[Frontiers In Bioscience, Landmark, 24, 427-434, Jan 1, 2019]

The mechanism of action of Lipiburn on fat metabolism

Bakr M. Rabie[1], Jeremy K. Ho[1]

[1]Tyjito Biotechnology Ltd., Central, Hong Kong, SAR, China

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1. ABSTRACT

Paeoniflorin (PF) is the active ingredient in Lipiburn, a fat loss supplement. PF is a component in *Paeonia Lactiflora* with multiple medicinal uses. Here, we studied the effect of Lipiburn on fat metabolism in differentiated 3T3-L1 adipocytes. Adipocytes exposed to Lipiburn exhibited significant increase in expression of beta-adrenergic receptors, HSL levels, and cAMP and exhibited increase in glycerol release. The data show that Lipiburn affects fat metabolism through the cAMP route by activating HSL which in turn breaks down triglycerides into glycerol and fatty acids.

2. INTRODUCTION

The active ingredient in Lipiburn, a patented targeted fat loss supplement, is paeoniflorin (PF),

a natural plant extract. The herb *Paeoniae Radix* (peony root) has been in medicinal use in China, Japan, Korea, and Europe by means of treating spasmodic conditions, pain, infections, and wounds (1-3).

The Paeonies can be classified into either the tree Peony (*Paeonia Moutan*) or the herbaceous varieties (2). Total glucosides of peony (TGP) of *P. Radix*, contains a plethora of compounds and with PF, in particular, comprising of over 90% of the abundance (3, 4). PF has been identified as the compound that attributes to most of TGP's pharmacological effects, including: anticoagulation, neuromuscular blocking, cognition enhancement, immunoregulation of lymphocytes, antihyperglycemia, analgesia, anti-inflammation, hepatoprotection, anti-atherosclerotic effects associated with lipid peroxidation inhibition, inhibition of hydrochloric acid secretion, anti-epileptic activity, appetite suppressant and metabolism stimulating activity, antimutagenic properties, protection of endothelium from negative effects of hyperlipidemia, platelet aggregation inhibition, and fibrinolysis (5-19).

In the present study, to determine the viability of Lipiburn, we investigated the effect of PF on fat metabolism and the mechanism by which PF regulates the β-adrenergic receptors on human fat cells. The process of natural fat breakdown involves activation of the β-adrenergic receptors which in turn activates the cAMP cascade. cAMP then activates the Protein Kinase A to add phosphate groups to the intracellular fat. Once fat is phosphorylated, the hormone sensitive lipase (HSL) is activated to break down the triglycerides into glycerol and fatty acids. Therefore, the objectives of this study were:

1. To examine the effect PF fat metabolism.
2. To examine the effect of PF on the β-adrenergic receptors.
3. To Identify the intracellular pathway and to measure the levels of expression of cAMP in the presence of PF.
4. To study the effect of PF on the levels of expression of HSL.
5. To measure the levels of glycerol release as an indication of fat metabolism.

3. MATERIALS AND METHODS

3.1. Glycerol release

Differentiated 3T3-L1 adipocytes were cultured in media containing 1 mol/L PF as test group, and media without PF as control. The amount of glycerol released into the medium was then measured over different time points. Abcam's Glycerol Assay Kit (ab65337) was used as it provides a sensitive assay to measure free glycerol concentration in the samples above. In the assay, glycerol is enzymatically oxidized to generate a product which reacts with the probe to generate color (λ= 570 nm). The 3T3-L1 cell line is an accepted model by those of skill in the art for natural fat loss. This cell line is a substrain of Swiss 3T3 mouse cell line 3T3-L1. This cell line propagated under normal conditions has a fibroblastic phenotype. However, when treated with a combination of dexamethasone, isobutylmethylxanthine (a non-specific inhibitor of phosphodiesterases) and insulin, these cells adopt a rounded phenotype and accumulate lipids intracellularly in the form of fat droplets.

3.2. cAMP

3T3-L1 adipocytes were cultured in medium containing 1 mol/L PF as test group and medium only as control. Cells were lysed by adding 0.1 N FICL, and intracellular cyclic AMP (cAMP) concentration was measured by ELISA. The concentration of cAMP released with different elapsed times (24 hours, 72 hours, 6 days, and 7 days elapsed) was identified.

3.3. Hormone sensitive lipase (HSL)

HSL is a multifunctional tissue lipase that plays a critical role in the process of fat metabolism. 3T3-L1 adipocytes were cultured in medium containing 1 mol/L PF as a test group and medium only as control. Total RNA was extracted and reverse transcribed to cDNA using RT PCR. Primers were used for polymerase chain reaction (PCR) amplification of mouse. HSL primers were selected based on published sequence (NM_010719) (expected PCR fragment: 409 bp): forward primer, 5'-GCTGGTGCAGAGAGACAC-3'; reverse primer, 5'-GAAAGCAGCGCGCACGCG-3'. For semi-quantitative analysis, the amplification cycles were chosen within the linear range (HSL: 24 cycles with denaturation at 58°C, GAPDH: 21 cycles with denaturation at 58°C). The levels of expression of HSL were identified over several time points.

3.4. Microarray analysis of obesity-related genes

In order to explore a more detailed mode of action of PF on adipolysis, microarray analysis was carried out on a panel of obesity-related genes. 3T3-L1 adipocytes were cultured in medium containing 1 mol/L PF as test group and medium only as control. Total RNA was extracted and Oligo GEArray was tested using SuperArray OMM-17. The GEArray included 111 obesity-related genes that were directly involved in the regulation of energy intake and expenditure. The genes included orexigenic peptides, hormones, and receptors, anorectic peptides, hormones, receptors, and central and peripheral signaling molecules involved in energy expenditure. Increases or decreases in change compared to control of more than 1.5-fold were regarded meaningful according to the diagnosis discipline, as described in the insert literature of a metabolic disease/ obesity gene panel product purchased from SuperArray Bioscience Corporation (Frederick, Maryland).

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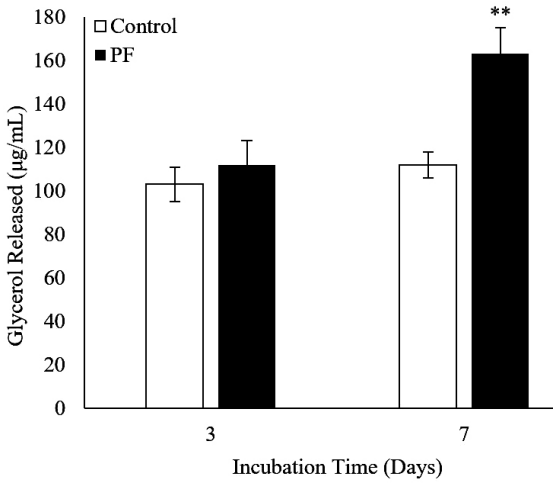

Figure 1. Glycerol released in the presence and absence of PF over different time points was measured using Abcam's Glycerol Assay Kit (ab65337). PF increased the release of glycerol significantly by the seventh day after exposure (** represents P<0.01, error bars represent ± SD).

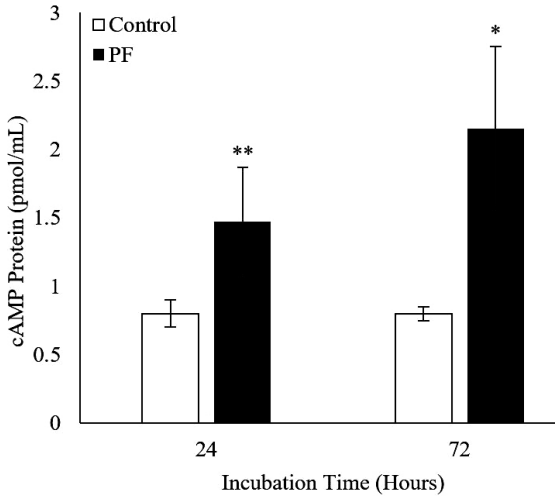

Figure 2. Intracellular cyclic AMP (cAMP) concentration was measured by ELISA test. Please note that the intracellular cAMP concentration was significantly increased by PF in the first (** represents P<0.01) and third days (* represents P<0.05) after treatment (error bars represent ± SD).

3.5. Comparison of the effect of PF and mesotherapy

The aforementioned four experiments were repeated with mesotherapy. The levels of glycerol release, expression of cAMP, and HSL were measured when 3T3 cells were cultured with PF and Mesotherapy solution.

4. RESULTS

4.1. Glycerol release

Results of the current study demonstrated that PF increased the release of glycerol significantly by the 7th day after exposure (p<0.01) (Figure 1), compared to glycerol release from 3T3-L1 adipocytes in the absence of the same amount of PF. It was found that PF increased adipolysis within one week after stimulation was initiated.

4.2. cAMP

Results of the current study demonstrated that intracellular cAMP concentration was significantly increased by PF in the first (P<0.01) and third days (P<0.05) after treatment (Figure 2). This was followed by significant increase in glycerol released on day 6 and 7 after exposure (P<0.01).

4.3. Hormone sensitive lipase (HSL)

Results of the current study demonstrated that PF significantly increased the expression of HSL in 3T3-L1 cells from the first 24 hours after exposure and continued to the third day (72 hours) of exposure (P<0.01), which followed the increase of cyclic AMP and gave rise to release of glycerol as a by-product of fat metabolism *in vitro* (Figure 3).

4.4. Microarray analysis of obesity-related genes

The present study demonstrated that one of the clinical indicators of the fat-burning and obesity fighting activity of the present formulations and/or preparation includes an increase in the expression levels of obesity-related genes, including the following:

4.4.1. Adrenergic receptor (AR)

The present data of microarray analysis after exposure to PF demonstrated a significant increase in the expression of Adrb1, Adrb2 and Adrb3, and no effect on Adra2b (Table 1). Adrb1 was activated earlier followed by Adbr3 then Adbr2. Adrb2 and Adrb3 were increased by 7.4-fold and 5.65-fold, respectively, at 7 days post exposure to PF.

4.4.2. Carboxypeptidase E (Cpe)

Cpe plays a vital role in fat metabolism. The present data of microarray analysis demonstrated a significant increase in the level of expression of Cpe on day 7 of exposure of 3T3-L1 adipocytes to PF. The expression of Cpe was increased by 2.67-fold compared to the control (Table 1).

4.4.3. Adiponectin receptor 1 (AdipoR1)

The present data of microarray analysis revealed that the expression of AdipoR1 was increased by 1.86-fold on day 7 of exposure of 3T3-L1 adipocytes to PF (Table 1).

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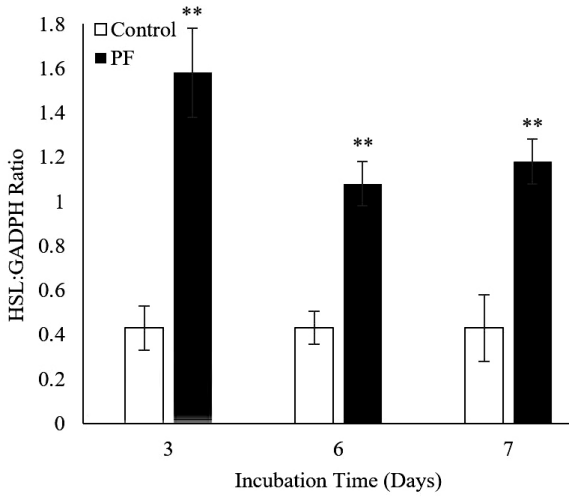

Figure 3. Semi-quantitative analysis, using RTPCR, demonstrated that PF significantly increased the expression of HSL in 3T3-L1 cells from the first 24 hours after exposure and continued to the third day (72 hours) of exposure (** represents P<0.01, error bars represent ± SD).

4.4.4. Peroxisome proliferators activated receptor gamma (Pparg)

It was shown by microarray that the level of expression of Pparg (PPAR-γ) was reduced by 1.54-fold on day 7 of exposure of 3T3-L1 adipocytes to PF (Table 1).

4.5. Comparison of the effect of PF and mesotherapy

The present study demonstrated that the effects of PF and mesotherapy on fat cells are distinct. More specifically, the present example demonstrated that the mode of action of PF on lipolysis is distinct from the action of mesotherapy.

The current study demonstrated that there is a significant increase in the levels of expression of HSL when fat cells are exposed to PF. Exposure of cells to mesotherapy did not demonstrate a significant release of HSL. The present study demonstrated as shown in the attached figures that the use of PF resulted in a significant increase in the release of glycerol, cAMP and HSL when compared to their levels when cells were exposed to mesotherapy.

5. DISCUSSION

5.1. Glycerol release

The present example demonstrated the utility of the present formulations and/or preparations for promoting adipolysis, or fat breakdown. The PF preparations are thus demonstrated to induce adipolysis indirectly by causing changes in gene expression that lead to triglyceride breakdown.

5.2. cAMP

The present example demonstrated the ability of Lipiburn to provide effective fat loss through PF action on cAMP levels. The major pathway leading to lipolysis involves activation of cAMP-dependent protein kinase A (PKA), which in turn activates other substrates such as HSL and perilipin. The agonists of β-AR bind to the β-AR receptor, which activates G-protein, G_s. The activation of G_s stimulates adenylate cyclase (AC) to produce cyclic AMP. Protein kinase A (PKA) is activated by cAMP to phosphorylate the lipid droplet surface protein, perilipin (PL). Hormone-sensitive lipase (HSL) docks onto phosphorylated PL and breaks down triglyceride into glycerol and free fatty acid. Glycerol is released into the extracellular space through aquaporin adipose (AQPad). Thus, an adipolysis promoting amount of PF may be described as an amount of PF that is sufficient to increase levels of cAMP in a culture of 3T3-L1 adipocytes.

In a study in 2001, Fredriksson and co-workers investigated whether PKA is involved in the adrenergically stimulated pathways which activate lipolysis. They used the PKA inhibitor H89. It was demonstrated that adrenergically induced lipolysis in brown adipocytes was inhibited by H89 and therefore concluded that these processes are mediated via PKA activation (20).

5.3. Hormone sensitive lipase (HSL)

The present example demonstrated that there was an increase in adipolysis *in vivo* by means of an increase in detectable levels of HSL. HSL is a multifunctional tissue lipase that plays a critical role in the process of fat metabolism. The enzyme has broad specificity, catalyzing the hydrolysis of tri-, di-, and monoacylglycerols, as well as cholesterol esters. HSL is thought to catalyze the major rate-limiting step in lipolysis. The lipase is acutely activated by cAMP-dependent phosphorylation, which also leads to its redistribution from the cytoplasm to the lipid droplet. Regulation of adipocyte HSL is the primary means by which lipolytic agents, such as catecholamines, stimulate the release of free fatty acids and thus control circulating levels. Thus, in some embodiments, an adipolysis promoting amount of PF may be described as an amount of PF that increases detectable amounts of hormone sensitive lipase (HSL) in a culture of 3T3-L1 adipocytes, compared to hormone sensitive lipase (HSL) levels in a culture of 3T3 adipocytes without the same amount of PF.

5.4. Microarray analysis of obesity-related genes

The present example demonstrated that one of the clinical indicators of the fat-burning and obesity fighting activity of the present formations and/

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Table 1. Ratio of expression signals in 3T3-L1 adipocytes exposed to Paeoniflorin (PF) compared to control

Receptor or substrate in 3T3-L1 adipocytes	PF treatment in days		
	3	6	7
β1-adrenergic receptor (Adrb1)	3.39	0.80	0.58
β2-adrenergic receptor (Adrb2)	0.74	1.96	7.40
β3-adrenergic receptor (Adrb3)	1.56	3.18	5.65
β-adrenergic receptor (Adra2b)	0.92	1.44	1.27
Carboxypeptidase E (Cpe)	0.79	1.04	2.67
Adiponectin receptor 1 (AdipoR1)	0.68	1.21	1.86
Peroxisome proliferators activated receptor gamma (PPAR-γ)	0.91	1.09	0.64

Note: Values of control are considered as 1.

or preparations includes an increase in the expression levels of obesity-related genes.

5.4.1. Adrenergic receptor (AR)

The present data of microarray analysis after exposure to PF demonstrated a significant increase in the expression of Adrb1, Adrb2 and Adrb3, and no effect on Adra2b. The adrenergic system plays a major role in the regulation of lipolysis in white adipose tissue, which is the major site of energy storage. Catecholamines are able to stimulate lipolysis by the activation of adipocyte β-adrenergic receptors (β1-, β2-, β3-AR). At the same time, catecholamines can also increase lipid storage through a2b-AR (21). Since β and a2b-AR coexist on the same fat cell, the ratio of functional alb- and β-AR present in adipose tissue may determine whether fat storage or release is activated by catecholamines (22). Results of the current study demonstrated a significant increase in the expression of Adrb1, Adrb2 and Adrb3, and no effect on Adra2b. Adrb1 was activated initially and was followed by Adrb3 then Adrb2. Adrb2 and Adrb3 were increased by 7.4-fold and 5.65-fold, respectively, at 7 days post-exposure to PF. Since the expression of Adra2b almost did not change during the whole study, while there was a significant increase in β receptor levels then the ratio of β-adrenergic receptors to a2b-adrenergic receptors is shown to be increased by PF. Therefore, lipolysis exceeds lipogenesis when exposed to PF. The three β-adrenergic receptor subtypes β1-, β2-, β-AR are members of a large family of G protein-coupled receptors, which function through the production of cAMP and the activation of HSL (22). When catecholamines interact with the β-ARs, β-ARs alternatively couple to G_s as well.

5.4.2. Carboxypeptidase E (Cpe)

Carboxypeptidase E (Cpe) is a key enzyme involved in the biosynthesis of peptide hormones and neurotransmitters, including insulin. Cpe plays a vital role in fat metabolism. The mutations in the gene of Cpe result in “fat mutation” (23). “Fat mutation” represents the first demonstration of an obesity-diabetes syndrome elicited by a genetic defect in a prohormone processing pathway (22). The fat mutation mouse does not express Cpe and presents as obese and hyperglycemic.

5.4.3. Adiponectin receptor 1 (AdipoR1)

Adiponectin has been shown to increase insulin sensitivity and decrease plasma glucose by increasing tissue fat oxidation. AdipoR1 serves as receptor for globular adiponectin and mediates increased AMP-activated protein kinase, glucose uptake and fatty-acid oxidation by adiponectin (24). The present data of microarray analysis revealed that the expression of AdipoR1 was increased by 1.86-fold on day 7 of exposure of 3T3-L1 adipocytes to PF. Therefore, this indicated a regulatory mechanism to the increased levels of fatty acids released as a result of lipolysis. The adipolysis promoting amount of PF may be described as an amount of PF that is sufficient to provide a decrease or reduction in the expression of AdipoR1 in a culture of 3T3-L1 adipocytes relative to the amount of AdipoR1 expression in a culture of 3T3-L1 adipocytes without the same amount of PF.

5.4.4. Peroxisome proliferators activated receptor gamma (PPARG)

PPAR-γ is a regulator of adipocyte differentiation. PPAR-γ has been implicated in the pathology of numerous diseases including obesity, diabetes, atherosclerosis and cancer (25). In other words, its reduction will inhibit the differentiation of new adipocytes and will reduce the adipogenesis process (25). It was shown by microarray that the level of expression of Pparg was reduced by 1.54-fold on day 7 of exposure of 3T3-L1 adipocytes to PF.

5.5. Comparison of the effect of PF and mesotherapy

The present study particularly demonstrated that the mode of action of PF on lipolysis is distinct

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from the action of mesotherapy. PF breaks down fat by activating fat cells to express more β-adrenergic receptors responsible for fat metabolism. Breaking down fat by PF is accompanied by an increase in cAMP. The increase in cAMP in turn increases the level of expression of HSL, which breaks down fat and causes a significant increase in glycerol released as a result of breaking down triglycerides into glycerol and fatty acids. Mesotherapy, on the other hand showed no effect on most of the measured parameters, except on slightly enhancing the HSL as a result of the presence of aminophylline, which is known to act on β-adrenergic receptors.

5.6. Overall effect of Lipiburn

Taken together, PF has been suggested to activate lipolysis and increase the number of β-adrenergic receptors and HSL. This is accomplished through the upregulation of cAMP, which breaks down triglycerides into glycerol and fatty acids. Since Lipiburn consists of PF, it provides a variety of preparations for use in enhancing lipolysis and fat reduction and/or loss *in vivo*. The preparations and/or compositions include as an active ingredient PF. PF is a natural, purified bioactive glucoside in Paeoniac Radix (PR), the roots of *Paeonia Pall*. PF, a natural plant extract purified to over 99%, is provided here as a natural organic potent lipolysis drug. Its mode of action is through significantly enhancing the expression of several obesity-related genes such as Adrb2, Adrb3, Cpe, AdipoR1 and PPAR-γ. Accordingly, the present preparations, formulations, methods, and techniques provide among other things, the following advantages, characteristics and features of Lipiburn:

1. A purified, natural and effective product (PF) that dissolves fat stored subcutaneously in the adipose tissue upon direct contact.
2. A predictable mode of PF's action. PF activates the expression of β-adrenergic receptors as well as other fat metabolism genes.
3. A fat-reducing product that is provided in a suitable carrier to deliver PF across the skin to the fat layers.
4. A method that employs electric current and a charged carrier to effectively deliver the preparation with the active agent, PF, to the fat cells and into the fat layers in target areas.
5. A method that delivers PF to fat cells through the use of albumin nanospheres driven through the skin by ultrasound.

In those embodiments where the preparation is suitable for injection, the preparation may be described as comprising a physiologically compatible carrier solution, such as saline and/or sterile water. In other embodiments, the injectable preparation will further include phosphatidylcholine, or any other of a variety of similar phospholipids and combinations of phospholipids. All things considered, by extension of these results, Lipiburn may therefore be a viable natural product that allows for the burning of fat, especially for middle-aged individuals in particular areas of the body, including "love handles", and can be delivered in a safe, easily assessible, and straightforward manner.

6. ACKNOWLEDGEMENTS

The authors declare that there was no conflict of interests regarding the publication of this paper.

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Key Words: Paeonia Lactiflora, Paeoniflorin, Lipiburn, fat metabolism, HSL, 3T3-L1 Adipocytes

Send correspondence to: Bakr M. Rabie, Tyjito Biotechnology Ltd., Central, Hong Kong, SAR, China, Tel: 852-90348584, E-mail: bkrabie@gmail.com

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