(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 December 2016 (08.12.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/196637 A1

(51) International Patent Classification:
A61P 3/04 (2006.01) *A61K 31/365* (2006.01)
A61K 31/19 (2006.01) *C07C 53/126* (2006.01)

(21) International Application Number:
PCT/US2016/035273

(22) International Filing Date:
1 June 2016 (01.06.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/169,334 1 June 2015 (01.06.2015) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2016/196637 A1

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

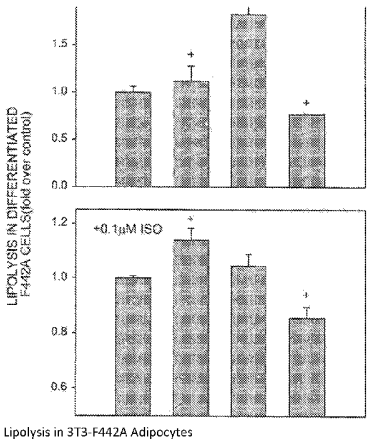


Figure 1: Lipolysis in 3T3-F442A Adipocytes

(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.

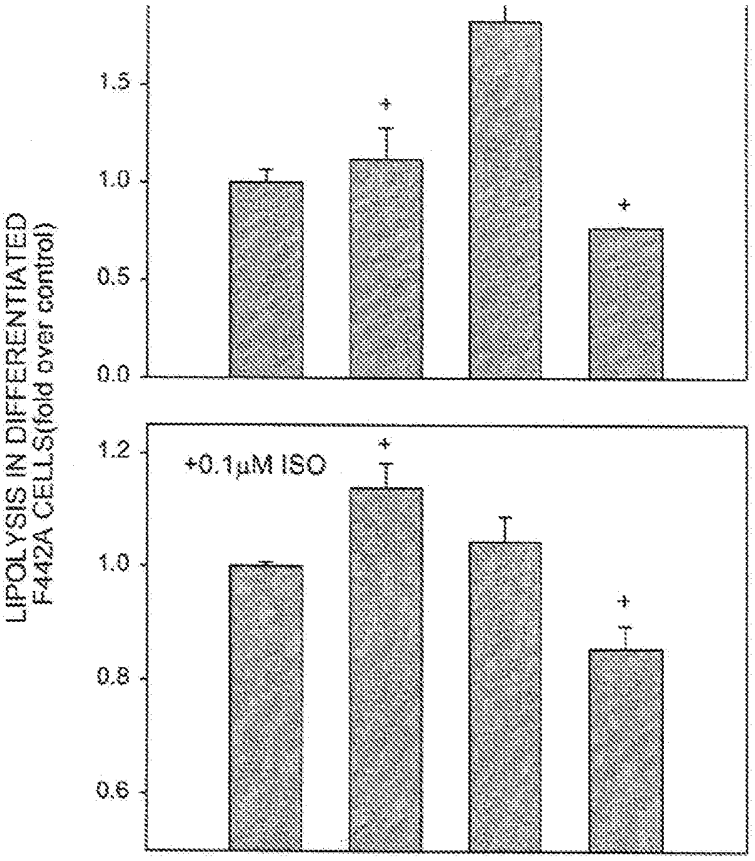


Figure 1: Lipolysis in 3T3-F442A Adipocytes

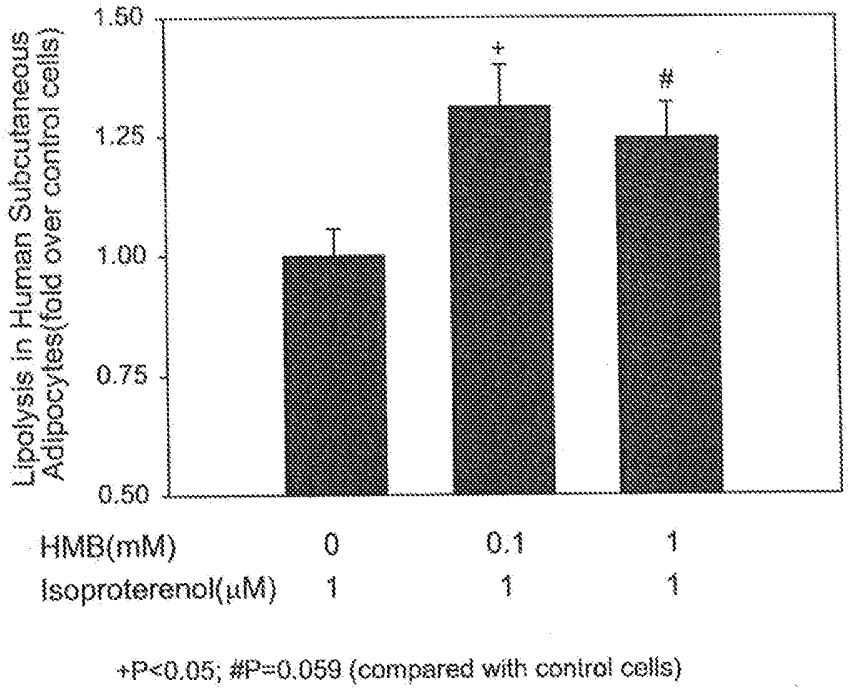
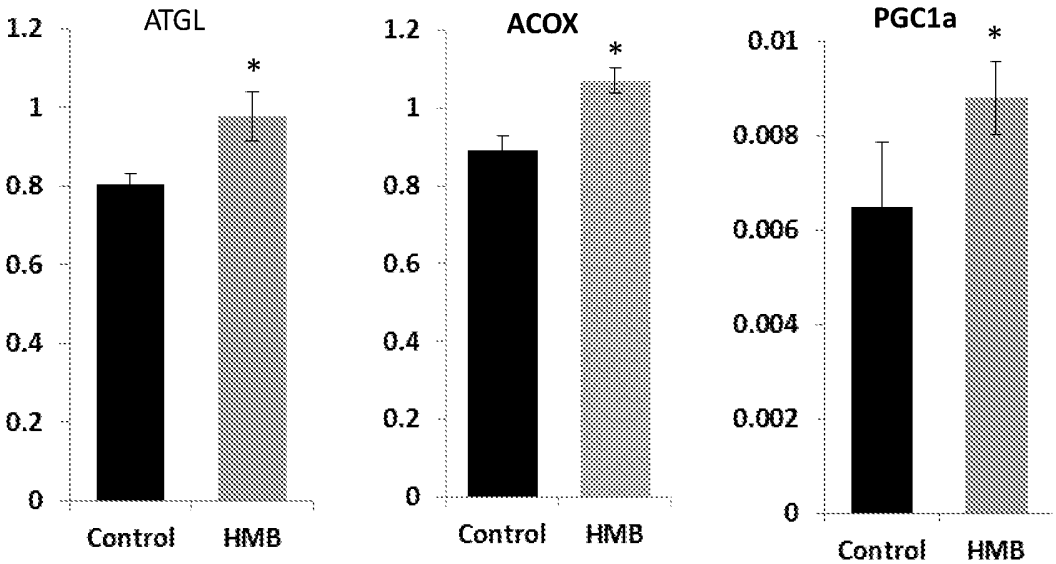


Figure 2. **Lipolysis in Human Subcutaneous Adipocytes**

Figure 3: Gene Expression



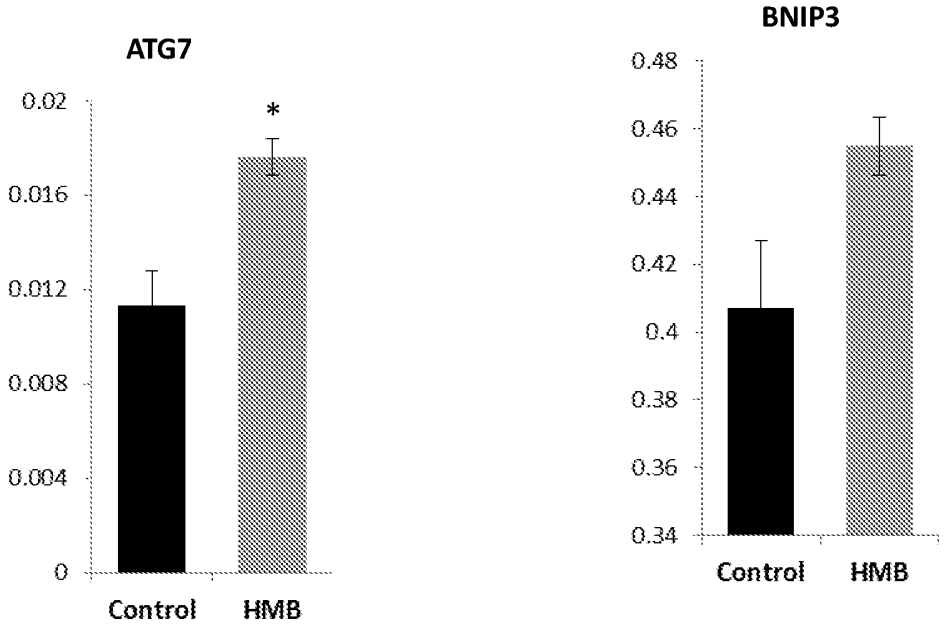


Figure 4: 48h HMB Treatment increases beta adrenergic stimulated lipolysis in OP9 adipocytes

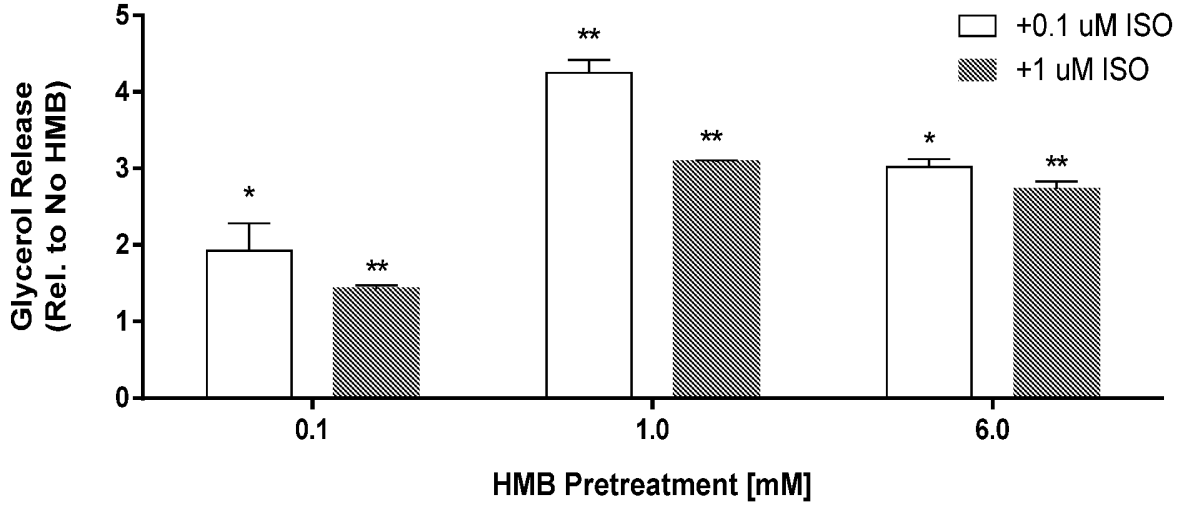
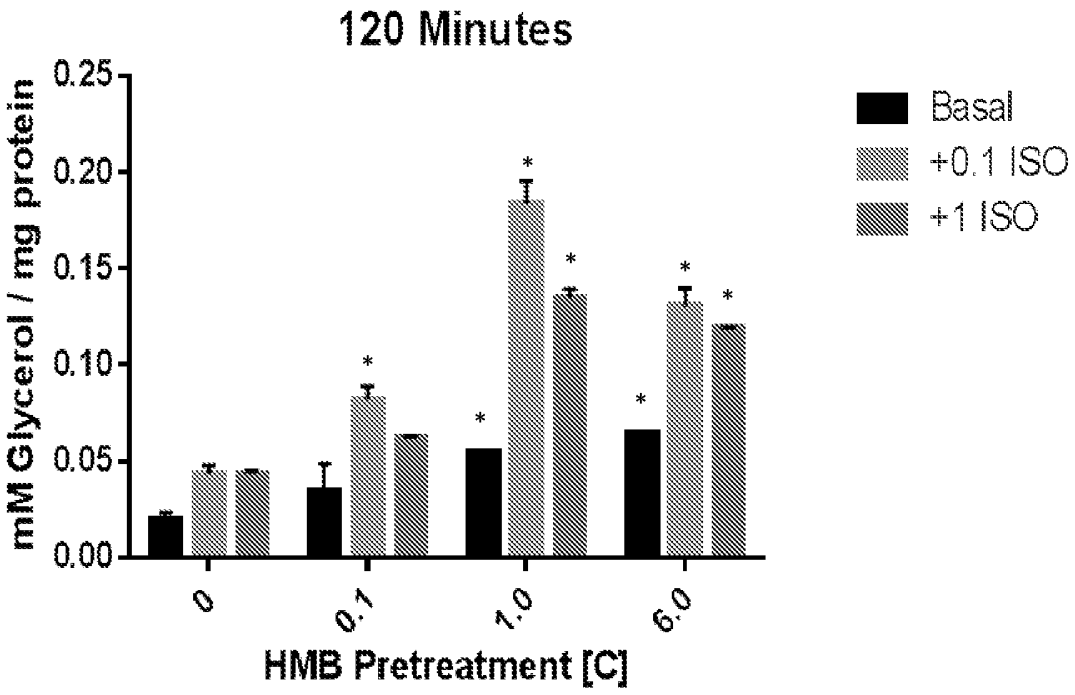


Figure 5: **HMB pretreatment increases basal lipolysis**



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
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"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

Form PCT/ISA/210 (second sheet) (January 2015)

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 107847759 A
(43)申请公布日 2018.03.27

(21)申请号 201680045057.0

(22)申请日 2016.06.01

(30)优先权数据
62/169,334 2015.06.01 US

(85)PCT国际申请进入国家阶段日
2018.01.31

(86)PCT国际申请的申请数据
PCT/US2016/035273 2016.06.01

(87)PCT国际申请的公布数据
WO2016/196637 EN 2016.12.08

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(51)Int.Cl.
A61P 3/04(2006.01)
A61K 31/19(2006.01)
A61K 31/365(2006.01)
C07C 53/126(2006.01)

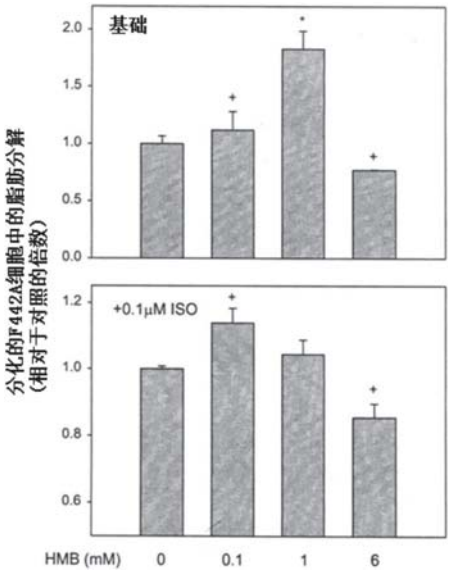
权利要求书1页 说明书9页 附图4页

(54)发明名称

用于减少脂肪量的β-羟基-β-甲基丁酸(HMB)的组合物和使用方法

(57)摘要

本发明提供了包括HMB的组合物。还描述了向动物施用HMB的方法。施用HMB以增强或促进脂解作用、增加脂肪细胞脂肪氧化、诱导脂肪细胞和肌肉线粒体生物发生、增加能量消耗、降低总体重和增加机体减脂。



CN 107847759 A

1.一种组合物，其包含约0.5g至约30g的β-羟基-β-甲基丁酸(HMB)，其中将所述组合物施用于对其有需要的动物产生至少一种选自包含以下的作用：增强的脂解作用、增加的脂肪细胞脂肪氧化、诱导的脂肪细胞生物发生、增加的能量消耗、脂肪量减少、总体重降低和增加的减脂。

2.权利要求1的组合物，其中所述HMB选自由以下组成的组：其游离酸形式、其盐、其酯、及其内酯。

3.权利要求2的组合物，其中所述盐选自由以下组成的组：钠盐、钾盐、镁盐、铬盐和钙盐。

4.用于减少动物体内脂肪量的组合物，所述组合物包含约0.5g至约30g促进所述动物体内脂解作用的β-羟基-β-甲基丁酸(HMB)。

5.权利要求4的组合物，其中施用HMB增加所述动物体内的肌肉量。

6.一种增加对其有需要的动物的脂肪组织中的脂解作用的方法，包括向所述动物施用约0.5g至约30g的β-羟基-β-甲基丁酸(HMB)的组合物，从而增加动物的脂解作用。

7.权利要求6的方法，其中所述HMB选自由以下组成的组：其游离酸形式、其盐、其酯、及其内酯。

8.权利要求7的方法，其中所述盐选自由以下组成的组：钠盐、钾盐、镁盐、铬盐和钙盐。

9.权利要求6的方法，其中所述动物不参与运动项目。

10.一种在对其有需要的动物中诱导减脂的方法，包括向动物施用有效量的包含β-羟基-β-甲基丁酸(HMB)的组合物。

11.权利要求10的方法，其中所述HMB选自由以下组成的组：其游离酸形式、其盐、其酯、及其内酯。

12.如权利要求11所述的方法，其中所述盐选自由以下组成的组：钠盐、钾盐、镁盐、铬盐和钙盐。

13.权利要求10的方法，其中HMB组合物的施用增加肌肉量。

14.一种促进对其有需要的动物从脂肪细胞中输出甘油的方法，包括向所述动物施用有效量的包含β-羟基-β-甲基丁酸(HMB)的组合物。

15.权利要求14的方法，其中所述HMB选自由以下组成的组：其游离酸形式、其盐、其酯、及其内酯。

16.权利要求15的方法，其中所述盐选自由以下组成的组：钠盐、钾盐、镁盐、铬盐和钙盐。

17.一种治疗或预防对其有需要的动物中与肥胖相关的至少一种合并症的方法，其包括向所述动物施用约0.5g至约30g的β-羟基-β-甲基丁酸(HMB)的组合物。

18.权利要求17的方法，其中所述合并症选自由以下组成的组：2型糖尿病、心血管疾病、慢性炎症和癌症。

用于减少脂肪量的β-羟基-β-甲基丁酸(HMB)的组合物和使用方法

[0001] 本申请要求于2015年6月1日提交的美国临时专利申请第62/169,334号的优先权，并且通过引用将临时申请并入本文。

[0002] 发明背景

[0003] 1.领域

[0004] 本发明涉及包括β-羟基-β-甲基丁酸(β-hydroxy-β-methylbutyrate(HMB))的组合物和使用HMB来增强脂解作用、增加机体减脂、减少脂肪细胞大小、增加脂肪细胞脂肪酸代谢、降低总体重和降低脂肪细胞炎症的方法。

[0005] 2.背景

[0006] 肥胖的高度流行是公共卫生和经济的主要负担，使得产生对肥胖和肥胖相关合并症(comorbidity)进行有效的营养管理的迫切需求。最近，慢性炎症已经被认为是许多代谢疾病的发病机制中的一个促成因素，部分源自脂肪组织。具体而言，脂肪细胞肥大驱动与代谢功能障碍相关的炎症基因谱(profile)。目前，国家优先考虑研发用于管理肥胖和脂肪组织炎症的有效工具，包括用于控制食欲和维持减肥期间最佳身体组成的营养产品。然而，减肥产品背后的科学迄今主要仅基于热量限制，而不是在脂肪组织的减少的同时维持非脂肪组织(lean tissue)。理想的减肥计划将优先推动脂肪组织的减少而不是肌肉组织。脂肪细胞代谢的提高导致脂肪细胞大小、氧化应激和炎症的减少，并促进心血管代谢健康。

[0007] 肥胖流行的上升是一个非常严重的健康危机，并且预计到2030年，大约50％的成年人会肥胖。肥胖与2型糖尿病(T2D)、心血管疾病(CVD)、高血压和许多癌症有关。除了对生活质量的破坏性影响之外，与正常体重人群相比，肥胖症和相关合并症使得每人平均每年医疗费用增加2,741美元(2005)，包括住院费用增加46％、门诊费用增加27％以及处方药支出增加80％。此外，全职员工生产力损失造成的成本估计为每年428亿美元。

[0008] 支链氨基酸，尤其是亮氨酸被认为是蛋白质代谢的关键调节因子。若干研究表明，亮氨酸的天然代谢物β-羟基-β-甲基丁酸(HMB)比亮氨酸更有效地导致肌肉蛋白质分解减少和促进肌肉蛋白质合成，其在年轻人和老年人中在健康和疾病期间转化为增加的非脂肪体重和改善的肌肉功能。

[0009] 亮氨酸缺乏饮食已经被证明引起腹部脂肪量急剧减少(Cheng et al.,Diabetes,2010Jan,59(1):17-25)，使得本发明的发现令人惊讶和意外。

[0010] HMB

[0011] α-酮异己酸盐(KIC)是亮氨酸的第一主要和活性代谢物。KIC代谢的次要产物是β-羟基-β-甲基丁酸(HMB)。已经发现HMB在各种应用的情况下是有用的。具体而言，在美国专利第5,360,613号(Nissen)中，HMB被描述为可用于降低总胆固醇和低密度脂蛋白胆固醇的血液水平。在美国专利第5,348,979号(Nissen等人)中，HMB被描述为可用于促进人体氮存留。美国专利第5,028,440号(Nissen)讨论了HMB增加动物的非脂肪组织发育的有用性。而且，在美国专利第4,992,470号(Nissen)中，HMB被描述为有效增强哺乳动物的免疫应答。美国专利第6,031,000号(Nissen等人)描述了HMB和至少一种氨基酸用于治疗疾病相关的消

耗的用途。

[0012] HMB用于抑制蛋白质水解的用途来源于亮氨酸具有节约蛋白质特征的观察。必需氨基酸亮氨酸既可用于蛋白质合成，也可诱导转氨基为α-酮酸(α-酮异己酸盐，KIC)。在一个途径中，KIC可被氧化成HMB，并且这占亮氨酸氧化的约5％。HMB在提高肌肉量和力量方面优于亮氨酸。当HMB以钙盐形式每天给予3.0g时，或者以0.038g•kg体重/天给予时，HMB可达到最佳效果，而亮氨酸需要每天超过30.0g。

[0013] 一旦产生或摄入，HMB似乎有两个命运。第一个命运是通过尿液的简单排泄。在摄入HMB后，其在尿液中的浓度增加，导致HMB的尿液损失约20-50％。另一个命运与HMB到HMB-CoA的活化有关。一旦转化为HMB-CoA，可发生进一步的代谢，HMB-CoA脱水至MC-CoA，或直接将HMB-CoA转化为HMG-CoA，HMG-CoA为细胞内胆固醇合成提供底物。一些研究已经表明HMB被并入胆固醇合成途径中，并且可成为用于受损细胞膜再生的新细胞膜的来源。人体研究已经表明，通过在最初的48小时内补充HMB可减轻激烈运动后的肌肉损伤(通过升高的血浆CPK(肌酸磷酸激酶)测量)。持续日常使用，HMB的保护作用持续达三周。许多研究显示HMB的有效剂量为每天3.0g CaHMB(钙HMB)(约38mg/kg体重/天)。这种剂量联合阻力训练增加肌肉量和肌肉力量，同时最小化与剧烈运动相关的肌肉损伤。HMB已经过安全测试，对健康的年轻人或老年人没有副作用。当补充给艾滋病和癌症患者时，HMB与L-精氨酸和L-谷氨酰胺组合也显示是安全的。

[0014] 最近研发了HMB的新型递送形式HMB游离酸。已经显示这种新的递送形式比CaHMB更快吸收并具有更大的组织清除率。在美国专利公开第20120053240号中描述了新的递送形式，其全部内容通过引用并入本文。

[0015] 目前的证据表明HMB通过高强度或长时间运动后加速骨骼肌的再生能力起作用。当训练和/或饮食控制时，HMB可以剂量依赖性方式降低骨骼肌损伤指数和蛋白质分解。最近，已研发出游离酸形式的HMB(HMB-FA)，其具有提高的生物利用度。最初的研究表明，与目前可得形式的HMB钙相比，该形式的HMB补充剂在施用后大约四分之一的时间内引起大约两倍的HMB血浆水平。此外，在快速大剂量阻力训练之前30分钟给予HMB-FA能够减弱肌肉损伤的指数，并且改善阻力训练的运动员的感知恢复。此外，快速摄入2.4g HMB-FA分别增加了骨骼肌蛋白质合成+70％，并将蛋白质分解减少了-56％。

[0016] HMB对肌肉的作用已有详细记录。已知HMB的补充会导致肌肉量和力量增加，并可导致有氧改善。虽然瘦肌肉量的增加改变了整个身体组成，但是已经假定HMB仅影响肌肉细胞以增加肌肉量，这反过来改变身体组成。在本发明所述的发现之前，尚不知道HMB对脂肪细胞和脂肪组织有任何直接作用。

[0017] 大多数减肥是由低热量饮食开始的。这种减肥包括脂肪量和非脂肪体重(即骨骼肌)的损失，其比例估计为2:1。运动导致体重减轻，其主要由脂肪量损失引起，肌肉量轻微或显著增加。现在认为运动中发生的显著数目的体重变化是由IL-6的经典(有益)途径介导的。令人惊讶的是，HMB具有与运动类似的效果，但运动不是其中变量；即使在没有运动的情况下也可看到脂肪量损失。

[0018] 本发明包括HMB组合物和使用HMB增强脂解作用、增加脂肪细胞脂肪氧化、诱导脂肪细胞和肌肉线粒体生物发生(biogenesis)、增加能量消耗、降低体重和增加机体减脂的方法。因此HMB可用于改善体型，如增加的非脂肪体重与减少的脂肪量一起所定义的。这可

导致总体重减轻。这些影响可在有或没有热量限制也不需要运动的情况下看到。

[0019] 使用HMB来增加脂解作用和/或降低脂肪量也减少了肥胖相关的疾病(例如2型糖尿病、心血管疾病、慢性炎症、癌症和其他相关合并症)。

[0020] 除了向人施用HMB以导致对脂肪细胞、脂肪组织和减脂的上述作用之外,本发明包括将HMB组合物施用于动物,并对脂肪细胞、脂肪组织和减脂具有相同的作用。伴侣动物肥胖率高;估计美国有54%的猫和狗超重或肥胖。因此,存在对于组合物和这些组合物用于导致动物减脂的方法的需要。

[0021] 发明概述

[0022] 本发明的一个目的是提供用于减脂和/或减轻体重的组合物。

[0023] 本发明的另一个目的是提供用于增加脂肪细胞和/或脂肪组织脂肪氧化的组合物。

[0024] 本发明的另一个目的是提供诱导脂肪细胞和肌肉线粒体生物发生的组合物。

[0025] 本发明的另一个目的是提供增加能量消耗的组合物。

[0026] 另外,本发明的目的是提供用于增强脂解作用的组合物。

[0027] 本发明的另一个目的是提供用于减脂的组合物的施用方法。

[0028] 本发明的另一个目的是提供增加脂肪细胞和肌肉脂肪氧化的组合物的施用方法。

[0029] 本发明的另一个目的是提供用于诱导脂肪细胞和肌肉线粒体生物发生的组合物的施用方法。

[0030] 本发明的另一个目的是提供用于增加能量消耗的组合物的施用方法。

[0031] 另外,本发明的目的是提供用于减脂的组合物的施用方法。

[0032] 本发明的另一个目的是提供用于降低体重的组合物的施用方法。

[0033] 本发明的另一个目的是提供用于增强脂解作用的组合物的施用方法。

[0034] 本发明的另一个目的是提供用于减少肥胖相关疾病(包括2型糖尿病、心血管疾病、慢性炎症、癌症和其他相关合并症)的组合物的施用方法。

[0035] 参照下面的说明书、附图和权利要求,本发明的这些和其它目的对于本领域技术人员将变得清楚。

[0036] 本发明旨在克服迄今为止遇到的困难。为此,提供了包括HMB的组合物。将所述组合物施用于对其有需要的对象。所有的方法包括将HMB施用于动物。包括在本发明中的对象包括人类和非人类哺乳动物。

[0037] 附图简述

[0038] 图1是显示3T3-F442A脂肪细胞中脂解作用的图。

[0039] 图2是显示人皮下脂肪细胞中脂解作用的图。

[0040] 图3显示了基因表达。

[0041] 图4是显示OP9脂肪细胞中脂解作用的图。

[0042] 图5是显示基础脂解作用(basal lypolysis)的图。

[0043] 发明详述

[0044] 令人惊讶且意外地发现HMB影响脂肪细胞和脂肪组织。本发明包括HMB组合物和HMB用于导致增强的脂解作用、脂肪细胞氧化、诱导脂肪细胞和肌肉线粒体生物发生、增加的能量消耗、降低的体重和增加的机体减脂的方法。尽管运动可与补充本发明的组合物和

方法一起进行,但是可在有或没有热量限制且不需要锻炼的情况下观察到这些效果。

[0045] 该组合物可用于寻求减脂的所有年龄组。该组合物也可用于人和非人哺乳动物,包括但不限于伴侣动物。

[0046] HMB

[0047] β-羟基-β-甲基丁酸或β-羟基-异戊酸可以其游离酸形式表示为$(CH_3)_2(OH)CCH_2COOH$。术语"HMB"是指具有上述化学式的游离酸和盐形式的化合物及其衍生物。尽管可在本发明的上下文中使用任何形式的HMB,但优选HMB选自游离酸、盐、酯和内酯。HMB酯包括甲酯和乙酯。HMB内酯包括异戊酰基内酯。HMB盐包括钠盐、钾盐、铬盐、钙盐、镁盐、碱金属盐和土金属盐。

[0048] 生产HMB及其衍生物的方法在本领域是公知的。例如,HMB可通过双丙酮醇的氧化来合成。在Coffman等人J.Am.Chem.Soc.80:2882-2887(1958)中描述了一个合适的步骤。如其中所述,HMB通过双丙酮醇的碱性次氯酸钠氧化来合成。产物以游离酸形式回收,其可被转化成盐。例如,HMB可通过类似于Coffman等人(1958)的步骤制备成其钙盐,其中HMB的游离酸用氢氧化钙中和并通过从乙醇水溶液中结晶回收。HMB的钙盐和游离酸形式可从Metabolic Technologies,Ames,Iowa购买获得。

[0049] 钙β-羟基-β-甲基丁酸(HMB)的补充

[0050] 二十多年前,HMB的钙盐被研发作为人类的营养补充剂。许多研究表明,CaHMB的补充联合阻力训练可增加肌肉量和肌肉力量,并减弱例如癌症和艾滋病等病症的肌肉量损失。Nissen和Sharp进行了补充剂与阻力训练联合使用的荟萃分析,并发现HMB是临床研究显示通过阻力训练显著增加力量和非脂肪体重的仅有的两种补充剂之一。研究表明,每kg体重每天38mg的CaHMB似乎是一个普通人的有效剂量。

[0051] 除了力量和肌肉量的增加,CaHMB的补充也降低了肌肉损伤和肌肉蛋白质降解的指标。人体研究表明,剧烈运动后的肌肉损伤(通过升高的血浆CPK(肌酸磷酸激酶)测量)随着补充HMB而降低。已显示持续日常使用,HMB的保护作用持续达至少三周。分离的大鼠肌肉的体外研究表明HMB是肌肉蛋白水解的有效抑制剂,特别是在应激期间。这些发现已经在人类中得到证实;例如,HMB抑制参与阻力训练的对象的肌肉蛋白水解。

[0052] 已经报道了HMB减少蛋白质分解和增加蛋白质合成的分子机制。Eley等人进行的体外研究显示HMB通过mTOR磷酸化刺激蛋白质合成。其他研究已经表明,当蛋白水解诱导因子(PIF)、脂多糖(LPS)和血管紧张素II刺激肌肉蛋白质分解代谢时,HMB通过减弱泛素-蛋白体蛋白水解途径的诱导来降低蛋白水解。还有其他研究已经证明HMB还减弱了半胱天冬酶-3和半胱天冬酶-8蛋白酶的活化。总的来说,这些研究表明补充HMB会导致瘦体重增加并伴随力量增加,这是通过蛋白水解减少和蛋白质合成增加的组合带来的。

[0053] HMB游离酸形式

[0054] 在大多数情况下,用于临床研究并作为增补剂(ergogenic aid)销售的HMB一直是钙盐形式。最近的进展已经允许HMB以游离酸形式制造以用作营养补充剂。最近,研发了新的游离酸形式的HMB,其显示出比CaHMB更快地吸收,导致更快和更高的血清HMB水平峰值以及对组织改善的血清清除。

[0055] 因此HMB游离酸可能是比钙盐形式更有效的施用HMB的方法,特别是在剧烈运动之前直接施用时。在快速训练之前30分钟开始加入的HMB游离酸比CaHMB更有效地减轻肌肉损

伤和改善炎症反应。然而，本领域的普通技术人员将认识到本发明包括任何形式的HMB。

[0056] 任何形式的HMB可以以一种方式结合到递送和/或施用形式中，从而导致约0.5g HMB到约30g HMB的典型剂量范围。

[0057] 当组合物以食用形式口服施用时，组合物优选为膳食补充剂、食品或药物介质的形式，更优选为膳食补充剂或食品的形式。在本发明的上下文中可使用任何合适的包括所述组合物的膳食补充剂或食品。本领域的普通技术人员将理解，无论形式(例如膳食补充剂、食品或药物介质)如何，组合物可包括氨基酸、蛋白质、肽、碳水化合物、脂肪、糖、矿物质和/或微量元素。

[0058] 为了制备作为膳食补充剂或食品的组合物，组合物通常以使得组合物基本均匀分布在膳食补充剂或食品中的方式组合或混合。或者，可以将组合物溶解在液体如水中。

[0059] 膳食补充剂的组成可以是粉末、凝胶、液体或者可制成片剂或装入胶囊。

[0060] 尽管在本发明的上下文中可使用包括所述组合物的任何合适的药物介质，但是优选地，所述组合物与合适的药物载体例如右旋糖或蔗糖组合。

[0061] 所述组合物可以以片剂、胶囊、软凝胶、丸剂、舌下凝胶或液体形式口服施用。所述组合物可与其他组分如蛋白质、游离形式的氨基酸、碳水化合物、糖、维生素(如维生素D、维生素C、维生素B_{12}、维生素B_6、维生素E和/或维生素K)和/或矿物质一起施用。

[0062] 此外，药物介质的组合物可以以任何合适的方式静脉内施用。为了通过静脉内输注施用，组合物优选为水溶性无毒形式。静脉内施用特别适用于正在经历静脉(IV)治疗的住院患者。例如，组合物可以溶解于施用于患者的IV溶液(例如盐水或葡萄糖溶液)中。而且，组合物可添加到营养IV溶液中，其可包括氨基酸、肽、蛋白质和/或脂质。待静脉内施用的组合物的量可与口服施用的水平相似。静脉内输注可比口服施用更可控和准确。

[0063] 计算施用组合物的频率的方法是本领域众所周知的，并且在本发明的上下文中可使用任何合适的施用频率(例如每天一次6g剂量或每天两次3g剂量)并且在任何合适的时间段内(例如，可以在五分钟的时间段内或在一小时的时间段内施用单剂量，或者可在延长的时间段内施用多剂量)。HMB可在延长的时间内施用，例如数周、数月或数年。

[0064] 在本发明的范围内可使用任何合适剂量的HMB。计算适当剂量的方法在本领域中是众所周知的。

[0065] 通常，HMB的量足以导致脂解作用增加、脂肪细胞脂肪氧化增加、诱导脂肪细胞和肌肉线粒体生物发生、能量消耗增加和减脂增加。这些影响可在有或没有热量限制且不需要锻炼的情况下看到。

[0066] 实验实施例

[0067] 以下实施例将更详细地说明本发明。容易理解的是，如本文实施例中一般描述和说明的本发明的组合物可以以各种制剂和剂型合成。因此，本发明的方法、制剂和组合物的目前优选的实施方案的以下更详细的描述并非如权利要求一样旨在限制本发明的范围，而仅仅代表本发明目前优选的实施方案。

[0068] 在3T3-F442A脂肪细胞中的脂解作用

[0069] 方法.鼠3T3-F442A前体脂肪细胞生长至汇合。汇合后，使3T3-F442A细胞分化成脂肪细胞并充满甘油三酯。通过从3T3-F442A脂肪细胞在孔板中的甘油释放评估脂解作用。将脂肪细胞单层用含有Hepes(KRH)、补充有2％无脂肪酸BSA、5mM葡萄糖、ADA和PIA的Krebs-

Ringer缓冲液孵育。另外，用0(对照)、0.1、1或6mM浓度的β-羟基-β-甲基丁酸(HMB)处理细胞。细胞在0.1μM异丙基肾上腺素不存在(基础)或存在的情况下孵育1小时。取孵育培养基的等分试样并冷冻直至甘油测定。甘油通过商业酶法测量。数据表示为相对于对照的倍数增加。

[0070] 结果.图1显示了HMB对分化的3T3-F442A脂肪细胞脂解作用的作用。在基础条件下，0.1和1mM浓度的HMB与对照相比增加脂解作用，但HMB在包含6.0mM水平下降低脂解作用。对异丙基肾上腺素刺激的反应，0.1mM HMB增加脂解作用，但HMB在包括6.0mM水平下降低脂解作用。这些数据表明，HMB可通过在基础条件和刺激时以剂量响应方式增加脂解作用活性来调节脂肪细胞的甘油三酯含量。

[0071] 人类皮下脂肪细胞中的脂解作用

[0072] 方法.从供体获得人皮下脂肪细胞并在体外孵育。通过甘油在孔板中的释放评估脂解作用。将脂肪细胞与包含Hepes(KRH)的补充有4％不含脂肪酸的BSA、5mM葡萄糖、ADA和PIA的Krebs-Ringer缓冲液一起孵育。此外，在1.0μM异丙基肾上腺素存在下，用0(对照)、0.1或1mM浓度的β-羟基-β-甲基丁酸(HMB)处理细胞2小时。取孵育培养基的等分试样并冷冻直至甘油测定。甘油通过商业酶法测量。数据表示为相对于对照的倍数增加。

[0073] 结果.图2显示了HMB对人皮下脂肪细胞中的脂解作用作用。对异丙基肾上腺素刺激的反应，0.1和1mM HMB与对照补充的脂肪细胞相比增加脂解作用。这些数据表明HMB可通过增加脂解作用活性来调节脂肪细胞的甘油三酯含量。这些发现表明，HMB可用于减少人体脂肪含量，并可加速肥胖人群的体重减轻。

[0074] HMB对分化的脂肪细胞基因表达的影响

[0075] 将SGBS人前脂肪细胞分化成脂肪细胞并用1mM HMB处理4天。人类Simpson-Golabi-Behmel综合征(SGBS)前脂肪细胞细胞株细胞起源于GSBS患者的脂肪组织样本。进行微阵列(mRNA)。数据显示经典(经典的)IL6信号传导途径中的显著提高(2.53倍)，其最近显示是抗炎和保护性的。类似地，通过测量mRNA，细胞因子受体相互作用和钙信号传导途径也升高。表1描述了结果。

[0076] 表1

[0077]

途径:	影响	p
IL6 信号传导	2.53	0.011
细胞因子、细胞因子受体相互作用	2.37	0.018
钙信号传导途径	2.09	0.037

[0078] 基因表达

[0079] 分化的脂肪细胞与1mM HMB一起孵育4天。提取RNA并通过qPCR测定基因表达。分析ATGL、ACOX、PGC1a、ATG7和BNIP3的基因表达。ATGL是脂肪甘油三酯脂肪酶(ATGL)，且被发现在催化甘油三酯水解的最初步骤中起主要作用。ATGL在人和小鼠的脂肪组织中高度表达。ACOX1或酰基-CoA氧化酶1是参与过氧化物酶体脂肪酸氧化的第一酶。当脂肪酸链太长而不能被线粒体处理时发生这种情况。产生的高电位电子转移到$O_2 \rightarrow H_2O_2$(+热)，其在过氧化氢酶的存在下产生→H_2O和O_2。PGC1α是能量代谢的关键调节因子，且在产生高能量脂肪组织的

棕色脂肪组织的发育过程中被发现是非常重要的。自噬相关蛋白7(ATG7)调节脂肪量。敲低ATG7会导致非功能性脂肪组织异常。提高的水平增加组织再循环。BNIP3是与ATG7类似的另一自噬相关蛋白,似乎也具有显著性。基因表达结果如图3所示。

[0080] 用HMB处理的SGBS脂肪细胞的磷酸激酶蛋白质阵列

[0081] 该方法用于以无偏见的方式测定已知影响重要信号传导蛋白活性的蛋白质磷酸化的变化。用或不用HMB(1mM)处理分化的人脂肪细胞4天。

[0082] 只显示与未处理细胞相比,所列出的蛋白质在所示的磷酸化位点的磷酸化显著增加的情况。进行蛋白质阵列,表2显示用HMB孵育分化的脂肪细胞后上调的磷酸化蛋白质,且表3显示下调的蛋白质

[0083] 表2

[0084]

HMB处理的人脂肪细胞基因上调			
HMB处理倍数变化	蛋白质	磷酸化位点	备注
1.255	GSK-3a/b	S21/S9	• 糖原合成酶激酶。 由胰岛素磷酸化
1.233	CREB	S133	• 增强脂肪形成
1.335	Lyn	Y397	• Src激酶(脂质代谢)
1.400	Fyn	Y420	• Src激酶(调节FA代谢)。
1.224	Yes	Y426	• Src激酶(调节内吞作用，例如EGFR)
1.262	FGR	Y412	• Src激酶，负调节(negative)细胞黏附/迁移

[0085] 表3

[0086]

HMB处理的人脂肪细胞基因下调			
HMB处理倍数变化	蛋白质	磷酸化位点	备注
0.804	STAT5a/b	Y694/Y699	• 被IL7、FAS激活
0.665	Stat3	Y705	• 被IL6、瘦素激活
0.732	RSK1/2/3	S380/S386/S377	• 高度保守的Ser/Thr激酶，调节多种细胞过程：生长、运动、存活等 • (ERK)/促分裂原活化蛋白激酶(MAPK)信号传导的效应物。可抑制葡萄糖转运
0.621	p53	S15	• 调节细胞周期。增强脂肪分化
0.834	P53	S46	• 调节细胞周期。增强脂肪分化。

[0087]

0.711	AKT1/2/3	S473	• AKT介导多种胰岛素效应和其他调节因子(如生长因子)对细胞生长的效应等。S473是2个活化位点之一。S473磷酸化是通过胰岛素经由PDK1和通过黏着斑激酶(FAK)响应细胞外信号(来自基质、细胞相互作用)而诱导的

[0088] 一个值得注意的发现是参与IL-6有益途径的Stat3的减少。此外,观察到AKT 1/2/3转录物(一种在丝氨酸473上被磷酸化并且与mTOR途径密切相关的蛋白质)的减少。这与苏氨酸308的AKT1/2/3没有变化形成对比,并且参与胰岛素信号传导途径。SRC激酶、Fyn、yes和lyn参与脂肪酸代谢。

[0089] 这些研究是在没有电刺激模拟运动的情况下进行的,表明即使在没有运动的情况下,补充HMB对减脂作用也是有效的。

[0090] HMB调节分化的脂肪细胞中基础和异丙基肾上腺素刺激的脂解作用。

[0091] 如上所述,观察到用HMB处理的细胞中ATGL表达的增加,因此直接在培养的脂肪细胞中测定HMB处理对基础和异丙基肾上腺素刺激的脂解作用的作用。使用Viswanadha和Londos的方案来优化测量鼠原代脂肪细胞中脂解作用的条件(J.Lipid Res.2006.47:1859-1864),其包括用腺苷脱氨酶(ADA)处理以除去内源性腺苷,并且然后加入耐ADA的PIA(苯异丙基腺苷)。

[0092] 测试了以下处理策略:HMB剂量为0.1、1和6mM;2种浓度的β-肾上腺素激动剂异丙基肾上腺素0.1和1μM,以及短期(紧接在脂解作用测定之前)与长期(48h)HMB处理(每个处理n=4)。0p9脂肪细胞用作SGBS细胞,因为SGBS细胞需要很长时间(几周)分化使得它们不方便使用。

[0093] 将细胞用不同浓度的HMB在OP9生长培养基中预处理48小时。预处理后,洗涤细胞并与异丙基肾上腺素(0.1或1.0μM)一起在脂肪分解缓冲液(含有4%BSA的Krebs ringer碳酸氢盐(KRB))中孵育。培养基还在37℃下含有腺苷脱氨酶(ADA)和苯基-异丙基腺苷(PIA)中孵育2小时。加入ADA以除去内源性腺苷,并且加入抗ADA的PIA用于低标准化的基础脂解作用。处理后,测定培养基的甘油含量,细胞用RIPA缓冲液溶解以测定蛋白质浓度,*p<0.05,**p<0.01。N=4。

[0094] 结果如图4和5所示:

[0095] a)在分化的OP9脂肪细胞中用HMB预处理48小时显著(2-4倍)增强异丙基肾上腺素刺激的脂解作用(图4)

[0096] b)在0.1mM HMB时,对0.1uM异丙基肾上腺素反应脂解作用增强了2倍(图4和5)。

[0097] c)在1mM HMB时,在0.1uM异丙基肾上腺素下观察到比没有HMB时增加4倍(图4和5)。

[0098] d)在6mM时,对0.1μM异丙基肾上腺素的反应增强3倍,因此1mM HMB的剂量显示最佳(图4)。

[0099] e)较高浓度的异丙基肾上腺素可降低最大脂解作用,如肾上腺素受体脱敏所预测的(图5)。

[0100] f)在用于脂解作用测定的上述条件下,即添加ADA,然后添加PIA以钳制基础脂解作用在低水平,HMB预处理也增加基础脂解作用约2倍(图4)。

[0101] 这些实验实施例证明,HMB对脂肪细胞和脂肪组织有直接的作用,并且表明补充HMB可用于减脂作用。

[0102] 前面的描述和附图包括本发明的说明性实施例。本文所述的前述实施例和方法可基于本领域技术人员的能力、经验和偏好而变化。仅仅以某种顺序列出该方法的步骤并不构成对该方法的步骤顺序的任何限制。前面的描述和附图仅解释和说明本发明,而本发明不限于此,除非权利要求是如此限定的。具有公开内容的本领域技术人员在不脱离本发明的范围的情况下将能够进行修改和变化。

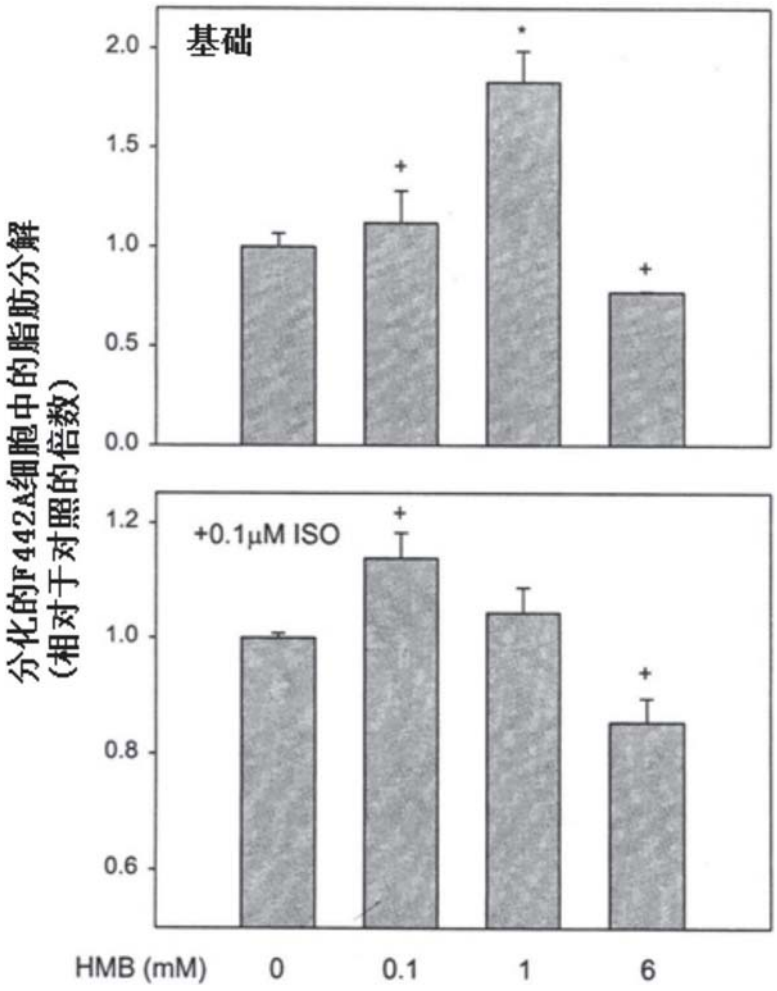


图1在3T3-F442A脂肪细胞中的脂解作用

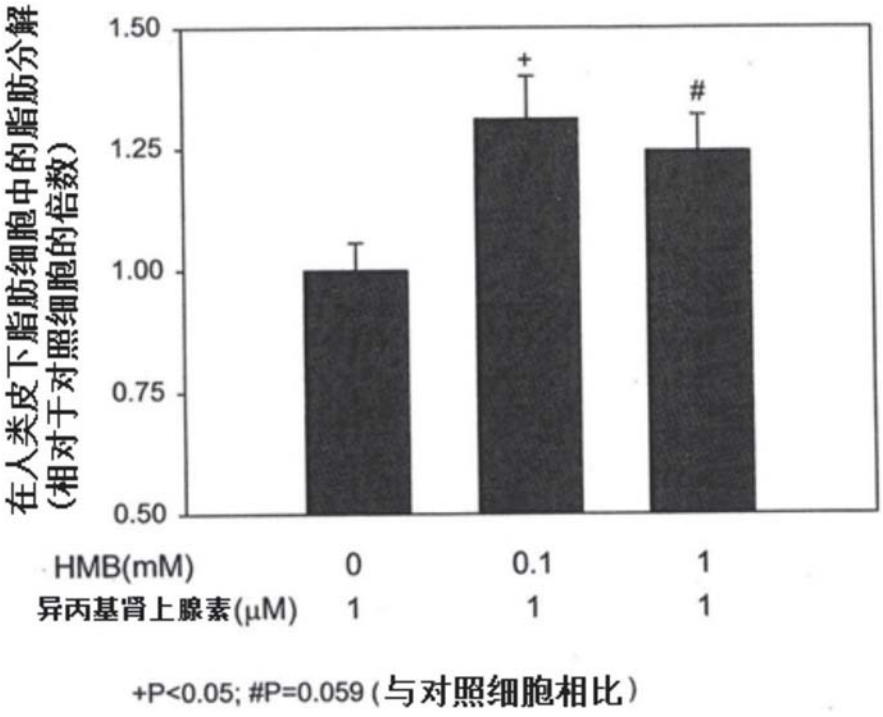


图2在人皮下脂肪细胞中的脂解作用

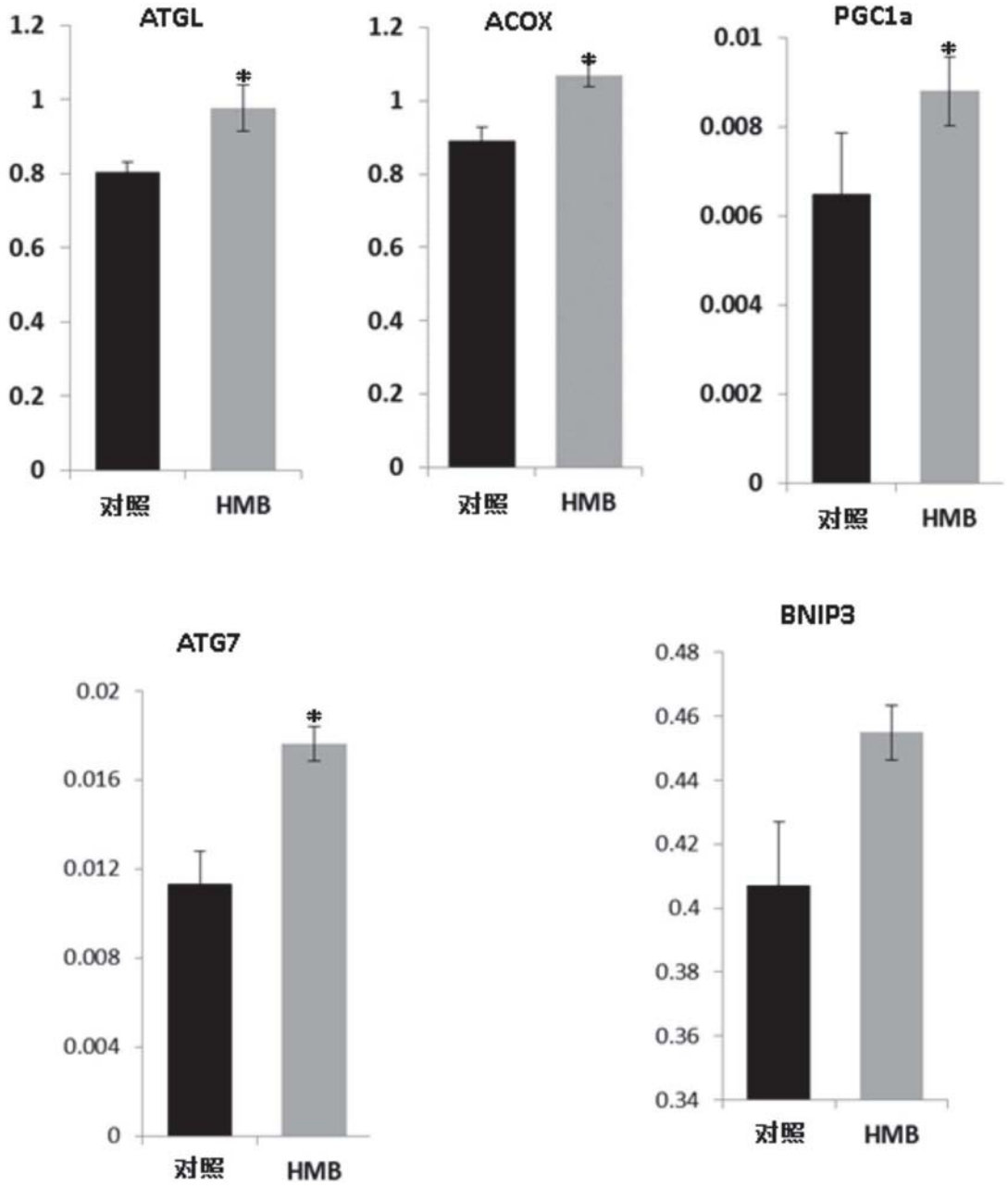

ATGL
1.2
1
0.8
0.6
0.4
0.2
0
对照
HMB
ACOX
1.2
1
0.8
0.6
0.4
0.2
0
对照
HMB
PGC1a
0.01
0.008
0.006
0.004
0.002
0
对照
HMB
ATG7
0.02
0.016
0.012
0.008
0.004
0
对照
HMB
BNIP3
0.48
0.46
0.44
0.42
0.4
0.38
0.36
0.34
对照
HMB

图3基图表达

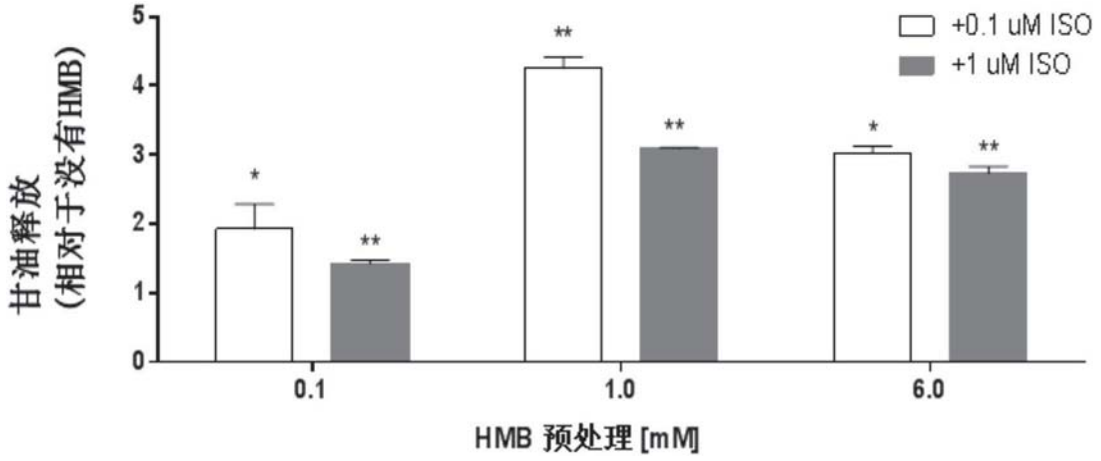


图4 48小时HMB处理在OP9脂肪细胞中增加β肾上腺素刺激的脂解作用

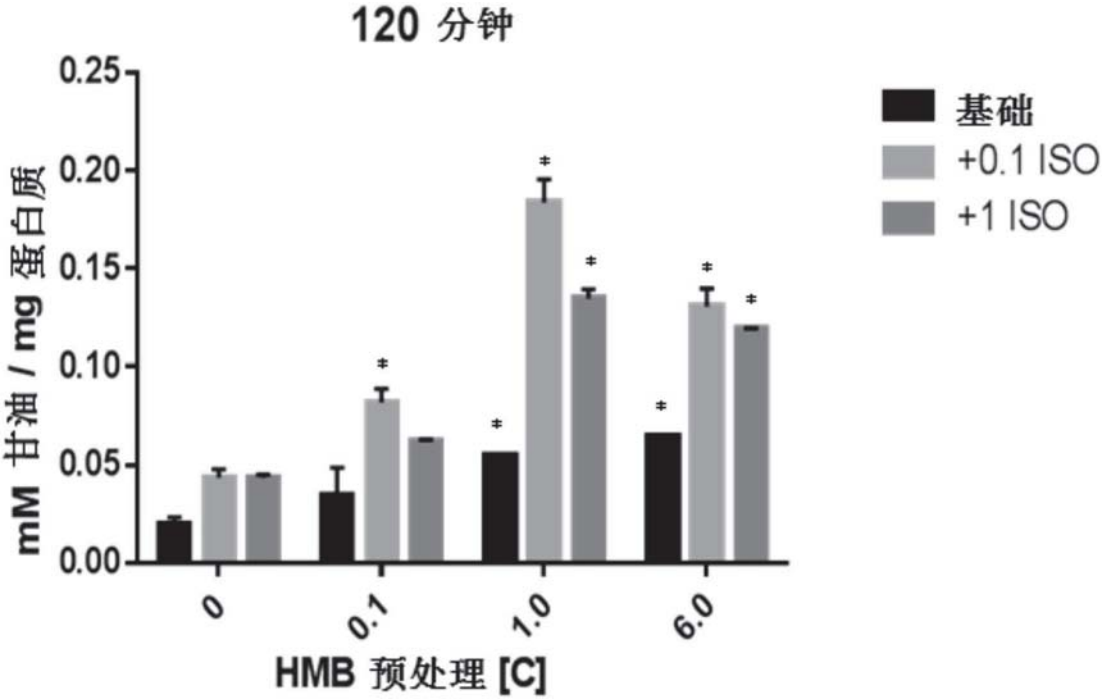


图5 HMB预处理增加基础脂解作用