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(54) Title: PROHIBITIN ATTENUATES INSULIN-STIMULATED GLUCOSE AND FATTY ACID OXIDATION IN ADIPOSE TISSUE BY INHIBITION OF PYRUVATE CARBOXYLASE

(57) Abstract: Many cancer cells show an increase in reliance on anaerobic glycolysis Prohibitin (PHB-I), which has been shown to be overexpressed in many cancer cells, has now been shown to inhibit glucose and fatty acid oxidation in adipocytes through inhibition of pyruvate carboxylase (PC) and consequent depletion of oxaloacetate which is necessary for the tricarboxylic acid cycle. The invention includes a method of identifying an agent that modulates PHB-I activity by measuring the level of inhibition of PC activity or EH domain containing 2 (EHD2) binding to PHB-I.
Prohibitin attenuates insulin-stimulated glucose and fatty acid oxidation in adipose tissue by inhibition of pyruvate carboxylase

PRIOR APPLICATION INFORMATION

This application claims the benefit of US Provisional Patent Application 60/757,012, filed January 9, 2006 and US Provisional Patent Application 60/790,792, filed April 11, 2006.

BACKGROUND OF THE INVENTION

Prohibitin (PHB-1) is a multifunctional phylogenetically conserved protein with a molecular mass of ~30kDa. Its best described function is as a chaperone protein involved in the stabilization of newly synthesized subunits of mitochondrial respiratory enzymes [1]. PHB-1 is essential for normal mitochondrial development and its deficiency in C. elegans is associated with inhibition of mitochondrial biogenesis and senescence [2].

In addition to its localization in the mitochondria, PHB-1 is also present in various other cellular compartments including the nucleus and the plasma membrane [3,4,5]. In the nucleus it may serve as a modulator of transcriptional activity [3,6]. At the plasma membrane it may function as a binding partner for as yet uncharacterized ligands. Plasma membrane bound PHB-1 is able to translocate peptides to the mitochondria as is evident from the observations of Kolonin and colleagues who demonstrated that a mitochondrial death peptide was able to bind to membrane associated PHB-1 and stimulate apoptosis in adipose tissue [7]. Recently PHB-1 has been found in the circulation [8]. The source of circulating PHB-1 is unclear but it may be shed from the plasma membrane [4,7], or released from adipocytes and possibly other cells in lipid droplets [9].

In an attempt to understand the role of circulating PHB-1 we investigated its effect on adipocyte metabolism and demonstrated that PHB-1 is a potent inhibitor of pyruvate carboxylase (PC).

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a method of identifying an agent capable of modulating prohibitin (PHB-1) activity comprising:
adding a test agent to a mixture comprising prohibitin and pyruvate carboxylase (PC); incubating the mixture under conditions promoting PHB-1 inhibition of PC; and determining if the test agent 1) reduces PHB-1 inhibition of PC, wherein PC activity is greater than PC activity in a PC-PHB-1 inhibited control; or 2) enhances PHB-1 inhibition of PC, wherein PC activity is lower than PC activity in a PC-PHB-1 inhibited control.

According to a second aspect of the invention, there is provided a method of identifying an agent capable of modulating prohibitin (PHB-1) activity comprising: adding a test agent to a mixture comprising prohibitin and EHD2;; incubating the mixture under conditions promoting interaction of prohibitin and EHD2; and determining if the test agent reduces PHB-1 interaction with EHD2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The effect of PHB-1 on insulin-stimulated glucose transport by adipocytes. 3H-2-deoxyglucose uptake was measured over a 15 minute period. The data are expressed in terms of the basal uptake, which was 4.1 ± 0.9 nmol/gram of fat. The number of independent experiments for each treatment group is indicated. NS indicated no significant differences between the group below the line. * indicates p < 0.05 for the difference between treatment groups and the basal group. A glycerol control was included to determine the effect of the small amount of glycerol (27μM) present in the recombinant PHB-1.

Figure 2. The effect of PHB-1 on insulin-stimulated glucose oxidation by adipocytes. 14CO2 production from uniformly labeled 14C-glucose was measured as described in the Experimental section. In the upper panel, data is expressed as the mean ± SEM 14CO2 produced per gram of fat tissue. The number of independent experiments for each treatment group is indicated. The subscripts a and b indicate significant difference from basal (histogram 1) and from the insulin only treatment group (histogram 2) respectively. In the lower panel the dose response curve for the inhibition of insulin induced glucose oxidation by PHB-1 is shown. Half-maximal inhibition was apparent at ~4nM PHB-1.
Figure 3. Lack of effect of PHB-I on insulin stimulated pyruvate oxidation by adipocytes. $^{14}$CO$_2$ production from 1-$^{14}$C-pyruvate was measured as described in the Experimental section. Data represent the mean ± SEM for 3 or 4 independent adipocyte cultures and has been expressed as a percentage of the mean insulin response which was 10.51± 1.57 µmol of $^{14}$CO$_2$/gram of fat tissue/2 hours.

Figure 4. The effect of PHB-1 on oleic acid oxidation by adipocytes. $^{14}$CO$_2$ production from 1-$^{14}$C-oleic acid was measured as described in the Experimental section. Data represent the mean ± SEM for the indicated number of independent adipocyte cultures and has been expressed as a percentage of the mean insulin response (28.5 ± 3.4 nmol of $^{14}$CO$_2$/gram of fat tissue/2 hours, histogram 4). Superchropts a,b and c represent p < 0.05 for the difference from basal (histogram 1), insulin only (histogram 4) or insulin plus pyruvate (histogram 7) respectively.

Figure 5. The effect of PHB-1 on PC activity in vitro. PC was purified from adipocytes. Incorporation of $^{14}$C into oxaloacetate was assessed using $^{14}$C-sodium bicarbonate as a substrate. Data is expressed as a percentage of the basal value which was 63.2 nmol of $^{14}$CO$_2$ incorporate /gram of fat tissue/min. This experiment was replicated on two occasions.

Figure 6. Identification of PC and EHD2 as binding partners for PHB-1. PHB-1 was cross-linked to adipocytes and after cell lysis rescued using Ni-agarose. The pellet was analyzed by SDS-PAGE and immunoblotting with anti-PHB-1 antibody (A), silver staining (B) or by blotting streptavidin-HRP only to identify biotin-containing proteins (C). The sizes of the major bands were determined by comparison with MW markers.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

Prohibitin (PHB-1) is a highly conserved protein involved in
mitochondrial biogenesis and function. Prohibitin (PHB-1) is a highly conserved protein involved in mitochondrial biogenesis and function. Mouse, rat, drosophila and yeast PHB-1 share 99, 96, 74 and 54 percent sequence identity respectively, with human PHB-1. It is secreted in lipid droplets from adipocytes and is present in the circulation. In adipose tissue it functions as a membrane receptor and can target binding partners to the mitochondria. Here we report that PHB-1 has a hitherto undescribed role as an inhibitor of pyruvate carboxylase (PC). As a consequence, PHB-1 can modulate insulin-stimulated glucose and fatty acid oxidation. PHB-1 had no effect on insulin-stimulated 2-deoxglucose uptake by isolated adipocytes but inhibited insulin-stimulated oxidation of $^{14}$C-glucose with a half maximal concentration of $\approx 4$ nM. PHB-1 also inhibited oleic acid oxidation in glucose depleted adipocytes via depletion of oxaloacetate. In vitro experiments using broken cell assays confirmed that PHB-1 inhibited PC. MALDI-TOF analysis of proteins identified by crosslinking of PHB-1 to adipocytes membranes indicated that PHB-1 is closely associated with PC and EH domain 2 (EHD2). Based upon these data we propose that PHB-1 is recycled between the extracellular space and the mitochondria by a mechanism involving lipid rafts and EHD2 and can modulate mitochondrial fuel metabolism by inhibition of PC.

Here we demonstrate that PHB-1 potently inhibits glucose and fatty acid oxidation in adipocytes. This effect is mediated by specific inhibition of PC and consequent depletion of oxaloacetate necessary for the tricarboxylic acid cycle. This effect occurred at physiologically relevant concentrations with a half-maximal effect observed in 4-5 nM range.

The inhibition of PC was demonstrable when PHB-1 was added to intact adipocytes indicating that a mechanism exists whereby extracellular PHB-1 can be translocated to the mitochondria to inhibit this enzyme. This observation is consistent with the previously published report of Kolonin et al., that mitochondrial death peptides targeted to plasma membrane associated PHB-1 was able to induce apoptosis in adipose tissue [7].

PC is predominantly located in the mitochondrial matrix close to the mitochondrial inner membrane [10]. It is activated by acetyl CoA and short-chain derivatives of CoA [10]. PHB-1 is also localized to the inner mitochondrial membrane at least in yeast [11] and appears to localize to a similar region of the
mitochondria in mammalian cells [1]. Mitochondrial PHB-1 forms a large molecular weight ring structure by association with a closely related protein PHB-2. It is possible that when complexed in this fashion PHB-1 is unable to inhibit PC. PHB-1 exists as various isoforms and posttranslational modifications such as phosphorylation have been suggested to be important in regulating the functional activity of PHB-1 [12]. The ability of fibroblasts to phosphorylate PHB-1 appears to decline with cellular senescence [13] which could be of potential relevance since an approximately 40% reduction in mitochondrial oxidative activity has been documented with aging in human subjects [14]. In light of our observations reported here, one could speculate that this decline in mitochondrial oxidative phosphorylation with aging is associated with changes in PHB-1 isoforms and increasing inhibition of PC with senescence.

A role for PHB-1 in switching from glycolytic to oxidative metabolism has been suggested by studies using TCM62 deficient S. cerevisiae mutants [15]. In yeast anaerobic glycolysis is used exclusively when a fermentable carbohydrate source is available but a diauxic shift to oxidative phosphorylation occurs when yeast are changed to a non-fermentable carbohydrate source. Yeast deficient in TCM62, which has a functional equivalent activity as PHB-1, have impaired ability to undergo this diauxic shift [15]. Our novel observations suggest that this impairment of the oxidative phosphorylation in TCM62 deficient yeast relates to the ability of PHB-1 to modulate PC.

PHB-1 is overexpressed in many cancer cells [5] and increased levels of PHB-1 have been demonstrated in the circulation of cancer patients [8]. Cancer cells are known to have an increased reliance on anaerobic glycolysis compared to normal non-transformed cells [16]. Nijtmans and colleagues have proposed that upregulation of PHB-1 in cancer cells is due to the presence of regulatory elements in the PHB-1 promoter that binds the Myc oncoprotein [1]. Upregulation of Myc expression, which is common in malignant cells, induces expression of the PHB-1 [17]. Nijtmans et al., suggest that increased expression of PHB-1 by Myc, which also induces expression of glycolytic enzymes and transporter proteins involved in carbohydrate metabolism [18], may be part of a coordinated response in rapidly proliferating cells to allow for reduced oxidative stress in the presence of enhanced anaerobic glycolysis [1,17]. This is an
appealing argument in light of our observation reported here that PHB-1 inhibits PC and shifts metabolism away from oxidative phosphorylation towards anaerobic glycolysis. Thus upregulation of PHB-1 in various cancers may by inhibiting PC be responsible for enhanced anaerobic glycolysis that provides a survival advantage to cancer cells under conditions of hypoxia which occurs when cancer growth outstrips angiogenesis. Consequently agents that modulate PHB-1 action, thereby preventing its inhibition of PC may prove to be useful therapeutic agents in the treatment of cancer and other disease associated with excessive cellular proliferation.

While our data does not directly address the mechanism whereby PHB-1 is transported to the mitochondria, we have shown that EHD2 is involved in this process. PHB-1 is known to be present on the cell membrane in lipid rafts associated with various receptors [4]. In HeLa cell PHB-1 is particularly abundant in the caveolin-1-rich fractions [5] suggesting that caveolin-1 may have a role in transporting PHB-1. Lipid rafts have been shown to be involved in internalization of various molecules including receptors and viral particles [19,20] and could potentially be internalized by a lipid raft and caveolin-dependent process. In addition to PC we identified EHD2 as a binding partner for PHB-1. PC, EHD2 and PHB-1 have been identified in lipid droplets released from 3T3L1 cells [9]. However EHD2 has not been implicated previously in the internalization of PHB-1. Interestingly PHB-1 and PC were identified only in lipid droplets from 3T3-L1 adipocytes incubated under conditions that stimulate lipolysis but were not identified in lipid droplets obtained under basal conditions [9]. EHD2 and its related protein EHD1 have been shown to be involved in endocytosis and vesicle recycling [21-23]. Back et al., have reported that the predicted structure of the C-terminal domain of PHB-1 has structural similarity to sytaxin 1A and T-Snare protein SS01, proteins that are important in docking and fusion necessary for vesicle mediated transport events [24]. While further studies are needed to clarify how PHB-1 is internalized our data suggests that EHD2 is likely to be involved in this process.

Since PHB-1 can function as an agent to translocate molecules from the plasma membrane to the mitochondria [7], the mechanism whereby PHB-1 is internalized is important since anti-apoptotic drugs targeted to PHB-1 on the membrane may be useful in the treatment of various diseases including but not limited to cancer
and obesity. The observations of Kolinin et al., [7] suggest that it is possible to obliterate fat tissue in rodent models of obesity by targeting mitochondrial death peptides to PHB-1 on the cell membrane. Agents that modulate the interaction of PHB-1 with EHD2 are important in this regard because they may have therapeutic utility in various disease states.

In one aspect of the invention, there is provided a method of identifying an agent capable of modulating prohibitin (PHB-1) activity comprising:

adding a test agent to a mixture comprising prohibitin and pyruvate carboxylase (PC);

incubating the mixture under conditions promoting PHB-1 inhibition of PC; and

determining if the test agent 1) reduces PHB-1 inhibition of PC, wherein PC activity is greater than PC activity in a PC-PHB-1 inhibited control; or 2) enhances PHB-1 inhibition of PC, wherein PC activity is lower than PC activity in a PC-PHB-1 inhibited control.

As will be appreciated by one of skill in the art, the mixture is incubated under conditions which would normally promote PHB-1 inhibition of PC, that is, would promote PHB-1 mediated inhibition of PC in the absence of the test agent or agent of interest.

As will be appreciated by one of skill in the art, the control(s) do not necessarily need to be repeated each time a test agent is assayed. That is, a level of PC activity considered to be 'inhibited' may be used.

It is further noted that many methods for determining PC activity are known in the art. An exemplary assay is shown in Figure 5 as discussed herein. As will be appreciated by one of skill in the art, such agents may be used to treat certain disease states, for example but by no means limited to cancer, obesity and aging.

In some embodiments, the test agents or agents of interest are small molecules from a combinatorial library. In a preferred embodiment, only agents that disrupt the interaction of PHB-1 with PC and thereby relieve the inhibition of PC by PHB-1 are screened for.

In yet other embodiments, the test agents comprise small peptides derived from PHB-1. As will be appreciated by one of skill in the art, these small
peptides may be synthetic peptides or may be fragments of PHB-1 prepared by
digestion with one or more proteases or other known chemical agents. As will be
appreciated by one of skill in the art, synthetic peptides may be derived from any
PHB-1 from any suitable organism, as discussed above.

In one aspect of the invention, there is provided a method of
identifying an agent capable of modulating prohibitin (PHB-1) activity comprising:
adding a test agent to a mixture comprising prohibitin and EHD2;
incubating the mixture under conditions promoting interaction of
prohibitin and EHD2; and
determining if the test agent reduces PHB-1 interaction with EHD2.

In some embodiments, a yeast two-hybrid system is used to identify
the regions of PHB-1 that interact with EHD2 and vice versa. In this manner, small
peptides and non-peptide molecules can be screened for the ability to disrupt the
interaction of PHB-1 with EHD2 and consequently inhibit translocation of PHB-1 to
the mitochondria.

It is of note that the conditions promoting interaction of prohibitin and
EHD2 are conditions under which prohibitin and EHD2 would normally interact with
one another in the absence of the test agent or agent of interest.

It is further noted that many means for detecting and quantifying
interaction between PHB-1 and EHD2 would be well known to one skilled in the
art.

As will be appreciated by one of skill in the art, following identification
of the region(s) of PHB-1 that interact with EHD2, these region(s) of PHB-1 can be
genetically or chemically linked to other agents, thereby transferring the ability of
PHB-1 to translocate agents to the mitochondria using the EHD2 mechanism. As
discussed above, the C-terminal domain of PHB-1 has structural similarity to
sytaxin 1A and T-Snare protein SS01, proteins that are important in docking and
fusion necessary for vesicle mediated transport events.

In summary we have demonstrated (i) that PHB-1 is a potent inhibitor
of PC and is likely to be involved in the mechanism whereby metabolism is
switched away from oxidative phosphorylation towards anaerobic glycolysis under
conditions of rapid proliferation associated with malignant transformation and that
PHB-1 is internalized via a mechanism that depends upon EHD2. This newly
identified role for PHB-1 may also be part of normal physiological regulation of mitochondrial function and may have implications for the decline in oxidative phosphorylation with senescence.

PHB localizes to many cellular compartments and may have distinct but possibly overlapping functions in each of these. Identification of PHB as a membrane receptor in the blood vessels of white adipose tissues and gastrointestinal cells provides evidence of additional potential functions of this very intriguing protein. While the role of PHB as a chaperone protein in mitochondria is now comparatively well established, the role of PHB as a tumor suppressor protein is still controversial. However the recent implication of PHB in the mechanism of action of antiestrogens, agents that are currently used therapeutically in breast cancer, opens a new avenue to explore for anti-cancer drugs which can induce apoptosis via PHB signalling. Other disease states may also benefit from targeting PHB using various mechanisms (Table 2).

The localization of PHB in the mitochondria, nucleus and plasma membrane as well as its potential to interact with tumor suppressor gene proteins, transcription factors and associated cofactors suggest that it has important biological roles which have yet to completely elucidated. Furthermore, all these new insights are occurring at a time when there is a resurgence of interest in mitochondrial dysfunction as a cause of premature aging and age-related disease such as diabetes, obesity and the metabolic syndrome. While a significant amount of work is still required to uncover the complexity and function of PHB in cell proliferation and apoptosis, the recent observations suggest that this protein will be of tremendous interest to the drug discovery industry involved in the development of therapeutic agents for common diseases such as obesity, diabetes and cancer, as discussed above.

MATERIALS AND METHODS

Reagents. All radioisotopes were purchased from Perkin-Elmer Life Sciences Inc., (Vaudreuil, PQ). Recombinant human His-tagged PHB-1 expressed in E. coli was obtained from AmProx Inc., (Carlsbad, CA, USA). It is greater than 90% pure and appears as a single band on Coomassie blue-stained gels. As supplied by the manufacturer, it contained 50% glycerol to prevent freeze-thaw damage. To control for the small amount of glycerol present after dilution, all
assays included a control where the glycerol at a final concentration of 0.2% v/v, ~
27 μM was added in the absence of PHB-1. Glycerol at these concentrations had no effect on any of the measured parameters. All other reagents were obtained from Sigma-Aldrich, Oakville, Ontario, Canada.

Mice. Male CD-1 mice, of 4-5 weeks of age, were allowed ad libitum
access to standard laboratory rodent chow and water and were maintained on 12 h
light: 12 h dark schedule (lights on at 0700 h). The animals were killed by cervical
dislocation between 9-10 AM and the epididymal fat pads from 4 mice were quickly
removed and used for each adipocyte preparation.

Adipocyte cultures. Adipocytes were isolated by the collagenase
technique as described by Rodbell [25]. Adipocytes were prepared by digestion of
adipose tissue at 37°C with continuous shaking at 180 rpm in KRBH buffer
containing 2 mM glucose, 2% fatty acid free BSA containing 2 mg/ml collagenase
type 2 at a concentration of 0.2 g of fat tissue/ml in a final volume of 5 ml. After 45
min the cells were centrifuged at 2000 rpm for 2 min to pellet non-fat cells and
undigested tissue. The adipocytes were washed in the appropriate buffer as
described below by centrifugation and removal of the infranatant. The final cell
suspension was at concentration of 2 ml of appropriate buffer per g fat processed.

Glucose transport. Experiments were performed in 15 ml disposable
polypropylene tubes in KRBH-ImM sodium pyruvate, 0.5% BSA, pH 7.4 (uptake
buffer) in a final volume of 1 ml containing 100 μl of adipocyte suspension (0.5 g of
fat tissue equivalent/ml) and insulin 79 nM unless otherwise stated. Cultures were
aerated for 20 seconds with O₂:CU₂ (95:5%) mixture. Tubes were capped and the
susensions were incubated for 30 min at 37°C with constant shaking at which
time PHB-1 was added at the indicated concentrations to duplicate tubes and the
tubes were again aerated and the incubation was continued for a further 30 min.
50 μl of uptake buffer containing 0.35 mg deoxyglucose and 30μCi of 2-³H-
deoxyglucose (5-10 Ci/mmol) was then added and the uptake was determined
over subsequent 15 min. At the end of the incubation period, 5 ml of ice cold
KRBH-ImM sodium pyruvate was added to each tube and the incubation mixture
was filtered through 8μm cellulose nitrate filters under mild vacuum. The filters
were then washed with 5 ml and again with 2.5 ml of same solution and dried
under vacuum. The dried filters were dissolved in 10 ml of Filter Count (Perkin-
Elmer Life Science Inc., Woodridge, Ontario, Canada) and radioactivity was determined in a Wallac Rack-Beta liquid scintillation counter.

Glucose and pyruvate oxidation. Experiments were performed in 15 ml disposable polypropylene tubes in a KRBH-2mM glucose, 2% BSA, pH 7.4 in a final volume of 1 ml containing 100 µl of adipocyte suspension (0.5 g of fat tissue equivalent/ml) and insulin 79 nM unless otherwise stated. PHB-1 was added at the indicated concentrations. Tubes were aerated for 20 second with O₂/CO₂ (95:5%) mixture and sealed with stoppers carrying plastic well inserts (Kontes Glass Co., Vineland, NJ, USA). The cultures were incubated for 1 hour at 37 °C with constant shaking (180 rpm), at which time 0.5 µCi of U-¹⁴C-glucose (303 mCi/mmol) was added and the incubation was continued for a further 2 h. Hyamine hydroxide, 0.1 ml was added to the center well and the incubation was terminated by the addition of 0.2 ml of 1N H₂SO₄. Radioactivity trapped in the center well was determined by liquid scintillation counting using Scintiverse (Fisher Scientific Inc. Edmonton, Alberta, Canada). The experiments where ¹⁴CO₂ production from pyruvate was measured were conducted as above with the exception that the final two washings of the cells were with KRBH-2mM pyruvate -2% BSA instead of glucose supplemented KRHB. Incubations were performed as above except 2mM pyruvate, 0.5 µCi ¹⁴C-pyruvate was substituted for glucose.

Oleic acid oxidation. Adipocytes were prepared as above with the exception that cells were washed twice in KRBH-1% BSA without glucose. The final cell suspension was at a concentration of 2 ml of KRBH-1% BSA per g of fat processed. Cold oleic acid was bound to fatty acid free BSA at a molar ratio of 2:1. Briefly, 0.8g of BSA was dissolved in 12.5 ml of KRB buffer. 7.6 mg of oleic acid was then added gradually with gentle stirring. To each 2.5 ml of the albumin-oleic acid solution, 125 µl of ¹⁴C-oleic acid (51mCi/mmol) was added and 200µl of this solution was used per ml of incubation. Therefore each ml of the incubation mixture contained 0.95 µCi of radioactivity and a final concentration of oleic acid of 0.398mM to give a specific activity of oleate of 5.3 x 10³ dpm/nmol. Insulin, pyruvate and PHB-1 were added as indicated. ¹⁴CO₂ production was determined as described above for the glucose oxidation experiments.

Membrane cross-linking of PHB-1. Recombinant His-tagged PHB-1 (1 µg) was incubated with adipocytes in PBS for 1 h on ice. Then DSS was added
and incubated for another 15 min. After quenching, cells were washed four times with ice cold PBS. Subsequently solubilized adipocyte membranes were prepared using a membrane preparation kit (Pierce, Rockford, IL) in the presence of protease inhibitors (aprotinin (2 µg/ml), 1 mM PMSF and 0.1 mM EDTA) according to manufacturer’s instructions. PHB-1 cross-linked proteins were precipitated with Ni-Agarose, centrifuged and the pellet washed 5 times in ice cold PBS. Cross-linked proteins were analyzed on two identical 10% SDS-PAGE gels. Separated proteins were electrophoretically transferred to nitro-cellulose membrane. Membranes were blocked in 5% milk, washed in TBST (0.5% T-20) and incubated with mouse monoclonal anti-PHB (1:500) for 1 h at room temperature. Membranes were washed three times in TBST and incubated with anti-mouse-HRP conjugate diluted to 1:5000 for 1 h at RT, or streptavidin-HRP at a 1:8000 dilution to identify PC. ECL was used to analyze the membrane. A second identical gel was processed for silver staining.

_In-gel tryptic digestion of PHB-1 cross-linked proteins._ Silver stained protein bands (corresponding to bands identified by Western blotting) were excised from the gel, further sliced into ~1 mm pieces and destained in silver destain solution containing 0.2% potassium ferricyanide and 0.1% sodium thiosulfate. After destaining, gel slices were washed in 100 mM ammonium bicarbonate and then in milli-Q water. Subsequently gel slices were alkylated by addition of 50 mM iodoacetamide and then digested _in situ_ with sequencing grade trypsin (Promega, Madison, WI, USA). The tryptic peptides were extracted and concentrated using C_{18} zip-tips and eluted with 0.1% trifluoroacetic acid in 30% acetonitrile followed by 0.1% trifluoroacetic acid in 75% acetonitrile. The eluates were dried under vacuum using a Speed Vac concentrator.

_Liquid chromatography-mass spectroscopy._ Peptide mixture were resuspended in 10 µl of 0.05% TFA and 0.5 µl was used for µHPLC-MALDI-QqTOF analysis coupled to an online system. Chromatographic separation was performed using an Agilent 1100 Series system. Sample (0.5 µl) were injected into 150 µm X 150 mm column (Vydac 218 TP C18, 5µ) and eluted with 1-80% acetonitrile (0.1% TFA) in 60 min. Major ion peaks of the total ion chromatogram were analyzed by mass spectrometry (MS) in Manitoba/Sciex prototype quodrupole/time of flight mass spectrometer (QqTOF). In this instrument ions are
produced by irradiation of the target using proton pulses from a 20-Hz nitrogen laser and mass accuracy within a few mDa in TOF spectra. Identification of the tryptic peptides was done by searching database against the peptide fingerprints using Global Proteome Machine (http://www.thegpm.org).

Streptavidin-agarose pull-down of PC. Streptavidin-agarose, 25 µl (Sigma) was added to 750 µl of adipocyte cell lysate in Tris buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 mM PMSF, 0.3 µM aprotinin, 0.5% Nonidet NP-40) and incubated for 2 h at 4°C with constant rotation. The mixture was centrifuged for 30 seconds at 7000 rpm and pellet was washed 5 times in ice cold TBS and finally resuspended in 50 µl TBS. For the assay of PC activity, 10 µl of this suspension was incubated in 100 µl of reaction buffer containing 2 mM Na-ATP, 2.5 mM NaHCO$_3$, 10 mM MgCl$_2$, 100 mM KCl, 1 mM DTT, 8 mM pyruvate, and 0.2 mM acetyl Co-A, biotin (50 µg/0.1 ml) and 1 µCi $^{14}$C-NaHCO$_3$/ml at 37°C for 30 min. The reaction was stopped by addition of 50 µl of 10% TCA followed by overnight air drying and liquid scintillation counting. To investigate the effect of PHB-1 on PC activity, the pull down mixture was preincubated with various concentrations of PHB-1 on ice for 30 min prior to the assay.

Statistical analysis. All data is expressed as the mean ± SEM of independent observations derived from separate adipocyte preparations. Because of variation in basal levels in the various experiments with different adipocyte preparations, the data has been expressed as a percentage of the insulin response seen in each experiment. An ANOVA was used to determine statistical differences between experimental groups. Where appropriate a post-hoc Dunnett's t-test was used to compare multiple groups with a single control group. The Bonferroni t-test was used where multiple comparison between groups were made.

RESULTS

Effects of PHB-1 on glucose transport and oxidation in adipocytes.

Insulin stimulated $^3$H-2-deoxyglucose uptake by ~ 2.5 fold. Basal uptake was 4.1 ± 0.9 nmol/gram of fat tissue/15 mins. PHB-1 had no effect on insulin stimulated glucose uptake by adipose tissue (Fig 1). Insulin increased $^{14}$CO$_2$ production from uniformly labeled $^{14}$C-glucose by adipocyte cultures by 5 fold (Fig. 2). This effect was markedly inhibited by PHB-1. A significant reduction was apparent with as little as ~ 2 nM and a half maximal effect was observed at ~
4 nM. In contrast PHB-1 had no significant effect on insulin stimulated $^{14}$CO$_2$
production from $^{14}$C-pyruvate labeled at the carbon 1 position indicating that PHB-1
did not inhibit pyruvate decarboxylation (Fig. 3).

In the absence of insulin and/or pyruvate minimal oleic acid oxidation
occurred because of depletion of oxaloacetate (Fig. 4, histogram 1). In glucose
deleted adipocytes insulin had a modest effect on $^{14}$CO$_2$ production from uniformly
labeled 1-$^{14}$C-oleic acid, $\sim$ 1.5 fold, $p < 0.05$. This effect was inhibited by PHB-1 ,
(Fig. 4, histograms 6 vs. 4, $p < 0.05$). The addition of pyruvate alone had minimal
effect on $^{14}$CO$_2$ production (histogram 2). However in the presence of insulin,
pyruvate increased oleic acid oxidation by $\sim$ 3 fold. Since the adipocytes were
prepared under glucose-depleted conditions, this enhancement of insulin-induced
oleic acid oxidation is presumably due to repletion of oxaloacetate from pyruvate.
This effect was also significantly inhibited by PHB-1 (histograms 8 vs. 7, $p < 0.05$).
The presence of insulin stimulates pyruvate dehydrogenase which results in the
generation of acetyl CoA and subsequent allosteric activation of PC [10] thus
allowing for the regeneration of oxaloacetate an important component of the
tricarboxylic cycle necessary for oleic acid oxidation.

PHB-1 inhibits PC in vitro.

PC, a biotin containing enzyme, was harvested from adipocyte lysate
using streptavidin-agarose pull-down. Incorporation of $^{14}$C-sodium bicarbonate into
oxaloacetate by PC was potently inhibited by PHB-1 with a half-maximal effective
concentration of $\sim$5 nM (Fig. 5).

Identification of PC and EHD2 as binding partners for PHB-1.

In an attempt to identify proteins which may mediate uptake of PHB-
1, we incubated mouse adipocytes with 6-his-tagged recombinant PHB-1 and
cross-linked with DSS. Following solubilization of the adipocytes membranes and
precipitation with nickel-agarose the cross-linked proteins were analyzed by
Western blotting. Anti-PHB-1 antibody identified two bands of $\sim$160 and 95 kDa
which were also easily identified on a silver stained gel run under identical
conditions (Fig. 6). When streptavidin-HRP was used as a probe, the 160 kDa
band was identified consistent with the presence of a biotin-containing protein.
These bands were excised, subjected to in gel trypsin digestion and analyzed by
MALDI-TOF. The larger of the bands was identified as PC while the smaller band
was identified as EHD2. The probability, coverage and peptides identified are shown in Table 1.

While the preferred embodiments of the invention have been described above, it will be recognized and understood that various modifications may be made therein, and the appended claims are intended to cover all such modifications which may fall within the spirit and scope of the invention.
REFERENCES


24. Back JW, Sanz MA, De Jong L, De Koning LJ, Nijtmans LG, De Koster CG,

Table 1. List of unique peptides identified after LC/MS-MS run by Global Proteome Machine (www.thegpm.org).

<table>
<thead>
<tr>
<th>Band (kDa)</th>
<th>Protein</th>
<th>Probability</th>
<th>Coverage</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Pyruvate carboxylase</td>
<td>-80.4</td>
<td>11</td>
<td>125ADFAQACDQDAGVR (SEQ ID No. 1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>138FIGPSPEWR (SEQ ID No. 2)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>274WEIAPATH LDPQLR (SEQ ID No. 3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>407LDNASAFQGAVISPHYDSLVLK (SEQ ID No. 4)</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td>572DAHQSI LLARTR (SEQ ID No. 5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>819GTPLDTEVPLER (SEQ ID No. 6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>929AEAEAQAEELSFPR (SEQ ID No. 7)</td>
</tr>
<tr>
<td>90</td>
<td>EH-domain containing protein (EHD-2)</td>
<td>-20.8</td>
<td>8.8</td>
<td>974IEGRPGASLPPLNLK (SEQ ID No. 8)</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td>1022DFATFGPLSDLNTR (SEQ ID No. 9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1062ALAVSDLNR (SEQ ID No. 10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1075QVFFELNGQLR (SEQ ID No. 11)</td>
</tr>
</tbody>
</table>

* Base -10 log of expectation that this assignment is stochastic. Low expect score (<-3.0) correspond to a confident identification.
Table 2. Potential sites and mechanisms to target the multiply functions of Prohibitin in various disease states.

<table>
<thead>
<tr>
<th>Types of Agents</th>
<th>Potential Mechanisms</th>
<th>Disease States</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial prohibitin</td>
<td>Small molecules, Peptides</td>
<td>Obesity, Diabetes, Metabolic Syndrome, Cancer, Anti-aging</td>
</tr>
<tr>
<td></td>
<td>Induction of apoptosis, Stabilization of mitochondrial function</td>
<td></td>
</tr>
<tr>
<td>Nuclear prohibitin</td>
<td>Small molecules, Morpholino sense and antisense Oligonucleotides, Viral and plasmid expression vectors</td>
<td>Breast and other cancers</td>
</tr>
<tr>
<td></td>
<td>Disruption of PHB/Brgl/Brm interactions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Disruption of PHB/E2F interactions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Disruption of PHB/pRB and PHB/p53 interactions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibition of PHB phosphorylation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibition of PHB translocation</td>
<td></td>
</tr>
<tr>
<td>Prohibitin 3' UTR</td>
<td>Morpholino sense and antisense Oligonucleotides, Viral and plasmid expression vectors</td>
<td>Breast and other cancers</td>
</tr>
<tr>
<td></td>
<td>Inhibition of translation, RNA degradation</td>
<td></td>
</tr>
<tr>
<td>Cell surface prohibitin</td>
<td>Small molecules, Peptides, Monoclonal antibodies, Morpholino sense and antisense Oligonucleotides, Viral and plasmid expression vectors</td>
<td>Intestinal infections, Inflammatory bowel disease, Obesity, Diabetes, Metabolic Syndrome, Cancer</td>
</tr>
<tr>
<td></td>
<td>Inhibition of inflammatory responses in gut, Vascular remodeling in adipose tissue, Anti-angiogenesis</td>
<td></td>
</tr>
</tbody>
</table>
CLAIMS

1. A method of identifying an agent capable of modulating prohibitin (PHB-1) activity comprising:
   adding a test agent to a mixture comprising prohibitin and pyruvate carboxylase (PC);
   incubating the mixture under conditions promoting PHB-1 inhibition of PC; and
   determining if the test agent 1) reduces PHB-1 inhibition of PC, wherein PC activity is greater than PC activity in a PC-PHB-1 inhibited control; or
   2) enhances PHB-1 inhibition of PC, wherein PC activity is lower than PC activity in a PC-PHB-1 inhibited control.

2. A method of identifying an agent capable of modulating prohibitin (PHB-1) activity comprising:
   adding a test agent to a mixture comprising prohibitin and EHD2;
   incubating the mixture under conditions promoting interaction of prohibitin and EHD2; and
   determining if the test agent reduces PHB-1 interaction with EHD2.
FIGURE 3
FIGURE 6
INTERNATIONAL SEARCH REPORT

International application No
PCT/CA2007/000030

A CLASSIFICATION OF SUBJECT MATTER
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)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Canadian Patent Database, PubMed, Delphion, GenomeQuest and Scopus  Keywords Prohibitm, PHB-I, pyruvate carboxylase, prohibitm inhibitors, EHD2, cancer, aerobic respiration, chaperone protein and glyconeogenesis

C DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>WANG S ET AL. Prohibitm, a potential tumor suppressor, interacts with RB and regulates E2F function ONCOGENE 1999 18 3501 - 3510 see pages 3501, 3504 and 3507</td>
<td>1 - 20</td>
</tr>
<tr>
<td>A</td>
<td>COATES P J ET AL. Mammalian prohibitm proteins respond to mitochondrial stress and decrease during cellular senescence EXP CELL RES 2001 265 262 - 273 see pages 262, 263 and 269</td>
<td>1 - 20</td>
</tr>
</tbody>
</table>

[X] 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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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
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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
30-March-2007 (30-03-2007)

Date of mailing of the international search report
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Ken Steinberg 819-934-7929
<table>
<thead>
<tr>
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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</tr>
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</table>