 2003 and 1999], the increase in cytokines expression is followed by the expression of transcriptional factor that is known as suppressor of cytokines signaling (SOCS3) to regulate the defensive mechanism of the body. Here, LPS induced SOCS3 expression but serotonin did not alter this induction and serotonin potentiates the expression of anti-inflammatory cytokines.

LPS inhibited TNF-α expression and in presence of serotonin a little additive inhibitory effect although it is not significant but clear (Fig. 2). The possible cause is the induction of adiponectin expression as the antagonistic effect of both adiponectin and TNF-α on each other was confirmed in study of Kapes & Loffler (2000). Therefore, adiponectin may be a local regulator of inflammation in the adipocytes and adipose tissue via its regulation of TNF, NF-kB and PPAR-γ2 transcriptional factors expression in line with a previous study (Ajuwon & Spurlock 2005). Pi-Sunyer, (2006) reported that sibutramine, a serotonin and norepinephrine reuptake inhibitor produces an increase in serum adiponectin and that is parallel with our results. Moreover, Arzt et al. (1991) showed that serotonin inhibits LPS-induced production of tumor necrosis factor by human
macrophages. This effect was blocked by the 5-HT2 receptor antagonist, ketanserin. There is also evidence that the production rate of pro-inflammatory cytokines, such as IL-1, IL-6 and IFNγ is increased in patients with major depression (Connor & Leonard 1998).

Serotonin is the major appetite-modulating neurotransmitter and a potent anorexigenic agent (Leibowitz et al., 1990). Acute administration of serotonin as well as fenfluramine, a serotonin reuptake inhibitor, induces an increase in metabolic rate of ~21% in lean rats which supported its role in obesity (Rothwell & Stock 1987). The potential functional interactions among serotonin and its receptors and different neuromodulatory systems could be involved in the physiological regulation of appetite (Guiterez et al., 2002).

Activation of TLR4 (a known cell receptor of LPS) initiates a cascade of intracellular events including the transcription factor nuclear factor KB and causes the release of a number of proinflammatory mediators, such as IL-1, IL-6, and IL-8 (Medzhitov et al., 1997). IL-8 is a chemokine produced by a wide variety of cell types in response to LPS (Yoshimura et al., 1987). So when serotonin incubated with LPS it induced additive stimulation for IL-1β and IL-8 expression (Fig. 1 and 2). Serotonin levels affect its function as reported by Cavaillon (1996). He reported that serotonin concentrations close to the physiological concentrations have immuno-stimulating effects, whereas higher concentration has immunosuppressive effects. Here, we used 1 µM serotonin and that concentration within physiological range and there is a possible interaction between LPS and serotonin receptors.

Serotonin effect on different cytokines production displays opposite effects even in cells of same function. This is true for TNF-α and L-1β, which are, respectively, decreased and increased by serotonin in human PBMC (Cloez-Tayarani et al., 2003), and human monocytes (Durk et al., 2005) and that parallel to our findings although no available information about the effect of serotonin in isolated adipocytes is established. Disruption in serotonin receptors (5-HT2c) indicated that central neural mechanisms in the regulation of feeding and autonomic outflow are not always in parallel and a dysfunction of autonomic neural circuits rather than feeding behaviour could be the primary causative factor in obesity (Nonogaki 1999).

It has been established that central serotonin is associated with food intake and body weight regulation and the pharmacological agents that increase its level in CNS inhibit food intake and promote weight loss (Heisler et al., 1999). Here in this study shows that serotonin may induced its effect through stimulation of leptin mRNA expression (Fig.3) although leptin inhibits serotonin release in rat brain as recorded by Clark et al. (2005).

The confirmative role of serotonin as anti-obesity factor is its effect on LPL and PPAR-γ expression (Fig. 4). LPL is the rate-limiting enzyme in the hydrolysis of serum triglycerides derived from the triglyceride-rich lipoprotein particles (very low density lipoproteins (VLDL) and chylomicrons (Knutson 2000). Here, the increase in LPL expression may be secondary to the increase in leptin expression as leptin induced its action through LPL as reported by Friedman and Halaas (1998). PPAR-γ is the nuclear transcriptional factor that stimulates lipogenesis so PPAR-γ agonist is the main components of differentiating medium of immature adipocytes. There is inverse correlation between leptin and PPAR-γ as leptin inhibition is occurred in presence of PPAR-γ agonists (Williams et al., 2000). We can conclude that serotonin induce its effects through the expression of other adipokines as leptin, LPL, adiponectin which inhibit PPAR-γ expression. We can conclude that serotonin is an immuno-modulatory neurotransmitter with anti-obesity like effects through its effect on obesity related genes.

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