Molecular pathways involved in metabolic control of CCL5 in adipocytes

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PhD Student: Federica Passaretti
To my family

“The family is a link to our past, a bridge to our future.”

Alex Haley
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<td>ANG-II</td>
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<tr>
<td>aP2</td>
<td>adipocyte protein 2</td>
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<td>apoE</td>
<td>apolipoprotein E</td>
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<td>ATF2</td>
<td>activating transcription factors 2</td>
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<td>ATM</td>
<td>adipose tissue macrophage</td>
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<td>BAT</td>
<td>brown adipose tissue</td>
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<td>BMI</td>
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<td>CNS</td>
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<td>DTH</td>
<td>delayed-type hypersensitivity</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>Elk-1</td>
<td>ETS domain-containing protein</td>
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<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
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<td>FA</td>
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<td>free fatty acid</td>
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<td>fibroblast growth factor</td>
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<td>insulin-like growth factor binding protein</td>
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<td>IGT</td>
<td>impaired glucose tolerant</td>
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<td>IkBα</td>
<td>nuclear factor kB inhibitor alpha</td>
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<td>insulin receptor substrate 1-4</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>lipoprotein lipase</td>
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<td>mitogen-activated protein kinase</td>
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<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
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<td>MIF</td>
<td>macrophage migration inhibitory factor</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>NF-κB</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<td>NK</td>
<td>natural killer</td>
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<td>NLS</td>
<td>nuclear localization signals</td>
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<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
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<td>PET/TC</td>
<td>positron emission tomography/computed tomography</td>
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<td>PI3K</td>
<td>phosphatidylinositol 3 kinase</td>
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<td>PKB/AKT</td>
<td>protein kinase B</td>
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<td>PPARs</td>
<td>peroxisome proliferator-activated receptors</td>
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<td>RANTES/CCL5</td>
<td>regulated on activation normal T cell expressed and secreted/CCL5</td>
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<tr>
<td>SCD1</td>
<td>stearoyl CoA desaturase</td>
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<tr>
<td>SYP</td>
<td>SH-PTP2 Src homology domain tyrosine phosphatase</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 Diabetes</td>
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<tr>
<td>TG</td>
<td>triglyceride</td>
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<td>transforming growth factor-beta</td>
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<td>TH</td>
<td>T helper</td>
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<td>TLR4</td>
<td>toll-like receptor 4</td>
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<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
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<td>TNF receptor 1</td>
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<td>T_REG</td>
<td>T regulatory</td>
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<tr>
<td>VMN</td>
<td>ventro-medial hypothalamic nucleus</td>
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<td>WAT</td>
<td>white adipose tissue</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Abstract

Obesity is a chronic disorder characterized by a tonic low-grade activation of the innate immune system that affects steady-state measures of metabolic homeostasis over time. In addition, obesity is often accompanied by elevations in tissue and circulating FFA concentrations. Systemic levels of FFAs can induce inflammatory cascades in adipocytes and macrophages through TLR4-dependent effect. Signaling through TLR4 activates a broad range of intracellular cascades that include stimulation of IKK-β, NF-kB, JNK and AP1. Indeed, in addition to store excess calories in the form of lipid, adipose tissue produces classical cytokines and chemokines such as MCP-1, IL-8 and CCL5. CCL5, as other chemokines, participates in mediating leukocyte infiltration of adipose tissue. Moreover circulating CCL5 concentrations are elevated in obesity, impaired glucose tolerance (IGT) and type 2 diabetes. In this study I have investigated the molecular mechanisms involved in the metabolic control of CCL5 expression in adipocytes. Cytokine/growth factor screening of conditioned media from 3T3-L1 pre-adipocytes and adipocytes revealed that adipocytes secreted higher amount of CCL5 compared to their undifferentiated precursors. Higher concentrations of glucose and fatty acids (oleate and palmitate) increased CCL5 secretion by 3T3-L1 adipocytes. Moreover, both oleate and palmitate enhanced CCL5 mRNA levels and induced an activation of JNK, NF-kB, MAPK and PI3K/AKT pathways. In cells treated with JSH23, a NF-kB inhibitor, the effect of FFAs on CCL5 mRNA levels was reduced thus indicating a direct involvement of NF-kB. Treatment of the cells with SP600125, a JNK inhibitor, also significantly reduced the stimulatory effect of oleate and palmitate on CCL5 mRNA and interestingly prevented FFA-induced NF-kB binding to CCL5 promoter. I have also obtained evidence that insulin exerted an inhibitory effect on CCL5 mRNA and counteracted fatty acid-induced stimulation. Both PD98059 and LY294002, inhibitors of MAPK and PI3K, respectively, increased CCL5 expression levels reverted anti-inflammatory effect of insulin in presence of fatty acids. Consistently, insulin exposure reduced NF-kB recruitment onto CCL5 promoter, and almost completely prevented fatty acid effect. In conclusion, oleate and palmitate induce CCL5 mRNA, possibly via JNK and NF-kB pathways. Fatty acid effect on CCL5 is largely prevented by insulin and may involve PI3K/AKT and MAPK.
SOMMARIO

L’obesità è una malattia cronica caratterizzata da un’infiammazione di basso grado e da elevate concentrazioni tissutali e plasmatiche di acidi grassi liberi. Gli acidi grassi possono indurre la stimolazione di recettori posti sulla membrana di adipociti e macrofagi, come ad esempio il TLR4, e attivare pathway infiammatori (IKK-β, NF-kB, JNK e AP1). Il tessuto adiposo immagazzina l’eccesso di calorie sotto forma di lipidi e produce numerose citochine e chemochine come ad esempio MCP-1, IL-8 e CCL5. CCL5, come altre chemochine, è coinvolta nei processi di infiltrazione leucocitaria del tessuto adiposo. Sono stati riscontrati elevati livelli circolanti di CCL5 in soggetti obesi, in individui con alterata tolleranza al glucosio e pazienti con diabete mellito di tipo 2. In questo studio, ho indagato i meccanismi molecolari coinvolti nel controllo metabolico dell’espressione di CCL5 negli adipociti. L’analisi di sovranaanti cellulari ha rivelato che adipociti murini 3T3-L1 rilasciano elevati livelli di CCL5 rispetto ai loro precursori indifferenziati. Il trattamento con alte concentrazioni di glucosio e acidi grassi (oleato e palmitato) aumenta la secrezione di CCL5 da parte degli adipociti 3T3-L1. Inoltre, sia oleato che palmitato incrementano i livelli di mRNA di CCL5 e inducono un’attivazione dei pathway di JNK, NF-kB, MAPK e PI3K/AKT. In cellule trattate con l’inibitore specifico di NF-kB, JSH23, l’effetto degli acidi grassi sui livelli di mRNA di CCL5 è ridotto, suggerendo un diretto coinvolgimento di NF-kB. Anche il trattamento con SP600125, inibitore specifico di JNK, riduce significativamente l’effetto di oleato e palmitato sul messaggero di CCL5 e, inoltre, previene il binding di NF-kB sul promotore di CCL5. Sono state anche ottenute evidenze che l’insulina esercita un effetto inibitorio sull’espressione di CCL5 e contrasta la stimolazione indotta dagli acidi grassi. Il trattamento con gli inibitori delle vie delle MAPK e della PI3K, PD98059 e LY294002, rispettivamente, aumenta l’espressione di CCL5 opponendosi all’effetto anti-infiammatorio dell’insulina. Infine, il trattamento con l’insulina riduce il reclutamento di NF-kB sul promotore di CCL5, e previene quasi completamente l’effetto degli acidi grassi. In conclusione, oleato e palmitato inducono il messaggero di CCL5 via JNK e NF-kB. L’insulina previene l’effetto degli acidi grassi su CCL5 e questo meccanismo potrebbe essere mediato da PI3K/AKT e MAPK.
1. BACKGROUND

1.1 Adipose tissue

Energy stores are depleted intermittently during sleep-wake cycles and over prolonged periods during famine or illness. To maintain vital cellular functions, human and other mammals consume more calories than is required for immediate metabolic needs and store excess calories as glycogen, protein and lipids. Adipose tissue, commonly called ‘fat’, is a type of loose connective tissue comprised of lipid-filled cells (adipocytes) surrounded by a matrix of collagen fibers, blood vessels, fibroblasts and immune cells (Ahima and Flier 2000). There are different kinds of adipose tissues that are generally regarded as connective tissues without a specific anatomy (Figure 1).

![Figure 1: Comparative image of two different kinds of adipose tissue. White adipose has little cytoplasm and has huge oil droplet (A). Brown adipose tissue has more capillaries and has much more cytoplasm (B).](image)

However, accumulating data support the idea that adipose tissues are organized to form a large organ with discrete anatomy, specific vascular and nerve supplies, complex cytology, and high physiological plasticity (Cinti 2011).

Among adipose tissue kind, Brown adipose tissue (BAT) is a specialized tissue whose main function is to produce heat. In small mammals, it is located in discrete depots and has largely been investigated for its role in thermogenesis (Richard et al. 2012). There are many evidences that BAT is not only found in small mammals but also in adult humans. A number of
studies involving imaging procedures such as positron emission tomography/computed tomography (PET/CT) scanning have revealed symmetrical distribution of metabolically active brown fat depots around cervical, clavicular, and paraspinal regions in humans (Richard et al. 2012). White adipose tissue (WAT), the predominant type of adipose tissue in humans, provides a virtually limitless storage site for triglycerides. It is characterized by adipocytes with a single lipid inclusion and eccentrically located nucleus, whereas brown adipocytes are polygonal cells with a roundish nucleus and several cytoplasmic lipid droplets (Cinti 2012). Thus, white and brown adipocytes are not only different in their morphology but also in their physiology: white adipocytes store energy for the metabolic needs of the organism, whereas brown adipocytes burn energy for thermogenesis (Cinti 2012). Both cell types are contained in the multiple depots of the adipose organ. White adipocytes of different sizes are present in subcutaneous depots (mainly large adipocytes) and visceral depots (mainly small adipocytes). Brown adipocytes in visceral depots are mainly found near aorta. Paucocilocular adipocytes, which are cells with intermediate morphology between that of white and brown adipocytes, are also present in the adipose organ. It should be noted that some groups also refer to ‘beige’ (Ishibashi and Seale 2010) or ‘brite’ (brown in white) (Petrovic et al. 2010) regions of white adipose tissue, containing brown or brown-like adipocytes. Adipocytes in visceral depots are sensitive to lipolytic stimuli, whereas adipocytes from structural depots (such as around the eyes and in the heel pads) do not release stored lipid easily.

Classically thought of as a tissue mass that stores excess energy and provides insulation and padding to the body, adipose tissue is now accepted to be an endocrine organ that secretes numerous hormones, growth factors, cytokines, matrix proteins, enzymes and complement factors (Figure 2). For instance, classical cytokines include tumor necrosis factor-α (TNF-α) and interleukin-1, 6, 10, and 18 (IL-1, IL-6, IL-10, IL-18), whereas monocyte chemoattractant protein-1 (MCP-1), macrophage migration inhibitory factor (MIF), regulated on activation normal T cell expressed and secreted/CCL5 (RANTES/CCL5) and interleukin-8 (IL-8) belong more to the subgroup of chemokines (D’Esposito et al. 2012). The diversity of secreted molecules includes also factors involved in lipid and glucose metabolism such as lipoprotein lipase (LPL), apolipoprotein E (apoE), cholesteryl ester transfer protein, peroxisome
proliferator-activated receptor (PPAR), glucocorticoids, sex steroids, prostaglandins, adipin, acylation stimulating protein, leptin, resistin, adiponectin/Acrp30/adipoQ, osteonectin, and cathepsins, among others. Finally, secreted growth factors include insulin-like growth factor 1 (IGF1), nerve growth factor (NGF), macrophage colony-stimulating factor, transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), heparin-binding epidermal growth factor, leukemia inhibitory factor, and bone morphogenetic proteins (Fruhbeck 2008).

Figure 2: Adipokines secreted by white adipose tissue. White adipose tissue (WAT) secretes various humoral factors called adipokines. Adipokines have important effects on lipid and glucose metabolism, and so on. Adapted from Ravussin. Pharmacogenomics J. 2002;2(1):4-7.

The secreted factors identified to date play a role in fat mass regulation and regulation of adipocyte differentiation, vascular and blood flow regulation, lipid and cholesterol metabolism, and immune system function. With such varied biological functional roles, it is likely that these adipokines have a significant role in disease processes known to be metabolic consequences of obesity, such as insulin resistance, cardiovascular disease, and altered lipid homeostasis (Poulus et al. 2009).
1.2 Obesity

Obesity is characterized by excessive fat accumulation in adipose tissue and other organs. It is a major cause of morbidity and mortality, associated with an increased risk of cardiovascular disease, metabolic syndrome and certain forms of cancer. The BMI (Body Mass Index), calculated as weight (kg) divided by height squared (m²), is used as a measurement of body fat in clinical and epidemiologic studies. The National Heart, Lung and Blood Institute and the world Health Organization (WHO) define overweight as a BMI equal to or greater than 25 Kg/m² and obese as a BMI equal to or greater than 30 Kg/m². The WHO estimates that 1.5 billion people are overweight and more than 500 million are obese worldwide (Gilbert and Slingerland 2013). Once considered a problem only in the USA and other high-income countries of the Western world, obesity has become a major contributor to the global burden of disease (Misra and Khurana 2008). The worldwide increase in the incidence of obesity and related chronic diseases has largely been driven by global trade liberalization, economic growth and rapid urbanization, which continue to fuel dramatic changes in living environments as well as in diets and lifestyles that promote positive energy balance. Positive energy balance occurs when individual’s caloric intake exceeds their energy expenditure, leading to weight gain (Haweks 2006; Malik et al. 2012). Indeed the balance between energy intake (food consumption) and energy expenditure (basal metabolic rate) is tightly regulated (Singla et al. 2010). Combined with reduction in physical activity, these factors are driving the global obesity epidemic (Fuster and Kelly 2010).

The global effect of the obesity epidemic was formally recognized by the WHO during a special obesity consultation in 1997. National survey data show that obesity epidemic began in the USA over 40 years ago, with the estimated prevalence having more than doubled in the USA from 1980 to 2010 and worldwide from 1980 to 2008. In the USA alone in 2009-2010, 35.5% of men and 35.8% of woman had obesity (Flegal et al. 2012). In 2008 it was estimated that 500 million adults had obesity, which represents 10-14% of the world’s population. Among women, obesity prevalence increased in all regions. The greatest magnitudes of increase were observed in central Latin America, North America and North Africa and the Middle East. The Caribbean, Oceania and Southern Africa had magnitudes of increase ranging
from around 14% to 18%. Other regions had increases ranging from <3% in east Asia to close to 7% in Western Europe. For men, obesity increased in all regions except South Asia. The greatest magnitude of increase was observed in North America, with an increase of <18%. In central Latin America, Southern Africa, Oceania, north Africa and the Middle East, Western Europe and the Caribbean prevalence increased by <9-15%. In the remaining regions, prevalence increased by between 1.4% in East Africa and <6% in Eastern Europe. In the 2008, the sex-specific prevalence of obesity was highest in North America (men: 29.2%) and in southern Africa (women: 36.5%) (Figure 3) (Malik et al. 2012).

Fig. 3: Global trends in the prevalence of obesity among women and men in 1980 and 2008 from select regions of the world. Estimates of obesity prevalence in women (A) and
men (B) are shown. Among women, obesity prevalence increased in all regions. For men, obesity increased in all regions except South Asia. Adapted from Finucane et al. *Lancet*. 2011 Feb 12;377(9765):557-67.

The prevalence of obesity is higher in adults than in children but the increase in the incidence of obesity among children has occurred more rapidly than that among adults in some countries, such as the USA, Brazil and China (Popkin et al. 2006). The worldwide prevalence of childhood overweight and obesity increased from 4.2% to 6.7% between 1990 and 2010 (de Onis et al. 2010). Genetic predisposition is a key contributing factor in obesity, as has been demonstrated by familial aggregation, twin and adoption studies. Estimates for a genetic basis for obesity range from approximately 40% to 70%. The idea that genetic loci alter body fat content has been substantiated by the identification of mutations that cause low- or high-fat phenotypes in rodents and humans (Friedman 2000).

The hypothalamus is the major nervous centre controlling food intake. It has two major areas that play an important role in maintaining the normal energy homeostasis of the body by controlling the hunger and satiety centres. The portion of hypothalamus, called the ventro-medial hypothalamic nucleus (VMN), is known as the ‘satiety centre’. Stimulation of VMN causes suppression of food intake, whereas a bilateral VMN lesion induces hyperphagia and obesity. On the contrary, stimulations or any lesion of lateral hypothalamic area, known as ‘hunger centre’, induce the opposite set of responses. Various neuropeptides (e.g. the melanocortin system, neuropeptide Y) and neurotransmitters (e.g. serotonin, dopamine, and noradrenaline) along with insulin and leptin molecules function in hypothalamus and thus coordinate the behavioral, physiological and metabolic responses (Singla et al. 2010). In addition to these long-term adiposity signals, short-term meal related signals are also transmitted to the central nervous system (CNS) through afferent nerves or gut-secreted peptides (e.g. cholecystokinin, ghrelin). In the CNS neurons are also directly sensitive to carbohydrates and fats (Arora et Anubhuti 2006).

Adipose tissue is major endocrine organ, producing various hormones that regulate body metabolism. An increase in the fat cell mass leads to an alteration of hormone-adipose tissue release, which can have various metabolic effects. The metabolic complications of obesity, often referred to as
the metabolic syndrome, consist of insulin resistance, often culminating in β-cell failure, impaired glucose tolerance and type 2 diabetes, dyslipidemia, hypertension and premature heart disease (Singla et al. 2010). Moreover obesity is characterized by a low-grade inflammation not only in adipose tissue but also in others organs.

### 1.3 Adipose tissue inflammation in obesity

Over the past decade, the search for a potential unifying mechanism behind the pathogenesis of obesity-associated diseases has revealed a close relationship between nutrient excess and derangements in the cellular and molecular mediators of immunity and inflammation. The inflammatory response triggered by obesity involves many component of the classical inflammatory response to pathogens and includes systemic increases in circulating inflammatory cytokines and acute phase proteins (e.g. C-reactive protein), recruitment of leukocytes to inflamed tissue, activation of tissue leucocytes and generation of reparative responses (e.g. fibrosis) (Spencer et al. 2010). The chronic nature of obesity produces a tonic low-grade activation of the innate immune system that affects steady-state measures of metabolic homeostasis over time. Superimposed on this chronic inflammation are recurrent acute episodes of nutrition-related immune activation induced by nutrient availability (fasting or high-fat meals) (Lumeng and Saltiel 2011). Adipose tissue can respond rapidly and dynamically to alternations in nutrient deprivation and excess through adipocyte hypertrophy and hyperplasia that can both contribute to adipose tissue expansion (Sun et al. 2011). It has been described that macrophage infiltration has a key role into expanding of adipose tissue, causing inflammation and linking obesity to insulin resistance. Several mechanisms including fatty acid flux, hypoxia, adipocyte cell death and augmented chemokine and adipokine secretion have been proposed to be initiators of macrophage infiltration (Weisberg et al. 2003; Xu et al. 2003). Plasma Free fatty acids (FFAs) concentrations are chronically elevated in human obesity because of the blunted capacity of insulin to inhibit lipolysis and the excessive consumption of dietary lipids (Cnop 2008). FFAs, stored in the form of triglycerides in adipose tissue, are released from hypertrophic adipocytes through lipolysis during fasting. Some of these FFAs are shunted to the liver and stored in lipid droplets, while some of them are
oxidized in other organs. However, many of them are locally re-esterified in adipocytes. Those FFAs that escape re-esterification play a critical role in several organs as a primary energy source during prolonged fasting (Sun et al. 2011). However, FFAs can also serve as ligands for the toll like receptor 4 (TLR4) complex, thereby activating the classical inflammatory response in the context of increased local extracellular lipid concentrations, which ultimately drives adipose tissue macrophage accumulation. Even though adipose tissue is not considered as a conventional tissue suffering from lipotoxic side effects, either high rates of lipolysis or an influx of saturated FFAs into adipocytes do cause temporary inflammation within the tissue (Shi et al. 2006). FFAs cause inflammation via TLR4 in all cellular fractions of adipose tissue. Thus, FFAs induce a pro-inflammatory pattern of adipokine production in primary adipocytes or adipocyte cell line (Nguyen et al. 2005; Shi et al. 2006). FFAs also activate macrophages, especially the CD11c⁺ subset, thereby exacerbating their pro-inflammatory activity (Suganami et al. 2007). Many of the metabolic stresses, through activation of TLR4, promote inflammation and induce several kinases, such as IkB kinase-β (IKK-β) and C-Jun N-terminal kinase (JNK), which may have key roles in pathogenesis of insulin resistance. Indeed, IKK-β activates the transcription factor nuclear factor-kB (NF-kB), and obesity induces the expression of NF-kB target genes, such as pro-inflammatory cytokines, in the liver and adipose tissue. These cytokines, including TNF-α, IL-6, IL-8 and IL-1β, may promote insulin resistance in the tissues where they are produced, such as the liver and adipose tissue, and may also be transported through the circulation to affect more distant sites, including the vessel walls, skeletal and cardiac muscle, the kidneys and circulating leukocytes. The other potentially important kinase, JNK, activates transcription factors such as ETS domain-containing protein (Elk-1), activating transcription factors 2 (ATF2) and Jun (Osborn and Olefsky 2012). IKK-β and JNK belong to group of kinases that phosphorylate on serine 307 the insulin receptor substrate (IRS) leading to an alteration of insulin signaling. Insulin signaling involves a complex signaling cascade downstream of the insulin receptor. This signaling cascade branches into two main pathways. The first is the phosphatidylinositol 3-kinase (PI3K)/AKT (also called protein kinase B (PKB)) pathway that is largely responsible for insulin action on glucose uptake, as well as other metabolic actions of insulin, including the suppression of gluconeogenesis. The second pathway is the Ras-
mitogen-activated protein kinase (MAPK) pathway, which mediates gene expression, but also interacts with PI3K-AKT pathway to control cell growth and differentiation. The common intermediate to these pathways is IRS, which include four distinct family members, IRS-1-4. Activation of the insulin receptor leads to tyrosine phosphorylation of IRS-1 thereby initiating signal transduction. When IRS-1 is alternatively phosphorylated on serine 307, its downstream signaling ability is diminished (Figure 4) (de Luca and Olefsky 2008).

Figure 4: Direct interaction of insulin signaling and inflammatory pathways. Insulin signaling cascade branches into two main pathways. The PI3K/AKT pathway mediates insulin action on nutrient metabolism including glucose uptake. The Ras/MAPK pathway mediates insulin’s effect on gene expression, but also interacts with the PI3K-AKT pathway to control cell growth and differentiation. Activation of the insulin receptor leads to tyrosine phosphorylation of IRS1 thereby initiating signal transduction. Stimulation of the NF-κB and AP-1 Fos/Jun inflammatory pathways results in the activation of the serine kinases, Ikkb and Jnk1, which reduce the signaling ability of IRS-1. Additional inflammation-related negative regulators of IRS proteins include the Socs proteins and NO, which are induced in inflammation, and promote IRS degradation. NO also reduces PI3K/AKT activity by s-nitrosylation of AKT.
Adipose tissue inflammation probably results from a complex crosstalk between adipocytes, macrophages and other immune cells, with contributions from both the innate and adaptive arms of immune system (Sell et al. 2012). An important finding that helped elucidate the cause of adipose tissue inflammation from obese mice and humans is the infiltration with a large numbers of macrophages. These adipose tissue macrophages (ATMs) can comprise up to 40% of the cells in obese adipose tissue (Osborn and Olefsky 2012). In the adipose tissue, macrophages have been shown to aggregate, forming crown-like structures (CLSs) surrounding necrotic adipocytes in advanced obesity. In this state they fuse to phagocyte the residual lipid droplet, forming large lipid-laden multinucleated syncytia in the process, a commonly accepted hallmark of chronic inflammation (Sun et al. 2011).

Figure 5: Immune cells mediate inflammation in adipose tissue. In the lean state, adipose tissue T\textsubscript{H}2 T cells, T\textsubscript{REG} cells, eosinophils and M2-like resident macrophages predominate. T\textsubscript{REG} cells secrete IL-10 and also stimulate IL-10 secretion from resident M2-like macrophages. Eosinophils secrete IL-4 and IL-13 and further contribute to the anti-inflammatory, insulin-sensitive phenotype. In obesity-induced inflammation, immune cells are recruited and contribute to adipose tissue inflammation. Monocytes respond to chemotactic signals and transmigrate into the adipose tissues and become polarized to the highly pro-inflammatory M1-like state. Once recruited, these M1-like macrophages secrete pro-inflammatory cytokines that work in a paracrine fashion. The eosinophil content declines in obese adipose tissue. Obesity also induces a shift in adipose tissue T cell populations with a
decrease in T\textsubscript{REG} content and an increase in CD4\textsuperscript{+} T\textsubscript{H}1 and CD8\textsuperscript{+} effector T cells, which secrete pro-inflammatory cytokines. B cell numbers also increase and activate T cells, which potentiate M1-like macrophage polarization, inflammation and insulin resistance. Cytokines and chemokines from the adipose tissue can also be released into the circulation and work in an endocrine manner to promote inflammation in other tissues.


Obesity is accompanied by a transformation in the polarized states of macrophages; from an anti-inflammatory “alternatively activated” M2 form that primarily accumulates during negative balance, to a more pro-inflammatory classically activated M1 form (Sun et al. 2011). The M1 population, CD11c\textsuperscript{+} macrophages, demonstrates a positive correlation with insulin resistance and dominates in states of overnutrition by targeting FFA-mediated increases in pro-inflammatory responses (Lumeng et al. 2007; Shoelson et al. 2006). This phenotypic switch of macrophages could be a key step of adipose tissue inflammation and might provide a link to the involvement of cells of adaptive immune system (Figure 5) (Sell et al. 2012).

In lean mice about 10% of the stroma-vascular fraction (SVF) from the visceral fat depot is composed of CD3\textsuperscript{+} T lymphocytes, with a 1:3 ratio of CD8\textsuperscript{+} to CD4\textsuperscript{+}. Adipocytes themselves could have immune-cell-like functions that lead to CD4\textsuperscript{+} T-cell activation, potentially triggering inflammation independently of macrophage infiltration. The CD4\textsuperscript{+} T-cell compartment could be subdivided into pro-inflammatory T-helper type 1 (T\textsubscript{H}1) and T\textsubscript{H}17 cells, and anti-inflammatory T\textsubscript{H}2 and T-regulatory cells (T\textsubscript{REG}) that express forkhead box protein P3 (mFoxp3) (Feuerer et al. 2009).

In mouse models of diet-induced obesity, T-cell populations differ in adipose tissue. A higher CD8\textsuperscript{+} and CD4\textsuperscript{+} T-cell ratio is found in visceral adipose tissue of high-fat diet fed mice compared with lean mice. Diet-induced obesity also leads to a dramatic increase in T\textsubscript{H}1-polarized cells, whereas the T\textsubscript{H}2-polarized fraction in significantly reduced by \(\approx 50\%\). After feeding with a high-fat diet, moreover, the T\textsubscript{H}17 subset in visceral adipose tissue represents <1% of the CD4\textsuperscript{+} population and T\textsubscript{REG} cells are reduced by 70%. Unlike visceral adipose tissue, the proportions of CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell subsets in subcutaneous adipose tissue were reduced, whereas T\textsubscript{REG} numbers did not change during obesity (Feuerer et al. 2009; Caspar-Bauguil et al. 2005).

Other immunity cells are implicated in inflammatory response to adipocyte hypertrophy such as CD8\textsuperscript{+}, natural killer (NK), NKT and B cells. CD8\textsuperscript{+} are
involved in macrophage differentiation, activation and migration, thereby initiating inflammatory cascades in adipose tissue that lead to insulin resistance. NK cells are present in large numbers in visceral adipose tissue where they might be involved in regulation of adipose tissue homeostasis and their number is decreased in a leptin-dependent manner. NKT cells are described as innate-like T lymphocytes, because they possess invariant pattern recognition receptors instead of antigen-specific T-cell receptors, and release a mixture of T\(\text{H}1\) and T\(\text{H}2\) cytokines such as IFN-\(\gamma\) and IL-4, thereby formation of subsequent adaptive immune responsive (Van Kaer 2007). Lymphocytes B are also involved in obesity-released inflammation. In fact, reconstitution with wild type B cells from high-fat-diet fed mice lead to impaired glucose metabolism and decreased insulin sensitivity as a result of the antigen-dependent activation of T cells (T\(\text{H}1\) and CD8\(^+\) T cells), pro-inflammatory M1 macrophages and secretion of pathogenic IgG antibodies from B cells. Treatment with a B cell-depleting antibody attenuates these effects, whereas transfer of IgG from mice with diet-induced obesity quickly induces insulin resistance and glucose tolerance (Winer et al. 2011).

Based on these studies in mouse models, lymphocytes infiltration in adipose tissue might occur in a chronological sequence that begins with B and T lymphocytes (T\(\text{H}1\) and CD8\(^+\) cells) being recruited during early obesity-induced inflammation by preadipocytes or adipocytes-derived chemokines like CCL5, MCP-1 (CCL2), CXCL12 (also known as SDF-1\(\alpha\)) and CXCL5 (Wu et al. 2007). These cells direct the local inflammatory process through the release of pro-inflammatory T\(\text{H}1\) cytokines such as IFN-\(\gamma\), which promote M1 macrophage recruitment by inducing pre-adipocytes and adipocytes to release chemokines and activated macrophages (Sell et al. 2012).

It has been suggested that a paracrine loop between adipocytes and macrophages establishes a vicious circle that aggravates the inflammatory changes in adipose tissue. Interactions between both cell types are critical. This paracrine loop involves FFAs and TNF-\(\alpha\) (Figure 6). Enlarged adipocytes release in excess saturated FAs that activate macrophages via TLR4 signaling. As a result, macrophages secrete the pro-inflammatory adipokine TNF-\(\alpha\), which in turn acts on TNF-\(\alpha\) receptor 1 (TNFR1) and induces inflammatory changes in hypertrophied adipocytes through activation of the NF-kB pathway and as well as enhanced FFA release (Suganami et al. 2007).
There is considerable evidence for the pathophysiological role of macrophage and hypertrophic adipocyte-derived chemotactic MCP-1/CCR2 pathways in the regulation of monocyte accumulation in obese adipose tissue. In particular, increased expression levels of MCP-1, CXCL14, MIP-1α, MCP-2, MCP-3, and CCL5 can be observed in obese and diabetes mice adipose tissue (Sun et al. 2011).

Figure 6: Cross-talk between adipocytes and macrophages of adipose tissue in obesity. TNF-α, which is mainly overproduced by macrophages appears to be a crucial contributor to adipokine dysregulation in adipocytes. Adipocytes of obese subjects overproduce adipokines in response to TNF-α. This hyper-responsiveness is mediated by TNF-α-receptor-1 (TNFR1) and by hyper-activation of NF-kB pathway. TNF-α also induces lipolysis (FFA release). Saturated FFAs in turn activate the TLR4/NF-kB pathway in both macrophages and adipocytes, thereby further increasing the production of TNF-α and other pro-inflammatory adipokines. Some of these adipokines exert chemoattractant activity through binding to specific receptors (CXCR and CCR) of macrophages leading to their infiltration in obese AT. Adapted from Maury and Brichard. Mol Cell Endocrinol. 2010 Jan 15;314(1):1-16.
Chapter 1

1.4 Regulated on Activation, Normal T cell Expressed and Secreted/CCL5 (RANTES/CCL5)

The chemokines are involved in many aspects of the immune response including lymphocyte chemo-attraction, adhesion, and activation. The family contains more than 40 members, which have been divided into subfamilies based on a conserved pattern of cysteines. Members of the CC subfamily have overlapping in vitro biological activities including chemo-attraction for monocytes and T cells (Makino et al. 2002). They also play a pivotal role in diseases such as autoimmune and allergic inflammatory disorders, cancer and organ transplant where excessive cellular recruitment plays a deleterious role. Their function is not limited to recruitment, since they also play a role in cellular activation, differentiation, degranulation and in processes such as organ development, angiogenesis, lymphogenesis and wound repair (Proudfoot 2006). CCL5 is a typical chemokine of 68 amino acids that is able to recruit leucocytes to sites of inflammation. It induces leucocyte migration by binding to specific receptors in the seven-transmembrane G-protein-coupled receptor (GCPR) family, namely CCR1, CCR3, CCR4 and CCR5. In common with other chemokines, there is a clear structural basis for the dimerization of CCL5, although dimer formation is not essential for its chemokine receptor interactions, and CCL5 can signal through its specific receptors as a monomer. However, a peculiar characteristic of CCL5 is its ability to self-aggregate, forming multimers at high concentrations. The amino acids involved in this self-aggregation, Glu66 and Glu26, are negatively charged residues. CCL5 also contains several positively charged residues on its surface, which makes it likely that ionic bindings are involved in the formation of multimers (Figure 7) (Appay and Rowland-Jones 2001).
Figure 7: Structure of a dimer of human CCL5. Positively charged residues are colored in blue and negatively charged residues are in red. The dimerization of CCL5 has a structural basis (white-colored residues belong to chain A, and yellow-colored residues belong to chain B) but, in addition, CCL5 self-aggregates to form multimers. The dimerization occurs at the N-terminal part of the molecule, which is also essential for receptor binding and signaling. The residues involved in the multimerization, Glu66 and Glu26, are found at the surface of the protein, suggesting that ionic binding with positively charged surface residues from other CCL5 molecules occurs and leads to self-aggregation. Changing these residues to serine abrogates aggregation.

CCL5 mediates the trafficking and homing of classical lymphoid cells such as T cells and monocytes, but also acts on a range of other cells, including basophils, eosinophils, nalural killer (NK) cells, dendritic cells and mast cells (Appay and Rowland-Jones 2001). It also promotes T cell adherence to activated endothelial cells (Taub et al. 1993) and stimulates T cell proliferation (Taub et al. 1996).

To better define CCL5 in vivo activity Makino et al. have used gene targeting to produce CCL5-deficient (-/-) mice. Analysis of these mice reveals that CCL5 plays a role in the delayed-type hypersensitivity (DTH) response, T cell proliferation, and the production of IL-2 and IFN-γ. One of the functional consequences of the absence of CCL5 is an impaired DTH reaction. Because CCL5 plays a role in several aspects of the immune response, the decreased DTH response is likely the consequence of the sum of this effects including...
decreased T cell priming and proliferation as well as possible changes in the T\(_{H1}\) response as indicated by the decreases in IFN-\(\gamma\) production (Makino et al. 2002).

When CCL5 was identified, it attracted particular attention because it was demonstrated to be a potent inhibitor of HIV replication \textit{in vitro}. In 1995 CCL5 was shown to be the most potent member of a trio of CC chemokines released by CD8\(^+\) T cells that were able to suppress the replication of non-syncitium-inducing (NSI) HIV-1 strains \textit{in vitro}. The others chemokines were macrophage inflammatory protein 1 alpha (MIP-1\(\alpha\)) and MIP-1\(\beta\). The three chemokines bind to the CCR5 receptor on the surface of CD4\(^+\) T cells, and this receptor is also the means by HIV gains entry to the cell. The HIV-suppressive effect of CCL5 appears to be critically dependent on the presence of glycosaminoglycans (GAG) on the target cell surface (Appay and Rowland-Jones 2001).

Protein and RNA levels of CCL5 are increased in a gender-dependent fashion in white adipose tissue of obesity (Wu et al. 2007). CCL5 is expressed by adipocytes and has been hypothesized to mediate leukocyte infiltration of adipose tissue in obesity (Poulain-Godefroy and Froguel 2007; Wu et al. 2007). Circulating CCL5 concentrations are elevated in obesity, impaired glucose tolerance (IGT) and type 2 diabetes (Nomura et al. 2000; Herder et al. 2005). Moreover, in the Finish Diabetes Prevention Study, high CCL5 levels were associated with resistance to lifestyle intervention and higher incidence of type 2 diabetes in the intervention group (Herder et al. 2006). Wu et al. found higher CCL5 mRNA levels in visceral compared with subcutaneous adipose tissue in obese humans. In their studies, Madani et al. showed CCL5 production by human subcutaneous adipose tissue \textit{in vivo} and release of this chemokine in novel depots, the gastric fat pad and epicardial fat. They demonstrated that while epicardial CCL5 was related to obesity, neither systemic CCL5 nor its release from the subcutaneous and the abdominal visceral adipose tissue is a good marker of adiposity. There is significant heterogeneity in the release of CCL5 from the various depots, a finding that suggests differential regulation by autocrine/paracrine factors (Madani et al. 2009).
2. AIM OF THE STUDY

Obesity is associated with insulin resistance, hyperglycaemia, dyslipidemia, hypertension and other components of the metabolic syndrome (Sell and Eckel 2009). Enlargement of adipose tissue is the consequence of a sedentary lifestyle and increased energy intake, and is associated with a prominent inflammatory response in the visceral compartment that leads to adipose tissue dysfunction (Sell et al. 2012). Adipocytes, in addition to their storage function, are also active endocrine cells that produce and release various adipokines such as adiponectin, visfatin, resistin, adipsin, cytokines (TNF-α, IL-6, IL-1, IL-10, IL-18) and chemokines including MCP-1, IL-8 and CCL5 (D’Esposito et al. 2012). Chemokines may be directly linked to a chronic state of low-grade inflammation and macrophage infiltration in adipose tissue (Sell and Eckel 2009). Macrophage infiltration has a key role into expanding of adipose tissue, causing inflammation and linking obesity to insulin resistance (Weisberg et al. 2003; Xu et al. 2003). It has been described that several mechanisms may contribute to the initial adipose macrophage infiltration and impair adipocyte insulin signaling such as stressed adipocytes secreting multiple chemokines and cytokines, fatty acid flux, hypoxia and adipocyte cell death (Jiao et al. 2011). Obesity is characterized by chronically elevated concentrations of plasma FFAs caused in part by the blunted capacity of insulin to inhibit lipolysis and the excessive consumption of dietary lipids (Cnop 2008). Excessive overnutrition and macrophage infiltration in adipose tissue lead to an inflammatory pathway activation and cytokine/chemokine production in adipocytes. Recent interest has focused on CCL5 chemokine, and in particular, its role in regulating the recruitment of inflammatory cells into adipose tissue. CCL5 levels have been found elevated in serum of obese, impaired glucose tolerance (IGT) and type 2 diabetes patients (Nomura et al. 2000; Herder et al. 2005; Wu et al. 2007). The aim of this study is to identify the molecular mechanisms involved in the metabolic control of CCL5 expression in adipocytes. In particular, I have evaluated whether metabolic perturbations such as different concentrations of glucose and free fatty acids may affect CCL5 adipocyte production. Moreover, I have investigated the role of different pathways normally activated by FFAs in adipocytes in the regulation of CCL5 production. Finally, I have observed the effect of insulin on FFA-induced CCL5 expression in adipocytes.
3. MATERIALS AND METHODS

3.1 Materials

Media, sera, antibiotics for cell culture were from Lonza (Basel, Switzerland). Antibodies against specific phospho-Serine473-AKT/PKB, phospho-Threonine183/Tyrosine185-JNK, AKT/PKB, JNK, ERK, IκBα, NF-kB, 14.3.3 and NF-kB activation inhibitor II (JSH23) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-Threonine202/Tyrosine204-ERK and phospho-Serine32-IκBα were obtained from Cell Signaling Technology (Denvers, MA, USA). JNK inhibitor (SP600125), MEK inhibitor (PD98059), PI3K inhibitor (LY294002), sodium oleate and sodium palmitate were purchased from Sigma-Aldrich (Steinhem, DE). Protein electrophoresis and real time RT-PCR reagents were purchased from Bio-Rad (Hercules, CA, USA), Western blotting and ECL reagents from Amersham Biosciences (Arlington Heights, IL, USA).

3.2 Cell culture

The 3T3-L1 murine fibroblast cells were available in host laboratory. They were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum (CS), 100 IU/ml penicillin, 100 IU/ml streptomycin, and 2% L-glutamine in a 5% humidified CO₂ incubator. 3T3-L1 cells were differentiated as previously described (Engelman et al. 1998). In brief, after 2 days from confluence (Day 0), the DMEM 10% FBS was changed and new DMEM 10% FBS was added to the cells with the addition of 160 nm insulin, 250 nm dexamethasone, and 0.5mm 3-isobutyl-1-methylxanthine. After 2 days (Day 2), this medium was replaced with fresh DMEM 10% FBS containing only 160 nm insulin. After another 2 days, the cells were then propagated in 10% FBS medium. Adipogenesis was scored by analysis of the expression of adipocyte-specific genes (aP2 and peroxisome proliferator-activated receptor γ (PPARγ)) (Chavey et al. 2003) and by lipid accumulation using microscopic analysis or oil red O staining (Ramirez-Zacarias et al. 1992).
3.3 Conditioned media system

The conditioned media was isolated in the following way: confluent 3T3-L1 adipocyte plates were identified, the 10% FBS removed, the plates washed two times with sterile phosphate-buffered saline (PBS), and 3–5 ml of serum-free DMEM 0.25%BSA (DMEM-BSA) added to the plates and incubated for approximately 8h. After the incubation, the DMEM-BSA was collected and centrifuged at 14000xg to remove cellular debris.

3.4 Bioplex cytokine and growth factor assay

Supernatants were collected after 8 h of plating the different cells. Concentrations of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC/IL-8, MCP-1, MIP-1α, MIP-1β, CCL5, TNF-α, PDGF, VEGF and IGF1 were determined using the Bioplex multiplex Mouse Cytokine and Growth factor assay kits (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. Briefly, 50 µl of the culture supernatant or cytokine standard was plated in a 96 well filter plate coated with a multiplex of beads coupled to antibodies against the above mentioned cytokines and incubated for 30 min on a platform shaker at 300 rpm at RT. After a series of washes to remove the unbound proteins, a mixture of biotinylated detection antibodies, each specific for a different epitope was added to the reaction resulting in the formation of antibodies assimilated around the target proteins. Streptavidin-phycoerythrin (streptavidin-PE) was then added to bind to the biotinylated detection antibodies on the bead surface. The data from the reaction were then collected and analyzed by using the Bio-Plex suspension array system (or Luminex 100 system) from Bio-Rad Laboratories (Hercules, CA).

3.5 ELISA

CCL5 protein secreted into the cell culture supernatants was quantified by ELISA, according to the manufacturer’s protocol (R&D System).
3.6 Real-time RT-PCR analysis

Total RNA was isolated from 3T3-L1 adipocytes by using the Rneasy Kit (Qiagen Sciences) according to the manufacturer’s instruction. For real-time RT-PCR analysis, 1 µg cell RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). PCR were analyzed using SYBR Green mix (Invitrogen). Reactions were performed using Platinum SYBR Green Quantitative PCR Super-UDG using an iCycler IQ multicolour Real-Time PCR Detection System (Biorad Hercules, CA). All reactions were performed in triplicate and ACTIN was used as an internal standards. Primer sequences used were as follows: CCL5 forward primer 5’- AGG AAC CGC CAA GTG TGT GCC -3’, reverse primer 5’- AGT GGC ATC CCC AAG CTG GC -3’; ACTIN forward primer 5’- CGC CCT AGG CAC CAG GTG GTG -3’, reverse primer 5’- TCG GTG AGC AGC ACA GAG TG TG-3’.

3.7 Western Blot analysis

3T3-L1 adipocytes were lysed in lysis buffer (50mM HEPES pH 7.6, 150mM NaCl, 10mM EDTA, 10mM Na₄P₂O₇, 2mM Na₃VO₄, 100mM NaF, 10% glycerol, 1mM PMSF, 100 IU/ml aprotinin, 20µM leupetin, 1% Triton X-100), for 2 h at 4 °C and lysates were centrifuged at 14,000 x g for 15 minutes to remove cellular debris. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blot as previously described (Laemmli 1970). Nitrocellulose membranes were probed with antibodies for specific proteins.

3.8 Chromatin Immunoprecipitation (ChIP) assay

The cross-linking solution, containing 1% formaldehyde, was added directly to cell culture media. The fixation proceeded for 10 min and was stopped by the addition of glycine to a final concentration of 125 mM. 3T3-L1 adipocytes were rinsed twice with cold phosphate buffered saline plus 1 mM phenylmethylsulfonyl fluoride and then scraped. Cells were collected by centrifugation at 800 X g for 5 min at 4 °C. Cells were swelled in cold cell lysis buffer containing 5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and inhibitors mixture (Sigma) and incubated on ice for 10 min. Samples were broken by sonication into
chromatin fragments of an average length of 500/1000 bp and then microcentrifuged at 16,000 X g for 10 min at 4 °C. The sonicated cell supernatant was diluted 8-fold in chromatin immunoprecipitation (ChIP) dilution buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris- HCl (pH 8.0), and 167 mM NaCl and precleared by adding salmon sperm and conjugating protein at equimolar concentration for 90 min at 4 °C. Precleared chromatin from 1X106 cells was incubated with 1 g of polyclonal antibody (anti-NF-kB) or no antibody and rotated at 4 °C for 16 h. Mouse IgG antibody (Upstate Biotecnology/Millipore) was used as a nonspecific antibody control. Immunoprecipitates were washed five times with radioimmune precipitation assay buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride, twice with LiCl buffer containing 0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0), and then 3 times with TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Before the first wash, the supernatant from there action lacking primary antibody was saved as total input of chromatin and was processed with the eluted immunoprecipitates beginning at the cross-link reversal step. Immunoprecipitates were eluted by adding 1% SDS, 0.1 M NaHCO3, and reverse cross-linked by the addition of NaCl to a final concentration of 200 mM and by heating at 65 °C for at least 4 h. Recovered material was treated with proteinase K, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated. The pellets were resuspended in 30µl of TE and analyzed by PCR using specific primers for the analyzed regions. The input sample was resuspended in 30µl of TE and diluted 1:10 before PCR.

Eluted DNA amplified by real time RT-PCR with specific oligos: forward primer: 5'-TTT GGC CAG AGA GGG AGT CAT CCT -3', reverse primer: 5'-AGT CCT CTG CAA GGG GTG CTC T -3'.

3.9 Statistical procedures

Data were analyzed with the Stat view software (Abacus-concepts) by one-factor analysis of variance. P values of less than 0.05 were considered statistically significant.
4. RESULTS

4.1 Analysis of cytokine and growth factor release by 3T3-L1 adipocytes and their precursors

For decades, adipose tissue was considered an inert mass of stored energy with some advantageous properties, such as its function as an insulating substance and as a mechanical support for more important structure (Rosen and MacDougald 2006). Adipose tissue is now accepted to be an endocrine organ that secretes numerous hormones, growth factors, matrix proteins, enzymes, cytokines and complement factors (Poulos et al. 2010).

<table>
<thead>
<tr>
<th>Cytokines and Growth Factors (pg/ml)</th>
<th>3T3-L1 CM (pg/ml)</th>
<th>3T3-L1 adipocyte CM (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.7±0.0008</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>IL-6</td>
<td>23.6±1.13</td>
<td>22.37±3.47</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.70±0.86</td>
<td>15±2.6*</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>3.73±0.38</td>
<td>5.59±0.80</td>
</tr>
<tr>
<td>KC/IL-8</td>
<td>989.14±100.62</td>
<td>10,343.72±1,551.53*</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>226.31±18.27</td>
<td>280±22.32</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>10.33±1.53</td>
<td>11.4±2</td>
</tr>
<tr>
<td>RANTES/CCL5</td>
<td>1,960±235.2</td>
<td>4,770±524.7*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>143.54±18.66</td>
<td>162.88±25.92</td>
</tr>
<tr>
<td>PDGF</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>bFGF</td>
<td>200±18</td>
<td>666.70±2.67*</td>
</tr>
<tr>
<td>VEGF</td>
<td>11,479.20±1,262.71</td>
<td>4,682.46±607.71*</td>
</tr>
<tr>
<td>IGF-1</td>
<td>79.37±0.88</td>
<td>334.37±40.41*</td>
</tr>
</tbody>
</table>

Table 1: Screening of cytokines and growth factors in 3T3-L1 pre-adipocyte and adipocyte CM. Supernatants from 3T3-L1 pre-adipocytes (3T3-L1 CM) and adipocytes (3T3-L1 adipocyte CM) were collected after 8h of incubation with serum-free medium and tested for 23 cytokines and 4 growth factors by Bioplex multiplex assay. The table represents the concentration (pg/ml) of some of these cytokines and growth factors. Asterisks denote statistically significant values (*p < 0.05).

In order to identify the factors released from adipocytes, I used ELISA/multiplex analysis. In particular, I analyzed the conditioned media for...
the content of growth factors and cytokines produced by both 3T3-L1 pre-adipocytes and adipocytes. The cells were incubated with serum-free medium. After 8 h conditioned media (CM) were collected and tested for 23 cytokines and 4 growth factors by using the Bioplex multiplex cytokine assay kit and the Bioplex multiplex growth factor assay kit. Table 1 shows that both 3T3-L1 fibroblasts and 3T3-L1 adipocytes released detectable amounts of IL-4, IL-6, IL-10, IFN-γ, KC/IL-8, MIP-1α, MIP-1β, CCL5 and TNF-α. However the levels of KC/IL-8 and CCL5 were significantly higher in 3T3-L1 adipocytes compared with their precursors. Moreover, levels of IL-10, an anti-inflammatory cytokine, were also significantly higher in 3T3-L1 adipocytes compared with fibroblasts. I also screened for growth factor release and found that VEGF, bFGF and IGF-1 were detectable in both pre-adipocytes and adipocytes. However, bFGF and IGF-1 concentrations were significantly higher in CM of differentiated cells compared to fibroblasts, while the amount of VEGF was significantly reduced in adipocyte CM.

4.2 Glucose and FFA effect on CCL5 secretion and mRNA levels

Many adipokines synthesized by adipocytes are candidates to attract inflammatory cells in adipose tissue. It has been shown that chemotactic proteins, particularly those of the chemokine family, were related in vivo to the metabolic syndrome, obesity and type 2 diabetes. Among chemokines, CCL5 is elevated in the serum of obese patients as compared with lean controls (Sell and Eckel 2009). Therefore, I examined whether perturbations of the metabolic environment may affect the CCL5 production by adipocytes. I evaluated the effect of free fatty acids and glucose on CCL5 secretion and mRNA levels in adipocytes. 3T3-L1 adipocytes were cultured for 24 h either in 5.5 mM glucose (LG, low glucose), a concentration corresponding to normal fasting glucose levels in humans, or in 25mM glucose (HG, high glucose), corresponding to the regular culture condition for this cell line, but resembling hyperglycaemia in humans. Moreover I added to culture media (LG) either 10 µM oleate or 100 µM palmitate, two major fatty acids whose plasma levels are increased in dysmetabolic conditions. Media were changed...
and cells were allowed to secrete factors into freshly added serum-free medium. After 8 h, media were collected and tested for the content of CCL5 chemokine. Moreover, I also evaluated CCL5 mRNA in adipocytes after 24 h of treatment with glucose and free fatty acids.

**Figure 8:** FFA- and glucose-induced CCL5 secretion and mRNA levels in adipocytes. 3T3-L1 adipocytes were incubated with either high glucose medium (25 mM-HG) or low glucose medium (5.5 mM-LG) in presence or absence of 10µM oleate (OLE (LG)) and 100µM palmitate (PAL (LG)). After 24 h media were collected and analyzed for the content of CCL5 chemokine by ELISA assay (A). CCL5 mRNA levels were determined by real-time RT-PCR; bars represent the mRNA levels in these cells and are relative to those in cells incubated with HG medium (B). Asterisks denote statistically significant values (*p < 0.05).

As shown 3T3-L1 adipocytes cultured in HG medium released significantly higher levels of CCL5 compared with those cultured in LG medium. Oleate and palmitate significantly increased CCL5 secretion (Figure 8A). No significant change was detected for CCL5 mRNA levels when cells were incubated in media containing different glucose concentrations while CCL5
mRNA levels were significantly increased by oleate and palmitate (Figure 8B).

### 4.3 Molecular pathway activation in adipocytes: FFA effect

Systemic levels of fatty acids (FAs) are increased in obesity and, through TLR4-dependent effects, FAs can induce the activation of proteins belonging to inflammatory cascades in adipocytes (Shi et al. 2006). In order to identify molecular pathways potentially involved in CCL5 regulation, I initially investigated the effect of oleate and palmitate on PI3K/AKT, JNK, MAPK and NF-κB pathways in adipocytes. I evaluated the phosphorylation of AKT and ERK, proteins that are downstream PI3K and MAPK signaling pathways respectively.

![Figure 9: Effect of oleate and palmitate on PI3K, MAPK, JNK and NF-κB signaling.](image)

3T3-L1 adipocytes were starved for 16 h and stimulated with either 10µM oleate or 100µM palmitate. After 24 h the cells were solubilized as described in Materials and Methods. Cell lysates were blotted with specific anti-phospho-Serine473-AKT/PKB antibody (pAKT), anti-phospho-Threonine183/Tyrosine185-JNK antibody (pJNK), anti-phospho-Threonine202/Tyrosine204-ERK antibody (pERK) and anti-phospho-Serine32-IkBα antibody (pIkBα) and then reblotted with anti-AKT/PKB (AKT), anti-JNK (JNK), anti-ERK (ERK) and anti-IkBα (IkBα). To ensure the equal protein transfer, membranes were blotted with an anti-14.3.3 antibody (14.3.3).
In addition, I evaluated the activation of inflammatory pathways such as JNK and NF-kB signaling pathways investigating the phosphorylation of JNK and of nuclear factor kB inhibitor alpha (IκBα), a protein that inhibits NF-κB by masking the nuclear localization signals (NLS) of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm. 3T3-L1 adipocytes were treated with either 10µM olate or 100µM palmitate for 24 h. As shown in Figure 9, both olate and palmitate induced the phosphorylation of AKT on Ser473, JNK on Thr183/Tyr185, ERK on Thr202/Tyr204 and IκBα on Ser32. The treatment with palmitate, almost invariably, induced higher phosphorylation of all the proteins compared to olate, but no statistically significant difference between olate- and palmitate-induced protein phosphorylation was detected.

4.4 Role of JNK and NF-kB pathways on CCL5 mRNA levels induced by FFAs

Specific inhibitors were used to characterize the potential role of olete- and palmitate-activated JNK and NF-kB on CCL5 regulation. 3T3-L1 adipocytes were pre-incubated with low glucose medium for 18 h. In order to block JNK and NF-kB pathways and then treated with either 20µM SP600125 or 5.5µM JSH23, JNK and NF-kB inhibitors respectively, for 1 h. Next, adipocytes were stimulated with either 10µM olate or 100µM palmitate for 24 h.

![Figure 10: Effect of FFAs on CCL5 mRNA levels in adipocytes treated with JNK and NF-kB inhibitors. 3T3-L1 adipocytes were incubated with LG medium in presence or absence of 20µM SP600125 and 5.5µM JSH23, JNK and NF-kB inhibitors respectively, for 1 h.](image)
h. Then adipocytes were incubated with either 10µM oleate or 100µM palmitate. After 24 h CCL5 mRNA levels were determined by real-time RT-PCR. Bars represent the mRNA levels in these cells and are relative to those in cells incubated with LG medium. * denote statistically significant values vs LG medium (p < 0.05). # denote statistically significant values vs OLE (p<0.05). § denote statistically significant values vs PAL (p<0.05).

As also previously shown, free fatty acids induced a significant increase of CCL5 mRNA levels. JNK and NF-kB inhibitions reduced CCL5 mRNA levels, impairing the effect of both oleate and palmitate. In particular, SP600125 inhibitor reduced oleate- and palmitate-induced CCL5 mRNA levels by 46.3% and 35.6% respectively, while JSH23 reduced oleate- and palmitate-induced CCL5 mRNA levels by 26.2% and 40.6% respectively (Figure 10).

Thus, JNK and NF-kB inhibitors reduced free fatty acid effect on CCL5 mRNA levels in adipocytes.

4.5 FFA effect on NF-kB binding to CCL5 promoter in adipocytes

NF-kB is a transcription factor normally involved in regulation of pro-inflammatory molecules. I hypothesized that oleate and palmitate activated NF-kB affecting CCL5 gene transcriptional control and that the NF-kB activation could be mediated by JNK. Indeed, I examined whether oleate and palmitate may affect NF-kB binding to CCL5 in presence or absence of the JNK inhibitor SP600125. Bioinformatic analysis revealed the presence of one binding site for NF-kB in the 2000 pair region upstream the CCL5 transcription initiation site (Figure 11A; positions nucleotide -175 to -41). To evaluate NF-kB binding to CCL5 promoter, I performed chromatin immunoprecipitation (ChIP) experiments in 3T3-L1 adipocytes. The cells, previously incubated in LG medium, were treated with 20µM SP600125 for 1 h and then stimulated with either 10µM oleate or 100µM palmitate for 24 h.
Figure 11: Effect of SP600125 on FFA-induced NFκB binding to CCL5 promoter. Schematic representation of 5’ sequence upstream the putative CCL5 transcription start site that includes the binding site for NF-κB (A). 3T3-L1 adipocytes were treated for 1 h with 20µM SP600125 and then incubated in presence or absence of 10µM oleate, 100µM palmitate. After 24 h the cells were processed to perform ChIP assay as described in Material and Methods. Soluble chromatin was immunoprecipitated with p65 NF-κB antibodies. Total and immunoprecipitated DNAs were amplified using primer pairs encompassing the CCL5 promoter by real-time RT-PCR. Bars represent the p65 binding levels and are relative to those in cells maintained in LG medium. Asterisks denote statistically significant values (*p < 0.05) (B).

I obtained evidence that oleate and palmitate significantly increased the NF-κB binding to CCL5 promoter by 3- and 3.7-fold, respectively (Fig. 11B). When the cells were treated with JNK inhibitor, there was a significant reduction of oleate- and palmitate-induced NF-κB binding by 61.3% and 51.2%, respectively (Figure 11B).

4.6 CCL5 mRNA levels induced by FFAs: role of ERK and AKT

I previously showed that, in 3T3-L1 adipocytes, oleate and palmitate activated the MAPK and PI3K/AKT signaling pathways increasing the phosphorylation of ERK and AKT, downstream proteins of these pathways,
respectively (Figure 8). To study the role of ERK and AKT in the control of CCL5 expression, I evaluated the CCL5 mRNA levels in adipocytes treated with specific inhibitors of MAPK and PI3K/AKT pathways. Cells were incubated with LG medium in presence or absence of either 25µM PD98059 (MEK inhibitor) or 25µM LY294002 (PI3K inhibitor) and then stimulated with either 10µM oleate or 100µM palmitate.

The inhibition of MAPK pathway in cells treated with oleate and palmitate induced a further significant increase of CCL5 mRNA levels by 2.6- and 1.3-fold, respectively and consistently similarly, LY294002 treatment induced a significant increase of CCL5 mRNA levels compared to oleate and palmitate, by 2.2- and 1.2-fold, respectively (Figure 12).

Thus, MAPK and PI3K inhibition lead to an increase of CCL5 mRNA levels.

![Figure 12](image_url)

Figure 12: CCL5 mRNA levels in adipocytes treated with FFAs in presence of MAPK and PI3K inhibitors. 25µM PD98059 and 25µM LY294002 in 3T3-L1 adipocytes maintained in LG medium were used for 1 h to block MEK and PI3K, respectively. Then adipocytes were incubated with either 10µM oleate or 100µM palmitate. After 24 h CCL5 mRNA levels were determined by real-time RT-PCR. Bars represent the mRNA levels in these cells and are relative to those in cells incubated with LG medium. * denote statistically significant values vs LG medium (p < 0.05). # denote statistically significant values vs OLE (p<0.05). § denote statistically significant values vs PAL (p<0.05).

Interestingly, the treatment of 3T3-L1 adipocytes with PD98059 and LY294002 induced a significant increase of CCL5 mRNA levels, even in the
absence of fatty acids suggesting a constitutive involvement of MAPK and PI3K in CCL5 regulation.

4.7 Insulin effect on CCL5 mRNA levels induced by fatty acids

Insulin is a pleiotropic hormone that has diverse functions including stimulation of nutrient transport into cells, regulation of gene expression, modification of enzymatic activity. Insulin signaling involves a complex signaling cascade downstream of the insulin receptor branched in two main pathways: MAPK and PI3K/AKT pathways (de Luca and Olefsky 2009). Indeed, I evaluated whether insulin, activating MAPK and PI3K/AKT pathways could reduce CCL5 production in adipocytes treated with free fatty acids.

First of all, I evaluated the effect of insulin on CCL5 mRNA levels in 3T3-L1 adipocytes in presence or absence of oleate or palmitate. In details, the cells were pre-incubated in LG medium and then treated with 10µM oleate, 100µM palmitate and 100nM insulin.

I observed that insulin significantly reduced CCL5 mRNA levels and inhibited both oleate and palmitate effect (Figure 13).

Figure 13: Insulin effect on CCL5 mRNA levels. 3T3-L1 adipocytes were incubated in LG medium and treated with 10µM oleate, 100µM palmitate and 100nM insulin for 24 h. The amount of CCL5 mRNA was determined by real-time RT-PCR analysis of total RNA isolated from adipocytes. Bars represent the mRNA levels in these cells and are relative to those in cells incubated with LG medium. Asterisks denote statistically significant values (*p < 0.05).
Next, I investigated whether the block of MAPK and PI3K/AKT pathways, by MEK (25µM PD98059) and PI3K (25µM LY294002) inhibitors, respectively, inhibited the effect of insulin and restored an increase of CCL5 mRNA levels in adipocytes in presence of palmitate. As shown in Figure 14, the treatment with PD98059 and LY294002, revert the effect of insulin both in the absence and in the presence of palmitate. Analogous results have been obtained with oleate (data not shown).

Thus, insulin, activating MAPK and PI3K/AKT pathways, reduced CCL5 mRNA levels counteracting palmitate and oleate effect.

Figure 14: Insulin effect on CCL5 mRNA levels mediated by MAPK and PI3K/AKT pathways. 3T3-L1 adipocytes were incubated in LG medium and treated with 25µM PD98059 (PD) and 25µM LY294002 (LY), MEK and PI3K inhibitors respectively. After 1 h adipocytes were incubated with 100µM palmitate and 100nM insulin for 24 h. The amount of CCL5 mRNA was determined by real-time RT-PCR analysis of total RNA isolated from adipocytes. Bars represent the mRNA levels in these cells and are relative to those in cells incubated with LG medium. Asterisks denote statistically significant values (*p < 0.05).
4.8 Effect of insulin on NF-kB binding to CCL5 promoter

Finally, I evaluated the effect of insulin on NF-kB binding to CCL5 promoter in adipocytes in presence or absence of free fatty acids. 3T3-L1 adipocytes were incubated with LG medium and then stimulated with 10µM oleate, 100µM palmitate and 100nM insulin for 24h. Then, adipocytes were processed to perform chromatin immunoprecipitation (ChIP) experiments. Bar graph in Figure 15 shows that levels of NF-kB binding to CCL5 promoter were decreased in cells treated with insulin by 35%. Moreover insulin prevented both oleate and palmitate effect almost completely abrogating NF-kB binding in presence of oleate and palmitate. Thus, in adipocytes, insulin affected CCL5 regulation by fatty acids reducing NF-kB recruitment onto CCL5 promoter. This result suggested a potential anti-inflammatory role of insulin.

![Figure 15: Insulin effect on FFA-induced NF-kB binding to CCL5 promoter.](image_url)

10µM oleate, 100µM palmitate and 100nM insulin were added to 3T3-L1 adipocytes pre-incubated with LG medium. After 24 h the cells were processed to perform ChIP assay as described in Material and Methods. Soluble chromatin was immunoprecipitated with p65 NF-kB antibodies. Total and immunoprecipitated DNAs were amplified using primer pairs encompassing the CCL5 promoter by real-time RT-PCR. Bars represent the p65 binding levels and are relative to those in cells maintained in LG medium. Asterisks denote statistically significant values (*p < 0.05).
5. DISCUSSION

In obesity, the expanded adipose tissue is characterized by significant inflammatory infiltration and, via the secretion of multiple adipokines, contributes to chronic, low-grade inflammation and to obesity-associated pathologies such as type 2 diabetes and cardiovascular disease (Berg and Scherer 2005; Fontana et al. 2007). Adipose tissue macrophages may contribute to maintaining the low-grade inflammatory state linked to obesity activating the inflammatory program in neighboring adipocytes (Osborn and Olefsky 2012; Clément 2010).

Overnutrition and obesity are often accompanied by elevations in tissue and circulating FFA concentrations, and saturated FFA can induce inflammatory cascades in vascular endothelial cells, adipocytes and myeloid-derived cells through activation of JNK and NF-kB pathways (Kim et al. 2005; de Luca and Olefsky 2008). At the level of adipocytes, FFAs induce secretion of several molecules (Vazquez-Vela et al 2008; D'Esposito et al 2012).

Recent interest has focused on a salient member of CC chemokine beta subfamily, RANTES/CCL5, and its emerging role in regulating the recruitment of inflammatory cells into tissues. Several studies show that CCL5 is implicated in atherogenesis, and that circulating levels of the chemokine are associated with impaired glucose tolerance, type 2 diabetes and other cardiovascular risk factors, indicating adipocyte CCL5 production as a molecular link between inflammation and obesity (Nomura et al. 2000; Herder et al. 2005; Matter and Handschin 2007; Wu et al. 2007; D'Esposito et al. 2012).

Thus, in this work I have investigated the molecular mechanisms involved in metabolic control of CCL5 adipocyte expression. To this end, initially, I have addressed whether different concentrations of glucose and fatty acids may affect adipocyte ability to secrete and express CCL5.

I have showed that 3T3-L1 fibroblasts and 3T3-L1 adipocytes released detectable amounts of IL-4, IL-6, IL-10, IFN-γ, KC/IL-8, MIP-1α, MIP-1β, CCL5 and TNF-α VEGF, bFGF and IGF-1. The secretory patterns obtained by 3T3-L1 adipocytes and fibroblasts were different and adipocytes secreted higher amount of several cytokines/growth factors compared to fibroblasts. In particular, the levels of CCL5 were significantly higher in 3T3-L1 adipocytes compared with their precursors. Interestingly, both glucose and fatty acids
(oleate and palmitate) increased adipocyte-released CCL5, suggesting that the metabolic environment could further modify adipocyte releasing properties. Moreover oleate and palmitate, while not glucose, also increased CCL5 mRNA levels. These data raise the possibility that glucose and fatty acids may regulate CCL5 at different levels. The mechanism responsible for these events has not been explained, but it may involve activation of the different molecular pathways in adipocytes.

To clarify the possible mechanism responsible of CCL5 regulation by fatty acids, I have investigated the role of JNK, NF-kB, MAPK and PI3K/AKT pathways. These pathways resulted activated by fatty acids. I have obtained that the treatment of adipocytes with palmitate, almost invariably, induced higher phosphorylation of JNK, IkBα, ERK and AKT compared to oleate, but no statistically significant difference between oleate- and palmitate-induced protein phosphorylation was detected. Inhibition of JNK and NF-kB pathways resulted in a decrease of CCL5 mRNA levels induced by oleate and palmitate. Indeed, in this study, I have demonstrated that both JNK and NF-kB pathways mediated the effect of fatty acids on CCL5 mRNA levels. Moreover, I hypothesized that oleate and palmitate activated NF-kB affecting CCL5 gene transcriptional control and that the NF-kB activation could be mediated by JNK. The block of JNK reduced NF-kB binding to CCL5 promoter affecting the ability of fatty acids to promote NF-kB recruitment onto CCL5 promoter.

These data confirmed the active involvement of NF-kB and JNK pathways in mediating the induction of CCL5 expression by fatty acids in adipocytes. At the opposite, inhibition of MAPK and PI3K/AKT pathways increased CCL5 mRNA levels in the presence of both oleate and palmitate. Interestingly, I have observed that even in the absence of fatty acids, the inhibitors of these pathways enhanced CCL5 mRNA levels suggesting that MAPK and PI3K/AKT pathways may also constitutively control CCL5 expression. These pathways represent two main pathways involved in a complex signaling cascade downstream of the insulin receptor (de Luca and Olefsky 2008). Indeed I have evaluated whether insulin, activating MAPK and PI3K/AKT pathways could reduce CCL5 production in adipocytes treated with oleate and palmitate.

Ghanim et al. have demonstrated that low-dose insulin infusion in type 2 diabetic patients suppressed expression and plasma concentrations of CCL5 and the expression of its receptors (Ghanim et al. 2010). Moreover Andersson
et al. have demonstrated an anti-inflammatory effect of insulin in 3T3-L1 adipocytes through inhibition of IL-6 signaling (Andersson et al. 2007). In my work I have showed that insulin reduced mRNA levels of CCL5 and counteracted the stimulatory effect of oleate and palmitate on CCL5 expression in adipocytes. Previous study reported that insulin inhibited NF-κB pathway and MCP-1 chemokine expression in Human Aortic Endothelial Cells (HAEC) (Aljada et al. 2001). Indeed I have evaluated the effect of insulin on NF-κB binding to CCL5 promoter in 3T3-L1 adipocytes in presence or absence of free fatty acids. Insulin decreased NF-κB binding to CCL5 promoter by 35%. Moreover insulin prevented both oleate and palmitate effect almost completely abrogating NF-κB binding in presence of oleate and palmitate. Thus, in adipocytes, insulin affected fatty acid regulation of CCL5 and reduced their ability of recruiting NF-κB onto CCL5 promoter.
6. CONCLUSIONS

In conclusion, I have described that fatty acids induce expression and secretion of CCL5 in adipocytes. This effect is mediated by JNK, which regulates CCL5 transcription through NF-kB. In adipocytes, CCL5 mRNA is constitutively controlled by MAPK and PI3K/AKT pathway. Insulin inhibits FFA effect in part through these pathways and prevents FFA-induced recruitment of NF-kB onto CCL5 promoter. Thus, CCL5 could be a good candidate for new potential therapeutic strategies in optimizing the prevention of insulin-resistance and type 2 diabetes, in future. Finally, understanding adipose tissue inflammation and the molecular mechanisms involved in CCL5 expression in adipocytes could facilitate targeting specific pathways for the treatment of obesity-related diseases.
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8. REFERENCES


Clément K. Adipose Tissue in Health and Disease. Edited by Todd Leff and James G. Granneman. Copyright 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.


Wu H, Ghosh S, Perrard XD, Feng L, Garcia GE, Perrard JL, Sweeney JF, Peterson LE, Chan L, Smith CW, Ballantyne CM. T-cell accumulation and regulated on

ABSTRACT. Background: The cellular abundance of the phosphoprotein enriched in diabetes (PED/PEA-15), a 15 kDa protein related to insulin resistance (IR), is increased in women with polycystic ovary syndrome (PCOS). Aim: To investigate whether metformin (MET) has additive effects on PED/PEA-15 protein levels. Material/subjects and methods: This is an open label, prospective clinical study over 6 months. Ten hyperandrogenic obese PCOS women [age: 24.6±1.6 yr; body mass index (BMI): 30.7±1.2 kg/m²] were treated with MET (1250 mg/day). Ten age- and BMI-matched normo-androgenic women were used as controls. Outcome measures are: PED/PEA-15 protein levels, fasting plasma glucose and insulin (FPI), reciprocal index of homeostasis model assessment of insulin resistance (1/HOMA-IR); quantitative insulin sensitivity check index (QUICKI); whole-body insulin sensitivity index (ISI); SHBG; total testosterone; free androgen index (FAI). Results: At baseline FPI and PED/PEA-15 protein levels were higher, while 1/HOMA-IR, QUICKI, and ISI were lower (p<0.001) in MET group than in controls. After treatment, independently of body weight and hyperandrogenism, FPI, and PED/PEA-15 protein levels decreased (p=0.001 and 0.004, respectively), while, 1/HOMA-IR, QUICKI, and ISI increased (p<0.001). PED/PEA-15 protein levels correlated significantly with ISI either before (r=0.636; p=0.048), and after treatment (r=0.758; p=0.011). Conclusions: PED/PEA-15 protein levels reduced after a short course of treatment with MET in a group hyperandrogenic obese PCOS women. This effect was independent of body weight and hyperandrogenism, and correlated with ISI, thus adding a further benefit to obese PCOS women. (J. Endocrinol. Invest. 33: 446-450, 2010) © 2010, Editrice Kurtis

INTRODUCTION

Insulin resistance (IR) plays an important role in the physiopathology of polycystic ovary syndrome (PCOS) (1). The association with IR is considered to be responsible for an increased susceptibility to Type 2 diabetes and cardiovascular disease (CVD) among adolescent and pre-menopausal women with PCOS also independent of obesity and androgens levels, especially in patients with familial history of Type 2 diabetes (2). A number of post-receptor molecular defects responsible for IR has been described in PCOS (3). The phosphoprotein enriched in diabetes (PED/PEA-15) is a 15 kDa cytosolic scaffold protein related to insulin sensitivity, found to be widely present in tissues, with an important role in controlling glucose metabolism (4). In particular, PED/PEA-15 is over-expressed in skeletal muscle and adipose tissue in patients with Type 2 diabetes or at risk for diabetes (5). PED/PEA-15 is also over-expressed in white blood cells (WBC) in about 30% of Type 2 diabetic patients and their first-degree relatives (6), as well as in PCOS women independently of obesity (7). Although not approved by the US Food and Drug Administration for use in PCOS, metformin (MET) is recommended as initial intervention in PCOS women who are overweight and obese, with the additional positive effect on improving glucose metabolism abnormality and menstrual cyclicity (8). Taking into account the high risk of PCOS women for developing Type 2 diabetes and CVD, and considering the central role of PED/PEA-15 protein on IR, the aim of this pilot clinical study was to evaluate the effects of MET on the PED/PEA-15 protein levels in association with IR indices and hyperandrogenism in obese PCOS women.

MATERIALS AND METHODS

Study design

This is an open label, prospective, clinical study based on the evaluation of MET effects in the treatment of obese PCOS women. The procedures used were in accordance with the guidelines of the Helsinki Declaration on human experimentation. The study was conducted without support from the pharmaceutical industry, after approval by the institutional review board of the University of Naples, Italy. The purpose of the protocol was explained to both the patients and the controls, and written consent was obtained at the beginning of the study.
Outcome measures

The primary outcome measure was PED/PEA-15 protein level in WBC lysates. Secondary outcomes were body weight (BW), fasting plasma glucose (FPG), fasting plasma insulin (FPI), insulin sensitivity indices (reciprocal index of homeostasis model assessment of IR (1/HOMA-IR)), quantitative insulin sensitivity check index (QUICKI), whole-body insulin sensitivity index (ISI), SHBG, total testosterone (T), free androgen index (FAI).

Subjects

Between December 2006 and January 2009, 10 obese PCOS women [age: 24.6±1.6 yr; body mass index (BMI): 30.7±1.2 kg/m²; range 30-33.8] were consecutively admitted to the Endocrinology Unit of the Department of Molecular and Clinical Endocrinology and Oncology of the Federico II University of Naples (Italy), and enrolled in this clinical study, according to following inclusion criteria: pre-menopausal women, with a strict age range (20-30 yr) with diagnosis of PCOS; anovulatory oligo-amenorrhea; comparable clinical/biochemical hyperandrogenism; BMI range: grade I obesity; caucasian ethnicity. To further minimize subjects variability, the presence of Type 2 diabetes or abnormal glucose tolerance was excluded by the oral glucose tolerance test (OGTT). Other exclusion criteria for all subjects were: smoking or alcohol consumption, pregnancy, hypothyroidism, hyperprolactinemia, Cushing’s syndrome, non-classical congenital adrenal hyperplasia; previous (within the last 6 months) use of oral contraceptives, glucocorticoids, anti-androgens, ovulation induction agents, anti-obesity drugs, or other hormonal drugs. None of the subjects was affected by any neoplastic, metabolic, hepatic, and cardiovascular disorder or other concurrent medical illness (i.e. renal disease, and malabsorptive disorders). Moreover, those with acute and chronic inflammations based on medical history, physical examination, and routine laboratory tests, including measurement of body temperature, WBC count and urinalysis, were excluded.

The diagnosis of PCOS was made according to the diagnostic criteria for PCOS (9). All PCOS women enrolled in the study had clinical and/or biochemical hyperandrogenism with IR, and showed increased PED/PEA-15 levels in WBC (Table 1).

Patients received a low-dose MET treatment (1250 mg/day). The duration of follow-up was 6 months. The control group consisted of 10 healthy female volunteers, who were age- and BMI-matched with patients (age: 23.6±3.2 yr; BMI: 30±0.6 kg/m²), came from the same geographical area, with regular menstrual cycles (defined as 26-32 days in length), normal androgen levels and insulin sensitivity, and normal PED/PEA-15 protein levels. Specifically, 100% of controls were selected in Naples and were young female doctors working in the Medical School of University “Federico II” in Naples who spontaneously agreed to be investigated. Their health status was determined by medical history, physical, and pelvic examination, and complete blood chemistry.

Methods

The ovulatory state was investigated by pelvic or transvaginal ultrasonography (TV-USG) and plasma progesterone (P) levels. Both procedures were performed during the luteal phase of the menstrual cycle (7 days before the expected menses). The presence of fluid in the cul-de-sac at pelvic or TV-USG and a plasma P assay >32 nmol/l (>10 ng/ml) were considered to be the criteria to show that ovulation had occurred (10). The controls were not genetically related to the PCOS group and without family history of diabetes.

Hirsutism was assessed using the Ferriman-Gallwey (FG) score (11). A progesterone challenge test (100 mg natural progesterone im) was performed, which induced uterine bleeding in all PCOS women. The normal glucose response to the OGTT was defined according to the “Report of the Expert Committee on the diagnosis and classification of diabetes mellitus” (12). All anthropometric measurements were taken with subjects wearing only light clothes and without shoes. In each woman, weight and height were measured to cal-

Table 1 - Baseline clinical and metabolic features of 10 healthy normal-weight controls (Controls) and within-group changes between baseline (T0) and after 6 months (T6) in clinical and metabolic features of 10 polycystic ovary syndrome (PCOS) women treated with metformin (MET).

<table>
<thead>
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<tr>
<td></td>
<td>T0</td>
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<td>FPG (mg/dl)</td>
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<td>395.0±155.9</td>
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</table>

p*: MET group T0 vs controls; p**: MET group T0 vs T6; FPG: fasting plasma glucose; FPI: fasting plasma insulin; 1/HOMA-IR: reciprocal index of homeostasis model assessment of insulin resistance; QUICKI: quantitative insulin sensitivity check index; ISI: whole-body insulin sensitivity index; FAI: free androgen index. Data are reported as mean±SD; PED/PEA-15 protein levels are expressed as arbitrary units.
calculate the BMI [weight (kg) divided by height squared (m²), kg/m²]. Height was measured to the nearest cm using a wall-mounted stadiometer. BW was determined to the nearest 50 g using a calibrated balance beam scale. Patients were given a standardized interview to obtain information about the duration of obesity, eating patterns, smoking habits, and physical exercise. In particular, subjects were also asked to make a daily record of the amount of physical activity (no exercise; ≤2-3 h/week; ≥2-3 h/week). Pre-admission food intake and dietary history were assessed by a skilled dietician who used a computer-assisted interview (Winfood 1.5, Medimatica srl, Martinisicuro, Italy). All PCOS women received a normocaloric diet.

**Assays**

Blood samples were obtained between 08:00 h and 09:00 h from an ante-cubital vein after an overnight fast, with the patient in the resting position. The OGTT was performed using 75 g dextrose. Blood samples were obtained at 0, 30, 60, 90, 120 min for plasma glucose and insulin measurements. FPG levels were determined by the glucose oxidase method immediately after the OGTT. FPI samples were promptly centrifuged, plasma was separated and stored at –20°C until assay. FPI, SHBG, and T levels were measured by a solid-phase chemiluminescent enzyme immunoassay using commercially available kits (Immune Diagnostic Products Co, Los Angeles, CA). The intra- and inter-assay coefficients of variations were less than 5·5% for the insulin and SHBG assays, and 10% for total T assay. FAI was calculated as an expression of peripheral androgen activity and estimated as total T and SHBG serum concentration, according to the formula FAI = T (nmol/l)/SHBG (nmol/l) × 100. The HOMA-IR was calculated from FPG and FPI according to the report by Matthews et al. (13), according to the formula [FPI (µU/ml) × FPG (mmol/l)]/22.5 and reciprocal index of HOMA-IR (1/HOMA-IR) was calculated. QUICKI was calculated as: 1/[(log(I₀)+log(G₀)] where I₀ is FPI (µU/ml) and G₀ is FPG (mg/dl). Whole-body ISI was calculated as: ϕ(FPG × FPI) × (mean glucose × mean insulin), according to Matsuda and DeFronzo (15). Cut-off points for 1/HOMA-IR, QUICKI, and ISI were ≤0.47, ≤0.33, ≤4.75, respectively (16).

PED/PEA-15 protein levels were measured at 3 and 6 months in WBC lysates obtained from 10 to 12 ml of freshly collected uncoagulated whole blood, after separation with dextran 6%, using Western blot analysis, as previously reported (6). For Western blot analysis WBC were solubilized at 4 C in TAT buffer, centrifuged at 500g for 20 min, and supernatant fractions were stored at –20°C until used. The amount of 50 µg of lysate proteins were heated at 100°C in Laemmli buffer. Proteins were separated by 15% SDS-PAGE and then transferred to 0·45-mm Immobilon-P membranes (Millipore, Bedford, MA). Filters were probed with PED/PEA-15 antiserum at 1:2000 dilution, revealed by enhanced chemiluminescence and autoradiography. The protein bands were quantified by laser densitometry and expressed as percentage of pixels (arbitrary units). As reported previously (6), PED/PEA-15 protein levels in WBC detected by Western blot analysis, correlated strongly with mRNA detected by real time RT-PCR (p<0.001), and with PED/PEA-15 protein amount in fat and skeletal muscle tissues (p<0.001). In addition, PED/PEA-15 protein stability was also assessed by repeated testing, at 2, 4, and 6 months after storage, displaying intra- and inter-assay coefficients of variation <8 and <15%, respectively.

**Statistical analysis**

All results are expressed as mean±SD. Fasting insulin and PED/PEA-15 protein values were not normally distributed and have been logarithmically transformed as loge. Differences between groups were analysed by paired t-test. Bivariate correlations between variables were examined using Pearson’s correlation coefficient. Values ≤5% were considered statistically significant. Data were stored and analysed using the Statistical Package for Social Science program (SPSS, release 13·01, Chicago, IL).

**RESULTS**

Clinical and metabolic features of 10 PCOS women and 10 age- and BMI-matched controls at baseline and after treatment are reported in Table 1. As expected, FPG, FPI, T levels, FAI, FG-score, and PED/PEA-15 protein expression were significantly higher in PCOS compared with controls, while insulin sensitivity indices and SHBG levels were significantly lower. All included subjects completed the study. During the follow-up, on the basis of reported dietary intake, all subjects maintained their normocaloric diet and their BW remained unchanged. All subjects had a normal physical activity level. After treatment, 1/HOMA-IR, QUICKI, and ISI increased, while FPI and PED/PEA-15 protein level were reduced; no difference were observed in T levels and FG-score; however, as SHBG levels increased, FAI was significantly reduced. PED/PEA-15 protein levels correlated significantly with ISI both before, as previously reported (7), than after treatment (Fig.1A and B, respectively), but no significant correlations were found with BMI. Moreover, while at baseline, there was no correlation between PED/PEA-15 protein levels, 1/HOMA-IR, and SHBG after treatment a negative correlation with these variables became evident (Fig. 1C and D, respectively).

**DISCUSSION**

Despite limitations due to the small sample size, this preliminary prospective study showed that PED/PEA-15 protein levels in hyperandrogenic obese PCOS women were significantly reduced after a 6 months treatment with MET. Interestingly, in line with the limited effects of MET on weight loss, as also reported in a recently published metaanalysis (17), the reduction in PED/PEA-15 protein levels was observed independently from any BW differences, and was also significantly correlated with the improvement of all mathematical indices for assessing insulin sensitivity evaluated in this study. In particular, PED/PEA-15 protein levels correlated with indices using the values of fasting glucose and insulin, thus primarily reflecting the hepatic insulin sensitivity, but that are likely influenced by BW and metabolic or hormonal factors; however PED/PEA-15 protein levels correlated also with
ISI, an OGTT-derived index that provides a better estimate of insulin sensitivity than fasting indices, as the plasma glucose responses are the results of peripheral glucose utilization and hepatic glucose production. To the best of our knowledge, this paper is the first report on the effect of MET treatment on PED/PEA-15 protein levels and its correlations with IR indices.

PED/PEA-15 is a 15 kDa ubiquitously protein implicated in a number of fundamental cellular functions, including apoptosis, proliferation, and glucose metabolism. In particular, PED/PEA-15 protein alteration was identified early during the natural history of Type 2 diabetes and might be involved in the progressive derangement of glucose tolerance, by affecting both insulin action and insulin secretion. We previously evaluated PED/PEA-15 protein levels in a group of obese and non-obese PCOS women evidencing that PCOS women presented an increased amount of PED/PEA-15 protein levels (7). This increase, similarly to healthy first-degree relatives of patients with Type 2 diabetes (6), was independent of BW, but was associated with hyperandrogenism. In particular, PED/PEA-15 protein levels showed a negative correlation with SHBG levels and a positive correlation with T levels and FAI (7). Thus, it was of interest to further evaluate in the present setting the relationships between PED/PEA-15 protein levels, IR, and androgens after MET treatment.

Concerning a possible relationship between PED/PEA-15 protein, IR, and androgens, a link between a hyperandrogenic, hyperinsulinemic environment in PCOS women, and the development of IR has been reported to occur, involving different steps of post-receptorial insulin signaling pathway (1, 18). Data obtained in our study showed that PED/PEA-15 protein levels reduction after treatment was not associated to the improvement of hyperandrogenism. After treatment, only FAI showed a slight but significant reduction associated with the increase in SHBG, likely reflecting the reduction of IR evidenced in this group (19) or, to a lesser extent, a direct effect of MET (20). Thus, although bidirectional influences between both variables are likely to take place in the progression of PCOS, our data supported the hypothesis of the independence of PED/PEA-15 protein pathway from hyperandrogenism. In that, the present study further extend the understanding of the mechanisms and consequences of PED/PEA-15 protein levels in PCOS pathogenesis.

Finally, a direct effect of MET on PED/PEA-15 protein could be hypothesized. In our laboratory is under investigation whether induction of PED/PEA-15 cellular abundance could be a direct effect of MET on its regulatory mechanisms (gene expression, protein degradation). However, preliminary data in skeletal muscle cells ap-
parently exclude this possibility. Alternatively, indirect effects may be responsible for this mechanism. Cloning of PED/PEA-15 promoter revealed nuclear factor-kB (NF-kB) binding consensus sequence (our unpublished data). NF-kB is a transcription factor, activated by tumour necrosis factor (TNF)-α, and involved in the regulation of genes that control inflammation, cell proliferation and survival. Thus, it might be speculated that PED/PEA-15 can be overexpressed via TNF-α/NF-kB pathways. Since MET, as previously reported, reduces TNF-α levels in PCOS (21), it could downregulate PED/PEA-15 by affecting TNF-α/NF-kB pathways. Despite limitations due to the small sample size, the strengths of this study include a) the subjects enrolled were homogenous in age and BMI, also sharing the same ethnicity, and all completed the study; b) the study has been carried out at a single Institution; c) the average time of the follow-up was sufficiently extended, and was as long as most PCOS trials; d) finally, the high statistical significance of the results obtained also in spite of the low number of subjects is an added value of our study and supported the association of MET with PED/PEA-15 protein pathway.

In conclusion, in line with well-known insulin-sensitizing action of MET (22), and apart from its consolidated use in the induction of ovulation (8), our findings provide support for the hypothesis that the reduction in PED/PEA-15 protein levels might represent a further puzzle piece in the complex framework of the effects on glucose metabolism exerted by MET, and suggests a novel adjunctive advantage of MET as therapeutic option for obese PCOS women. However, as long as most PCOS trials, further investigations with long-term follow-up, and larger sample series are still needed to confirm this hypothesis, and the evaluation of the possible direct or indirect effects of MET on PED/PEA-15 expression and/or degradation in target tissue will be the aim of our future researches.

ACKNOWLEDGMENTS

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Serum 25-Hydroxyvitamin D Levels, phosphoprotein enriched in diabetes gene product (PED/PEA-15) and leptin-to-adiponectin ratio in women with PCOS

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Abstract

Background: Polycystic ovary syndrome (PCOS) is frequently associated with hypovitaminosis D. Vitamin D is endowed with pleiotropic effects, including insulin resistance (IR) and apoptotic pathway. Disruption of the complex mechanism that regulated ovarian apoptosis has been reported in PCOS. Phosphoprotein enriched in diabetes gene product (PED/PEA-15), an anti-apoptotic protein involved in type 2 diabetes mellitus (T2DM), is overexpressed in PCOS women, independently of obesity. Leptin-to-adiponectin ratio (L/A) is a biomarker of IR and low-grade inflammation in PCOS. The aim of the study was to investigate the levels of 25-hydroxy vitamin D (25(OH)D), and L/A, in association with PED/PEA-15 protein abundance, in both lean and overweight/obese (o/o) women with PCOS.

Patients and Methods: PED/PEA-15 protein abundance and circulating levels of 25(OH)D, L/A, sex hormone-binding globulin, and testosterone were evaluated in 90 untreated PCOS patients (25 ± 4 yrs; range 18-34) and 40 healthy controls age and BMI comparable, from the same geographical area. FAI (free androgen index) and the homeostasis model assessment of insulin resistance (HOMA-IR) index were calculated.

Results: In o/o PCOS, 25(OH)D levels were significantly lower, and L/A values were significantly higher than in lean PCOS (p < 0.001), while there were no differences in PED/PEA-15 protein abundance. An inverse correlation was observed between 25(OH)D and BMI, PED/PEA-15 protein abundance, insulin, HOMA-IR, FAI (p < 0.001), and L/A (p < 0.05). At the multivariate analysis, in o/o PCOS L/A, insulin and 25(OH)D were the major determinant of PED/PEA-15 protein abundance (β = 0.45, β = 0.41, and β = -0.25, respectively).

Conclusions: Lower 25(OH)D and higher L/A were associated to PED/PEA-15 protein abundance in PCOS, suggesting their involvement in the ovarian imbalance between pro-and anti-apoptotic mechanisms, with high L/A and insulin and low 25(OH)D levels as the main determinants of PED/PEA-15 protein variability. Further studies, involving also different apoptotic pathways or inflammatory cytokines and granulosa cells are mandatory to better define the possible bidirectional relationships between 25(OH)D, PED/PEA-15 protein abundance, leptin and adiponectin in PCOS pathogenesis.

Keywords: 25-hydroxyvitamin D, PED/PEA-15, Leptin-to-adiponectin ratio, PCOS, apoptosis

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Background

Obesity, predominantly intra-abdominal visceral adipose tissue, is observed in 30%-75% of women with polycystic ovary syndrome (PCOS) [1], with hyperandrogenism and insulin resistance (IR) as other common features of this syndrome [2]. Obesity is considered to be a risk factor for hypovitaminosis D [3]. A number of cross-sectional studies have evidenced the association between obesity, hypovitaminosis D and PCOS cohorts [4-8], although hypovitaminosis D have been considered as the consequence of obesity and IR per se [7].

Vitamin D is endowed with pleiotropic effects on a wide spectrum of intracellular regulatory mechanisms, including insulin metabolism [9], or intrinsic apoptotic pathway, on both classical and nonclassical tissues, such as ovary [10]. Dysregulation of the complex mechanism that regulated ovarian apoptosis has been reported in PCOS [11].

Phosphoprotein enriched in diabetes gene product (PED/PEA-15) is an anti-apoptotic protein involved in IR and type 2 diabetes mellitus (T2DM) [12]. We previously reported that PED/PEA-15 is overexpressed in T2DM patients [13] and in PCOS women [14]. In both groups this association was independent of obesity, thus suggesting that PED/PEA-15 overexpression might represent a T2DM or PCOS specific feature, likely linked to IR, although also different pathogenetic mechanisms influencing PED/PEA-15 expression in PCOS, such as apoptosis, have not been excluded.

Circulating levels of leptin and adiponectin, two adipose tissue-derived hormones with opposing associations with the metabolic syndrome (MetS) and coronary heart disease, are altered in PCOS [15], thus contributing through the low-grade chronic inflammation to the long-term metabolic consequence of the syndrome [16], and possibly, to the dysregulation between apoptotic and antiapoptotic mechanisms [17]. Finally, the ratio of leptin-to-adiponectin (L/A), has been reported to represent a better marker for obesity, IR, and MetS than each single adipokine [18], in particular in female population [19]. Although there is still some debate, a number of recent studies supported a role for L/A as a biomarker of both IR and low-grade inflammation also in women with PCOS [20].

Taking into account their involvement in obesity and apoptosis, the aim of our study was to investigate the balance between circulating levels of 25-hydroxy vitamin D (25(OH)D), leptin, and adiponectin, and PED/PEA-15 protein abundance, in both lean and overweight/obese (o/o) women with PCOS. The procedures used were in accordance with the guidelines of the Helsinki Declaration on human experimentation. The study was conducted without support from the pharmaceutical industry, after approval by the institutional review board of the University of Naples, Italy. The purpose of the protocol was explained to both the patients and the controls, and written consent was obtained at the beginning of the study.

Subjects

Ninety untreated PCOS patients (25 ± 4 yrs; range 18-34) were consecutively admitted to the Endocrinology Unit of the Department of Molecular and Clinical Endocrinology and Oncology of the Federico II University of Naples (Italy), and enrolled in this observational clinical study according to following inclusion criteria: premenopausal women, with a strict age range (20-40 yr) with diagnosis of PCOS; anovulatory oligo-amenorrhea; comparable clinical/biochemical hyperandrogenism; caucasian ethnicity. All subjects included in the study resided in the Naples metropolitan area (latitude 40° 49’ N; elevation 17 m) and were evaluated from March trough June 2011.

To further minimize subjects variability, the presence of T2DM or abnormal glucose tolerance was excluded by the oral glucose tolerance test (OGTT). Other exclusion criteria for all subjects were: smoking or alcohol consumption, pregnancy, hypothyroidism, hyperprolactinemia, Cushing’s syndrome, non-classical congenital adrenal hyperplasia; previous (within the last 6 months) use of oral contraceptives, glucocorticoids, anti-androgens, ovulation induction agents, anti-obesity drugs, or other hormonal drugs. None of the subjects was affected by any neoplastic, metabolic, hepatic, and cardiovascular disorder or other concurrent medical illness (i.e. renal disease, and malabsorptive disorders). Moreover, those with acute inflammations based on medical history, physical examination, and routine laboratory tests, including measurement of body temperature, white blood cell count (WBC) and urinalysis, were excluded. The diagnosis of PCOS was made according to the diagnostic criteria for PCOS [21]. All PCOS women enrolled in the study had clinical and/or biochemical hyperandrogenism with IR. Forty young women, among clerks, and medical and paramedical personnel of the Department of Molecular and Clinical Endocrinology and Oncology of the University “Federico II” of Naples, age and BMI comparable with the patients, from the same geographical area, with regular menstrual cycles (defined as 26-32 days in length), agreed to participate in this study and were used as controls. Exclusion criteria for controls were the same of the patients. All participants gave their informed consent before enrolment.

Materials and methods

Study design

This is an observational clinical study based on the evaluation of 25(OH)D, leptin, and adiponectin in both
Methods

The ovulatory state was investigated by pelvic or translabial ultrasonography (TV-USG) and plasma progesterone (P) levels. Both procedures were performed during the luteal phase of the menstrual cycle (7 days before the expected menses). The presence of fluid in the cul-de-sac at pelvic or TV-USG and a plasma P assay >32 nmol/l (>10 ng/ml) were considered to be the criteria to show that ovulation had occurred [22]. The controls were not genetically related to the PCOS group and without family history of diabetes.

Hirsutism was assessed using the Ferriman–Gallwey (FG)-score, with a score >8 indicative of hyperandrogenism [23]. A progesterone challenge test (100 mg natural progesterone im) was performed, which induced uterine bleeding in all PCOS women. The normal glucose response to the OGTT was defined according to the “Report of the Expert Committee on the diagnosis and classification of diabetes mellitus” [24].

All anthropometric measurements were taken with subjects wearing only light clothes and without shoes. In each woman, weight and height were measured to calculate the BMI [weight (kg) divided by height squared (m²), kg/m²]. Height was measured to the nearest cm using a wall-mounted stadiometer. Body weight was determined to the nearest 50 g using a calibrated balance beam scale. The degree of normal weight, overweight, or obesity was established on the basis of BMI cut-off points of 18-24.9 (lean subjects), 25-29.9, 30-34.9, 35-39.9 and > 40 kg/m², respectively (%/o).

Assays

Blood samples, obtained between 08:00 h and 09:00 h from an ante-cubital vein after an overnight fast, with the patient in the resting position, were promptly centrifuged, and serum separated and stored at -20°C until assay. The OGTT was performed using 75 g dextrose. Blood samples were obtained at 0, 30, 60, 90, 120 min for plasma glucose and insulin measurements. Fasting plasma glucose (FPG) levels were determined by the glucose oxidase method immediately after the OGTT. Circulating levels of 25(OH)D (LIAISON; DiaSorin, Saluggia (VC), Italy), fasting plasma insulin (FPI), sex hormone-binding globulin (SHBG), and testosterone (T) levels (Immulite, Diagnostic Products Co, Los Angeles, CA; n.v. 0.2-1.2 nmol/l) were measured by solid-phase chemiluminescent enzyme immunoassays. The intra-assay coefficients of variation (CV) of variations were less than 5% for the 25(OH)D, insulin, and SHBG assays, and 10% for total T assay. FAI (free androgen index) was calculated as an expression of peripheral androgen activity and estimated as total T and SHBG serum concentration, according to the formula FAI = T (nmol/l)/SHBG (nmol/l) × 100. Leptin and total adiponectin (low, middle, and high molecular weight) levels were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits (AvisBion: Orgenium Laboratories, Helsinki, Finland), with sensitivity 1 and 3 ng/ml, respectively; intra- and inter-assay CVs were less than 10% and 12%, respectively, for adiponectin and leptin. Vit D deficiency and insufficiency were defined as 10-30 and <10 ng/ml, respectively. The homeostasis model assessment of insulin resistance (HoMA-IR) index was calculated from FPG and FPI according to the report by the formula [FPI (μU/ml) × FPG (nmol/l)]/22.5 [25]. As a stringent measure of IR, a value of HoMA-IR > 2.0 was set (deriver by the mean ± 2 SD of our lean population), in accordance with a previous cutoff [26].

PED/PEA-15 protein levels were measured in WBC lysates obtained from 10 to 12 ml of freshly collected uncoagulated whole blood, after separation with dextran 6%, using Western blot analysis, as previously reported [13]. For Western blot analysis WBC were solubilized at 4°C in TAT buffer, centrifuged at 500 g for 20 min, and supernatant fractions were stored at -20°C until used. The amount of 50 μg of lyse proteins were heated at 100°C in Laemmli buffer. Proteins were separated by 15% SDS-PAGE and then transferred to 0.45 mm Immobilon-P membranes (Millipore, Bedford, MA). Filters were probed with PED/PEA-15 antiserum at 1:2000 dilution, revealed by enhanced chemiluminescence and autoradiography. The protein bands were quantified by laser densitometry and expressed as arbitrary units. As reported previously [13], PED/PEA-15 protein levels in WBC detected by Western blot analysis, correlated strongly with mRNA detected by real time RT-PCR (p < 0.001). In addition, PED/PEA-15 protein stability was also assessed by repeated testing, at 2, 4, and 6 months after storage, displaying intra- and inter-assay coefficients of variation <8 and <15%, respectively.

Statistical analysis

All results are expressed as mean ± SD. Fasting 25(OH)D, insulin, PED/PEA-15 protein abundance, leptin, and adiponectin values were not normally distributed and have been logarithmically transformed. Differences between lean and overweight/obese subjects in both PCOS and Control women were analysed by unpaired t test. Bivariate correlations between variables were examined using Pearson’s correlation coefficient. Only variables significant on univariate analysis were included in the multivariate analysis. Three multiple linear regression analysis (stepwise model, p to include <0.005, p to remove >0.1, maxstep 15), were calculated with PED/PEA-15, as dependent variable and BMI, insulin, 25(OH)D, L/A and FAI as independent variables considering PCOS women as a whole, or according to BMI (lean
and o/o). To avoid multicollinearity, variables with a tolerance of 0.2 were excluded. Values ≤5% were considered statistically significant. Data were stored and analysed using the Statistical Package for Social Science program (SPSS, release 13.01, Chicago, IL).

**Results and Discussion**

Metabolic and hormonal characteristics of the study population were reported in Table 1. Testosterone levels and FG score in PCOS women were 2.8 ± 1.1 nmol/l and 16 ± 6, respectively. Grouping PCOS women according to BMI, there were 42 lean and 48 o/o subjects. Subgroup analysis of o/o and lean women revealed differences between groups. In particular, apart from the expected differences in insulin, HoMA-IR, and androgens, 25(OH)D and adiponectin levels were significantly lower, while leptin levels and L/A values were significantly higher in o/o PCOS compared with lean PCOS. No differences in PED/PEA-15 protein abundance were evident between lean and o/o PCOS women.

Correlations between variables in the study population were reported in Figure 1. In PCOS women, an inverse correlation was observed between 25(OH)D and BMI, and PED/PEA-15 protein abundance (b), insulin (c), HoMA-IR (d), and FG score (e) (r= -0.474, -0.553, -0.380, -0.407, -0.374, respectively; p < 0.001), and L/A (f) (r= -0.306, p < 0.05). At the multivariate analysis, with PED/PEA-15 protein abundance as dependent variable only insulin and 25(OH)D remained in the model (β = 0.39 and β = -0.32, respectively) (Table 2). The results of the analysis were different, however, in the two group of PCOS women, as the major determinants of PED/PEA-15 protein abundance were BMI and insulin in lean PCOS (β = 0.65 and β = 0.26, respectively) (Table 3), and L/A, insulin and 25(OH)D in o/o PCOS (β = 0.45, β = 0.41 and β = -0.25, respectively) (Table 4).

Our data evidenced that in women with PCOS low levels of 25(OH)D and PED/PEA-15 protein abundance are associated to high insulin, HoMA-IR, and L/A values. According to previous data hypovitaminosis D was evident in all PCOS women, with 25(OH)D levels being significantly lower in o/o than in lean PCOS women. Insulin was the major determinant of PED/PEA-15 protein abundance, explaining about 26% of PED/PEA-15 protein abundance variability, while 25(OH)D levels added another 7% of its variability. In that our data confirmed the association between insulin and 25(OH)D [7], but evidenced also a novel association between 25(OH)D, PED/PEA-15 protein abundance, and L/A in PCOS women.

A number of cross-sectional studies have evidenced the association between hypovitaminosis D and PCOS cohorts [4-8], although low serum 25(OH)D concentrations have been considered as the consequence of obesity and IR per se [5-7]. Also an altered L/A has been already reported in PCOS women, as a biomarker of either IR and low-grade inflammation [15,17,27,28]. However, to the best of our knowledge, all these variables in PCOS women have been investigated in the setting of IR or MS, while it is well-known that either 25(OH)D or low-grade inflammation display strict relationships with apoptotic and antiapoptotic mechanisms [29]. Thus, the association of 25(OH)D and L/A ratio with the anti-apoptotic protein PED/PEA-15 might suggest a different scenario in PCOS women.

**Table 1** Metabolic and endocrine characteristics of the study population grouped according to BMI.

<table>
<thead>
<tr>
<th></th>
<th>Lean (42)</th>
<th>Overweight/Obese (48)</th>
<th>p</th>
<th>Lean (20)</th>
<th>Overweight/Obese (20)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>24.1 ± 4.6</td>
<td>24.8 ± 4.0</td>
<td>NS</td>
<td>23.9 ± 3.6</td>
<td>25.4 ± 4.6</td>
<td>NS</td>
</tr>
<tr>
<td>BMI</td>
<td>22.1 ± 2.6</td>
<td>33.1 ± 5.8</td>
<td>&lt;0.001</td>
<td>22.1 ± 1.6</td>
<td>34.9 ± 4.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>25(OH)D (ng/ml)</td>
<td>15.1 ± 7.6</td>
<td>11.1 ± 5.8</td>
<td>0.021</td>
<td>48.9 ± 11.3</td>
<td>10.2 ± 4.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PED/PEA-15</td>
<td>290 ± 102</td>
<td>295 ± 113</td>
<td>0.745</td>
<td>153 ± 29</td>
<td>177 ± 47</td>
<td>0.092</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>25.8 ± 6.6</td>
<td>403.9 ± 9.1</td>
<td>&lt;0.001</td>
<td>9.9 ± 48</td>
<td>294 ± 66</td>
<td>0.003</td>
</tr>
<tr>
<td>Adiponectin (ng/ml)</td>
<td>13.2 ± 5.7</td>
<td>94.6 ± 6.3</td>
<td>&lt;0.001</td>
<td>174.3 ± 5.3</td>
<td>11.6 ± 25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>11.9 ± 4.6</td>
<td>171.9 ± 9.4</td>
<td>0.014</td>
<td>7.6 ± 1.9</td>
<td>11.5 ± 20</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leptin-to-adiponectin ratio</td>
<td>25.2 ± 18</td>
<td>58 ± 27</td>
<td>&lt;0.001</td>
<td>0.6 ± 0.4</td>
<td>20 ± 1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HoMA-IR</td>
<td>2.4 ± 1</td>
<td>3.8 ± 2.2</td>
<td>&lt;0.001</td>
<td>1.4 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>45.7 ± 7.2</td>
<td>41.3 ± 6.9</td>
<td>0.001</td>
<td>69.5 ± 3.1</td>
<td>63.3 ± 10.5</td>
<td>0.016</td>
</tr>
<tr>
<td>FAI</td>
<td>4.6 ± 0.8</td>
<td>8.7 ± 3.8</td>
<td>&lt;0.001</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD. BMI, body mass index; 25(OH)D, 25(OH) Vitamin D; PED/PEA-15, PED/PEA-15 abundance; HoMA-IR, homeostasis model assessment of insulin resistance; SHBG, sex hormone binding globulin; FAI, free androgen index; FG, Ferriman-Gallwey. PED/PEA-15 protein levels are expressed as arbitrary units. Fasting 25(OH)D, insulin, PED/PEA-15 protein abundance, leptin, and adiponectin values were not normally distributed and have been logarithmically transformed. Differences between lean and overweight/obese subjects in both PCOS and Control women were analysed by unpaired t-test. Controls were age and BMI comparable.
Loss of the ovarian apoptotic mechanism has been reported to account PCOS appearance [11,30,31], with hyperandrogenism as one of the proposed mechanisms affecting the balance between the ovarian expression of pro and anti-apoptotic proteins [32]. In particular, follicular atresia is associated with an imbalance in the antiapoptotic effect of the Bcl-2 family members [33], while gonadotropin treatment inhibits granulosa cell apoptosis and follicular atresia through the reduction of the expression of the proapoptotic protein Bax [32]. Recent studies have revealed that Vit D is involved in the control of various cellular processes, including apoptosis, via Bcl-family up-regulation and Bax down-regulation, but the mechanisms underlying this action have not been fully explored [10,34]. Similarly, leptin and adiponectin exerted opposite effects also in the complex control of apoptosis-antiapoptosis mechanisms. In particular, leptin acts as a mitogenic factor in a variety of cell types, including ovary [35], where it has been found to interfere with ovulation rate [36]. On the other hand, adiponectin, whose receptors are expressed in ovary in human [37], has been recently reported to activate the Mitogen-Activated Protein Kinase (MAPK) cascade in granulosa cells, in the context of the classical adiponectin anti-apoptotic effects [37-39].

<table>
<thead>
<tr>
<th>Independent variables:</th>
<th>BMI, insulin, 25(OH)D, L/A, FAI</th>
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<tbody>
<tr>
<td>Step</td>
<td>Variables inserted in the model</td>
</tr>
<tr>
<td>1</td>
<td>insulin</td>
</tr>
<tr>
<td>2</td>
<td>insulin, 25(OH)D</td>
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</tbody>
</table>

Variables excluded from the model: BMI, L/A, FAI

Variations in PED/PEA-15 abundance explained by stepwise model (p to include < 0.05, p to remove > 0.1), with selected variables in PCOS women (n = 90). BMI, body mass index; 25(OH)D, 25(OH) Vitamin D; PED/PEA-15, L/A, leptin-to-adiponectin ratio; FAI, free androgen index.

<table>
<thead>
<tr>
<th>Independent variables:</th>
<th>BMI, insulin, 25(OH)D, L/A, FAI</th>
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<tbody>
<tr>
<td>Step</td>
<td>Variables inserted in the model</td>
</tr>
<tr>
<td>1</td>
<td>BMI</td>
</tr>
<tr>
<td>2</td>
<td>BMI, insulin</td>
</tr>
</tbody>
</table>

Variables excluded from the model: 25(OH)D, L/A, FAI

Variations in PED/PEA-15 abundance explained by stepwise model (p to include < 0.05, p to remove > 0.1) in lean PCOS women (n = 42). BMI, body mass index; 25(OH)D, 25(OH) Vitamin D; PED/PEA-15, L/A, leptin-to-adiponectin ratio; FAI, free androgen index.
In that, our data evidenced that, hypovitaminosis D and impaired L/A played an adjunctive role to insulin in influencing the increase in the anti-apoptotic protein PED/PEA-15 abundance and, therefore, contributing to alter the equilibrium between anti and pro-apoptotic factors in PCOS.

Of interest, grouping PCOS women according to BMI there were different associations between the study variables. In particular, in lean PCOS BMI explained 52% of PED/PEA-15 protein abundance variability, and insulin added only another 6% of its variability; in o/o PCOS women L/A, insulin and 25(OH)D explained 37%, 22% and 5% of PED/PEA-15 protein abundance variability, respectively. Thus, it is tempting to speculate that in PCOS women, along with the progressive increase in BMI, A/L and IR induced by increased amount of dysfunctional adipocytes are involved in the higher activation of anti-apoptotic pathways, such as PED/PEA-15 protein.

The alteration of the dynamics of the loss of preantral follicle by atresia might contribute to increase androgen secretion from atresic follicles, that on turn, resulted not only in further increase in follicle atresia [32], but also in IR and PED/PEA-15 protein abundance. In line with hypothesis, we found opposite correlations between 25(OH)D, PED/PEA-15 protein and L/A with FAI, the main biochemical endocrine PCOS features. In particular, 25(OH)D exhibited an inverse, while PED/PEA-15 protein and L/A showed a positive correlation with FAI. Apart from a clear effect of gender differences [40], the inverse association of 25(OH)D with testosterone and FAI in the setting of PCOS, despite some controversies [8], and the positive association of PED/PEA-15 protein abundance with hyperandrogenism, were according to previously published data [14], further suggesting the involvement of bidirectional influences between both variables in the progression of the disease.

Due to the cross-sectional design of the study, these results must be regarded as preliminary data and cannot be generalized beyond the cases studied, nor we can draw conclusions on the natural progression of these relationships over time. Moreover, we evaluated PED/PEA-15 protein abundance in WBC and not in ovary cells; thus, although we previously reported that PED/PEA-15 protein levels in WBC correlated strongly with mRNA detected by real time RT-PCR PED/PEA-15 protein amount in fat and in skeletal muscle tissues [13], we need to be aware of possible unexplored differences between WBC and ovary cell PED/PEA-15 protein expression. Taking into account that laparoscopy is not routinely performed in PCOS for ethical considerations, we did not obtain granulosa cells preparations from women with PCOS in this preliminary investigation.

Table 4 Multiple linear regression analysis in overweight/obese PCOS women.

<table>
<thead>
<tr>
<th>Independent variables:</th>
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<tbody>
<tr>
<td>BMI, insulin, 25(OH)D, L/A, FAI</td>
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<td></td>
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<tr>
<td>Step</td>
<td>Variables inserted in the model</td>
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</tr>
<tr>
<td>2</td>
<td>L/A</td>
<td>&lt;0.001</td>
<td>0.58</td>
</tr>
<tr>
<td>3</td>
<td>Insulin</td>
<td>&lt;0.001</td>
<td>0.63</td>
</tr>
<tr>
<td>4</td>
<td>25(OH)D</td>
<td>0.015</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Variations in PED/PEA-15 abundance explained by stepwise model (p to include < 0.05, p to remove > 0.1) with selected variables in o/o PCOS women (n = 48). BMI, body mass index; 25(OH)D, 25(OH) Vitamin D; PED/PEA-15, L/A, leptin-to-adiponectin ratio; FAI, free androgen index.

Abbreviations
PCOS: polycystic ovary syndrome; 25(OH)D: 25-hydroxy vitamin D; IR: insulin resistance; PED/PEA-15: phosphoprotein enriched in diabetes gene product; T2DM: type 2 diabetes mellitus; MetS: metabolic syndrome; L/A: leptin-to-adiponectin ratio; TV-USG: transvaginal ultrasonography; P: plasma progesterone; FG score: Ferriman-Gallwey score; BMI: body mass index; OGTT: oral glucose tolerance test; FPG: fasting plasma glucose; FPI: fasting plasma insulin; SHBG: sex hormone-binding globulin; T: testosterone; FAI: free androgen index.

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Authors’ contributions
SS and RV are first authors of the manuscript as they equally contributed to the study, participated in its design and coordination, and helped to draft the manuscript. FO and CDS performed the clinical investigation. BV, CP, FP, and FP gathered the data. CA and BF critically revised the manuscript.
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Page contributed to the study design, to performing statistics, and drafting manuscript. All Authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Adipocyte-released insulin-like growth factor-1 is regulated by glucose and fatty acids and controls breast cancer cell growth in vitro

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Abstract

Aims/hypothesis Type 2 diabetes and obesity are associated with increased risk of site-specific cancers. We have investigated whether metabolic alterations at the level of adipose-derived differentiating cells may affect specific phenotypes of breast cancer cells.

Methods Growth profiles of breast cancer cell lines were evaluated in co-cultures with differentiated adipocytes or their precursor cells and upon treatment with adipocyte conditioned media. Production and release of cytokines and growth factors were assessed by real-time RT-PCR and multiplex-based ELISA assays.

Results Co-cultures with either differentiated mouse 3T3-L1 or human mammary adipocytes increased viability of MCF-7 cells to a greater extent, when compared with their undifferentiated precursors. Adipocytes cultured in 25 mmol/l glucose were twofold more effective in promoting cell growth, compared with those grown in 5.5 mmol/l glucose, and activated mitogenic pathways in MCF-7 cells. Growth-promoting action was also enhanced when adipocytes were incubated in the presence of palmitate or oleate. Interestingly, 3T3-L1 and human adipocytes released higher amounts of keratinocyte-derived chemokine/IL-8, the protein ‘regulated upon activation, normally T expressed, and secreted’ (RANTES), and IGF-1, compared with their precursor cells. Their levels were reduced upon incubation with low glucose and enhanced by fatty acids. Moreover, both undifferentiated cells and differentiated adipocytes from obese individuals displayed about twofold higher IGF-1 release and MCF-7 cell growth induction than lean individuals. Finally, inhibition of the IGF-1 pathway almost completely prevented the growth-promoting effect of adipocytes on breast cancer cells.

Conclusions/interpretation IGF-1 release by adipocytes is regulated by glucose and fatty acids and may contribute to the control of cancer cell growth in obese individuals.

Keywords Adipocytes · Breast cancer · IGF-1

Abbreviations Adipo 3T3-L1 3T3-L1 adipocytes
AU Arbitrary units
bFGF Basic fibroblast growth factor
CM Conditioned medium
ER Oestrogen (estrogen) receptor
Introduction

It has recently become clear that obesity and type 2 diabetes are associated with an increased frequency of many cancers [1, 2]. For instance, increased adiposity is associated with an increased risk of several cancers [3]. Similarly, epidemiological data link type 2 diabetes with an increased incidence of multiple types of cancer, including breast, colorectal, hepatocellular, endometrial and pancreatic malignancies [4, 5]. Both obesity and diabetes can also lead to poorer treatment outcome and increase cancer-related mortality rates [6–8].

The adipocyte may represent a candidate to integrate energy and nutrient metabolism with cancer cell growth by regulating cell functions through a complex network of endocrine, paracrine and autocrine signals [9–11]. Indeed, in addition to storing excess calories in the form of lipid, adipose tissue is an active endocrine organ that can have far-reaching effects on the physiology of other tissues [12]. It has been shown that several hormones, growth factors and cytokines are produced or generated in white adipose tissue [13]. Recent interest has centred on the role of adipokines, chiefly leptin and adiponectin, and inflammatory cytokines [14]. However, all of the adipocyte factors may be envisioned as contributing factors for cancer onset or progression [15]. Moreover, adipocytes are largely represented in the microenvironment of several tumours, possibly providing a number of signals and resources to tumour cells.

The adipocyte represents one of the most abundant cell types surrounding breast cancer cells and may prove to be a key player in the stromal–ductal epithelial cell interactions within the mammary microenvironment [16]. In this regard, several in-vitro and in-vivo studies demonstrated that adipocytes can directly influence breast tumour growth [17].

Obesity has been consistently shown to increase the rate of breast cancer occurrence in postmenopausal women by 30–50% [11]. Studies of breast cancer mortality rates and survival illustrate that adiposity is associated both with poorer survival and increased likelihood of recurrence among those with the disease, regardless of menopausal status and after adjustment for disease stage and treatment. Very obese women (BMI ≥40.0) have breast cancer death rates that are three times higher than very lean (BMI<20.5) women [3, 18]. Moreover, case–control and cohort studies showed that women with diabetes may have a 20% increased risk of breast cancer [19] and increased cancer-related mortality rate [20].

Nevertheless, whether adipocyte-derived factors are directly involved in breast cancer onset or progression is still poorly understood [21]. The effect on tumour phenotype of metabolic alterations at the level of the adipose tissue is also unclear.

Here, we show that adipocytes may integrate inputs from the metabolic environment and promote growth of breast cancer cells. Indeed, both glucose and fatty acids enhance the ability of adipocytes to produce factors, including IL-8, RANTES (regulated upon activation, normally T expressed, and secreted) and IGF-1, involved in the control of cancer cell phenotypes. Finally, both stromal vascular fraction (SVF) cells and differentiated adipocytes from obese individuals release more IGF-1 than those from lean individuals, suggesting that obesity per se may enhance breast cancer cell growth.

Methods

Materials Media, sera and antibiotics for cell culture were from Lonza (Basel, Switzerland). Antibodies against phospho-Ser473 protein kinase B (PKB/Akt1), forms of extracellular signal-regulated kinase (ERK), signal transducer and activator of transcription (STAT)3 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-Thr202/Tyr204 ERK and phospho-Tyr 705 STAT3 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). PKB/Akt antibody was from Millipore (Billerica, MA, USA). Anti-leptin antibodies were a generous gift from G. Matarese (CNR, Naples, Italy). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
(SDS-PAGE) reagents were from Bio-Rad (Hercules, CA, USA). All the other chemicals were from Sigma-Aldrich (St Louis, MO, USA).

Cell cultures MCF-7 (ERα positive) and MDA-MB-231 (ERα negative) human breast cancer cells and 3T3-L1 mouse fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mmol/l glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. 3T3-L1 differentiation was achieved as previously described [22]. Human adipose tissue specimens were obtained from mammary adipose tissue and from abdominal subcutaneous adipose tissue biopsies. In detail, mammary adipose tissue specimens were obtained from women (n=12; age 25–63 years; BMI 24.2–29) undergoing surgical mammary reduction. All women were otherwise healthy and free from metabolic or endocrine diseases. Abdominal adipose tissue was obtained from obese (n=10; age 45–65 years; BMI 30–35) and lean women (n=10; age 32–60 years; BMI 20–25) by abdominal biopsy in the periumbilical region under local anaesthesia (2% lidocaine). Informed consent was obtained from every study participant before the surgical procedure. This procedure was approved by the ethical committee of the University of Naples. Adipose tissue was digested with collagenase and then adipocyte-derived SVF cells were isolated and differentiated as previously reported [23]. Conditioned media were obtained by incubating the cells for 8 h with serum-free DMEM containing 0.25% BSA after two washes with PBS. After the incubation, medium was collected and centrifuged at 14,000 g to remove cellular debris and placed onto recipient cells for incubation, medium was collected and centrifuged at 14,000 g cell RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCRs were analysed using SYBR Green mix (Invitrogen). Reactions were performed using Platinum SYBR Green Quantitative PCR Super-UDG using an iCycler IQ multicolor Real-Time PCR Detection System (Bio-Rad). All reactions were performed in triplicate and β-actin was used as an internal standard. Primer sequences used are described in electronic supplementary material (ESM) Table 1.

Immunoblot procedure Total cell lysates were obtained and separated by SDS-PAGE as previously described [27].

Phosphoprotein assay Protein lysates were prepared by using Cell lysis kit (Bio-Rad) and the presence of phosphorylated Akt/PKB, ERK, STAT3 and IGF-1R was detected by Bio-Plex Phospho-Set473 Akt1/PKB, Phospho-Thr202/Tyr204 ERK, Phospho-Tyr705 STAT3 and Phospho-Tyr1131 IGF-1R Assay kits and the Phosphoprotein Testing Reagent kit (Bio-Rad) according to the manufacturer’s protocol. The total proteins for Akt1/PKB, ERK and STAT3 were tested using the Bio-Plex multiplex Mouse and Human Cytokine and Growth factor kits (Bio-Rad) according to the manufacturer’s protocol.

Real-time RT-PCR analysis Total RNA was isolated from 3T3-L1 and human pre-adipocytes and adipocytes by using the Rneasy Kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer’s instruction. For real-time RT-PCR analysis, 1 µg cell RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCRs were analysed using SYBR Green mix (Invitrogen). Reactions were performed using Platinum SYBR Green Quantitative PCR Super-UDG using an iCycler IQ multicolor Real-Time PCR Detection System (Bio-Rad). All reactions were performed in triplicate and β-actin was used as an internal standard. Primer sequences used are described in electronic supplementary material (ESM) Table 1.

Flow cytometry MCF-7 cells were kept in the presence or in the absence of serum or incubated with conditioned media, as indicated. Cell cycle phases were then assayed by cytometric analysis as previously described [27].

Cytokine and growth factor assay 3T3-L1 and human pre-adipocyte and adipocyte conditioned media were screened for the concentration of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, eotaxin, granulocyte-colony stimulating factor (G-CSF), granulocyte–macrophage colony stimulating factor (GM-CSF), IFN-γ, keratinocyte-derived chemokine (KC)/IL-8, monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1-alpha/beta (MIP-1α, MIP-1β), RANTES, TNF-α, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and IGF-1, using the Bio-Plex multiplex Mouse and Human Cytokine and Growth factor kits (Bio-Rad) according to the manufacturer’s protocol.

Statistical analysis Data were analysed with Statview software (Abacus concepts) by one-factor analysis of variance.
A *p*-value of less than 0.05 was considered statistically significant.

**Results**

*Adipocytes promote breast cancer cell survival* We first analysed the impact of adipocyte-secreted factors on breast cancer cell growth, in comparison with factors released by undifferentiated fibroblasts. Conditioned medium (CM) from 3T3-L1 adipocytes induced a time-dependent increase of cell growth, which was significantly more effective than that induced by CM from 3T3-L1 fibroblasts (Fig. 1a) and similar to that observed for cells incubated with 10% FBS-medium (Fig. 1a). Consistent results were obtained with the ERα-negative MDA-MB-231 cells (Fig. 1b), suggesting that the effect was independent of oestrogen release. To investigate whether human adipocytes were as effective as murine cell lines in promoting breast cancer cell growth, we obtained adipose tissue from breast surgery. As shown in Fig. 1c, CM from adipocytes, either differentiated from SVF or directly isolated from mammmary specimens, induced growth of MCF-7 cells, by 1.5 fold and twofold, respectively, while CM from undifferentiated SVF cells had no significant effect. Similar data were also obtained with MDA-MB-231 cells (data not shown). Consistently, as assessed by sulforhodamine assay,

![Graphs showing cell proliferation and cell viability](image)

**Fig. 1** Effect of adipocyte-released factors on breast cancer cell growth. (a) Undifferentiated mouse 3T3-L1 cells or mature 3T3-L1 adipocytes were incubated with serum-free DMEM for 8 h. Media were collected from undifferentiated 3T3-L1 cells and from mature 3T3-L1 adipocytes (respectively, 3T3-L1 CM [grey diamonds] and Adipo 3T3-L1 CM [black squares]) and added to serum-starved MCF-7 breast cancer cells for 6, 12, 24 and 48 h. As a control, MCF-7 cells were also incubated with DMEM without serum supplementation (DMEM-BSA [white triangles]) or with 10% FBS (DMEM 10% FBS [white circles]). The cells were then counted and the results reported as fold increase over basal (cell count in DMEM-BSA). (b) 3T3-L1 CM and Adipo 3T3-L1 CM were added to serum-starved MDA-MB-231 breast cancer cells for 24 h. As a control, MDA-MB-231 cells were also incubated with DMEM-BSA or with DMEM 10% FBS. Cells were then counted as described in the Methods and the results reported as fold increase over basal (cell count in DMEM-BSA). (c) Adipose-derived SVF, differentiated adipocytes (hAdipo) or mature adipocytes (Isol. Adipo) isolated from breast surgery (n=12) were incubated with serum-free medium for 8 h. Media were collected (CM) and added to serum-starved MCF-7 cells for 24 h. As a control, MCF-7 cells were also incubated with medium without serum supplementation (basal medium). Cells were then counted and the results reported as fold increase over basal (cell count in basal medium). (d) 3T3-L1 CM, Adipo 3T3-L1 CM, SVF CM and hAdipo CM were added to serum-starved MCF-7 for 48 h. Cell viability was then assessed by sulforhodamine assay as described in the Methods and the results reported as percentage of viable cells compared with cells in DMEM 10% FBS, considered as 100% viable cells. (e) MCF-7 cells were seeded in the upper chamber of a transwell culture system with or without 3T3-L1, Adipo 3T3-L1, SVF or hAdipo in the lower chamber and cell viability was determined by crystal violet staining as described in the Methods. The results are reported as percentage of stained cells. *p*<0.05, **p**<0.01, ***p***<0.001 compared with basal; †*p*<0.05; ††*p*<0.01 compared with 3T3-L1 or SVF CM. (f) MCF-7 cells were starved in serum-free DMEM for 18 h and incubated with 3T3-L1 CM, Adipo 3T3-L1 CM, SVF CM and hAdipo CM for 24 h. Cells were stained with propidium iodide and the ratio of cells in sub-G1 phase was determined by cytometric analysis, as described in the Methods. *p*<0.05, comparing indicated data For all the panels in the figure, data in the graphs represent the mean ± SD of at least four independent triplicate experiments.
cell viability was increased by 3T3-L1 and human mammary adipocyte CM by 1.6 and 2.6 fold, respectively compared with their undifferentiated precursors (Fig. 1d).

Again, when co-cultured with 3T3-L1 and human adipocytes, MCF-7 displayed higher viability, comparable with that observed for cells cultured in DMEM–10% FBS. A much lower effect was observed when MCF-7 cells were co-cultured with either 3T3-L1 fibroblasts or SVF undifferentiated cells (Fig. 1e).

To further investigate whether growth-promoting action was dependent on oestrogen release by mature adipocytes, MCF-7 cells were incubated with mammary adipocyte CM in the presence of 1 μmol/l Tamoxifen, a selective oestrogen receptor antagonist (ESM Fig. 1). No significant effect was elicited by tamoxifen on the adipocyte growth-promoting action. Also, exposure of the cells to anti-leptin antibodies did not change the effect of adipocyte CM on cancer cell growth (ESM Fig. 1).

Moreover, incubation of MCF-7 cells with CM from 3T3-L1 fibroblasts only slightly decreased the number of cell deaths induced by growth factor deprivation, reducing the number of cells in sub-G1 phase by 25% (Fig. 1f). Interestingly, cell death was substantially rescued by incubation with CM from 3T3-L1 adipocytes, at levels similar to those achieved with 10%FBS medium (Fig. 1f). Again, the number of cell deaths was decreased following incubation with CM from mammary adipocytes, and from undifferentiated SVF cells (Fig. 1f), although the effect of the latter CM was lower.

Adipocyte factors induce MAPK, PI3K and JAK/STAT activation in MCF-7 cells To gain further insight into the molecular mechanism by which adipocytes promote breast cancer cell growth, we tested the ability of adipocyte conditioned media to induce mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and JAK/STAT activation. To this aim, MCF-7 cells were treated with CM from 3T3-L1 or from mammary adipocytes for 12 h (ESM Fig. 2). Western blot and phosphoprotein assay revealed that CM induced the phosphorylation of ERK, Akt and STAT3 (ESM Fig. 2).

Release of cytokines/chemokines and growth factors by 3T3-L1 and human mammary adipocytes Based on the observed induction of signalling pathways, we have screened for the ability of 3T3-L1 and mammary adipocytes to release cytokines/chemokines and growth factors. Both 3T3-L1 fibroblasts and undifferentiated SVF cells released detectable amounts of IL-4, IL-6, IL-10, IFN-γ, KC/IL-8, MIP-1α, MIP-1β, RANTES and TNF-α; MIP-1α was exclusively detected in the medium of 3T3-L1 pre-adipocytes and not of human SVF cells. However, the levels of KC/IL-8 and RANTES were higher both in 3T3-L1 and human adipocytes, compared with their respective precursors. Moreover, increased levels of IL-6 and MIP-1β were detected in the CM of human adipocytes while not in the CM of 3T3-L1 adipocytes.

We also screened for growth factor release and found that VEGF, bFGF, PDGF and IGF-1 were detectable in both pre-adipocytes and adipocytes. However, while there was no significant difference in the content of PDGF and bFGF, the amount of VEGF was significantly reduced in the CM of both 3T3-L1 and human mammary adipocytes. At variance, IGF-1 was about fivefold higher in the CM of differentiated 3T3-L1 and mammary adipocytes (Table 1).

Similar to protein levels in the conditioned media, KC (also known as Cxcl1)/IL-8, RANTES and IGF-1 mRNA levels were higher by 5, 6 and 6.5 fold, and 4, 4.5 and 7.5 fold, respectively, in differentiated 3T3-L1 cells and in human adipocytes, compared with their undifferentiated counterparts (Fig. 2).

Glucose and fatty acids modify adipocyte growth-promoting action Next, 3T3-L1 adipocytes were cultured for 24 h either in 5.5 mmol/l glucose (LG, low glucose), a concentration corresponding to normal fasting glucose levels in humans, or in 25 mmol/l glucose (HG, high glucose), corresponding to the regular culture condition for this cell line, but resembling hyperglycaemia in humans. Moreover, we added to culture media either 0.5 μmol/l oleate or 10 μmol/l palmitate, two major fatty acids whose plasma levels are increased in dysmetabolic conditions. Media were changed and cells were allowed to secrete factors into freshly added serum-free medium. After 8 h, media were collected and tested for the content of cytokines/chemokines and growth factors. No significant change was detected for IL-4, IL-6, IL-10, IFN-γ, MIP-1α, MIP-1β, TNF-α, PDGF, bFGF and VEGF (data not shown). However, 3T3-L1 adipocytes cultured in HG medium released significantly higher levels of KC (Fig. 3a), RANTES (Fig. 3b) and IGF-1 (Fig. 3c), compared with those cultured in LG medium. Very similar results were obtained for IL-8 (Fig. 3d), RANTES (Fig. 3e) and IGF-1 (Fig. 3f) in differentiated mammary adipocytes. Interestingly, culturing 3T3-L1 and human adipocytes with either oleate or palmitate in LG medium was accompanied by a significant increase of KC/IL-8, RANTES and IGF-1 (Fig. 3a–f).

We next addressed whether regulation of those adipocyte-produced factors occurred at the transcriptional level. KC and Igf-1 mRNA levels were about twofold higher in 3T3-L1 adipocytes cultured in HG medium compared with LG medium (Fig. 4). Similarly, in differentiated human adipocytes, both IL-8 and IGF-1 levels were higher in HG medium than in LG medium. At variance, RANTES (also known as CCL5) mRNA levels were comparable in HG and LG media, both in 3T3-L1 and human adipocytes.
However, when oleate or palmitate were added to LG medium, significant increases of RANTES and IGF-1 mRNA levels, while not of Kc/IL-8, were observed both in 3T3-L1 and human adipocytes. Thus, these data suggest that glucose may upregulate Kc/IL-8 and IGF-1, while fatty acids may induce RANTES and IGF-1 at mRNA levels.

Therefore, we tested whether glucose and fatty acids may change the growth-promoting action of murine and human adipocytes. To this end, MCF-7 cells were incubated for 24 h in the presence of either LG or HG conditioned media from 3T3-L1 and human mammary adipocytes. Pre-incubation of adipocytes (both 3T3-L1 and human) with HG medium enhanced, by about twofold, their ability to induce MCF-7 cell growth, compared with LG medium (Fig. 5). Moreover, the presence of palmitate or oleate in the LG medium (from both 3T3-L1 and human adipocytes) significantly increased breast cancer cell growth (Fig. 5).

Induction of breast cancer cell growth by adipocytes from lean and obese individuals

SVF cells were isolated from abdominal liposuctions carried out in lean (n=10) and obese (n=10) individuals. These cells were differentiated into mature adipocytes and conditioned media were collected as previously described (see Methods). As for mammary-derived specimens (see Fig. 1c), abdominal subcutaneous differentiated adipocytes induced MCF-7 cell growth to a larger extent, compared with undifferentiated SVF cells from both lean and obese individuals. However, CM from both undifferentiated SVF cells and differentiated adipocytes obtained from obese individuals were significantly more effective than those obtained from lean individuals in inducing MCF-7 cell growth.

Table 1  Adipocyte-released cytokines and growth factors

<table>
<thead>
<tr>
<th>Cytokine/growth factor</th>
<th>3T3-L1 CM (pg/ml)</th>
<th>Adipo 3T3-L1 CM (pg/ml)</th>
<th>SVF CM (pg/ml)</th>
<th>hAdipo CM (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.7±0.008</td>
<td>0.10±0.02</td>
<td>1.12±0.66</td>
<td>1.36±0.66</td>
</tr>
<tr>
<td>IL-6</td>
<td>23.6±1.13</td>
<td>22.37±3.47</td>
<td>2,705.58±541</td>
<td>5,005.06±1,200†</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.70±0.86</td>
<td>15±2.6*</td>
<td>10.47±2.24</td>
<td>6.53±1.66†††</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>3.73±0.38</td>
<td>5.59±0.80</td>
<td>46.70±9.2</td>
<td>41.64±19.8</td>
</tr>
<tr>
<td>KC/IL-8</td>
<td>989.14±100.62</td>
<td>10,343.72±1,551.53*</td>
<td>2,975.59±300</td>
<td>15,647.85±190.2†</td>
</tr>
<tr>
<td>MIP-1a</td>
<td>226.31±18.27</td>
<td>280±22.32</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>10.33±1.53</td>
<td>11.4±2</td>
<td>3.37±1.02</td>
<td>25.88±9.01††</td>
</tr>
<tr>
<td>RANTES</td>
<td>1,960±235.2</td>
<td>4,770±524.7*</td>
<td>12.27±1.5</td>
<td>52.99±10†††</td>
</tr>
<tr>
<td>TNF-α</td>
<td>143.54±18.66</td>
<td>162.88±25.92</td>
<td>1.54±0.80</td>
<td>2.17±1.10</td>
</tr>
<tr>
<td>PDGF</td>
<td>ND</td>
<td>ND</td>
<td>1.20±0.90</td>
<td>2.65±1.32</td>
</tr>
<tr>
<td>bFGF</td>
<td>200±18</td>
<td>660.70±2.67*</td>
<td>19.37±3.87</td>
<td>25.58±5.37</td>
</tr>
<tr>
<td>VEGF</td>
<td>11,479.20±1,262.71</td>
<td>4,682.46±607.71*</td>
<td>526.53±105.30</td>
<td>295.58±65.02††</td>
</tr>
<tr>
<td>IGF-1</td>
<td>79.37±0.88</td>
<td>334.37±40.41*</td>
<td>28.85±0.50</td>
<td>136.66±5.7††</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD of at least four independent triplicate experiments. Supernatants from mouse 3T3-L1 pre-adipocytes (3T3-L1 CM), 3T3-L1 adipocytes (Adipo 3T3-L1 CM), human SVF (SVF CM) and differentiated adipocytes (hAdipo CM were collected for 8 h and tested by using the Bio-Plex multiplex cytokine assay kit and the Bio-Plex multiplex growth factor assay kit as described in the Methods.

*p<0.05 for 3T3-L1 adipocytes vs 3T3-L1 CM; †p<0.05, ††p<0.01, †††p<0.001 for human adipocytes vs SVF CM
counterparts (Table 2), both for lean and obese individuals. No difference in IL-4, IL-8, IL-10, IFN-γ, MIP-1α, MIP-1β, RANTES, TNF-α, PDGF, bFGF and VEGF content was found in the media of SVF cells and mature adipocytes from lean and obese individuals. Nevertheless, IL-6 and IGF-1 were significantly more abundant in CM of obese compared with lean adipocytes (Table 2). Moreover, only IGF-1 levels were higher in SVF cells from obese individuals than in those from lean individuals.

Effect of IGF-1 pathway inhibition on cell growth

To address the biological relevance of IGF-1 as an adipocyte-derived growth-promoting factor, MCF-7 cells were treated with HG CM in the presence of 10 μmol/l AG1024, a specific inhibitor of IGF-1R kinase activity. AG1024 almost completely prevented the effect of 3T3-L1 CM on MCF-7 cell growth (Fig. 7a). Very similar results were obtained for the prevention of the effect induced by mammary (Fig. 7b) as well as subcutaneous abdominal adipocytes, both from lean and from obese individuals (Fig. 7c). Indeed, the induction of MCF-7 cell growth by lean and obese adipocytes was reduced at comparable levels, following treatment with AG1024 (Fig. 7c). Moreover, the increased growth induced by SVF cells from obese individuals was fully rescued by AG1024 (Fig. 7c) and returned to levels similar to those achieved by SVF cells from lean individuals. Similarly, conditioned media from obese adipocytes induced IGF-1R phosphorylation twofold more effectively than those from lean individuals (Fig. 7d). Also, only SVF cells from obese, and not from lean, individuals were able to increase IGF-1R phosphorylation. Finally, these effects were completely blocked by AG1024, indicating a major involvement of IGF-1 (Fig. 7d).

Discussion

Convincing evidence links obesity and diabetes to the risk of several forms of cancer [4, 11, 14]. We now describe that differentiated adipocytes, both of mouse and of human origin, have a significant impact in enhancing breast cancer cell survival and proliferation. Consistent with previous reports [16], the effect of adipocytes is larger than that of their precursor cells. Interestingly, however, their growth-promoting action is paralleled by a specific secretory pattern of inflammatory cytokines, chemokines and growth factors, which is also affected by culture conditions. In particular, both glucose and fatty acids enhanced the ability of
adipocytes to induce the growth of breast cancer cells. When cultured in 25 mmol/l glucose (HG), a condition mimicking hyperglycaemia, both 3T3-L1 and human breast mammary adipocytes were more effective in inducing growth of MCF-7 and MDA-MB-231 cells, compared with when they were cultured in 5.5 mmol/l glucose (LG), which represents normal glycaemia in humans.

Incubation of adipocytes with fatty acids also led to increased growth-promoting action, suggesting that metabolic perturbations may change their secretory pattern. This effect was observed with palmitate or oleate, either individually or in combination (data not shown). Higher concentrations of fatty acids (up to 100 μmol/l palmitate and 10 μmol/l oleate) had a slightly higher effect on both cancer cell growth and secretory profile (data not shown). Moreover, it is known that high concentrations of fatty acids impair insulin sensitivity [28] and this may further affect adipocyte functions.

It has been reported that adipocyte-derived molecules can affect cancer cell growth. For instance, leptin has been shown to induce proliferation of breast cancer cells [29]. However, we found that glucose and fatty acids had no effect on leptin release by mammary adipocytes (data not shown). Nevertheless, treatment of MCF-7 cells with anti-leptin blocking antibodies did not rescue the effect of HG adipocyte CM on cell growth (ESM Fig. 1). Adipocyte-derived oestrogens have also been related to breast cancer development and progression [30]. It seems unlikely that oestrogens could mediate the effect of glucose and fatty acids, however, since adipocyte CM elicited a similar effect in the oestrogen-independent MDA-MB-231 cells and it was unchanged following tamoxifen treatment of MCF-7 cells. We therefore tested whether glucose and fatty acids could modify the release of cytokines, chemokines and growth factors by adipocytes. The secretory patterns obtained by mouse 3T3-L1 and human mammary adipocytes were somewhat different. For example, MIP-1α and TNF-α were more abundant in 3T3-L1 cells, while IL-6 and IFN-γ were more abundant in human cells. However, IL-8 and RANTES were released to a larger extent by both differentiated 3T3-L1 cells and human adipocytes, compared with their precursors, indicating that common changes were present in the differentiation process. The increased release of IL-8 and RANTES was paralleled by a similarly sized increase in their mRNA levels.

Interestingly, both glucose and fatty acids increased the release of IL-8 and RANTES, suggesting that the metabolic
environment could further modify adipocyte releasing properties. Similar results were also found for IGF-1, which was more abundant in the medium of mature adipocytes and upregulated by glucose and fatty acids. bFGF also tended to be detected in higher amounts in the mature adipocytes, while VEGF levels were more elevated in the undifferentiated precursors. However, neither bFGF nor VEGF were significantly regulated by nutrients.

We have obtained evidence that glucose and fatty acids may regulate IL-8, RANTES and IGF-1 at different levels. Culturing adipocytes in HG is accompanied by a significant upregulation of IL-8 and IGF-1, while not of RANTES mRNA, compared with low glucose. At variance, both oleate and palmitate enhance IGF-1 and RANTES, while not IL-8 mRNA levels. Altogether, these data raise the possibility that, beside regulating gene expression, nutrients may control adipocyte secretion process.

Thus, these findings are consistent with the hypothesis that adipocytes, in an appropriate metabolic environment, may contribute to tumour growth and progression by releasing factors that may function to promote cancer cell proliferation. In particular, RANTES, IL-8 and IGF-1 represent good candidates since: (1) they are produced in the adipocytes and released in the culture medium; (2) their production/release is induced by nutritional factors, such as glucose and fatty acids; and (3) they have been implicated in the control of cancer cell phenotypes. For instance, RANTES and IL-8 are elevated in plasma of obese individuals [31–33] and have been largely implicated in obesity-related health complications [34–36] as well as in the mechanisms of cancer progression [37–39].

IGF-1 also plays a pivotal role in the progression of many forms of cancer, including breast cancer [40–42]. Indeed IGF-1 is involved in the development of normal mammary gland and in the onset and/or progression of mammary tumours [42], as well as in the development of drug resistance [43]. It was recently reported that, in pancreatic cancer, autocrine production of IGF-1 by cancer cells enhances the production of RANTES by stromal cells, thereby providing a paracrine loop to facilitate tumour progression [44]. Moreover, expression profile studies indicate that IL-8 and IGF-1 are produced at higher levels in tissues from patients with high-grade breast cancer than in tissues from patients with low-grade breast cancer [45].

We have also found that adipocytes obtained from subcutaneous fat specimens from obese individuals were capable of producing larger amounts of IGF-1 compared with lean controls. This is consistent with higher circulating IGF-1 levels often found in obesity [46]. Surprisingly, the difference was also significant when IGF-1 was measured in the conditioned media of undifferentiated SVF cells from obese individuals. Thus, IGF-1 upregulation appears to occur early...
during adipocyte differentiation, is enhanced in obesity and is maintained in cultured mesenchymal stem cells. This could possibly be due to epigenetic changes in IGF-1 gene expression regulation [47]. Interestingly, however, upregulation was only observed for IGF-1 and not for other chemokines and growth factors, both in undifferentiated SVF and in mature adipocytes.

Table 2  Cytokines and growth factors released by adipocytes from lean and obese individuals

<table>
<thead>
<tr>
<th>Cytokine/growth factor</th>
<th>BMI 21–25</th>
<th>BMI 30–35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVF CM (pg/ml)</td>
<td>hAdipo CM (pg/ml)</td>
</tr>
<tr>
<td>IL-2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.09±0.60</td>
<td>1.49±0.53</td>
</tr>
<tr>
<td>IL-6</td>
<td>2,446.87±489.37</td>
<td>6,420.31±1,348.26*</td>
</tr>
<tr>
<td>IL-10</td>
<td>11.40±2.66</td>
<td>6.75±1.48*</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>45.82±9.62</td>
<td>46.7±10.27</td>
</tr>
<tr>
<td>IL-8</td>
<td>2,556.45±350.45</td>
<td>25,019.32±1,264.48**</td>
</tr>
<tr>
<td>IL-10</td>
<td>17.98±3.40</td>
<td>73.65±9.68*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>204.08±15.35</td>
<td>291.65±35.82</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.48±1.35</td>
<td>3.04±0.88</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>28.63±11.90</td>
<td>36.88±12.66</td>
</tr>
<tr>
<td>IL-10</td>
<td>620.53±165.61</td>
<td>178.54±85.01**</td>
</tr>
<tr>
<td>VEGF</td>
<td>15.8±5.46</td>
<td>666±40.42*</td>
</tr>
</tbody>
</table>

SVF cells and differentiated SVF (hAdipo) isolated from obese (BMI 30–35; n=10) and lean women (BMI 20–25; n=10) were incubated with serum-free medium for 8 h. Media were collected (CM) and analysed by using the Bio-Plex multiplex human cytokine assay kit and the Bio-Plex multiplex human growth factor assay kit. Determinations were done in triplicate

*p<0.05, **p<0.01 for adipocytes vs SVF CM within the same group; †p<0.05; ††p<0.01 for SVF or hAdipo CM of one group vs the other

ND, no data

IGF-1 in the adipose tissue may act either as an autocrine factor [48], regulating adipose tissue development and homeostasis, or in a paracrine manner, regulating survival and function of the surrounding cells, including cancer cells. Inhibition of IGF-1 function, indeed, almost completely abolished the effect of adipocyte-released factors, thereby indicating that IGF-1 is a pivotal factor in adipocyte

![Fig. 7](image-url)  Effect of IGF-1 pathway inhibition on cell growth. Conditioned media collected from mouse 3T3-L1 adipocytes (a) and mammary adipocytes (b) and subcutaneous abdominal SVF and adipocytes isolated from obese (BMI 30–35) and lean women (BMI 20–25) (c) were added to serum-starved MCF-7 cells for 24 h in the presence or absence of 10 μmol/l AG1024. Cells were then counted and the results reported as fold increase over basal (cell count in serum-free medium). White columns, SVF CM; light grey columns, SVF CM + Ag1024; dark grey columns, human adipocyte (hAdipo) CM; black columns, hAdipo CM + Ag1024. (d) MCF-7 cells were exposed to conditioned media collected from subcutaneous abdominal SVF and adipocytes isolated from obese (BMI 30–35) and lean women (BMI 20–25) for 24 h and proteins extracted were tested for the presence of phosphor-ylated IGF-1R by using the Bio-Plex phosphoprotein assay kit as described in Methods. White columns, SVF CM; light grey columns, SVF CM + Ag1024; dark grey columns, hAdipo CM; black columns, hAdipo CM + Ag1024. *p<0.05, **p<0.01, comparing indicated data. Data in the bar graphs represent the mean ± SD of at least four independent triplicate experiments.
regulation of cancer cell growth. However, these results are limited to breast cancer models, in which the importance of the local environment, including adipocyte function, has been largely documented. Further studies are needed to assess the relevance of adipose-related factors in the control of other cancer types. Moreover, the communications between cancer cells and adipocytes may be bidirectional. Release of TNF-α from cancer cells may affect adipocyte differentiation and function, possibly increasing NEFA release, to provide additional feed for the cancer cells [49, 50].

Thus, we have described that IGF-1 release by adipocytes is enhanced in obesity and is regulated by metabolic perturbations. Other factors, such as RANTES and IL-8, which are not primarily upregulated in obese individuals, are also enhanced by glucose and fatty acids and may as well contribute to adipocyte control of breast cancer cell growth.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement VD’E was the main contributor in terms of conception, design, acquisition and interpretation of data and in drafting the article. FP, AH, DL, DT, GM, LC and CM mainly contributed in conceptual design and acquisition of data. FB and US mainly contributed in terms of conceptual design, analysis and discussion of the results. PF mainly contributed in terms of conceptual design, interpretation and discussion of the results and supervision of the overall work. All the authors critically revised the article and approved the final version.

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References

Bisphenol A in polycystic ovary syndrome and its association with liver–spleen axis


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Summary

Context Bisphenol A, one of the highest-volume chemicals currently available, is known to act as endocrine disruptor and alters several metabolic functions, including inflammatory pathways. Elevated serum levels of bisphenol A have been found in women with polycystic ovary syndrome (PCOS) and a role of low-grade chronic inflammation has been recently reported in the pathogenesis of this syndrome. Increased spleen volume, a reliable and stable index of chronic inflammation, was strictly associated with the severity of hepatic steatosis (HS) in obese subjects, determining the so-called liver–spleen axis.

Objective To evaluate the contribution of increased serum bisphenol A levels to low-grade chronic inflammation, HS and hyperandrogenism in women with PCOS.

Design, setting and participants Forty lean and overweight/obese premenopausal women with PCOS and 20 healthy age-matched women were consecutively enrolled in a cross-sectional study from 2009 to 2011 at the Federico II University Hospital in Naples.

Measurements Bisphenol A, homeostasis model assessment of insulin resistance (HoMA-IR), laboratory liver tests, testosterone, sex hormone-binding globulin, free androgen index (FAI), C-reactive protein, interleukin-6, and the ultrasound quantification of HS and spleen longitudinal diameter.

Results Independently of body weight, higher bisphenol A levels in PCOS women were associated with higher grades of insulin resistance, HS, FAI and inflammation, spleen size showing the best correlation. At multivariate analysis, spleen size and FAI were the best predictors of bisphenol A (β coefficients 0.379, P = 0.007 and 0.343, P = 0.014, respectively).

Conclusions In premenopausal women with PCOS, we evidenced an association of serum bisphenol A levels with HS and markers of low-grade inflammation, in particular with spleen size, unravelling the presence of the liver–spleen axis in this syndrome.

Introduction

Bisphenol A is one of the highest-volume chemicals currently available. This lipophilic compound is in fact detectable worldwide at nanomolar levels in human serum and is known to act as endocrine disruptor.1 Bisphenol A, at very low doses, alters several metabolic functions, including oxidative stress, a key component of inflammatory reactions, thus contributing to the global rise in the prevalence of obesity, type 2 diabetes and cardiovascular diseases.2,3 It is not known, however, whether bisphenol A is associated with inflammation markers in humans. A role for bisphenol A as endocrine disruptor has been recently proposed in the pathogenesis of polycystic ovary syndrome (PCOS), the most common endocrinopathy among women of reproductive age, characterized by hyperandrogenism, insulin resistance and chronic anovulation.5 In particular, a bidirectional underlying mechanism has been hypothesized to occur in the relationship between PCOS and bisphenol A, involving both androgens6 and bisphenol A metabolism.7 The existence of low-grade chronic inflammation8–11 and the association with autoimmune diseases12 have also been reported in PCOS. The increased androgen bioavailability estimated by the free androgen index (FAI) has been extensively examined in the mechanisms underlying the low-grade chronic inflammation in PCOS.10,11 On the other hand, the incidence of autoimmune thyroid diseases, such as Hashimoto’s thyroiditis, has been reported to be threefold higher in women with PCOS than in the general female population.13
So far, no single circulating marker has been detected to specifically reflect the presence and/or severity of inflammation. Nevertheless, a key role in low-grade chronic inflammation has been depicted for Interleukin (IL)-6, which is produced by macrophages infiltrating adipose tissue and the spleen. This pro-inflammatory cytokine induces the production of C-reactive protein (CRP) by the liver. Increased spleen volume has been recently proposed as a reliable and stable index of chronic inflammation strictly associated with severity of nonalcoholic fatty liver disease, otherwise known as hepatic steatosis (HS), in obesity, determining the so-called liver–spleen axis.

The aim of this pilot study was to evaluate in premenopausal women with PCOS the possible contribution of increased serum bisphenol A levels to the concurrent presence of low-grade chronic inflammation, HS and hyperandrogenism, by analysing the size of the spleen, an organ whose role in the mechanisms of inflammation/immune disorders, is well known.

Materials and methods

Study design

This is a cross-sectional study carried out in accordance with the guidelines of the Helsinki Declaration on human experimentation, after approval by the Institutional Review Board of the University of Naples, Italy (#231/05, 20 February 2006). All participants gave their informed consent before enrolment.

Subjects

Forty women with a diagnosis of PCOS, from 1st November 2009 to 31st October 2011, were consecutively selected from a pool of patients referred to the outpatient clinic of our University Hospital. The diagnosis of PCOS was based on the Rott-PCOS criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004), as reported elsewhere and were enrolled according to the following criteria: premenopausal status; anovulatory oligo-amenorrhoea; Caucasian ethnicity. None of the participants were neither on hypocaloric diet nor taking weight loss drugs at least 3 weeks before the admission.

Controls

Twenty healthy, normal weight women among clerks, paramedical and medical personnel of our Department, matched for age with the patients, accounting for the possible progressive increase in bisphenol A exposure with age, from the same geographical area, with regular menstrual cycles (defined as 26–32 days in length) and no hyperandrogenaemia, hirsutism or acne, were recruited; they all agreed to participate in this study and acted as controls to set range of bisphenol A.

Exclusion criteria

The following exclusion criteria were defined: smoking or alcohol consumption, pregnancy, hypothyroidism, hyperprolactinaemia, Cushing’s syndrome, nonclassical congenital adrenal hyperplasia; previous (within the last 6 months) use of oral contraceptives, insulin sensitizing agents, glucocorticoids, anti-androgens, ovulation inducing agents, anti-obesity drugs; the presence of any acute viral, bacterial or fungal infection, any type of chronic liver disease, arthritis, bronchial asthma; chronic inflammatory bowel; cancer.

Methods

Ovulatory state and hirsutism, assessed using the Ferriman–Gallwey score, were investigated as previously reported elsewhere. The degree of normal weight, overweight or obesity was established on the basis of body mass index (BMI) cut-off points of 18–24.9 (lean subjects), 25–40 kg/m², respectively (overweight/obese: O/O).

Assays

Fasting plasma glucose (FPG) levels were determined by the glucose oxidase method. Fasting plasma insulin (FPI), sex hormone-binding globulin (SHBG) and testosterone levels (Immulite, Diagnosti c Products Co, Los Angeles, CA, USA; manufacturer’s range 0.2–1.2 nmol/l) were measured by solid-phase chemiluminescent enzyme immunoassays. The intra-assay coefficients (CV) of variations were less than 5.5% for insulin and SHBG assays and 10% for total T assay. FAI was calculated according to the formula = Testosterone (nmol/l)/SHBG (nmol/l) × 100. Among women with PCOS, the cut-off values used were 3.5 nmol/l for testosterone (sensitivity 92.6 and specificity 92.3) and 8.1 for FAI (sensitivity 100 and specificity 100) to identify less vs more severe biochemical hyperandrogenism. The homoeostasis model assessment index of insulin resistance (HoMA-IR) was calculated according to the formula [FPI (μU/ml) × FPG (mM)]/22.5, as previously reported. As stringent measure of insulin resistance, a value of HoMA-IR > 2.0 was set. Liver enzymes (alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, γ-GT) were measured by in-house procedures. CRP was determined with a nephelometric assay with CardioPhase from Siemens Healthcare Diagnostics (Marburg, Germany). IL-6 was measured by an enzyme-linked immunosorbent assay kit (Bioresource, Camarillo, CA, USA). Its sensitivity was <2 pg/ml, and the range was 7.8–500 pg/ml. The intra-assay CV for low concentration was <5%.

Bisphenol A analysis

Serum concentrations of bisphenol A were determined using a kit from IBL Co., Ltd., Gunma, Japan, characterized by a measurement range of 0.3–100 ng/ml bisphenol A and based on a competitive ELISA protocol using the anti-rabbit IgG antibody solid-phase method. All standards and samples were measured in duplicate. The assay shows 100% cross-reactivity with bisphenol A, 85% with bisphenol A–glucuronide, 68% with bisphenol A–Na-sulphate and 8.5% with bisphenol. The ranges of the intra- and interassay coefficients of variation were <14% and 5%, respectively. The bisphenol A values were considered.
increased when they were above the 95th percentile of those detected in controls (0.45 ng/ml).

**US analyses**

Sonographic measurements were performed in both control and PCOS women by the same operator, blinded to patients’ data, using a Vivid system (General Electric Healthcare Company, Milan, Italy). Briefly, spleen longitudinal diameter, the best single measurement well related to spleen size, was measured by postero-lateral scanning. Maximum and crano-caudal lengths were measured and then averaged. A cut-off for spleen longitudinal diameter was set at 103 mm. To avoid confounders that could modify echo intensity and thus bias the comparisons, the mean brightness levels of both liver and the right kidney were obtained on the same longitudinal plane. The levels of brightness of liver and right kidney were calculated three times directly from the frozen images.

**Statistical analysis**

Results are expressed as mean ± SD or as median plus range according to variable distributions. Differences between groups were analysed by unpaired t-test or Mann–Whitney U-test when appropriate. Frequencies were analysed by chi-squared. Correlations between variables were performed using Spearman’s rho correlation coefficient. Multiple linear regression analysis (stepwise method) was calculated using the predictive value of bisphenol A levels on HoMA index, HS severity, FAI or spleen size in the whole study population. To avoid multicollinearity, variables with a tolerance of 0.2 were excluded. Values ≤5% were considered statistically significant. Data were stored and analysed using the MedCalc® package (Version 12.3.0 © 1993-2012 MedCalc Software bvba - MedCalc Software, Mariakerke, Belgium).

**Results**

The characteristics of our study population, that is, control and PCOS women, are expressed in Table 1. Apart from the significant differences in anthropometric and metabolic data between the two groups, serum bisphenol A levels and low-grade inflammation markers were higher in PCOS women than in controls. Interestingly, bisphenol A values were higher in all subjects with increased spleen size (4 controls and 26 PCOS) than in those with normal spleen size: 0.1 (0.1–1.0) vs 1.3 (0.1–6) ng/ml; P < 0.001. However, as bisphenol A levels were higher than the cut-off value in only one control compared to the 29 (73%) women with PCOS, the subsequent subgroup analyses were performed taking into account only PCOS women.

**Subgroup analyses**

Comparisons between the main study variables according to the serum bisphenol A cut-off value (bisphenol A+ and bisphenol A−) are reported in Fig. 1. In particular, while age and BMI were not significantly different between the two groups, the subgroup of PCOS women with higher serum bisphenol A levels displayed more severe insulin resistance and hyperandrogenism, increased prevalence of HS, and higher CRP, IL-6 and spleen size than the bisphenol A− counterpart (Fig. 1).

Grouping PCOS for BMI (lean 22.2 ± 2.2 vs O/O 34.0 ± 6.7; P < 0.001), there were no significant differences in terms of age. Conversely, HoMA index and FAI remained higher in O/O

| Table 1. Comparisons of bisphenol A plasma levels, anthropometric, metabolic, endocrine variables and inflammatory markers in the study population of control and polycystic ovary syndrome (PCOS) women |
|----------------------------------|---------|---------------|---|
|                                | Controls (n = 20) | PCOS women (n = 40) | P-values |
| Age (years)                     | 26.2 ± 3.9       | 27.7 ± 6.8       | 0.382 |
| Bisphenol A (ng/ml)             | 0.1 (0.1–0.6)    | 0.7 (0.1–6)      | <0.0001* |
| No. patients with bisphenol A >45 ng/ml | 1            | 29 (73%)        | <0.0001* |
| BMI (kg/m²)                     | 22.1 ± 1.8       | 28.1 ± 7.7       | 0.001 |
| Waist circumference (cm)        | 73.8 ± 4.5       | 87.1 ± 15.2      | <0.0001 |
| Plasma glucose (mM)             | 4.4 ± 0.3        | 5.0 ± 0.5        | <0.0001 |
| Insulin (psu)                   | 50.2 (35.9–71.8) | 86.1 (40.9–243.9) | <0.0001 |
| HoMA-IR                         | 1.4 ± 0.3        | 3.0 ± 1.2        | <0.0001 |
| No. patients with HoMA-IR > 2   | 0                | 27 (68%)         | <0.0001 |
| Testosterone (nm)               | 0.6 ± 0.1        | 3.8 ± 0.8        | <0.0001 |
| SHBG (nm)                       | 69.5 ± 3.1       | 40.0 ± 5.4       | <0.0001 |
| FAI                             | 0.8 ± 0.2        | 9.7 ± 2.9        | <0.0001 |
| AST (U/l)                       | 24 ± 7           | 34 ± 7           | <0.0001 |
| ALT (U/l)                       | 24 ± 9           | 38 ± 10          | <0.0001 |
| γGT (μM/μl)                     | 29 (7–40)        | 42 (9–140)       | <0.0001 |
| No. patients with hepatic steatosis | 0           | 26 (65%)        | <0.0001 |
| CRP (mg/ml)                     | 0.2 (0.1–2.0)    | 1.7 (0.8–4)      | <0.0001 |
| IL-6 (pg/ml)                    | 5.7 (3.5–8.3)    | 10.6 (2.1–79.4)  | <0.0001 |
| Spleen size (mm)                | 99.0 ± 6.8       | 110.0 ± 7.6      | <0.0001 |
| No. patients with spleen size   | 4 (20%)          | 26 (65%)        | 0.003§ |

Values are expressed as mean ± SD or median plus range. Differences between control and PCOS women were analysed by unpaired t-test or Mann–Whitney U-test when appropriate. Frequencies were analysed by chi-squared. As a stringent measure of IR, a value of HoMA-IR > 2.0 was set, as reported elsewhere. Hepatic steatosis (HS) and spleen size were determined by ultrasound. A cut-off for spleen longitudinal diameter was set at 103 mm. BMI, body mass index; HoMA-IR, homoeostasis model assessment of insulin resistance; SHBG, sex hormone-binding globulin; FAI, free androgen index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGT, gamma-glutamyltransferase; CRP, C-reactive protein; IL-6, interleukin-6.

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PCOS than in their lean counterpart, while differences in HS prevalence, CRP and IL-6 levels and spleen size were no longer evident (Fig. 2).

Again, grouping PCOS for insulin resistance, HS, FAI or spleen size, bisphenol A levels were higher in those with insulin resistance (1.39 ± 1.35 vs 0.57 ± 1.11 ng/ml; \( P = 0.0003 \)), HS (1.66 ± 1.56 vs 0.54 ± 0.47 ng/ml; \( P = 0.027 \)), more severe hyperandrogenism evaluated only by FAI (0.62 ± 0.54 vs 1.62 ± 1.50 ng/ml; \( P = 0.025 \)), and with larger spleen size (1.64 ± 1.50 vs 0.23 ± 0.26 ng/ml; \( P < 0.0001 \)) than in their counterparts without insulin resistance, HS, less severe hyperandrogenism or normal spleen (Fig. 3).

**Correlation analysis**

The correlations between bisphenol A and transaminases or γ-GT and BMI are shown in Fig. 4a–d. At the multivariate analysis, adjusted for BMI, with HoMA index, HS severity, FAI or spleen size as independent variables in the whole study population, spleen size and FAI were the strongest predictors of bisphenol A levels (\( \beta \) coefficients 0.379, \( P = 0.007 \) and 0.343, \( P = 0.014 \), respectively).

**Discussion**

The main finding of the present study was the strong association of bisphenol A levels with HS and the markers of low-grade inflammation in premenopausal women with PCOS, in particular with spleen size, thus unravelling the presence of the liver–spleen axis in this syndrome. In particular, independently of age and BMI, higher serum bisphenol A levels identified a quite different subgroup of PCOS women with more severe insulin resistance and hyperandrogenism, increased prevalence of HS, and evidence of a low-grade chronic inflammation status. Noteworthy, spleen size was the unique discriminating parameter of high circulating bisphenol A concentrations, in as much as the highest bisphenol A values were associated with the largest spleen sizes. To the best of our knowledge, this association is novel within the context of PCOS and was still evident when PCOS women were grouped according to insulin resistance or HS.

In line with the well-known influence of oestrogens on several effectors of the immune system, there is a considerable burden of evidence in vitro and in animal models that they may exert also immunotoxic effects. In particular, bisphenol A has been reported to stimulate cytokine productions and proliferative responses of murine spleen and thymus cells in vitro. Moreover, bisphenol A was involved in autoantibody production by B1 cells. Thus, it is possible that oestrogen-mimicking environmental factors, such as bisphenol A, may cause increased incidence of autoimmune diseases.

The first observation of increased levels of bisphenol A in women with ovarian dysfunction was reported by Takeuchi et al. in 2004 on a small group of women with PCOS; this finding was subsequently confirmed by Kandaraki et al. in 2011. In particular, these authors found that serum bisphenol A levels in women with PCOS were significantly higher in comparison with controls independently of the degree of obesity. Accordingly, despite a weak positive correlation between bisphenol A and BMI, otherwise no more evident at multivariate analysis, our data confirm the lack of differences in serum bisphenol A levels between lean and O/O women with PCOS. This finding is apparently in contrast with the positive association between urinary bisphenol A concentration and the prevalence of obesity very recently reported...
in a cross-sectional study on a large sample of Chinese adult population. This discrepancy could be likely due to differences in the setting of the population study (general vs PCOS), in the age of the population samples, mainly taking into account the possible progressive increase in bisphenol A exposure with age and, last but not least, in the assay methods used (serum vs urine). In fact,
as also previously reported,
the urinary excretion of bisphenol A is affected by the androgen-related increase in the glucuronidation of bisphenol A by liver microsomes. Conversely, serum bisphenol A assays, evaluating the total (conjugated and unconjugated) bisphenol A, could minimize the effect of androgens on bisphenol A metabolism.

Interestingly, in the group of women with PCOS in our study population, the increased bisphenol A levels were highly correlated with HS and laboratory liver tests, as well as with the markers of low-grade chronic inflammation, such as CRP, IL-6 and spleen enlargement. A number of endocrine disruptors have been considered responsible for the alterations similar to those encountered in nonalcoholic fatty liver disease, either directly through a hepatotoxic effect and/or indirectly by triggering hepatic and systemic insulin resistance. Moreover, the association between bisphenol A and HS has been previously reported in animal models, and has been related to increased expression of lipogenic genes, thus contributing to HS. In addition, in animal models, bisphenol A caused oxidative damage in the liver by disturbing the balance between reactive oxygen species and the antioxidant defence system, independently of oestrogen receptors. In the United States, bisphenol A concentrations were associated with cardiovascular diseases and metabolic disorders, including altered blood markers of liver function.

Finally, a significant association of bisphenol A with oxidative stress and inflammation markers has been reported among postmenopausal women, likely due to their low oestrogen levels compared with available oestrogen receptors.

The prevalence of HS was reported to be increased in women with PCOS. Although insulin resistance has been accounted for the common underlying pathophysiological mechanism, the relative role of obesity in this association still remains unravelled. The low-grade chronic inflammation has been involved in the pathogenesis of HS through the autocrine/paracrine/endocrine production of adipokines. Apart from its clinical significance in the pathogenesis of HS and its link to both obesity, namely visceral obesity, and insulin resistance, the low-grade inflammation process has been emphasized as a main pathophysiological mechanism in PCOS. This has been shown to occur also independently of obesity, and recently, the role of hyperandrogenism per se, in particular the increased androgen bioavailability estimated by FAI, has been extensively examined as grassroot of low-grade chronic inflammation in PCOS.

Except for low-grade chronic inflammation and HS, our data are also largely confirmatory of the association in PCOS women between bisphenol A and androgens or HoMA index. Accordingly, Diamanti-Kandarakis et al., evidenced the positive correlation between bisphenol A and insulin resistance using the Matsuda index, a different surrogate index of insulin sensitivity. This association was in line with experimental data evidencing insulin resistance in intact animals exposed to relevant doses of bisphenol A owing to disturbance of β-cell physiology.

In line with the suggested role of bisphenol A as endocrine disruptor for the ‘environmental obesogen hypothesis’, a possible pathway involving bisphenol A, low-grade inflammation, hyperandrogenism and liver-spleen axis in PCOS pathogenesis might be hypothesized. Besides the direct hepatotoxic and adipogenic effects, bisphenol A could act as pro-inflammatory primer, via macrophage activation and pro-inflammatory cytokine hypersecretion. Within this context, the spleen enlargement could represent a marker of this process, possibly linked to the recently discovered immune/autoimmune derangement in women with PCOS. The possible exposure in utero to low-chronic bisphenol A doses might trigger not only obesity and insulin resistance, but might also contribute to the low-grade chronic inflammation in women who will develop PCOS, per se or through hyperandrogenism.

In conclusion, our study adds a further tile to the emerging evidence of the role of environmental exposure to bisphenol A on polycystic ovary syndrome, by describing a so far unreported association between bisphenol A exposure, inflammation, liver-spleen axis and hyperandrogenism in the polycystic ovary syndrome setting, a major health condition for women.

Acknowledgements

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Competing interests/financial disclosure

The authors declare that they have no competing interests.

Authors’ contribution

TG and VR are the first authors of the manuscript as they equally contributed to the study, participated in its design and co-ordination and helped to draft the manuscript. TG performed the statistics and ultrasonography. DSC and PG performed the clinical investigation. D’E V and PF performed the laboratory analyses. BV and OF collected the data. OF, FP and CA critically revised the manuscript. VR and SS conceived the study, analysed the statistical data and wrote the manuscript. All authors read and approved the final manuscript.

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the Androgen Excess and PCOS Society. Fertility and Sterility, 94, 690–693.
ORIGINAL ARTICLE

Growth-promoting action and growth factor release by different platelet derivatives

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Abstract
Platelet derivatives are commonly used in wound healing and tissue regeneration. Different procedures of platelet preparation may differentially affect growth factor release and cell growth. Preparation of platelet-rich fibrin (PRF) is accompanied by release of growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor β1 (TGFβ1), and several cytokines. When compared with the standard procedure for platelet-rich plasma (PRP), PRF released 2-fold less PDGF, but >15-fold and >2-fold VEGF and TGFβ1, respectively. Also, the release of several cytokines (IL-4, IL-6, IL-8, IL-10, IFNγ, MIP-1α, MIP-1β and TNFα) was significantly increased in PRF-conditioned medium (CM), compared to PRP-CM. Incubation of both human skin fibroblasts and human umbilical vein endothelial cells (HUVECs) with PRF-derived membrane (mPRF) or with PRF-CM enhanced cell proliferation by >2-fold (p < 0.05). Interestingly, PRF elicited fibroblast growth at a higher extent compared to PRF. At variance, PRF effect on HUVEC growth was significantly greater than that of PRP, consistent with a higher concentration of VEGF in the PRF-CM. Thus, the procedure of PRP preparation leads to a larger release of PDGF, as a possible result of platelet degranulation, while PRF enhances the release of proangiogenic factors.

Introduction
Platelet products have been extensively used for clinical and surgical applications which require tissue regeneration [1]. Thus, platelet derivatives represent promising therapeutic tools offering opportunities for periodontal, oral, maxillo-facial, orthopedic and dermatological procedures [2, 3]. Indeed, platelets represent a known source of cytokines and growth factors involved in wound healing and tissue repair [4, 5]. Many platelet-derived factors are considered important players in wound healing processes. In particular, beside their known functions in hemostasis and clot formation, platelet granules contain growth factors, including platelet-derived growth factor (PDGF), transforming growth factor β (TGFβ), IGF-1, involved in cell proliferation and differentiation [6]. It should also be considered that, among platelet-released factors, no individual growth factor has proven per se effectiveness both in soft and in hard tissue regeneration [7]. Therefore, most probably, the mixture of more platelet-derived factors contained in platelet releasates, could be responsible for the tissue-regeneration potential of platelet derivatives [8]. There is also a large body of evidence that the cross-talk between factors released by platelets and those released by recipient cells mediates the propagation of the tissue repair mechanisms [9]. Indeed, many cell types, including blood cells, fibroblasts and endothelial cells participate to the final healing process and each cell type may specifically affect the function of the other cell types, both by cell-cell and cell-matrix contacts and by producing and releasing soluble factors [10]. Thus, understanding the complex mechanisms regulating tissue repair and regeneration, is still incomplete.

Platelet-rich plasma (PRP) has long been used as a source of platelet growth factors [11]. Several different products have been developed in the last years [6]. All available PRP procedures have some points in common. A first centrifugation step is needed to separate red blood cells (RBC), buffy coat and platelet-poor plasma (PPP). Then, different procedures have been used to discard RBC and PPP and to collect the buffy coat. The platelets could be eventually activated by thrombin and calcium and applied to the injured/surgical site [12]. More recently, an alternative approach has also been adopted. It requires blood collection in tubes without anticoagulant and immediate centrifugation, while PRP preparation leads to a larger release of PDGF, as a possible result of platelet degranulation, while PRF enhances the release of proangiogenic factors.
Methods

Subject recruitment and preparation of biomaterials

Fourteen healthy blood donors (M/F:6/8; age 24–40 years) were enrolled in the study. All were non-smokers, non-obese (BMI range: 20.4–26.3) and with a platelet count >180,000/mm³. None of them was under any medication for the last 21 days. Informed consent was obtained from every subject before blood drawing. The protocol has been approved by the Ethical Committee of the University of Naples.

Blood was drawn from each individual and two 9-ml aliquots were obtained. One aliquot was collected in tubes without anticoagulant for the preparation of leukocyte- and platelet-rich fibrin (L-PRF). The other was collected in a vacutainer tube (Vacutainer; Becton Dickinson, East Rutherford, NJ) containing 10% trisodium citrate anticoagulant solution for the preparation of PRP.

L-PRF was prepared through a single 12-min step of centrifugation of whole blood (PRF production kit, Process, Nice, France) according to manufacturer’s instruction. Each 9 ml tube produced one L-PRF clot. Where indicated, the L-PRF clot was separated from the RBC base and condensed through sterile gauzes in order to obtain a membrane-like structure (mPRF) [13].

For PRP preparation, the whole blood was initially centrifuged at 350 g for 15 min. The supernatant was transferred into another tube and a second centrifugation step was performed for 10 min at 980 × g. After centrifugation, the upper fraction containing PPP was discarded and the lower fraction containing PRP was used for the experimental procedures [9, 15, 16]. For platelet gel preparations, autologous thrombin (0.1 NIH unit/ml final concentration) and calcium gluconate (10 mg/ml final concentration) were added to PRP for 5 min at room temperature to allow clot formation.

Conditioned media (CM) were obtained by incubating the platelet preparations for 24 hours with serum-free Dulbecco’s modified Eagle medium (DMEM)-F12 (1:1). 0.25% BSA was added to the medium in order to prevent osmotic cell death. After the incubation, the medium was collected and centrifuged at 14,000 × g to remove cellular debris and analyzed for cytokines and growth factor content or placed onto recipient cells for different times, as described below.

Determination of cytokines and growth factors released by platelet-based biomaterials

PRP, PRF and mPRF CM were screened for the concentration of IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFNγ, MIP-1α, MIP-1β, RANTES, TNFα, bFGF, PDGF, vascular endothelial growth factor (VEGF) using the Bioplex Multiplex human cytokine assay kit and the Bioplex Multiplex human growth factor kit (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions.

Cell culture and growth

Skin fibroblasts were obtained by punch biopsy and cultures established as described previously [17]. The cells were grown at 37 °C in DMEM supplemented with 10% fetal calf serum in a 5% CO₂–95% air-humidified atmosphere. For the experimental procedures, the cultures were used between the 8th and 15th passage, and, for each individual experiment, cells were maintained in culture for an equal number of generations. Primary human umbilical vein endothelial cells (HUVECs) were obtained and cultured as previously described. HUVECs were cultured under 37 °C and 5% CO₂–95% air-humidified atmosphere in the endothelial cell medium (ECM, Sciencell) according to the manufacturer’s instruction. The ECM was consisted of 500 ml of basal medium, 25 ml of fetal bovine serum, 5 ml of endothelial cell growth supplement and 5 ml of penicillin/streptomycin solution. For all experiments HUVEC up to passage five were used [18].

For cell growth determination, the studies were performed as previously described [19]. Briefly, either skin fibroblasts or HUVEC cells were seeded in 6-well culture plates in a complete medium. The following day, the cells were starved in serum-free DMEM 0.25% BSA for 16 hours and incubated with either platelet preparations or CM obtained as described above for different times. Cell count was performed by both Bürker chamber counting and the TC10™ Automated Cell Counter (Bio-Rad, Hercules, CA) according to the manufacturer’s instruction.

Statistical analysis

Data were analyzed with Statview software (Abacus concepts) by one-factor analysis of variance. p values of <0.05 were considered statistically significant.

Results

Release of cytokines and growth factors by PRF

We have first evaluated the ability of PRF, obtained as described in ‘‘Methods’’ section to release cytokines/chemokines. To this end, PRF has been allowed to release factors into serum-free medium for 24 hours. PRF released detectable amounts of IL-2, IL-4, IL-6, IL-8, IL-10, IFNγ, MIP-1α, MIP-1β, RANTES and TNFα (Table I). Similarly, detectable levels of bFGF, PDGF, VEGF and TGFβ1 were found in PRF-CM (Table II).

Comparison of PRF- and PRP-released factors

Next we have compared the release of cytokines/chemokines and growth factors by PRF (as membrane, mPRF – see ‘‘Methods’’ section) and PRP (as platelet gel). The use of mPRF was preferred to that of PRF since, when the latter was incubated in serum-free

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Concentration (pg/ml)</th>
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<tbody>
<tr>
<td>IL-2</td>
<td>5.39 ± 0.67</td>
</tr>
<tr>
<td>IL-4</td>
<td>9.63 ± 0.77</td>
</tr>
<tr>
<td>IL-6</td>
<td>13423.71 ± 3192.81</td>
</tr>
<tr>
<td>IL-8</td>
<td>51497.47 ± 7724</td>
</tr>
<tr>
<td>IL-10</td>
<td>38.57 ± 3.20</td>
</tr>
<tr>
<td>IFNγ</td>
<td>539.74 ± 89</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>641.21 ± 70</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>595.16 ± 58</td>
</tr>
<tr>
<td>RANTES</td>
<td>1837.97 ± 190</td>
</tr>
<tr>
<td>TNFα</td>
<td>85.54 ± 7.90</td>
</tr>
</tbody>
</table>

Table II. Growth factors released by PRF. PRF was incubated with serum-free DMEM-F12 (1:1). After 24 hours, the media were collected and tested by using the Bioplex multiplex cytokine assay kit as described in ‘‘Methods’’ section.

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Concentration (pg/ml)</th>
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<tbody>
<tr>
<td>bFGF</td>
<td>7.66 ± 0.65</td>
</tr>
<tr>
<td>PDGF</td>
<td>2189.09 ± 225</td>
</tr>
<tr>
<td>VEGF</td>
<td>1376.3 ± 129</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>265667.50 ± 39851.60</td>
</tr>
</tbody>
</table>
medium, many cells (mainly RBC) and cellular debris were found floating, possibly interfering with the evaluation of the function of PRF-released factors. PRF and PRP were obtained by equal amounts of blood drawn by the same individual. mPRF and PRP gels were then applied onto culture dishes and incubated with serum-free medium for 24 hours to obtain CM. The amount of several inflammatory cytokines such as IL-6, IL-8, IL-10, IFN\(\gamma\), MIP-1\(\alpha\), MIP-1\(\beta\), TNF\(\alpha\), was higher in the mPRF-CM compared to PRP-CM. At variance, the levels of RANTES were ~3-fold higher in PRP-CM compared to mPRF-CM (Table III). Concerning the concentration of growth factors, the levels of bFGF were detectable in small amounts compared to the other growth factors both in mPRF-CM and in PRP-CM (Table IV). VEGF and TGF\(\beta\) levels were 11- and 2.6-fold higher, respectively, in mPRF-CM compared to PRP-CM. Instead, the amount of PDGF was ~2-fold lower in mPRF-CM compared to PRP-CM (Table IV).

**Induction of cell growth by PRF- and PRP-released factors**

In order to address whether PRF and PRP may have different effects on different cell types, we tested the ability of those preparations to induce the growth of primary cultures of human fibroblasts and vascular endothelial cells (HUVEC). To this end, PRF gel and mPRF were directly applied onto the culture plate containing either skin fibroblasts or endothelial cells. Indeed, clinical applications of PRF and PRP often require fibroblast proliferation and angiogenesis [1, 11]. Interestingly, PRP gel and mPRF-induced proliferation of both cell types. However, PRP was significantly more effective than mPRF in inducing fibroblast growth (Figure 1). Very similar results were also obtained with mesenchymal stem cells obtained from human subcutaneous adipose tissue (data not shown). At variance, mPRF effect on proliferation of endothelial cells was slightly higher than that of PRP, suggesting a prevalent pro-angiogenic function (Figure 2).

In order to verify if released factors rather than the particulate fraction of mPRF and PRP were responsible for the growth promoting action, mPRF-CM and PRP-CM were obtained as previously described and added onto cultured human fibroblasts for 24 ours. Again, both mPRF- and PRP-released factors induced fibroblast growth with PRP significantly more effective than mPRF in inducing fibroblast growth (Figure 3). Next, we have evaluated the effect of mPRF-CM and PRP-CM on the growth of endothelial cells. Interestingly, mPRF induced growth of HUVEC to a significantly greater extent compared to PRP releasate (Figure 4).

**Discussion**

It is well established that platelet derivatives may play a key role in soft and hard tissue regeneration and in enhancing hemostasis in patients receiving anti-coagulating agents [1, 3, 8, 11, 13, 20–22]. Nevertheless, many procedures have been used to obtain platelet factors alone or in combination with other factors eventually derived from white blood cells or circulating stem cells [12, 23]. Much less is known about the biological activity of

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**Table III. Cytokines released by mPRF and PRP.** mPRF and PRP (as platelet gel) were incubated with serum-free DMEM-F12 (1:1). After 24 hours, the media were collected and tested by using the Bioplex multiplex cytokine assay kit as described in ‘‘Methods’’ section.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>mPRF (pg/ml)</th>
<th>PRP (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>3.05 ± 0.45</td>
<td>2.34 ± 0.30</td>
</tr>
<tr>
<td>IL-4</td>
<td>10.56 ± 0.1</td>
<td>1.78 ± 0.43***</td>
</tr>
<tr>
<td>IL-6</td>
<td>10093.55 ± 1064.03</td>
<td>6.20 ± 0.55**</td>
</tr>
<tr>
<td>IL-8</td>
<td>4340.37 ± 5011</td>
<td>1303.01 ± 126***</td>
</tr>
<tr>
<td>IL-10</td>
<td>79.01 ± 7.9#</td>
<td>3.91 ± 0.42***</td>
</tr>
<tr>
<td>IFN(\gamma)</td>
<td>484.04 ± 57.6</td>
<td>37.30 ± 3.40*</td>
</tr>
<tr>
<td>MIP-1(\alpha)</td>
<td>479.56 ± 28.6</td>
<td>0.25 ± 0.03**</td>
</tr>
<tr>
<td>MIP-1(\beta)</td>
<td>822.18 ± 82.2#</td>
<td>6.90 ± 0.57***</td>
</tr>
<tr>
<td>RANTES</td>
<td>2818.57 ± 281#</td>
<td>5505.29 ± 530*</td>
</tr>
<tr>
<td>TNF(\alpha)</td>
<td>73.55 ± 5.3</td>
<td>2.21 ± 0.35*</td>
</tr>
</tbody>
</table>

*denotes statistically significant differences (*\(p<0.05\); **\(p<0.01\); ***\(p<0.001\)). # denotes statistically significant differences of mPRF vs. PRF conditioned media (#\(p<0.05\)).

**Table IV. Growth factors released by mPRF and PRP.** mPRF and PRP (as platelet gel) were incubated with serum-free DMEM-F12 (1:1). After 24 hours, the media were collected and tested by using the Bioplex multiplex growth factor assay kit as described in ‘‘Methods’’ section.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>mPRF (pg/ml)</th>
<th>PRP (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bFGF</td>
<td>7.29 ± 0.80</td>
<td>1.40 ± 0.14***</td>
</tr>
<tr>
<td>PDGF</td>
<td>2225.69 ± 333.85</td>
<td>4708.19 ± 601.89*</td>
</tr>
<tr>
<td>VEGF</td>
<td>2330.89 ± 233#</td>
<td>125.48 ± 12.5***</td>
</tr>
<tr>
<td>TGF(\beta)</td>
<td>261886 ± 9282.9</td>
<td>102817.60 ± 15422.64*</td>
</tr>
</tbody>
</table>

*denotes statistically significant differences of PRP vs. mPRF conditioned media (*\(p<0.05\); **\(p<0.01\); ***\(p<0.001\)). # denotes statistically significant differences of mPRF vs. PRF conditioned media (#\(p<0.05\)).

---

**Figure 1.** Effect of mPRF and PRP on fibroblast cell growth. mPRF and PRP gels were directly applied for 24 hours onto the culture plates containing serum-starved skin fibroblasts, previously obtained by punch biopsy (n = 10). As a control, fibroblasts have been incubated with DMEM F12 (1:1) without serum supplementation (MEDIUM BSA) or with 10% fetal bovine serum (MEDIUM 10% FBS). The cells were then counted as described in ‘‘Methods’’ section. Results are presented as fold-increase over basal (cell count in MEDIUM BSA). * denotes statistically significant values (*\(p<0.05\); **\(p<0.01\); ***\(p<0.001\)).

**Figure 2.** Effect of mPRF and PRP on endothelial cell growth. mPRF and PRP gels were directly applied onto the culture plates containing serum-starved primary human umbilical vein endothelial cells (HUVEC) for 24 hours. As a control, endothelial cells were incubated with DMEM F12 (1:1) without serum supplementation (MEDIUM BSA) or with 10% fetal bovine serum (MEDIUM 10% FBS). The cells were then counted as described in ‘‘Methods’’ section. Results are presented as fold-increase over basal (cell count in MEDIUM BSA). * denotes statistically significant values (**\(p<0.001\)).
the individual platelet preparations, in particular with the aim of defining their involvement in selected clinical applications.

In this work, we have addressed whether different procedures to obtain platelet products may lead to the release of a differential spectrum of molecules and may differentially control the growth of specific cell types. To this end, we have determined the concentration of several growth factors and cytokines/chemokines in the media exposed to either PRP gel or PRF membranes. Indeed, platelet gels were obtained by treating PRP with calcium and thrombin and PRF membranes were obtained by condensing PRF clots (see “Methods” section). Interestingly, while PRP released a significantly higher amounts of PDGF and RANTES, the amount of several cytokines, typically involved in wound healing and re-vascularization, was more abundant in the releasate of PRF membranes. It is conceivable that the enrichment of platelets obtained in the standard procedure for PRP is responsible for the higher release of PDGF [9, 24]. Also, RANTES, a pro-inflammatory chemokine, is very abundant in platelets [25]. On the other end, the clot obtained by the PRF procedure is most likely enriched in white blood cells, which may represent major producers of inflammatory cytokines (IL-6, IL-8, IL-10, IFNγ, MIP-1α, MIP-1β, TNFα) and of pro-angiogenic factors (VEGF and TGFβ1).

It should also be noticed that mPRF released higher levels of IL-10, MIP-1β, RANTES and VEGF, compared to PRF clot. The mechanism responsible for these events has not been completely explained, but it may involve activation of the cells embedded in the clot, following condensation and mechanical stress.

The differences in the release of specific cytokines and growth factors prompted us to investigate whether mPRF and PRP may have differential growth effect in fibroblasts and endothelial cells. According to previous reports, platelet derived products can increase cellular survival and proliferation [26–28]. Indeed, both preparation procedures were able to induce cell growth (either in fibroblast or in endothelial cells). Interestingly, however, direct application of PRP gel was significantly more effective in inducing fibroblast growth, compared to mPRF. Similarly, when PRP was enabled to release factors and CM added to fibroblasts, greater effectiveness than with mPRF CM was observed. On the other hand, growth of endothelial cells was slightly more effective following direct application of mPRF, compared to PRP, while, the effect of mPRF CM on HUVEC growth was significantly more pronounced than that of PRP CM.

These data indicate that different procedures to obtain platelet products display quantitative differences in the content of growth factors and cytokines. This may be relevant for the choice of the appropriate tool. For example, standard procedures for PRP, more efficiently release PDGF and RANTES, most likely because of the higher enrichment in platelets occurring trough the preparation and, perhaps, following the activation with thrombin and calcium which facilitates platelet degranulation. On the other hand, PRF procedures allow a better yield of several growth factors (bFGF, VEGF, TGFβ1) and cytokines, which derive from white blood cells and, possibly, circulating progenitor cells, which are embedded in the fibrin clot.

This is a relevant issue, since it may recommend the use of one or the other method, based on the specific goal to achieve. Indeed, if the expansion of connective tissue is mainly required, the use of a higher concentration of PDGF, to increase the number of the fibroblast component, is more indicated. Alternatively, if reduced angiogenesis is a major obstacle to tissue regeneration, one may choose preparations containing higher levels of bFGF and VEGF, thereby stimulating endothelial cell recruitment and vessel formation. In addition, procedures for PRF production enable the achievement of an higher concentration of several cytokines, which may play important roles in many events involved in the tissue regeneration process.

Conclusion

We have reported that different procedures to obtain blood derivatives for regenerative medicine applications may yield different products. When platelet activation is achieved via thrombin stimulation, platelets release greater amounts of PDGF and other factors, mainly involved in fibroblast growth. At variance, when clot is directly and rapidly obtained without anticoagulation, the enrichment of VEGF and pro-angiogenic cytokines, possibly released by the embedded white blood cells, facilitates endothelial cell growth.

Acknowledgements

The authors are grateful to Dr. E. D’Agostino (Blood bank, Federico II University Hospital, Naples, Italy) for helpful discussion and critical reading of the manuscript and Drs G. Perruolo (CNR, Naples, Italy).
M. Nigro and G. Aurioso (Blood bank, Federico II University Hospital, Naples, Italy) for technical help and for platelet preparation.

Declaration of interest

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References

Bisphenol-A Impairs Insulin Action and Up-Regulates Inflammatory Pathways in Human Subcutaneous Adipocytes and 3T3-L1 Cells

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1 Istituto di Endocrinologia ed Oncologia Sperimentale (IEOS-CNR), Naples, Italy, 2 Dipartimento di Scienze Mediche Traslazionali, Università degli Studi di Napoli “Federico II”, Naples, Italy, 3 Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, Salerno, Italy

Abstract

Current evidence indicates that chemical pollutants may interfere with the homeostatic control of nutrient metabolism, thereby contributing to the increased prevalence of metabolic disorders. Bisphenol-A (BPA) is a lipophilic compound contained in plastic which is considered a candidate for impairing energy and glucose metabolism. We have investigated the impact of low doses of BPA on adipocyte metabolic functions. Human adipocytes derived from subcutaneous adipose tissue and differentiated 3T3-L1 cells were incubated with BPA, in order to evaluate the effect on glucose utilization, insulin sensitivity and cytokine secretion. Treatment with 1nM BPA significantly inhibited insulin-stimulated glucose utilization, without grossly interfering with adipocyte differentiation. Accordingly, mRNA levels of the adipogenic markers PPARγ and GLUT4 were unchanged upon BPA exposure. BPA treatment also impaired insulin-activated receptor phosphorylation and signaling. Moreover, adipocyte incubation with BPA was accompanied by increased release of IL-6 and IFN-γ, as assessed by multiplex ELISA assays, and by activation of JNK, STAT3 and NFkB pathways. Treatment of the cells with the JNK inhibitor SP600125 almost fully reverted BPA effect on insulin signaling and glucose utilization. In conclusion, low doses of BPA interfere with inflammatory/insulin signaling pathways, leading to impairment of adipose cell function.

Introduction

Overweight, obesity and insulin resistance epidemics are significant human health problems in adults, but also in children and adolescents [1,2]. They are associated with increased risk of diseases related to metabolic dysfunctions, including metabolic syndrome (MS), type 2 diabetes mellitus (T2D), coronary heart disease (CHD), and some forms of cancer [3–6]. Chronic inflammation often accompanies obesity and related disorders, suggesting that inflammatory factors might be a crucial culprit connecting adipose tissue dysfunction with insulin resistance and diabetes as well as with cardiovascular disease and cancer [5–9].

Environmental signals, including chemical pollutants, are now considered contributing factors for insulin resistance [10–13]. Most of chemical pollutants potentially accumulate into adipose tissue [14], and could be responsible, in addition to the well-known dietary and lifestyle habits, for overweight and obesity epidemic, driving the parallel T2D epidemic [13,15–17].

The “environmental obesogen hypothesis” has been associated to the adipose tissue inflammatory phenotype, with hypersecretion of pro-inflammatory and decrease of anti-inflammatory cytokines, and considered as a major contributing factor for the decreased insulin sensitivity [7–10]. Indeed, the exponential rise of obesity-related pathologies worldwide is associated with the marked increase of toxic chemicals in the environment. In the modern world, in fact, the environment has been largely affected by an ever increasing number of synthetic chemical lipophilic pollutants, such as pesticides, organophosphates, polychlorinated bisphenyls, phthalates, solvents etc., that permeate the diet, the air and the ground [15,16,18–20]. An even larger impact could be exerted by those which are resistant to biological and chemical degradation, also termed Persistent Organic Pollutants (POPs), most of them also classified as endocrine disruptors [15–20].

Bisphenol-A (BPA) represents a potential obesogen compound and has been studied for its estrogen mimetic activity and endocrine disruption [21–24]. BPA is found in products containing polycarbonate plastics and in resins, as lining for metal cans and as an additive in other widely used plastics [25–27]. It is a lipophilic compound detectable at nanomolar levels not only in food and tap water, in rivers, lakes and sea, but also in human blood samples and urine worldwide, as well as in the placenta, amniotic fluid of pregnant women and in human milk [28,29]. In animal models, BPA has been shown to disrupt the major weight controlling hormones, such as thyroid hormones, estrogens, testosterone, corticosteroids, growth hormone and leptin, and to

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Competing Interests: The authors have declared that no competing interests exist.

alter adipogenesis, beta-cell and endocrine pancreas function [24,30–32]. The relationship between BPA, inflammation and insulin sensitivity in adipose tissue is still not well understood.

Thus, we have investigated whether BPA, at nanomolar doses, consistent with those found in the human bloodstream and/or in environment, could interfere with adipose tissue function. We found that, in cultured adipose cells derived from human subcutaneous tissue and in 3T3-L1 adipocytes, BPA exposure impaired insulin sensitivity and glucose utilization and enhanced the release of pro-inflammatory compounds, even in the absence of major derangement of adipocyte differentiation.

### Materials and Methods

#### Materials

Media, sera, and antibiotics for cell culture were from Lonza (Lonza Group Ltd, Basel, Switzerland). Antibodies against phospho-Ser473PKB/Akt1, ERK, phospho-Thr183/Tyr185 c-Jun N-terminal kinase (JNK), STAT3, NFκB, Laminin A/C and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-Thr202/Tyr204 ERK, Phospho-IGF1-Receptor beta Tyr1131/Insulin Receptor beta Tyr1146, Insulin Receptor beta and Phospho-Tyr705 STAT3 antibodies were obtained from Cell Signaling Technology (Danvers, MA). PKB/Akt antibody was

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<td><strong>PPARγ Forward</strong> 5’-TGG TGC CTTGCTCATGC-3’</td>
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<td><strong>PPARγ Reverse</strong> 5’-CTG TGG TAA AGG GCT TGA TGTC-3’</td>
</tr>
<tr>
<td><strong>GLUT4 Forward</strong> 5’-CAG AAG GTG ATT GAA CAG AG-3’</td>
</tr>
<tr>
<td><strong>GLUT4 Reverse</strong> 5’-AAT GAT GCC AAT GAG AAA GG-3’</td>
</tr>
<tr>
<td><strong>GLUT1 Forward</strong> 5’-GGG AAT GTG CTC ATC TTG GA-3’</td>
</tr>
<tr>
<td><strong>GLUT1 Reverse</strong> 5’-TGA GGC TCT GTG TGG TTC TG-3’</td>
</tr>
<tr>
<td><strong>LEPTIN Forward</strong> 5’-ACTCCACATGCTTGACTC-3’</td>
</tr>
<tr>
<td><strong>LEPTIN Reverse</strong> 5’-CTTACCTCACTCTCTTCG-3’</td>
</tr>
<tr>
<td><strong>β-actin Forward</strong> 5’-GGG CCT AGG CAC CAG GGT GTG-3’</td>
</tr>
<tr>
<td><strong>β-actin Reverse</strong> 5’-TCG GTG AGC ACA GGA GGG TG-3’</td>
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<td><strong>GLUT4 Reverse</strong> 5’-AAT GAT GCC AAT GAG AAA GG-3’</td>
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<td><strong>GLUT1 Forward</strong> 5’-GGG AAT GTG CTC ATC TTG GA-3’</td>
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**Figure 1. Effect of BPA on adipocyte glucose utilization.** Human adipocytes (a) and 3T3-L1 adipocytes (b) were incubated in serum free-media with 1 nM or 100 nM BPA and 100 nM insulin for 24 and 48h as indicated. Next, supernatants were collected and glucose consumption was determined as described in Materials and Methods. Bars represent the mean ± SD of three independent experiments. Data were analyzed with Statview software (Abacus concepts) by one-factor analysis of variance. p values of less than 0.05 were considered statistically significant. Asterisks indicate statistically significant differences (* p<0.05). Error bars indicate mean ± S.D.

doi:10.1371/journal.pone.0082099.t001

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doi:10.1371/journal.pone.0082099.g001
from Millipore (Billerica, MA). BPA in ethanol was a generous gift of Prof. C. Crescenzi (Department of Pharmaceutical Science, University of Salerno, Italy). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were from Bio-Rad (Hercules, CA). All the other chemicals were from Sigma-Aldrich (St. Louis, MO).

**Cell cultures**

Human adipose tissue samples were obtained by abdominal biopsy in the periumbilical region under local anesthesia (2% lidocaine) from patients undergoing elective abdominal surgery for gall bladder disease. The study was approved by the Ethic Committee of the University of Napoli “Federico II”. The written informed consent with respect to taking the samples and making the cell lines was obtained before the biopirical procedure. The study protocol was conducted in accordance to the principles of the Declaration of Helsinki as revised in 2000. Adipose tissue was digested with collagenase and Adipose-derived Stromal Vascular Fraction cells (SVF) were isolated and differentiated as previously reported [6].

**Conditioned media**

3T3-L1 mouse embryonic fibroblasts (ATCC-CL-173, American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mmol/l glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin. Cultures were maintained in humidified atmosphere of 95% air and 5% CO2 at 37 C. 3T3-L1 differentiation has been achieved as previously described [6].

Conditioned media were obtained by incubating the cells for 8 h with serum-free DMEM 0.25% BSA after two washes with PBS. After the incubation, medium was collected and centrifuged at 14000g to remove cellular debris and analyzed for cytokine content, as described below.

**Glucose Utilization**

For glucose utilization studies, the method previously described [33] was modified for human adipocytes and 3T3-L1 adipocytes. Adipocytes were incubated in serum-free media containing 0.25% BSA in the absence or presence of 1 nM–100 nM BPA and 100 nM insulin for 24–48h. Glucose concentration was measured in the medium before and after the incubation.

The difference in glucose concentration was considered to be utilized by the cells. Quantitative analysis of glucose concentration was performed with a ABX Pentra 400 clinical chemistry analyzer using the reagent ABX Pentra Glucose CP (ABX-Horiba, Montpellier, France) according to the manufacturer’s instructions.

**Immunoblot procedure**

Total cell lysates and, where indicated, cytosolic and nuclear fractions were obtained, separated by SDS-PAGE and immunobotted with specific antibodies as previously described [34–35].

**Cytokine Assay**

Human and 3T3-L1 adipocytes conditioned media were screened for the concentration of IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC/IL-8, MCP-1, MIP-1a, MIP-1β, RANTES and TNF-α using the Bioplex multiplex Mouse and Human Cytokine kits (Bio-Rad) according to the manufacturer’s protocol as previously described [6].

**Real-time RT-PCR analysis**

Table 1. Total RNA was isolated from 3T3-L1 adipocytes by using the RNeasy Kit (Qiagen Sciences) according to the manufacturer’s instruction. For real-time RT-PCR analysis, 1 μg cell RNA was reverse transcribed using Super Script II Reverse Transcriptase (Invitrogen). PCR were analyzed using SYBR...
Green mix (Invitrogen). Reactions were performed using Platinum SYBR Green Quantitative PCR Super-UDG using an iCycler IQmulticolor Real-Time PCR Detection System (Bio-Rad). All reactions were performed in triplicate and β-actin was used as an internal standard. Primer sequences used were described in Table 1.

Figure 3. Effect of BPA on insulin transduction pathways. Human (a) and 3T3-L1 adipocytes (c) were incubated with 1 nM BPA for 24 and 48 h as indicated and exposed to 100 nM insulin for 10 min and then solubilized as described in Materials and Methods. Cell lysates (50 μg protein/sample) were blotted with phospho- Tyr1146 Insulin Receptor (pIR), phospho- Ser473/Akt/PKB and phospho-Thr202/Tyr204/Extracellular signal-Regulated Kinases (pERK) antibodies and then reblotted with anti-IR, Akt/PKB and ERK antibodies. To ensure the equal protein transfer, membranes were blotted with actin antibodies. The filters were revealed by ECL and autoradiography. The autoradiographs shown are representative of four independent experiments. b-d) Filters obtained in a and c have been analyzed by laser densitometry as described under Materials and Methods. Data were analyzed with Statview software (Abacus concepts) by one-factor analysis of variance. * p values of less than 0.05 were considered statistically significant. Asterisks indicate statistically significant differences (*p<0.05). Error bars indicate mean ± S.D.
BPA down-regulates insulin-stimulated glucose utilization in differentiated adipocytes

We have first investigated whether BPA could affect glucose utilization in differentiated adipocytes. To this end, human adipocytes, obtained following differentiation of adipose tissue-derived stromal vascular fraction (SVF) cells, were incubated with BPA (1 nM or 100 nM), for 8, 24 and 48h, in the absence or in the presence of insulin (100 nM). Following BPA treatment, glucose utilization tended to be increased, at each time point (data not shown). As expected, in the absence of BPA, insulin induced a significant 4.5 fold increase of glucose utilization. Insulin stimulatory effect was still detectable after 8h incubation with 1 nM BPA, while after 24h of incubation with BPA, insulin effect on glucose utilization was reduced by 55% (Fig 1a). Within the same time frame, 100 nM BPA decreased insulin stimulatory action by >70%. Prolonging BPA pre-exposure to 48h, further reduced insulin effect. Consistent results were obtained following identical treatment of differentiated 3T3-L1 adipocytes (Fig 1b).

Thus, 1 nM BPA treatment for 24h was sufficient to inhibit insulin effect on glucose utilization both in differentiated human adipocytes and in 3T3-L1 cells. Therefore, for the next experimental settings we have used 1 nM BPA, as the lowest non toxic dose.

No relevant morphological abnormalities were observed following BPA exposure (up to 48 h) both for human and 3T3-L1 adipocytes.

Moreover, 1 nM BPA did not roughly affect adipocyte differentiation. Indeed, when 3T3-L1 pre-adipocytes were incubated with BPA along with the differentiation mix, during early (day 2), medium (day 6) and late (day 10) adipogenesis phases, PPARγ (Fig 2a) and GLUT4 (Fig 2b) mRNA levels were unchanged. At variance, GLUT1 levels were increased, following BPA exposure (Fig 2c). Consistent results were obtained with human pre-adipocytes (data not shown).

BPA inhibits insulin receptor phosphorylation and downstream signalling in differentiated adipocytes

We have therefore analyzed the effect of BPA on insulin signalling in mature adipocytes. Human (Fig 3a-b) and 3T3-L1 (Fig 3c-d) adipocytes were pre-incubated with 1 nM BPA for 24h and 48h and then stimulated with insulin for 10 min. BPA exposure led to a time-dependent decrease of insulin-stimulated

---

**Table 2. BPA interference on adipocyte-released cytokines.**

<table>
<thead>
<tr>
<th>Abiop3T3-L1(pg/ml)</th>
<th>Human Adipo + BPA (pg/ml)</th>
<th>Adipo3T3-L1(pg/ml)</th>
<th>Adipo3T3-L1 + BPA (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>ND</td>
<td>60.58±9</td>
<td>55.36±7.4</td>
</tr>
<tr>
<td>IL-2</td>
<td>9.63±1.2</td>
<td>9.68±0.8</td>
<td>ND</td>
</tr>
<tr>
<td>IL-4</td>
<td>8.23±0.9</td>
<td>8.55±0.9</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>IL-6</td>
<td>689.7±72.5</td>
<td>884.9±93.4*</td>
<td>0.98±0.03</td>
</tr>
<tr>
<td>IL-10</td>
<td>85.01±9.2</td>
<td>83.15±9.7</td>
<td>3.70±0.4</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>112.31±15.6</td>
<td>114.41±13.1</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>393.79±42.6</td>
<td>481.71±45.7*</td>
<td>0.25±0.09</td>
</tr>
<tr>
<td>KC/IL-8</td>
<td>56.81±6.9</td>
<td>63.63±6.1</td>
<td>20566±215</td>
</tr>
<tr>
<td>MIP-1a</td>
<td>2.44±0.9</td>
<td>2.52±0.7</td>
<td>26.22±3.2</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>13.68±1.9</td>
<td>14.07±2</td>
<td>2.35±0.52</td>
</tr>
<tr>
<td>RANTES</td>
<td>25.08±3.1</td>
<td>25.94±2.3</td>
<td>362.75±39.2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>11.12±1.3</td>
<td>11.37±1</td>
<td>255.91±27.1</td>
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</table>

Supernatants from 3T3-L1 and human adipocytes treated with or without 1 nM BPA for 24h were collected and tested by using the Bioplex multiplex cytokine assay kit as described in Materials and Methods. Data were analyzed with Statview software (Abacus concepts) by one-factor analysis of variance. p values of less than 0.05 were considered statistically significant. Asterisks indicate statistically significant differences (* p<0.05; ** p<0.01).

doi:10.1371/journal.pone.0082099.t002
insulin receptor (IR) tyrosine phosphorylation and to a significant reduction of PKB/Akt and ERK1/2 phosphorylation. No significant BPA-dependent change was observed for IR, PKB/Akt and ERK1/2 total protein or basal phosphorylation levels.

**BPA exposure affects adipocyte secretion and induces activation of inflammation-related pathways in adipocytes**

We assessed whether BPA could affect the expression of specific adipokines. To this end, we have measured leptin mRNA levels in 3T3-L1 cells. A significant decrease in leptin mRNA levels was found following treatment with 1 nM BPA, compared the control cells (Fig.4).

We have next analyzed the impact of 1 nM BPA on adipocyte secretory function, in terms of release of inflammatory cytokines (Table 2). Both human and 3T3-L1 differentiated adipocytes displayed higher levels of IL-6 and IFN-γ. No significant difference was found for several other cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, KC/IL-8, MIP-1α, MIP-1β, RANTES and TNF-α) with the exception of MIP-1α, which was significantly elevated in BPA-treated 3T3-L1, while not in human adipocytes. Some other cytokines (IL-1α, IL-3, IL-5, IL-9, IL-12 p40, IL-12p70, IL-13, IL-17, Eotaxin, G-CSF, MCP-1) were not detected
inhibition occurring after BPA treatment alone. This action appeared inhibited by only 30%, compared to 55% inhibitory effect. Indeed, in the presence of SP600125 insulin signalling pathway, the compound reverted BPA STAT3 phosphorylation, however. Interestingly, the treatment decreased cytosolic abundance (Fig.6).

Figure 6. Effect of BPA on NFkB activation. 3T3-L1 adipocytes were incubated with 1 nM BPA for 24h. Next, subcellular fractionation(-nuclear and cytoplasmic) was performed and proteins were extracted and subjected to SDS-PAGE and immunoblotted with anti-NFkB antibody. Actin and Laminin A/C were used as loading control for the cytosolic and nuclear fractions, respectively. Blots were revealed by ECL and autoradiography. The autoradiographs shown are representative of four independent experiments.

doi:10.1371/journal.pone.0082099.g006

in the conditioned media of either human and 3T3-L1 differentiated adipocytes.

This led us to hypothesize that BPA may elicit an inflammatory-like response in adipocytes. We also observed a significantly increased detection of the phosphorylated forms of JNK and STAT3 in human (Fig. 5a-b) and 3T3-L1 (Fig. 5c-d) adipocytes upon treatment with BPA for 24h.

Consistently, 1 nM BPA treatment led to a significantly increased detection of NF-kB in nuclear extracts, paralleled by a decreased cytosolic abundance (Fig.6).

Next, human adipocytes were treated with 20μM SP600125. At this concentration the compound inhibited BPA-stimulated JNK phosphorylation (Fig. 7a-b). We also observed a slight inhibition of STAT3 phosphorylation, however. Interestingly, the treatment with SP600125 largely rescued insulin effect on IR, PKB/Akt and ERK1/2 phosphorylation in BPA-stimulated cells (Fig.7c-d). We therefore tested the effect of SP600125 on glucose utilization on BPA-and insulin-stimulated cells (Fig. 8). Consistent with the effect on insulin signalling pathway, the compound reverted BPA inhibitory effect. Indeed, in the presence of SP600125 insulin action appeared inhibited by only 30%, compared to 55% inhibition occurring after BPA treatment alone.

Discussion

Many environmental pollutants, including BPA, are lipophilic compounds with small size and with the ability to mimic or block the natural action of endogenous hormones [26]. For this capability, they may affect several endogenous pathways, including those regulating energy and glucose metabolism, thereby contributing to insulin resistance and metabolic dysfunction [36].

Moreover, environmental pollutants, as well as excess nutrients, can trigger inflammatory signals. There is a large body of evidence indicating that obesity is accompanied by inflammation in metabolically relevant tissues, particularly in adipose tissue [7–10,37]. It is therefore possible that pollutants may participate in deranging the function of adipose tissue, contributing to the inflammatory condition and to insulin resistance.

We have now provided evidence that nanomolar BPA concentrations may induce an inflammation-like response in human adipocytes. BPA exposure of human adipocytes increases the release of inflammatory factors, such as IL-6 and IFN-γ, raising the possibility that it may directly elicit a pseudo-inflammatory response in adipose tissue. Another consistent finding is the activation of typical inflammatory pathways. Indeed, JNK, JAK/STAT and NF-kB pathways have been found activated in BPA-treated adipocytes. Thus, both secretion of cytokines and activation of intracellular pathways, suggestive of an inflammatory response, occur after treatment with very low doses of BPA, comparable to those commonly found in the environment and in biological samples [28,29]. The direct stimulation of BPA on inflammatory pathways in adipocytes could also be accompanied, in vivo, by an effect on inflammatory infiltrates in the adipose tissue [8–10,38].

It has been hypothesized that BPA can trigger Toll-like Receptors (TLR), which in turn may induce JNK and NF-kB pathways, leading to up-regulation of pro-inflammatory factors, including IL-6 and IFN-γ [38,39]. Alternatively, BPA may act through estrogen receptors both at genomic and at non-genomic level [39,40]. Recent evidence also indicates that BPA specifically binds G protein-coupled receptor 30 (GPR30), a novel non-classical membrane ER, by which estrogenic compounds might induce biological effects in different cell types, including adipocytes [39,41,42].

Thus, one might speculate that BPA elicits intracellular signalling pathways (either via TLR, ER or GPR30) leading to up-regulation of IL-6 and IFN-γ, which in turn may contribute to activate typical inflammatory pathways, such as JNK and JAK/STAT. Consistent with our findings, other groups have shown that IL-6 release is increased following BPA exposure of human pre-adipocytes [39,43]. To the best of our knowledge, this is the first report of IFN-γ release elicited by BPA in adipocytes, while it has been described in dendritic cells and in CD4+ lymphocytes [39,43].

In parallel with the pseudo-inflammatory profile, we observed that BPA-treated adipocytes were less sensitive to insulin in terms of glucose utilization. Interestingly, the effect was already detectable upon treatment of the cells with 1 nM BPA. At this concentration, we failed to observe any gross abnormality in adipocyte morphology and differentiation markers, including GLUT4, suggesting that the alteration specifically occurred at the level of the insulin signalling machinery. Other laboratories have reported that BPA stimulated an increase in GLUT4 and glucose uptake in adipocytic cell models [44]. However, these in vitro effects required doses considerably higher than those found in human tissues [28]. Nonetheless, we observed increased basal glucose utilization, accompanied by increased levels of GLUT1. The possibility of reduced total glucose uptake is, therefore, not supported by data. At variance, we observed reduced insulin-stimulated tyrosine phosphorylation of insulin receptor and a consistent reduction of downstream signalling. In agreement with Kidani et al [45], these alterations can be responsible for a worsening in insulin signalling via PKB/Akt and ERK, causing a reduction in insulin sensitivity and in insulin-mediated glucose uptake in adipose tissue. This also raises the possibility that BPA-induced insulin sensitivity may contribute to worsen the pro-inflammatory profile. Indeed, an emerging body of evidence suggests that insulin suppresses the inflammatory process, not only through preventing hyperglycemia but also by modulating
Moreover, the decrease of leptin levels observed in BPA-treated adipocytes may be due to reduced insulin promotion of leptin gene expression, as previously reported in human adipocytes [47,48]. Interestingly, however, inhibition of JNK activity almost completely restored insulin receptor signalling and largely rescued insulin-stimulated glucose utilization in BPA-treated adipocytes, suggesting a primary involvement of inflammatory factors. Although no gross effect on adipocyte morphology and differentiation was observed, we cannot exclude the possibility that pre-natal or early post-natal exposure to BPA may affect adipogenesis as well, as has also been reported [49–53].

Figure 7. Effect of JAK2/STAT3 and JNK inhibition on BPA-impaired inflammatory and insulin pathways. Human adipocytes were incubated with 1 nM BPA for 24h and exposed to 20 μM SP600125 for 1 h. a) Cell lysates (50 μg protein/sample) were blotted with phospho-JNK and Phospho-Tyr705 STAT3 antibodies and then rebotted with anti-JNK and STAT3 antibodies. c) Cells were treated with 100 nM insulin for 10 min and then solubilized. Cell lysates (50 μg protein/sample) were blotted with phospho-IR, phospho- Ser473 Akt/PKB and phospho-Thr202 ERK and then rebotted with anti-IR, Akt/PKB and ERK antibodies. To ensure the equal protein transfer, membranes were blotted with actin antibodies. The filters were revealed by ECL and autoradiography. The autoradiographs shown are representative of four independent experiments. b-d) Filters obtained in a and c have been analyzed by laser densitometry as described under Materials and Methods. Data were analyzed with Statview software (Abacus concepts) by one-factor analysis of variance. p values of less than 0.05 were considered statistically significant. Asterisks indicate statistically significant differences (* p<0.05). Error bars indicate mean ± S.D.

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and glucose consumption was determined as described in Materials and Methods. Bars represent the mean ± SD of three independent experiments. Data were analyzed with Statview software (Abacus Concepts) by one-factor analysis of variance. *p values of less than 0.05 were considered statistically significant. Asterisks indicate statistically significant differences (* p < 0.05). Error bars indicate mean ± S.D.

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Thus, exposure to BPA impairs insulin sensitivity and induces the release of inflammatory factors in adipocytes. One possible mechanism is that BPA activates JNK, via TLRs or ERs, and this may directly impair insulin action [39,54]. Alternatively, the release of IL-6 and IFN-γ may, in turn, contribute to JNK activation in the adipocytes and either directly or indirectly down-regulate insulin-stimulated glucose uptake [34].

It should be also pointed out that an associations between serum and urinary concentrations of persistent organic pollutants and diabetes have been described [13,35,36]. Moreover, we have recently reported that women with Polycystic Ovary Syndrome (PCOS), characterized by insulin resistance and low-grade chronic inflammation, displayed elevated serum BPA levels [57]. These observations indicate a relevant role for BPA in impairing energy and nutrient metabolism, and, together with our current report, suggest that adipocytes may integrate BPA signals and further derange insulin sensitivity and inflammatory pathways.

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Author Contributions

Conceived and designed the experiments: RV PF FB FO. Performed the experiments: RV VDE FP AL SC ML. Analyzed the data: RV VDE GP AL SC FO. Contributed reagents/materials/analysis tools: SC FO. Wrote the paper: RV VDE PF.

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