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(54) Title: COMPOSITIONS AND METHODS OF USE OF BETA-HYDROXY-BETA-METHYLBUTYRATE (HMB) FOR DECREASING FAT MASS

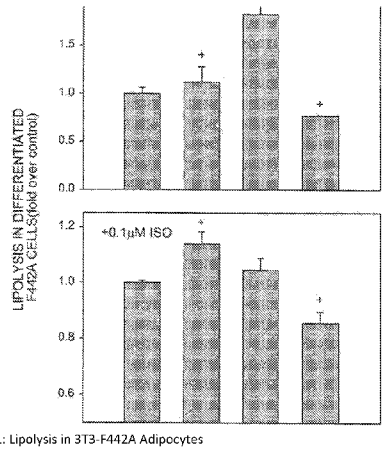


Figure 1: Lipolysis in 3T3-F442A Adipocytes

(57) Abstract: The present invention provides a composition comprising HMB. Methods of administering HMB to an animal are also described. HMB is administered to enhance or promote lipolysis, increase adipocyte fat oxidation, induce adipocyte and muscle mitochondrial biogenesis, increase energy expenditure, decrease total body weight and increase body fat loss.

COMPOSITIONS AND METHODS OF USE OF β-HYDROXY-β-METHYLBUTYRATE (HMB) FOR DECREASING FAT MASS

This application claims priority to United States Provisional Patent Application No. 62/169,334 filed June 1, 2015 and herein incorporates the provisional application by reference.

Background of the Invention

1. Field

The present invention relates to a composition comprising β-hydroxy-β-methylbutyrate (HMB) and methods of using HMB to enhance lipolysis, increase body fat loss, reduce adipocyte size, increase adipocyte fatty acid metabolism, reduce total body weight, and lower adipocyte inflammation.

2. Background

The high prevalence of obesity is a major public health and economic burden driving an urgent need for effective nutritional management of obesity and obesity-associated co-morbidities. Recently, chronic inflammation has been recognized as a contributing factor in the pathogenesis of many metabolic diseases, originating, in part, from adipose tissue. Specifically, adipocyte hypertrophy drives an inflammatory gene profile associated with metabolic dysfunction. Currently, there is a national priority to develop effective tools for the management of obesity and adipose tissue inflammation, including nutritional products for the control of appetite and maintenance of optimal body composition during weight loss. However, the science behind weight loss products has thus far been primarily based solely upon caloric restriction, and

not the maintenance of lean tissue versus fat tissue loss. The ideal weight loss program would drive preferential loss of adipose tissue over muscle tissue. Improvements in adipocyte metabolism lead to reductions in adipocyte size, oxidative stress, and inflammation, and promotion of cardio-metabolic health.

The increase in the prevalence of obesity is a health crisis of extraordinary magnitude, and it is projected that by 2030, approximately 50% of the adult population will be obese. Obesity is linked to type 2 diabetes (T2D), cardiovascular disease (CVD), hypertension, and many cancers. In addition to devastating impact on quality of life, obesity and associated co-morbidities increase average annual medical costs per person by $2,741 (2005 dollars) compared to costs for normal weight people, including 46% increased inpatient costs, 27% more outpatient costs, and 80% increased spending on prescription drugs. Additionally, costs attributable to lost productivity for full time employees are estimated to be $42.8 billion annually.

Branch chain amino acids, and leucine in particular, are recognized as key regulators of protein metabolism. Several studies have shown that β-hydroxy-β-methylbutyrate (HMB), a natural metabolite of leucine, is more effective than leucine leading to reductions in muscle protein breakdown and promoting muscle protein synthesis, translating into increased lean body mass and improved muscle function in both young and older adults, during health and disease.

A leucine-deficient diet has been demonstrated to cause a dramatic reduction in abdominal fat mass (*Cheng et al., Diabetes, 2010 Jan, 59(1):17-25*), making the findings of the present invention surprising and unexpected.

HMB

Alpha-ketoisocaproate (KIC) is the first major and active metabolite of leucine. A minor product of KIC metabolism is β-hydroxy-β-methylbutyrate (HMB). HMB has been found to be useful within the context of a variety of applications. Specifically, in U.S. Patent No. 5,360,613 (Nissen), HMB is described as useful for reducing blood levels of total cholesterol and low-density lipoprotein cholesterol. In U.S. Patent No. 5,348,979 (Nissen et al.), HMB is described as useful for promoting nitrogen retention in humans. U.S. Patent No. 5,028,440 (Nissen) discusses the usefulness of HMB to increase lean tissue development in animals. Also, in U.S. Patent No. 4,992,470 (Nissen), HMB is described as effective in enhancing the immune response of mammals. U.S. Patent No. 6,031,000 (Nissen et al.) describes use of HMB and at least one amino acid to treat disease-associated wasting.

The use of HMB to suppress proteolysis originates from the observations that leucine has protein-sparing characteristics. The essential amino acid leucine can either be used for protein synthesis or transaminated to the α-ketoacid (α-ketoisocaproate, KIC). In one pathway, KIC can be oxidized to HMB and this account for approximately 5% of leucine oxidation. HMB is superior to leucine in enhancing muscle mass and strength. The optimal effects of HMB can be achieved at 3.0 grams per day when given as calcium salt of HMB, or 0.038g·kg of body weight^{-1}·day, while those of leucine require over 30.0 grams per day.

Once produced or ingested, HMB appears to have two fates. The first fate is simple excretion in urine. After HMB is fed, urine concentrations increase, resulting in an approximate 20-50% loss of HMB to urine. Another fate relates to the activation of HMB to HMB-CoA. Once converted to HMB-CoA, further metabolism may occur, either dehydration of HMB-CoA to MC-CoA, or a direct conversion of HMB-CoA to HMG-CoA, which provides substrates for

intracellular cholesterol synthesis. Several studies have shown that HMB is incorporated into the cholesterol synthetic pathway and could be a source for new cell membranes that are used for the regeneration of damaged cell membranes. Human studies have shown that muscle damage following intense exercise, measured by elevated plasma CPK (creatine phosphokinase), is reduced with HMB supplementation within the first 48 hrs. The protective effect of HMB lasts up to three weeks with continued daily use. Numerous studies have shown an effective dose of HMB to be 3.0 grams per day as CaHMB (calcium HMB) (~38 mg·/kg body weight^{-1}day^{-1}). This dosage increases muscle mass and strength gains associated with resistance training, while minimizing muscle damage associated with strenuous exercise. HMB has been tested for safety, showing no side effects in healthy young or old adults. HMB in combination with L-arginine and L-glutamine has also been shown to be safe when supplemented to AIDS and cancer patients.

Recently, HMB free acid, a new delivery form of HMB, has been developed. This new delivery form has been shown to be absorbed quicker and have greater tissue clearance than CaHMB. The new delivery form is described in U.S. Patent Publication Serial No. 20120053240 which is herein incorporated by reference in its entirety.

Current evidence suggests that HMB acts by speeding regenerative capacity of skeletal muscle following high intensity or prolonged exercise. When training and/or diet are controlled, HMB can lower indices of skeletal muscle damage and protein breakdown in a dose-dependent fashion. Recently, HMB in a free acid form (HMB-FA) has been developed with improved bioavailability. Initial studies have shown that this form of HMB supplementation results in approximately double the plasma levels of HMB in about one-quarter the time after

administration when compared with the presently available form, calcium HMB. Further, HMB-FA given 30 minutes prior to an acute bout of high volume resistance training was able to attenuate indices of muscle damage and improve perceived recovery in resistance trained athletes. Moreover, acute ingestion of 2.4 grams of HMB-FA increases skeletal muscle protein synthesis and decreases protein breakdown by +70 % and – 56 % respectively.

The effects of HMB on muscle are well documented. It is known that HMB supplementation leads to increased muscle mass and strength and can result in aerobic improvement. While increases in lean muscle mass change overall body composition, it has been assumed that HMB only affects muscle cells to increase muscle mass which in turn changes body composition. Prior to the discoveries described in the present invention, it was not known that HMB had any direct effect on adipocytes and adipose tissue.

Most weight loss is initiated by hypocaloric diets. This weight loss comprises losses in both fat mass and lean body mass (i.e. skeletal muscle), in a ratio that is estimated to be 2:1. Exercise results in weight loss that is primarily accounted for by fat mass losses, with minor or significant increases in muscle mass. A significant number of the body mass changes that occur in exercise are now thought to be mediated by the classical (beneficial) pathway of IL-6. Surprisingly, HMB has a similar effect to that of exercise but without the exercise being a variable; fat mass loss is seen even in the absence of exercise.

The present invention comprises a composition of HMB and methods of use of HMB to result in enhanced lipolysis, increased adipocyte fat oxidation, induced adipocyte and muscle mitochondrial biogenesis, increased energy expenditure, reductions in body weight and increased body fat loss. HMB can thus be used for improvement of body contour, as defined by enhanced

lean body mass in conjunction with decreased fat mass. This can result in a reduction of total body weight. These effects are seen with or without caloric restriction and without requiring exercise.

Use of HMB to increase lipolysis and/or decrease fat mass also decreases the associated morbidities of obesity, such as Type 2 diabetes, cardiovascular disease, chronic inflammation, cancer and other associated comorbidities.

In addition to administering HMB to humans to result in the above-described effects on adipocytes, adipose tissue and fat loss, the present invention includes administering a composition of HMB to animals with the same effects on adipocytes, adipose tissue and fat loss. There is a high rate of obesity among companion animals; an estimated 54% of cats and dogs in the United States are overweight or obese. Thus a need exists for compositions and methods of use of these compositions to result in fat loss in animals.

Summary of the Invention

One object of the present invention is to provide a composition for use in fat loss and/or reductions in body weight.

A further object of the present invention is to provide a composition for use in increasing adipocyte and/or adipose tissue fat oxidation.

Another object of the present invention is to provide a composition to induce adipocyte and muscle mitochondrial biogenesis.

An additional object of the present invention is to provide a composition to increase energy expenditure.

Additionally, an object of the present invention is to provide a composition for use in enhancing lipolysis.

Another object of the present invention is to provide methods of administering a composition for use in fat loss.

An additional object of the present invention is to provide methods of administering a composition for increasing adipocyte and muscle fat oxidation.

Another object of the present invention is to provide methods of administering a composition for use in inducing adipocyte and muscle mitochondrial biogenesis.

A further object of the present invention is to provide methods of administering a composition for use in increasing energy expenditure.

Additionally, an object of the present invention is to provide methods of administering a composition for use in fat loss.

Another object of the present invention is to provide methods of administering a composition for use in reductions in body weight.

A further object of the present invention is to provide methods of administering a composition for use in enhancing lipolysis.

An additional object of the present invention is to provide methods of administering a composition for use in decreasing the associated morbidities of obesity, including Type 2 diabetes, cardiovascular disease, chronic inflammation, cancer and other associated comorbidities.

These and other objects of the present invention will become apparent to those skilled in the art upon reference to the following specification, drawings, and claims.

The present invention intends to overcome the difficulties encountered heretofore. To that end, a composition comprising HMB is provided. The composition is administered to a subject in need thereof. All methods comprise administering to the animal, HMB. The subjects included in this invention include humans and non-human mammals.

Brief Description of the Drawings

Figure 1 is a graph showing lipolysis in 3T3-F442A Adipocytes.

Figure 2 is a graph showing lipolysis in Human Subcutaneous Adipocytes.

Figure 3 shows gene expression.

Figure 4 is a graph showing lipolysis in OP9 Adipocytes.

Figure 5 is a graph showing basal lipolysis.

Detailed Description of the Invention

It has been surprisingly and unexpectedly discovered that HMB affects adipocytes and adipose tissue. The present invention comprises a composition of HMB and methods of use of HMB to result in increased enhanced lipolysis, adipocyte fat oxidation, induced adipocyte and muscle mitochondrial biogenesis, increased energy expenditure, reductions in body weight, and increased fat loss. These effects are seen with or without caloric restriction and without requiring exercise, although exercise can be conducted in conjunction with supplementation with the composition and methods of the present invention.

This composition can be used on all age groups seeking fat loss. This composition can also be used in humans and non-human mammals, including but not limited to companion animals.

HMB

β-hydroxy-β-methylbutyric acid, or β-hydroxy-isovaleric acid, can be represented in its free acid form as $(CH_3)_2(OH)CCH_2COOH$. The term "HMB" refers to the compound having the foregoing chemical formula, in both its free acid and salt forms, and derivatives thereof. While any form of HMB can be used within the context of the present invention, preferably HMB is selected from the group comprising a free acid, a salt, an ester, and a lactone. HMB esters include methyl and ethyl esters. HMB lactones include isovalaryl lactone. HMB salts include sodium salt, potassium salt, chromium salt, calcium salt, magnesium salt, alkali metal salts, and earth metal salts.

Methods for producing HMB and its derivatives are well-known in the art. For example, HMB can be synthesized by oxidation of diacetone alcohol. One suitable procedure is described by Coffman et al., *J. Am. Chem. Soc.* 80: 2882-2887 (1958). As described therein, HMB is synthesized by an alkaline sodium hypochlorite oxidation of diacetone alcohol. The product is recovered in free acid form, which can be converted to a salt. For example, HMB can be prepared as its calcium salt by a procedure similar to that of Coffman et al. (1958) in which the free acid of HMB is neutralized with calcium hydroxide and recovered by crystallization from an aqueous ethanol solution. The calcium salt and free-acid forms of HMB are commercially available from Metabolic Technologies, Ames, Iowa.

Calcium β-hydroxy-β-methylbutyrate (HMB) Supplementation

More than 2 decades ago, the calcium salt of HMB was developed as a nutritional supplement for humans. Numerous studies have shown that CaHMB supplementation improves muscle mass and strength gains in conjunction with resistance-exercise training, and attenuates loss of muscle mass in conditions such as cancer and AIDS. Nissen and Sharp performed a meta-analysis of supplements used in conjunction with resistance training and found that HMB was one of only two supplements that had clinical studies showing significant increases in strength and lean mass with resistance training. Studies have shown that 38 mg of CaHMB per kg of body weight per day appears to be an efficacious dosage for an average person.

In addition to strength and muscle mass gains, CaHMB supplementation also decreases indicators of muscle damage and muscle protein degradation. Human studies have shown that muscle damage following intense exercise, measured by elevated plasma CPK (creatine phosphokinase), is reduced with HMB supplementation. The protective effect of HMB has been shown to manifest itself for at least three weeks with continued daily use. *In vitro* studies in isolated rat muscle show that HMB is a potent inhibitor of muscle proteolysis especially during periods of stress. These findings have been confirmed in humans; for example, HMB inhibits muscle proteolysis in subjects engaging in resistance training.

The molecular mechanisms by which HMB decreases protein breakdown and increases protein synthesis have been reported. Eley et al conducted *in vitro* studies which have shown that HMB stimulates protein synthesis through mTOR phosphorylation. Other studies have shown HMB decreases proteolysis through attenuation of the induction of the ubiquitin-proteosome proteolytic pathway when muscle protein catabolism is stimulated by proteolysis inducing factor (PIF), lipopolysaccharide (LPS), and angiotensin II. Still other studies have

demonstrated that HMB also attenuates the activation of caspases-3 and -8 proteases. Taken together these studies indicate that HMB supplementation results in increased lean mass and the accompanying strength gains through a combination of decreased proteolysis and increased protein synthesis.

HMB Free Acid form

In most instances, the HMB utilized in clinical studies and marketed as an ergogenic aid has been in the calcium salt form. Recent advances have allowed the HMB to be manufactured in a free acid form for use as a nutritional supplement. Recently, a new free acid form of HMB was developed, which was shown to be more rapidly absorbed than CaHMB, resulting in quicker and higher peak serum HMB levels and improved serum clearance to the tissues.

HMB free acid may therefore be a more efficacious method of administering HMB than the calcium salt form, particularly when administered directly preceding intense exercise. HMB free acid initiated 30 min prior to an acute bout of exercise was more efficacious in attenuating muscle damage and ameliorating inflammatory response than CaHMB. One of ordinary skill in the art, however, will recognize that this current invention encompasses HMB in any form.

HMB in any form may be incorporated into the delivery and/or administration form in a fashion so as to result in a typical dosage range of about 0.5 grams HMB to about 30 grams HMB.

When the composition is administered orally in an edible form, the composition is preferably in the form of a dietary supplement, foodstuff or pharmaceutical medium, more preferably in the form of a dietary supplement or foodstuff. Any suitable dietary supplement or foodstuff comprising the composition can be utilized within the context of the present invention.

One of ordinary skill in the art will understand that the composition, regardless of the form (such as a dietary supplement, foodstuff or a pharmaceutical medium), may include amino acids, proteins, peptides, carbohydrates, fats, sugars, minerals and/or trace elements.

In order to prepare the composition as a dietary supplement or foodstuff, the composition will normally be combined or mixed in such a way that the composition is substantially uniformly distributed in the dietary supplement or foodstuff. Alternatively, the composition can be dissolved in a liquid, such as water.

The composition of the dietary supplement may be a powder, a gel, a liquid or may be tabulated or encapsulated.

Although any suitable pharmaceutical medium comprising the composition can be utilized within the context of the present invention, preferably, the composition is combined with a suitable pharmaceutical carrier, such as dextrose or sucrose.

The composition can be administered orally as a tablet, capsule, softgel, pill, sublingual gel, or as a liquid. The composition can be administered with other components, such as protein, free form amino acids, carbohydrates, sugars, vitamins (such as vitamin D, vitamin C, vitamin B_{12}, vitamin B_6, vitamin E, and/or vitamin K) and/or minerals.

Furthermore, the composition of the pharmaceutical medium can be intravenously administered in any suitable manner. For administration via intravenous infusion, the composition is preferably in a water-soluble non-toxic form. Intravenous administration is particularly suitable for hospitalized patients that are undergoing intravenous (IV) therapy. For example, the composition can be dissolved in an IV solution (e.g., a saline or glucose solution) being administered to the patient. Also, the composition can be added to nutritional IV solutions,

which may include amino acids, peptides, proteins and/or lipids. The amounts of the composition to be administered intravenously can be similar to levels used in oral administration. Intravenous infusion may be more controlled and accurate than oral administration.

Methods of calculating the frequency by which the composition is administered are well-known in the art and any suitable frequency of administration can be used within the context of the present invention (e.g., one 6 g dose per day or two 3 g doses per day) and over any suitable time period (e.g., a single dose can be administered over a five minute time period or over a one hour time period, or, alternatively, multiple doses can be administered over an extended time period). HMB can be administered over an extended period of time, such as weeks, months or years.

Any suitable dose of HMB can be used within the context of the present invention. Methods of calculating proper doses are well known in the art.

In general, an amount of HMB in the levels sufficient to result in enhanced lipolysis, increased adipocyte fat oxidation, induced adipocyte and muscle mitochondrial biogenesis, increased energy expenditure and increased fat loss. These effects are seen with or without caloric restriction and without requiring exercise.

Experimental Examples

The following examples will illustrate the invention in further detail. It will be readily understood that the composition of the present invention, as generally described and illustrated in the Examples herein, could be synthesized in a variety of formulations and dosage forms. Thus, the following more detailed description of the presently preferred embodiments of the methods, formulations and compositions of the present invention are not intended to limit the scope of the

invention, as claimed, but it is merely representative of the presently preferred embodiments of the invention.

Lipolysis in 3T3-F442A Adipocytes

Methods. Murine 3T3-F442A preadipocytes were grown until confluence. After confluence, 3T3-F442A cells were allowed to differentiate into adipocytes and become filled with triglycerides. Lipolysis was assessed as glycerol release from 3T3-F442A adipocytes in well plates. Adipocyte monolayers were incubated with Krebs-Ringer buffer containing Hepes (KRH), supplemented with 2% fatty acid-free BSA, 5 m*M* glucose, ADA and PIA. In addition, cells were treated with β-hydroxy-β-methylbutyrate (HMB) at either 0 (control), 0.1, 1, or 6 m*M* concentrations. Cells were incubated in the absence (Basal) or in the presence of 0.1 μ*M* isoproterenol for 1 hour. Aliquots of the incubation medium were removed and frozen until glycerol determination. Glycerol was measured by a commercial enzymatic method. Data were presented as the fold increase over control.

Results. The effect of HMB on lipolysis in differentiated 3T3-F442A adipocytes is shown in Figure 1. Under basal conditions, HMB at 0.1 and 1 mM concentrations increased lipolysis above control, but HMB decreased lipolysis at the 6.0 mM included level. In response to the isoproterenol stimulation, 0.1 mM HMB increased lipolysis but HMB decreased lipolysis at the 6.0 mM included level. These data indicate that HMB can regulate the triglyceride content of adipocytes by increasing the lipolytic activity in a dose responsive manner in both a basal condition and when stimulated.

Lipolysis in Human Subcutaneous Adipocytes

Methods. Human subcutaneous adipocytes were obtained from donors and incubated *in vitro*. Lipolysis was assessed as glycerol release in well plates. Adipocyte were incubated with Krebs-Ringer buffer containing Hepes (KRH), supplemented with 4% fatty acid-free BSA, 5 m*M* glucose, ADA and PIA. In addition, cells were treated with β-hydroxy-β-methylbutyrate (HMB) at either 0 (control), 0.1, or 1 m*M* concentrations in the presence of 1.0 μ*M* isoproterenol for 2 hours. Aliquots of the incubation medium were removed and frozen until glycerol determination. Glycerol was measured by a commercial enzymatic method. Data were presented as the fold increase over control.

Results. The effect of HMB on lipolysis in human subcutaneous adipocytes is shown in Figure 2. In response to the isoproterenol stimulation, 0.1 and 1 mM HMB increased lipolysis above that of control supplemented adipocytes. These data indicate that HMB can regulate the triglyceride content of adipocytes by increasing the lipolytic activity. These findings demonstrate that HMB may be used to reduce fat content in humans and can accelerate weight loss in obese humans.

Effect of HMB on Gene Expression in Differentiated Adipocytes

SGBS human pre-adipocytes were differentiated into adipocytes and treated with 1mM HMB for 4 days. The human Simpson-Golabi-Behmel syndrome (SGBS) preadipocyte cell strain cells originate from an adipose tissue specimen of a patient with GSBS. Microarrays (mRNAs) were performed. The data show significant elevation (2.53 fold) in the canonical (classic) IL6 signaling pathway, which has recently been shown to be anti-inflammatory and protective.

Similarly, cytokine receptor interaction and calcium signaling pathways are also elevated as measured by mRNAs. Table 1 describes the results.

Table 1

Pathway:	**Effect**	**p**
IL6 signaling	2.53	0.011
Cytokine, cytokine receptor interaction	2.37	0.018
Calcium signaling pathway	2.09	0.037

Gene Expression

Differentiated adipocytes were incubated for 4 days with 1mM of HMB. RNA was extracted and gene expression was determined by qPCR. Gene expression for ATGL, ACOX, PGC1a, ATG7 and BNIP3 were analyzed. ATGL is an adipose triglyceride lipase (ATGL) and was found to play a major role in catalyzing the initial step in triglyceride hydrolysis. ATGL is highly expressed in adipose tissue of humans and mice. ACOX1, or acyl-CoA oxidase 1 is the first enzyme involved in peroxisomal fatty acid oxidation. This occurs when the fatty acid chains are too long to be handled by the mitochondria. The high-potential electrons generated are transferred to $O_2 \rightarrow H_2O_2$ (+heat) which in the presence of catalase $\rightarrow H_20$ and O_2. PGC1α is a key regulator of energy metabolism and is found to be very important in the development of brown adipose tissue which is the high energy yielding adipose tissue. Autophagy-related protein 7 (ATG7) regulates fat mass. Knocking ATG7 down will result in abnormal non-functional adipose tissue. Elevated levels increase tissue recycling. BNIP3 is another autophagy-related protein like ATG7 that appears to be significant. The gene expression results appear in Figure 3.

Phosphokinase *Protein Array of SGBS Adipocytes Treated with HMB*

This approach was used to determine in non-biased way changes in protein phosphorylation that are known to influence activity of important signaling proteins. Differentiated human adipocytes were treated for 4 days with or without HMB (1mM).

Shown are only the significant increases above untreated cells in phosphorylation of the listed protein at the indicated phosphorylation site(s). Protein arrays were performed and Table 2 shows that the phosphorylated proteins that up-regulated following incubation of differentiated adipocytes with HMB and Table 3 shows the proteins that were down-regulated

Table 2

Human adipocyte genes up-regulated with HMB			
Fold Change with HMB	**Protein**	**Phosphorylation-Site**	**Comments**
1.255	GSK-3a/b	S21/S9	• Glycogen Synthase Kinase. Phosphorylated by insulin
1.233	CREB	S133	• Enhances adipogenesis
1.335	Lyn	Y397	• Src kinase (lipid metabolism)
1.400	Fyn	Y420	• Src kinase (regulates FA metabolism).
1.224	Yes	Y426	• Src kinase (regulates endocytosis, e.g. EGFR)
1.262	FGR	Y412	• Src kinase, negative on cell adhesion/migration

Table 3

Human adipocyte genes down-regulated with HMB			
Fold Change with HMB	**Protein**	**Phosphorylation-Site**	**Comments**
0.804	STAT5a/b	Y694/Y699	• Activated by IL7, FAS
0.665	Stat3	Y705	• Activated by IL6, leptin
0.732	RSK1/2/3	S380/S386/S377	• Highly conserved Ser/Thr kinase, regulates diverse cellular processes: growth, motility, survival etc. • Effector of (ERK)/mitogen-

			activated protein kinase (MAPK) signaling. Might inhibits glucose transport
0.621	p53	S15	• Regulates cell cycle. Enhances adipose differentiation.
0.834	P53	S46	• Regulates cell cycle. Enhances adipose differentiation.
0.711	AKT1/2/3	S473	• AKT mediates many insulin effects and effects of other regulators (e.g. growth factors) on cell growth etc. S473 is 1 of 2 activation sites. S473 phosphorylation is induced by insulin via PDK1 and by Focal Adhesion Kinase (FAK) in response to extracellular signals (from matrix, cell interaction).

One notable finding is that of a decrease in Stat 3 which is involved in the beneficial pathway of IL-6. In addition, a decrease in AKT 1/2/3 transcript, a protein that is phosphorylated at serine 473 and is intimately involved with the mTOR pathway, was seen. This contrasts with no change in AKT1/2/3 which is at Threonine 308 and is involved in the insulin signaling pathway. The SRC kinases, Fyn, yes and lyn are involved in fatty acid metabolism.

These studies were conducted in the absence of electrical stimuli simulating exercise, demonstrating that HMB supplementation is effective for fat loss even in the absence of exercise.

HMB regulation of basal and isoproterenol stimulated lipolysis in differentiated adipocytes.

As noted above, an increase in expression of ATGL in cells treated with HMB was observed, so the effect of HMB treatment on basal and isoproterenol stimulated lipolysis was determined directly in cultured adipocytes. The protocol of Viswanadha and Londos was used for

optimization of conditions for measuring lipolysis in murine primary adipoctyes *(J. Lipid Res. 2006. 47: 1859–1864)* that include treatment with adenosine deaminase (ADA) to remove endogenous adenosine and then addition of ADA resistant PIA (phenylisopropyl-adenosine).

The following treatment strategies were tested: HMB doses of 0.1, 1 and 6mM; 2 concentrations of the beta-adrenergic agonist isoproterenol 0.1 and 1uM, and short term (immediately before lipolysis assay) vs. long term (48h) HMB treatment (n=4 per treatment). Op9 adipocytes were used as SGBS cells take a long time (several weeks) to differentiate making them inconvenient to use.

Cells were pre-treated with varying concentrations of HMB in OP9 growth medium for 48 hrs. Following pre-treatment, cells were washed and incubated with Isoproterenol (0.1 or 1.0 uM) in lipolysis buffer: Krebs ringer bicarbonate (KRB) with 4% BSA. The medium also contained adenosine deaminase (ADA) and phenyl-isopropyl-adenosine (PIA) for 2 hrs at 37° C. ADA was added to remove endogenous adenosine and PIA, which is resistant to ADA, was used to have low standardized basal lipolysis. Following treatment, media was assayed for glycerol content and cells were lysed with RIPA buffer to determine protein concentration, *$p < 0.05$, ** $p < 0.01$. $N = 4$.

The results are shown in Figures 4 and 5:

a) Pre-treatment with HMB for 48h dramatically (2-4 fold) enhanced isoproterenol-stimulated lipolysis in differentiated OP9 adipocytes (Figure 4)

b) At 0.1mM HMB, lipolysis was enhanced by 2 fold in response to 0.1uM isoproterenol (Figure 4 and 5).

c) At 1mM HMB, a 4-fold increase over no HMB was observed at 0.1uM isoproterenol (Figure 4 and 5).

d) At 6mM the response to 0.1 uM isoproterenol was enhanced 3-fold so the dose of 1 mM HMB appears optimal (Figure 4).

e) Higher concentrations of isoproterenol reduced maximal lipolysis as would be predicted by desensitization of adrenergic receptors (Figure 5).

f) Under the above conditions used for the lipolysis assay, i.e. addition of ADA followed by PIA to clamp basal lipolysis at low level, HMB pretreatment also increased basal lipolysis about 2-fold (Figure 4).

These experimental examples demonstrate that HMB has a direct effect on adipocytes and adipose tissue and show that supplementation with HMB can be used for fat loss.

The foregoing description and drawings comprise illustrative embodiments of the present inventions. The foregoing embodiments and the methods described herein may vary based on the ability, experience, and preference of those skilled in the art. Merely listing the steps of the method in a certain order does not constitute any limitation on the order of the steps of the method. The foregoing description and drawings merely explain and illustrate the invention, and the invention is not limited thereto, except insofar as the claims are so limited. Those skilled in the art who have the disclosure before them will be able to make modifications and variations therein without departing from the scope of the invention.

Claiming:

1. A composition comprising from about 0.5 g to about 30 g of β-hydroxy-β-methylbutyric acid (HMB) wherein administration of the composition to an animal in need thereof has at least one effect selected from the group comprising enhanced lipolysis, increased adipocyte fat oxidation, induced adipocyte biogenesis, increased energy expenditure, reductions in fat mass, reductions in total body weight and increased fat loss.

2. The composition of claim 1, wherein said HMB is selected from the group consisting of its free acid form, its salt, its ester, and its lactone.

3. The composition of claim 2, wherein said salt is selected from the group consisting of a sodium salt, a potassium salt, a magnesium salt, a chromium salt and a calcium salt.

4. A composition for reducing the amount of fat in the body of an animal, said composition comprising from about 0.5 g to about 30 g of β-hydroxy-β-methylbutyric acid (HMB) which promotes lipolysis in the body of said animal.

5. The composition of claim 4 wherein administration of HMB increases muscle mass in the body of said animal.

6. A method of increasing lipolysis in adipose tissue of an animal in need thereof comprising administering to said animal a composition of from about 0.5 g to about 30 g of β-hydroxy-β-methylbutyric acid (HMB) thereby increasing lipolysis of animal.

7. The method of claim 6, wherein said HMB is selected from the group consisting of its free acid form, its salt, its ester, and its lactone.

8. The method of claim 7, wherein said salt is selected from the group consisting of a sodium salt, a potassium salt, a magnesium salt, a chromium salt and a calcium salt.

9. The method of claim 6, wherein the animal is not participating in an exercise program.

10. A method of inducing fat loss in an animal in need thereof comprising administering to the animal an effective amount of a composition comprising β-hydroxy-β-methylbutyric acid (HMB).

11. The method of claim 10, wherein said HMB is selected from the group consisting of its free acid form, its salt, its ester, and its lactone.

12. The method of claim 11, wherein said salt is selected from the group consisting of a sodium salt, a potassium salt, a magnesium salt, a chromium salt and a calcium salt.

13. The method of claim 10, wherein administration of the composition of HMB increases muscle mass.

14. A method of promoting the export of glycerol from adipocytes in an animal in need thereof comprising administering to the animal an effective amount of a composition comprising β-hydroxy-β-methylbutyric acid (HMB).

15. The method of claim 14, wherein said HMB is selected from the group consisting of its free acid form, its salt, its ester, and its lactone.

16. The method of claim 15, wherein said salt is selected from the group consisting of a sodium salt, a potassium salt, a magnesium salt, a chromium salt and a calcium salt.

17. A method of treating or preventing at least one of the comorbidities associated with obesity in an animal in need thereof comprising administering to the animal a composition of from about 0.5 g to about 30 g of β-hydroxy-β-methylbutyric acid (HMB).

18. The method of claim 17, wherein the comorbidities are selected from the group consisting of Type 2 diabetes, cardiovascular disease, chronic inflammation, and cancer.

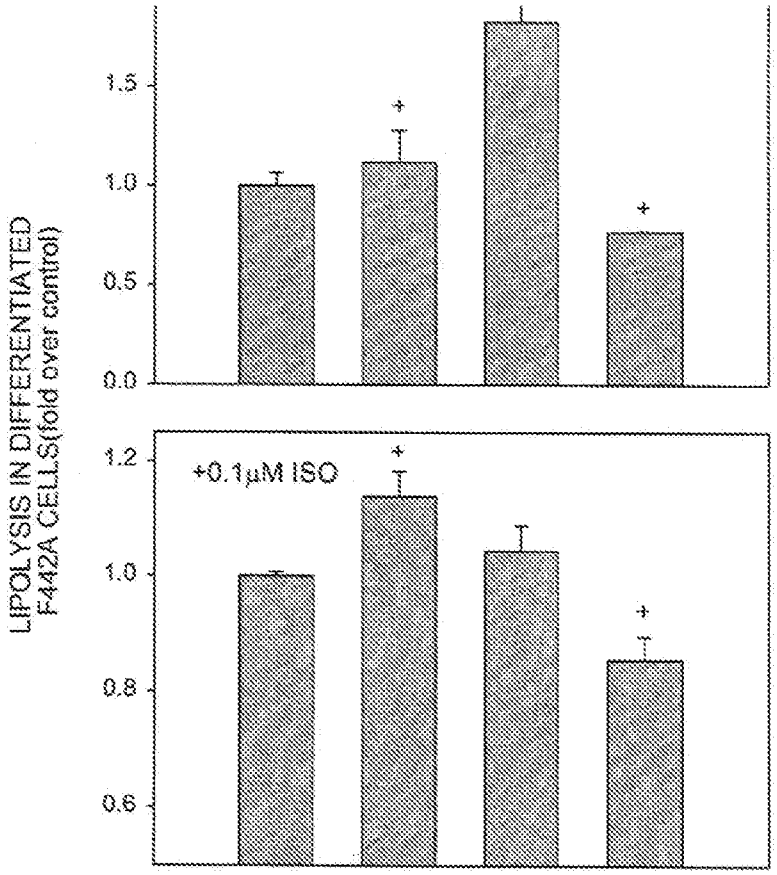


Figure 1: Lipolysis in 3T3-F442A Adipocytes

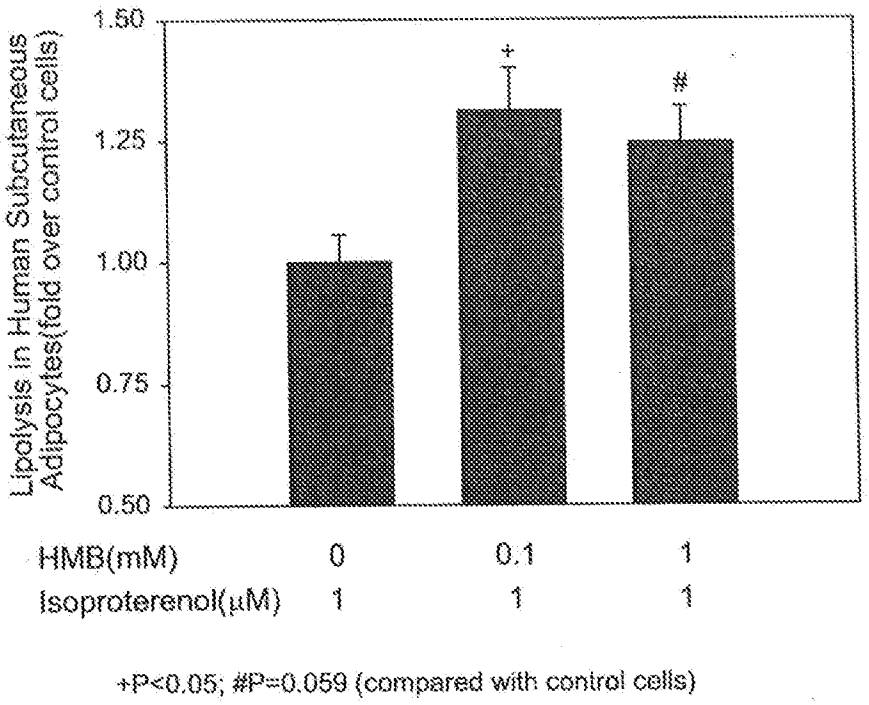


Figure 2. **Lipolysis in Human Subcutaneous Adipocytes**

Figure 3: Gene Expression

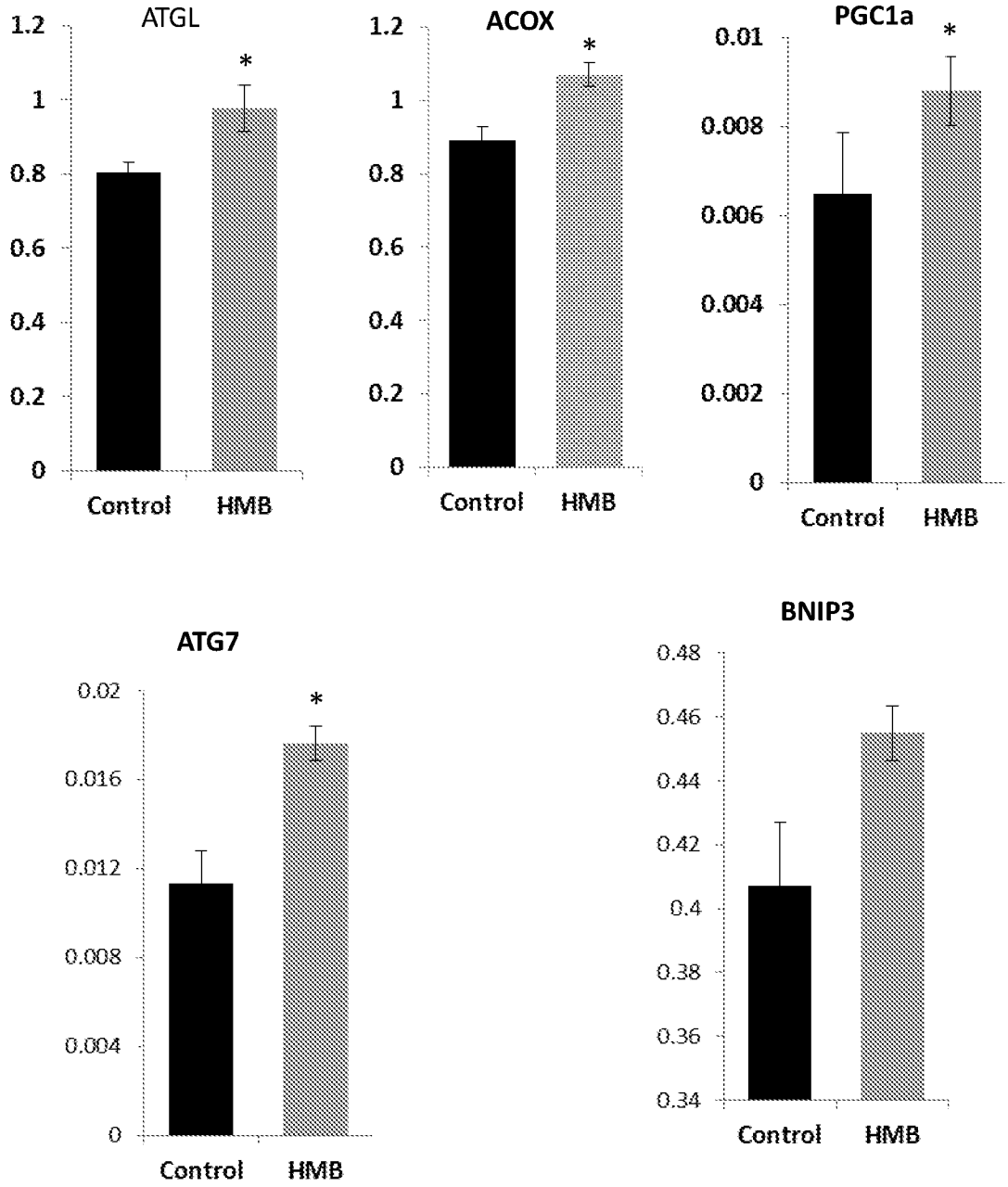


Figure 4: 48h HMB Treatment increases beta adrenergic stimulated lipolysis in OP9 adipocytes

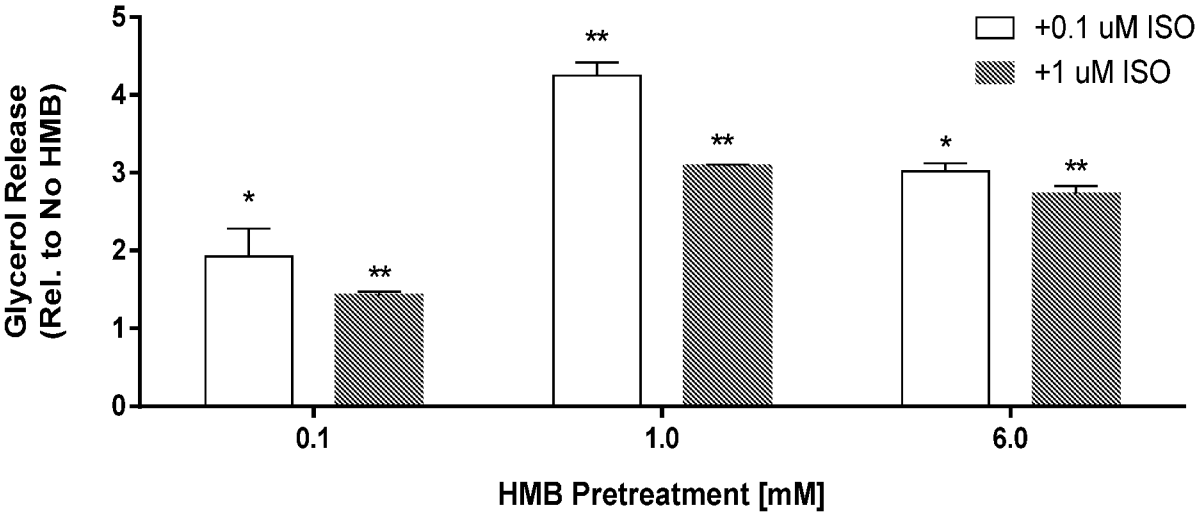
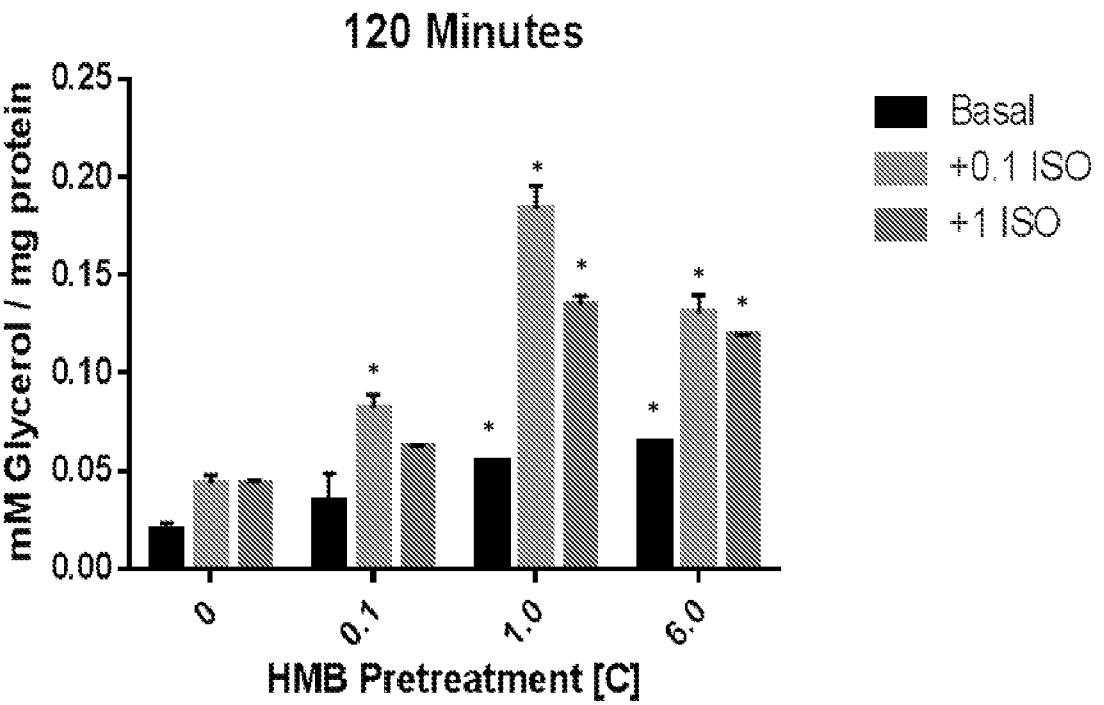


Figure 5: **HMB pretreatment increases basal lipolysis**



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/035273

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61P 3/04; A61K 31/19; A61K 31/365; C07C 53/126 (2016.01)
CPC - A61K 31/19; A61K 31/365; C07C 53/126 (2016.05)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC - A61K 31/19; A61K 31/365; A61P 3/04; C07C 53/126
CPC - A61K 31/19; A61K 31/365; C07C 53/126

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/557; 562/579; IPC - A61K 31/19; A61K 31/365; A61P 3/04; C07C 53/126; CPC - A61K 31/19; A61K 31/365; C07C 53/126 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, PubChem
Search terms used: hydroxymethyl butyric acid, hydroxyisovaleric, 625-08-1, HMB, fat loss, adipocyte, fat oxidation, biogenesis, lipolysis, obesity, comorbidities, exercise, weight loss, reduction, muscle mass, adipose volume, glycerol

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2014/0148488 A1 (NUSIRT SCIENCES, INC) 29 May 2014 (29.05.2014) entire document	1-8, 10-18 ---------- 9
Y	US 8,815,280 B2 (RATHMACHER et al) 26 August 2014 (26.08.2014) entire document	9
A	US 8,541,383 B2 (GOKARAJU et al) 24 September 2013 (24.09.2013) entire document	1-18
A	FERREIRA et al. The Effects of Supplementation of beta-Hydroxy-beta-Melthylbutyrate on Inflammatory Markers in High Performance Athletes. Journal of Exercise Physiology-online 16 (1): 53-63, 2013. entire document	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 2016	14 SEP 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774