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Chemical Knockout of Pantothenate Kinase Reveals the Metabolic and Genetic Program Responsible for Hepatic Coenzyme A Homeostasis

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Summary

Coenzyme A (CoA) is the major acyl group carrier in intermediary metabolism. Hopantenate (HoPan), a competitive inhibitor of the pantothenate kinases, was used to chemically antagonize CoA biosynthesis. HoPan dramatically reduced liver CoA levels and the mice developed severe hypoglycemia. Insulin and corticosterone levels were reduced, glucagon levels were elevated in HoPan-treated mice and fasting accelerated the HoPan-induced hypoglycemia. Metabolic profiling revealed a large increase in carnitine, particularly acetylcarnitine, illustrating the role of carnitine in buffering acyl groups to maintain the unesterified CoASH level. HoPan treatment triggered significant changes in hepatic gene expression that substantially increased the thioesterases, which liberate CoASH from acyl-CoA, and increased pyruvate dehydrogenase kinase 1, which prevents the conversion of CoASH to acetyl-CoA. These results identify the metabolic re-arrangements that maintain the CoASH pool which is critical to mitochondrial functions, including gluconeogenesis, fatty acid oxidation, and the tricarboxylic acid and urea cycles.

Introduction

Coenzyme A (CoA) is an essential cofactor that carries carboxylic acid substrates and supports a multitude of oxidative and synthetic metabolic reactions, including those involved in the citric acid cycle, sterol biosynthesis, amino acid metabolism, fatty acid biosynthesis and oxidation (Leonardi et al., 2005). CoA is derived from vitamin B_5 (pantothenate), cysteine and ATP. Pantothenate kinase (PanK) catalyzes the first committed step and is the rate-controlling enzyme in CoA biosynthesis (Jackowski and Rock, 1981;Leonardi et al., 2005;Robishaw et al., 1982). PanK expression levels define the upper threshold of the cellular CoA content (Rock

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et al., 2000;Song and Jackowski, 1992;Zhang et al., 2005), and PanK biochemical activities are feedback regulated differentially by non-esterified CoA (CoASH) or CoA thioesters (Halvorsen and Skrede, 1982;Rock et al., 2002;Rock et al., 2000;Song and Jackowski, 1994;Vallari et al., 1987;Zhang et al., 2005), providing a mechanism to coordinate the rate of CoA synthesis with the demand for the cofactor in metabolic pathways. Loss of the feedback regulation by mutation at a single PanK residue results in run-away CoA production (Rock et al., 2003).

The discovery of multiple PanK isoforms encoded by four genes in humans (Hörtnagel et al., 2003;Ni et al., 2002;Ramaswamy et al., 2004;Zhang et al., 2006;Zhou et al., 2001) and mice (Rock et al., 2002) suggests a complexity associated with diet-, drug- and disease-induced responses of the PanK activities which, in turn, regulate CoA availability. Liver CoA levels are apparently important in metabolic function, as hepatic total PanK activity and CoA content are altered in response to nutritional state (Kondrup and Grunnet, 1973;Lund et al., 1986;Smith et al., 1978;Smith and Savage, Jr., 1980;Voltti et al., 1979), insulin (Robishaw et al., 1982), glucagon or glucocorticoids (Smith and Savage, Jr., 1980), fibrate drugs (Bhuiyan et al., 1988;Halvorsen, 1983;Savolainen et al., 1977;Skrede and Halvorsen, 1979;Voltti et al., 1979), and diabetes (Reibel et al., 1981a;Reibel et al., 1981b). The human PanK2 is a mitochondrial isoform (Hörtnagel et al., 2003;Johnson et al., 2004;Kotzbauer et al., 2005) that is sensitive to inhibition by sub-micromolar acetyl-CoA (Zhang et al., 2006) and mutations that inactivate this isoform are associated with a neurodegenerative disorder (Hörtnagel et al., 2003;Johnson et al., 2004;Kotzbauer et al., 2005;Zhou et al., 2001). Loss of one or more of the PanKs would presumably lead to chronically reduced tissue CoA levels, but details about the molecular sequelae that accompany such a disorder are lacking. We investigated the biochemical and genetic alterations imposed on animals by reduced CoA using a pantothenate antimetabolite, hopantenate (HoPan, Figure 1A) to chemically ablate CoA biosynthesis. The data show that unesterified CoASH is the most important component of the CoA pool that is necessary for mitochondrial function. The experiments also illustrate for the first time the metabolic and genetic processes that re-align in an effort to maintain the level of CoASH. These data provide insight into the molecular shifts that may contribute to a loss of physiological function due to CoA deficiency.

Results

Biochemistry of HoPan Action

HoPan is a structural analog of pantothenate containing an extra methylene group (Figure 1A), and we identified this molecule as a pantothenate kinase inhibitor as part of an enzyme based screen of pantothenamides and related structures (Virga et al., 2006). The PanK1α, PanK1β, PanK2, and PanK3 proteins were expressed in 293T cells to evaluate their sensitivities to HoPan (Fig. S1). The putative PanK4 was not enzymatically active when expressed in HEK 293T cells (Fig. S1). HoPan inhibited all active PanK isoforms with IC_{50}s between 50 μM and 150 μM in our *in vitro* assays (Figure 1B). Purified *E. coli* PanK (CoaA), the prototypical type I pantothenate kinase, was refractory to inhibition (Figure 1B). HoPan inhibition of all the mammalian PanKs was consistent with the high degree of similarity among their catalytic domains (Rock et al., 2000;Rock et al., 2002;Zhang et al., 2005;Zhang et al., 2006;Zhou et al., 2001). Likewise, the PanK from *Aspergillus nidulans*, which belongs to the same PanK family as the mammalian isoforms (Calder et al., 1999), was inhibited by HoPan (Virga et al., 2006).

Analysis of the steady-state kinetics of the PanK reaction revealed competitive inhibition by HoPan with respect to the natural pantothenate substrate, as illustrated with PanK1α in Figure 1C. Lineweaver-Burk plots of 1/v vs. 1/[ATP] demonstrated that HoPan was a non-competitive inhibitor with respect to ATP (Figure 1D). The mode of inhibition was the same for PanK1β

(Figure S2). Inhibition of PanK was stereoselective for the (*R*)-isomer of HoPan (Figure 1E). We tested the reactivity of the inhibitor HoPan using [γ-^{32}P]ATP and PanK1α and detected phosphorylated HoPan formation (Figure 1F), revealing that HoPan was a pantothenate antimetabolite.

Physiological Effects of HoPan

HoPan was not toxic to cultured HEK 293T, HepG2 or PC12 cells at concentrations up to 800 μM (Figure 2A). These experiments were performed in pantothenate-free DMEM supplemented with 1 μM pantothenate and dialyzed serum (up to 800:1 ratio of inhibitor to substrate). Consistent with its inhibitory effect on the PanKs in vitro, however, HoPan inhibited pantothenate incorporation into CoA in isolated primary hepatocytes, resulting in a blockade of CoA production (Figure 2B). Surprisingly, the experiments also revealed the breakdown of pantothenate to β-alanine (Figure 2B, inset), a reaction that has not been previously reported in mammals. β-Alanine migrated in the same position as 4′-phosphopantetheine in the acidic solvent system (Figure 2B), but clearly separated from the phosphorylated metabolites that remain on the origin in the basic solvent system (Figure 2B, inset). Hepatocytes labeled with [^{3}H]HoPan accumulated P-[^{3}H]HoPan, demonstrating that HoPan was phosphorylated in vivo and was not metabolized to a CoA analog (Figure 2C). Also, a compound with the chromatographic properties of the HoPan synthesis precursor and degradation product, γ-aminoisobutryic acid, was detected in the hepatocytes (Figure 2C, inset). The fact that no [^{3}H] Pan-derived intermediate other than β-alanine accumulated in the HoPan-treated cells indicated that PanK was the only enzyme of CoA synthesis that was inhibited by HoPan treatment. Nonetheless, we tested the effects of HoPan and P-HoPan on the bifunctional CoA synthase in vitro. The results showed that neither compound inhibited the two activities of CoA synthase (data not shown). In contrast to immortalized cultured cells, animals rely heavily on oxidative metabolism, gluconeogenesis is critical for survival and both processes depend on CoA. Thus, we administered HoPan to male and female mice (100 μg/g/day), all of which expired within 5 and 15 days, respectively (Figure 2D). Control male and female mice given HoPan plus an equal amount of pantothenate supplement survived the 16-day course of the experiment, demonstrating that the end-result of HoPan treatment was specifically due to interference with pantothenate metabolism.

HoPan administration resulted in a precipitous decrease in liver total CoA levels which were the highest among the four tissues examined and dropped by 73% (Figure 2E). Analysis of the distribution of CoA species showed that CoASH (nonesterified CoA) was the most significantly reduced in HoPan-treated liver. Kidney CoASH levels were lower than liver, but also exhibited a significant drop following HoPan (Figure 2F). Control male and female livers (HoPan + pantothenate) had between 110 and 125 nmole/g wet weight of CoA, which were the same as in mice maintained on chow without HoPan and regardless of pantothenate enrichment. The rapid CoA depletion in male liver implied that hepatic CoA had a high turnover rate compared to brain and heart. Assuming that CoA production was completely inhibited following the first dose of HoPan, the half life for liver CoA was estimated to be 24–20 hours in male mice and ~90 hours in females. The different turnover rates of CoA could account for the sexual diphorphism we observed in the HoPan-treated mice. In addition, possible different pharmacokinetics (absorption, excretion and degradation of HoPan) in male and female mice could also explain the sexual diphorphism.

The livers from HoPan-treated mice exhibited distinct pathology characterized by marked vacuolization arising from swollen mitochondria (Figure 3). The liver sections stained with toluidine blue showed more lipid droplets, shrunken nuclei and pronounced vacuoles in the cytoplasm in the HoPan-treated mice (Figure 3A and 3B). Transmission electron microscopy revealed that the apparent vacuoles were swollen mitochondria (Figure 3C-3F). Also, the

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glycogen storage granules evident in the control liver (Figure 3E) were missing from the HoPan-treated liver (Figure 3F). All of the HoPan-treated animals also had elevated serum triglycerides, indicating defective utilization of this fuel source by the liver. The brain, kidney, and heart exhibited normal histology in HoPan-treated male mice (not shown), pointing to the liver as the most sensitive target organ. Control sera from mice treated with HoPan + Pan had 93 ± 47 mg/dL triglyceride, whereas sera from treated males had 230 ± 177 mg/dL ($p<0.05$) (Table S1). The triglycerides in mice treated with H_2O vehicle was 76 ± 14 mg/dL (Table S1), similar to the level of the mice supplemented with pantothenate, demonstrating that pantothenate supplement completely countered the effect of HoPan. Serum triglycerides in female control mice increased from 55.3 ± 17.8 mg/dL in controls to 93.7 ± 78 mg/dL in HoPan-treated animals (Table S1). This increase was not statistically significant due to the variability in the data from the treated females.

Mechanism of HoPan Toxicity

Mice that succumbed to HoPan had low to undetectable (<20 mg/dL) glucose levels at or near the time they expired (Figure 4A), implicating hypoglycemia as a primary cause for death. A pyruvate tolerance test showed that the low blood glucose in the experimental animals was due to a defect in gluconeogenesis. Glucose rose significantly in control animals following the injection of pyruvate; however, the HoPan-treated animals failed to convert the pyruvate into glucose (Figure 4B). Treated mice had lower levels of insulin (Figure 4C), and elevated levels of glucagon (Figure 4D) and cortiscosterone (Figure 4E), indicating a normal hormonal response to hypoglycemia (Cryer, 1993). These data indicated a metabolic imbalance in liver gluconeogenesis due to inadequate CoA rather than a hormonal deficiency. Also, HoPan moderately decreased lactate levels in both male (8.3 ± 1.6 to 5.7 ± 2.4 mmol/ml) and female (7.5 ± 3.8 to 5.3 ± 2.1 mmol/ml) mice (Table S1), arguing against lactic acidosis as a cause of death. We evaluated the response of female mice to fasting mid-way through the two-week HoPan-treatment program to investigate their ability to switch to fatty acids as a fuel source. Animals treated with the antimetabolite had serum glucose (254.7 ± 79.7 mg/dL; $n = 15$) similar to controls (297.5 ± 52.8 ml/dL; $n = 11$), but were unable to maintain blood glucose during a 48-h fast (61.1 ± 15.1 mg/dL; $n = 31$) and all 31 animals expired by 30 h.

Alterations in Intermediary Metabolism and Gene Expression

The carnitine and organic acid compositions of HoPan-treated livers revealed several metabolic derangements created by reduced CoA (Figure 4F and 4G). Total acylcarnitines increased significantly. All but one of the acylcarnitine species was either increased or remained the same in HoPan-treated mice (Table S3) and the largest increases were in the levels of acetyl- and proprionylcarnitine. Free carnitine was also elevated, but not nearly to the same extent as the acylcarnitines (Table S3). Total organic acids did not appear to change in HoPan-treated mice due to the dominance of this pool by pyruvate. However, the second most abundant acid, lactate, decreased 50%, consistent with decreased serum lactate levels in HoPan-treated mice despite impaired gluconeogenesis. The TCA cycle intermediates succinate, fumarate, and malate were all decreased (Figure 4G; Table S3). In addition, urea cycle intermediates ornithine and citrulline significantly accumulated in HoPan-treated mice (Figure 4G).

Microarray analysis showed that more than 3,500 probe sets exhibited greater than 2 fold change with $p<0.05$ in the HoPan-treated liver compared to the controls. A multiple hypothesis testing was performed and the q value was set at 0.05 to limit the false discovery rate to 5% (Benjamini and Hochberg, 1995;Benjamini and Yekutieli, 2005). This analysis yielded 939 probe sets. Replicate probe sets representing the same genes were averaged to generate 772 unique probe sets, of which 502 had annotated biological processes indicating hepatic CoA depletion had a major effect on gene expression. Gene ontology analysis using the NetAffx™ Analysis Center (Liu et al., 2003) was carried out to obtain a global view of the hepatic genes affected by CoA depletion and correlate the significantly regulated probe sets with their biological functions. The most significantly perturbed physiological processes were lipid metabolism, organic acid/carboxylic acid metabolism and protein metabolism (Figure 5). Among lipid metabolism genes, those responsible for biosynthesis were significantly repressed, whereas the expression of catabolic genes was elevated. For example, among the most highly induced genes were the acyl-CoA thioesterases (between 13–45 fold), whereas fatty acid synthase expression was reduced 5-fold (Table S4). Analysis of the array data by sorting gene ontology based on the subcellular location of the affected genes revealed that the proteins altered in the processes shown in Figure 5 were mainly associated with two membrane-bound organelles, the endoplasmic reticulum and mitochondria. This correlation arose from the association of many of the lipid metabolism genes with the endoplasmic reticulum, whereas the genes that affected organic acid metabolism are associated with the mitochondria.

A comparison between the gene expression array analyses and metabolic profiling of the HoPan-treated mouse livers revealed relationships between gene expression (Table S4) and the perturbation in organic acid levels (Figure 4G). The reduced levels of the TCA cycle intermediates succinate and fumarate correlated with a 2.9-fold increase in expression of the downstream enzyme fumarate hydratase (*Fh1*). The microarray data also provided a basis for understanding the dysregulation of the urea cycle exemplified by increased ornithine and citrulline intermediates following HoPan treatment. The expression of ornithine carbamoyltransferase (*Otc*), which utilizes ornithine, was depressed 8.5-fold and the expression of arginase (*Arg2*), which produces ornithine, increased 2.7-fold consistent with the increased ornithine level (Figure 4G). The higher level of citrulline was linked to a 2.6-fold increase in the expression of the citrulline forming enzyme nitric oxide synthase (*Nos3*). *PanK1* was the only pantothenate kinase gene that was upregulated (5.9-fold) in response to the blockade of CoA synthesis by HoPan, and none of enzymes downstream of PanK in the CoA biosynthetic pathway was induced. Other biological processes that were affected by reduced CoA were the response to stress, development and the immune response. A complete list of the genes significantly altered by HoPan treatment is presented in Table S4.

Discussion

The metabolomic and gene expression analysis identifies a program of adjustments focused on preserving the CoASH concentration (Figure 6). CoASH is required for several key mitochondrial reactions including pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and fatty acid βoxidation. The conversion of CoASH to acetyl-CoA from glycolysis via pyruvate is reduced by the 136-fold upregulation of pyruvate dehydrogenase kinase 1, which phosphorylates and inactivates pyruvate dehydrogenase. Acyl-CoA thioesterases 1, 2 and 3 are significantly up-regulated (13–45 fold) illustrating a genetic adjustment to convert acyl-CoA to CoASH. Our metabolic profiling results provide experimental verification for the proposed role of carnitine as a sink to off-load acyl moieties from acyl-CoA, thereby liberating CoASH to support cycling within mitochondria (Bieber, 1988;Ramsay and Zammit, 2004). The significant accumulation of acylcarnitines (5.1-fold) and the upregulation of carnitine palmitoyltransferase (4.7-fold) and carnitine acetyltransferase (2.7-fold) following HoPan administration are consistent with the function of carnitine in accepting acyl groups from acylated CoA and releasing the CoASH form to support gluconeogenesis and the urea cycle. The inability of HoPan-treated mice to produce glucose from liver and kidney is reflected by their inability to survive a brief fast and their failure in a pyruvate tolerance test. The impairment of gluconeogenesis in HoPan-treated mice is reminiscent of the phenotype of mouse models of fatty acid β-oxidation disorders; however, the HoPan phenotype differs in that animals with acyl-CoA dehydrogenase deficiencies accumulate serum lactate (Schuler and Wood, 2002). Acylcarnitines are formed within the mitochondria and released into the cytosol. Unlike CoA thioesters, acylcarnitines pass through biological membranes and are excreted in the urine, a diagnostic parameter for a number of inborn errors in mitochondrial function (Schuler and Wood, 2002;Sim et al., 2002a;Sim et al., 2002b).

Our data show that liver actively controls the intracellular CoA concentration through a balance of synthesis and degradation. The synthetic arm of this regulatory cycle is governed by the four isoforms of pantothenate kinase, which are all expressed in liver. Each of these isoforms has a distinctive sensitivity to feedback inhibition by diverse CoA species (Rock et al., 2002;Zhang et al., 2005;Zhang et al., 2006), enabling the CoA biosynthetic flux to rapidly respond to branches of intermediary metabolism that differentially impact components of the CoA pool. Less is known about the genetic regulation of PanK expression, but the upregulation of hepatic PanK1α expression by fibrates (Ramaswamy et al., 2004) is an example of the modulation of gene expression levels by nutritional regulators. Adjustment of hepatic CoA levels in response to fasting or feeding conditions, diet, disease or treatment with hypolipidemic drugs support the importance of adjusting CoA levels in the metabolic adaptation of liver to different fuel sources (Bhuiyan et al., 1988;Halvorsen, 1983;Halvorsen and Skrede, 1982;Kondrup and Grunnet, 1973;Lund et al., 1986;Reibel et al., 1981a;Reibel et al., 1981b;Robishaw et al., 1982;Savolainen et al., 1977;Skrede and Halvorsen, 1979;Smith et al., 1978;Smith and Savage, Jr., 1980;Voltti et al., 1979). Rapid turnover is a key feature of important metabolic regulatory cycles, and the abrupt cessation of CoA synthesis by HoPan leads to a precipitous decline in hepatic CoA levels as the degradative branch of the regulatory cycle continues. Nudt7 is a peroxisomal protein that hydrolyzes CoASH and its thioesters (Gasmi and McLennan, 2001). Nudt7 is highly expressed in liver and kidney, in contrast to brain and heart (Ofman et al., 2006), and this expression pattern correlates with liver and kidney exhibiting the most rapid decline in the total CoA pool in HoPan-treated animals, with the heart and brain being relatively unaffected (Figure 2F). Subcellular fractionation of mouse kidney showed that virtually all of the CoA hydrolase activity was associated with the peroxisomes, pointing to Nudt7 as the predominant enzyme responsible for CoA turnover (Ofman et al., 2006). The sexual dimorphism in the maintenance of hepatic CoA levels noted in this study may be due to differential expression of the pantothenate kinase isoforms in males and females, differences in the rates CoA degradation, differences in the pharmacokinetic parameters of HoPan absorption, metabolism and excretion, or a combination of these processes.

Significance

CoA is a key cofactor in intermediary metabolism and its tissue levels are tightly regulated by the rate of biosynthesis at the pantothenate kinase step. This work integrates chemical genetics, metabolomics and genomics to provide a comprehensive view of the abnormalities that arise from a defect in pantothenate kinase. Regulation of the CoaSH pool is critical to support mitochondria functions including gluconeogenesis, fatty acid oxidation, and the urea cycle, and multiple biochemical and genetic mechanisms are engaged to maintain CoASH. The carnitine transacylation systems play an important role in supporting CoASH levels by off-loading acyl groups from acyl-CoA. The alterations in intermediary metabolism in HoPan-treated animals serves as a model for the milder disruption of CoA homeostasis that underlies the abnormalities in pantothenate kinase-associated neurodegeneration (PKAN), a hereditary disorder arising from a deficiency in the mitochondrial PanK2 isozyme (Hörtnagel et al., 2003;Zhou et al., 2001).

These results also provide insight to understanding the toxicity of HoPan in humans. HoPan was originally developed as an antipsychotic drug to deliver the neurotransmitter γamino-iso-butryic acid to patients. HoPan showed some efficacy (Task Force for Evaluation of Children's Behaviors, 1974); however, the side-effects were so severe that therapy was discontinued. The adverse side effects induced hypoglycemia, hepatic steatosis and organic acid excretion (Matsumoto et al., 1990;Matsumoto et al., 1991;Nakanishi et al., 1990;Noda et al., 1991;Ohsuga et al., 1989). We attribute these side effects to the interference of HoPan with hepatic CoA metabolism, and our findings suggest that pantothenate supplementation would overcome these side effects, as it does in our mouse model.

Experimental procedures

Preparation of pantothenate kinases

Mouse PanK1α and PanK1β expression vectors were described previously along with the procedure for transfecting and assaying cell lysates (Rock et al., 2002). The mouse cDNAs homologous to the human PanK2 and PanK3 proteins (Zhou et al., 2001) were cloned into pcDNA3.1(–) (Invitrogen) and the human cDNA encoding PanK4 (Zhou et al., 2001) was cloned with an amino-terminal His-tag into pcDNA3.1/HisA (Invitrogen). Protein concentrations were determined by the method of Bradford (Bradford, 1976) using γ-globulin as a standard. A peptide SKDNYKRVTGTSLGC was synthesized and coupled to keyhole limpet hemocyanin by the Hartwell Center for Biotechnology, St. Jude Children's Research Hospital, and was sent to Rockland Inc. (Gilbertsville, PA) for raising rabbit polyclonal antiserum against PanK1, PanK2 and PanK3. Affinity purified polyclonal antibody (Jackowski, 1994;Rock et al., 2002) was used at a dilution of 1:500 (stock is 1.4 mg/ml)to detect proteins by western blot. The His-tagged hPanK4 protein was detected with anti-His rabbit IgG (Santa Cruz Biotechnology) used as the primary antibody at a dilution of 1:500 (stock is 0.2 mg/ml). Horseradish peroxidase-labeled anti-rabbit IgG was used as the secondary antibody and was diluted to 1:5,000 prior to incubation with the membrane for 1 h at room temperature. The fluorescent signal was detected using a Typhoon 9200 (Amersham) and analyzed with ImageQuant 5.2 (Molecular Dynamics).

Synthesis of HoPan: (4-(2,4-dihydroxy-3,3-dimethylbutylamido)butyric acid)

In two separate reaction vials, 4-aminobutyric acid (340 mg, 3.3 mmol) and diethylamine (345 μl, 3.3 mmol) were dissolved in methanol (10 ml). Then to the first reaction vial (*R*)-pantolactone (390 mg, 3 mmol) was added and to the second vial (*S*)-pantolactone (390 mg, 3 mmol) was added. The reactions were stirred overnight at 60°C. The reactions were then stirred overnight at 60°C. The reaction mixtures were evaporated to dryness and dissolved in 20 ml of water. Each mixture was passed over an Amberlite® IR-120 (H+) ion exchange column (2 cm x 10 cm) and eluted with water until neutrality was obtained. These solutions were then washed three times with dichloromethane to remove any excess pantolactone. The extracted solutions were then evaporated to dryness to yield the (*R*)-4-(2,4-dihydroxy-3,3-dimethylbutylamido)butyric acid and (*S*)-4-(2,4-dihydroxy-3,3-dimethylbutylamido)butyric acid products, respectively as oily residues that crystallized upon cooling. The Ca^{++} salts of each product were obtained by dissolving each in 5 ml of methanol with the addition of 0.6 eq of calcium hydroxide according to the protocol of Kopelevich *et al.* (Kopelevich et al., 1971). The solutions were gently warmed to 40°C and filtered to remove any undissolved solid. The filtered solutions were evaporated to dryness to yield each product as a white amorphous powder.

Calcium (*R*)-4-(2,4-dihydroxy-3,3-dimethylbutylamido)butyrate: 406 mg (53% yield) a white powder. ^{1}H NMR (500 MHz, D_2O) δ 3.84 (s, 1H), 3.37 (d, *J*=11.2 Hz, 1H), 3.25 (d, *J*=11.2 Hz, 1H), 3.09 (t, *J*=6.8 Hz, 2H), 2.07 (t, *J*=7.8 Hz, 2H), 1.63 (qin, *J*=7.3 Hz, 2H), 0.79 (s, 3H), 0.76 (s, 3H); MS (ESI^-) 231.9 (M – 1).

Calcium (*S*)-4-(2,4-dihydroxy-3,3-dimethylbutylamido)butyrate: 389 mg (51% yield) a white powder. ^{1}H NMR (500 MHz, CH_3OD) δ 3.95 (s, 1H), 3.49 (d, *J*=11.0 Hz, 1H), 3.43 (d, *J*=11.0 Hz, 1H), 3.28 (t, *J*=6.8 Hz, 2H), 2.26 (t, *J*=7.1 Hz, 2H), 1.82 (qin, *J*=6.8 Hz, 2H), 0.97 (s, 3H), 0.95 (s, 3H); MS (ESI^-) 231.8 (M – 1).

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To 4-amino-n-[2,3-^{3}H]butyric acid (1 mCi, 96.0 Ci/mmol) in 2% aqueous ethanol (1 ml) was added cold 4-amino-butyric acid (1mg, 10μmol) in methanol (100 μl) and the mixture was concentrated to dryness *in vacuo* using a Speedvac. The residue was resuspended in a solution of diethylamine (1.3 μl, 13 μmol) in methanol (100 μl) and to which was added (*R*)-pantolactone (1.7 mg, 13 μmol) in methanol (130 μl). The reaction mixture was heated overnight at 60°C and then concentrated to dryness. The resulting residue was resuspended in water (200 μl) and extracted with dichloromethane (200 μl) to remove any unreacted pantolactone. The aqueous phase was passed through a mini-column of freshly activated Amberlite IR-120 (H^+) ion exchange resin and the eluent was concentrated to dryness to afford [^{3}H]Ho-Pan (4-(2,4-dihydroxy-3,3-dimethylbutylamido)-[2,3-^{3}H]butyric acid.

Pantothenate Kinase Assays

The standard pantothenate kinase assays were performed as described (Rock et al., 2002;Rock et al., 2003). HoPan phosphorylation was determined in a similar reaction mix containing pantothenate (90 μM) or HoPan (400 μM), [γ-^{32}P]ATP (0.25 mM; specific activity 1 Ci/mmol, Amersham), $MgCl_2$ (10 mM), Tris-HCl (0.1 M, pH 7.5), and 40 μg protein from a soluble PanK cell extract for in a total volume of 40 μl. A 10 μl aliquot of the reaction mixture was spotted onto an activated Silica Gel H plate (Analtech), which was developed in butanol/acetic acid/H_2O (5:2:4, v/v/v), and product quantitated using the PhosphoIMager (Molecular Dynamics).

HoPan toxicity in cell cultures and hepatocyte labeling

HepG2 (American Type Culture Collection; ATCC), PC12 (ATCC), or HEK 293T cells were cultured in pantothenate-free DMEM medium containing 1 μM pantothenate and 10% dialyzed FBS in the presence of various concentrations of HoPan. After two doubling times (48 h for HepG2 cells, 30 h for HEK 293T cells, 192 h for PC12 cells) viable cells were counted, which excluded trypan blue.

Whole livers of 2 4-mo old C57BL6 male mice were harvested and rinsed in ice cold buffer A (each liter contains 3.9 g of NaCl, 0.5 g of KCl, 24 g of HEPES, 2.7 g of glucose, the pH was adjusted to 7.6 with NaOH). The liver was chopped with a Vibrotome tissue chopper at a setting of 0.5 mm and resuspend in 20 ml erythrocyte buffer (15 ml of buffer A plus 5 ml of erythrocyte lysis buffer (Qiagen)) in a 125 ml Erlenmeyer flask, gently mixed in the buffer and allowed to settle. The supernatant was carefully pour off and discarded, and the procedure with the erythrocyte buffer was repeated twice. The tissue pieces were then resuspended in 30 ml of digestion buffer (buffer A plus 0.7 g/liter $CaCl_2$, 0.5 mg/ml type I collagenase and 6 μg/ml deoxyribonuclease) and incubated in a water bath shaker at 37°C at 120 rpm for 20 minutes. The tissue pieces were allowed to settle on ice and the supernatant was transferred to a centrifuge tube. The tissue pieces were resuspended in another 30 ml of digestion buffer, the procedures were repeated twice and the 3 supernatants were combined. Cell suspension from the supernatants was filtered through Spectra/Mesh Nylon (41 μm size, Fisher Scientific), and liver cells in the filtrate were centrifuged down at room temperature at 100 x g for 2 minutes. The cell pellet was resuspended in wash buffer (each liter contains 8 g of NaCl, 0.35 g of KCl, 0.16 g $MgSO_4$, 0.18 g of $CaCl_2$, 2.4 g of HEPES, 15 g of bovine serum albumin, the pH was adjusted to 7.4 with NaOH) containing 10% erythrocyte lysis buffer (v/v) and centrifuged down three times.

Mouse liver cells were resupended in phosphate buffered saline containing 0.5% BSA to the density of about 1 x 10^7 cells/ml. For each labeling experiment, 75 μl of the cell suspension was used. The cells were labeled with 2 μM of [^{3}H]pantothenate (50 Ci/mmol) in the presence of 5 μM of hopantenate or PBS (for control). For labeling with [^{3}H]hopantenate (90 mCi/mmol), 500 μl of the cell suspension was used. After 6-hour incubation at 37°C, cells were harvested and washed twice with phosphate-buffered saline. The cells were lysed by sonication in 50 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM DTT, 5 mM EDTA, and 50 mM NaF). The identity of the intracellular metabolites was determined by fractionating the cell lysates by thin-layer chromatography on Silica Gel H plates developed in either solvent I (butanol:acetic acid:water,5:2:3, v/v) or solvent II (ethanol:28% ammonium hydroxide, 4:1, v/v) using the standards described previously (Jackowski and Rock, 1981). Sections (0.5 cm) were scraped from the plate and the radioactivity was quantitiated in 3 ml of scintillation fluid using a liquid scintillation counter.

Animal Experiments

C57BL/6 mice (6-weeks old) were purchased from the Jackson Laboratory and fed on a diet without panthothenate supplement (TD 95248, Harlan) for 2-weeks prior to and during experimentation. Mice were maintained at a room temperature of 72° ± 2°F, room humidity of 50% ± 10%, and a 12-h light, 12-h dark cycle, with the dark cycle starting at 1800 h. For time-to-death assays, calcium (*R*)-HoPan was dissolved in water and administered to 8-week old mice daily by stomach gavage. Control mice were given water or HoPan plus equal amount of pantothenate. In the fasting experiment, after 8-week female mice were administered HoPan at 100 μg/g/day by gavage for a week, one group of mice was starved while the other group was fed as usual. HoPan was administered daily to both groups during the fasting period. Water was supplied *ad libitum*. Blood was drawn either by retro orbital bleed or by cardiac puncture for glucose, lactate and triglyceride tests performed by the Diagnostic Lab of the Animal Research Center, St. Jude Children's Research Hospital. Serum samples from HoPan-treated and control mice were sent to Ani Lytics, Inc for insulin and glucagon measurements. Serum corticosterone levels were determined using the Corticosterone EIA kit according to the manufacture's instruction (Cayman Chemical). All procedures were performed according to St. Jude Children's Research Hospital Institutional Animal Care and Use Committee approved protocols. Liver tissues were fixed in 10% buffered formalin and processed by dehydration in 70% ethanol, absolute ethanol, and then xylene. Tissues were infiltrated and embedded in paraffin, cut at 4 μM, mounted on microscope slides and stained with hematoxylin and eosin.

Pyruvate tolerance test

Female C57BL/6 mice (6-weeks old) fed on low-pantothenate diet (TD 95248, Harlan) for 2 weeks prior to and during experimentation. Calcium (*R*)-HoPan was dissolved in water and administered to 8-week old mice daily by stomach gavage. Controls were given water. After one-week of treatment, the pyruvate tolerance test was performed. Two-month-old female mice (5 per group) were fasted for 14 hr before receiving an intraperitoneal dose of pyruvate (in saline) at 2 g/kg body weight. Plasma glucose levels were measured from the tail blood using a FreeStyle blood glucose monitoring system (TheraSense, Inc) at 0, 15, 30, 45, 60, 90, and 180 min after pyruvate infusion.

Measurement of CoA species in mouse tissues

Extraction of different CoA species from mouse tissues and CoA assays were performed as described with modifications (Knights and Drew, 1988;Rabier et al., 1983). Briefly, mouse tissue (0.1 g) was homogenized in 0.4 ml of chilled 6% perchloric acid containing 28 mM DTT. The precipitant containing the acid insoluble long-chain acyl-CoAs, such as palmitoyl-CoA, was removed by centrifugation (1,500 x g, 4°C, 10 min) and saved for long-chain acyl-CoA determination. Free CoA and short-chain acyl-CoAs, such as acetyl-CoA and malonyl-CoA, were acid soluble and remained in the supernatant, which was split into two tubes, one for free CoA measurement, the other for both free and short-chain acyl-CoAs. To extract free CoA from the supernatant, 0.1 volume of 1 M Tris and 0.3 volume of 2 M KOH were added before the pH was adjusted to 6.5~8.5 with 0.6 N HCl (and 200 mM KOH if necessary). The mix was

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centrifuged (1,500 x g, 4°C, 10 min) and the supernatant was saved for CoA determination. To extract free CoA and short-chain acyl-CoA from the other half of the supernatant, 1 M KOH was used to adjust the pH to 11~12. The mix was incubated at room temperature for 1 h, neutralized with 0.6 N HCl to pH 6.5~8.5, and centrifuged (1,500 x g, 4°C, 10 min). The resultant supernatant was extracted with equal volume of hexane three times before being used in CoA determination assay. Long-chain acyl-CoAs were extracted from the initial perchloric acid precipitated pellet, which was washed with 0.6% perchloric acid containing 5 mM DTT and then washed with 5 mM DTT. The washed pellet was resuspended in 300 μl of 5 mM DTT and the pH was adjusted to 11~12 with 1 M KOH. The mixture was incubated at room temperature for 1 h, neutralized with 0.6 N HCl to pH 6.5~8.5, and centrifuged (1,500 x g, 4°C, 10 min). The resultant supernatant was extracted with equal volume of hexane three times before being used in CoA determination assay. The reaction mixture for the CoA measurement contained 200 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 0.1% Triton X-100, 2 mM EDTA, 20 mM NaF, 2.5 mM ATP, 10 μM [1-^{14}C]lauric acid, 100 ng of *E. coli* acyl-CoA synthetase (FadD) and tissue extract (5 to 20 μl) containing 25–400 pmol of CoA, in a total volume of 100 μl. The reaction was initiated by the addition of FadD, and the reaction mixture was incubated at 35°C for 30 min, followed by the addition of 325 μl of methanol/chloroform/n-heptane (1.41:1.25:1, v/v/v) and 25 μl of 0.4 M acetic acid. After the mixture was mixed vigorously and centrifuged, [1-^{14}C]lauroyl-CoA in the upper phase was quantitated by counting in 3 ml of ScintiSafe 30% using a Beckman LS 6500 scintillation counter. A standard curve using commercial CoA was used to calculate the amount of CoA species in mouse tissue extracts.

Determination of Liver Triglyceride Content

Mouse liver was weighed and approximately 50 mg was extracted as described (Bligh and Dyer, 1959). Lipid mass was detected following chromatography on silica rods by flame ionization using an Iatroscan (Iatron Laboratories, Tokyo) with PEAK SIMPLE software (SRI Instruments, Torrance, CA). Peaks were identified by co-migration with authentic standards. Triglyceride mass was calculated using a standard curve prepared from a neutral lipid mixture (#1129; Matreya, Inc.).

Metabolomics

Specimens of powdered liver tissue were homogenized in deionized water, and tissue extracts were prepared as previously described (An et al., 2004;Jensen et al., 2006). Measurement of acylcarnitines, organic acids and amino acids in tissue extracts was done by direct-injection electrospray tandem mass spectrometry, using a Quattro Micro LC-MS system (Waters-Micromass) equipped with a model HTS-PAL autosampler (Leap Technologies), a model 1100 HPLC solvent delivery system (Agilent Technologies) and a data system running MassLynx software.

Transcriptional profiling

Affymetrix Microarray Analysis of Gene Expression in Livers of HoPan-treated Mice. Samples from the same livers used for the measurement of the distribution of CoA species were used to determine the effect of HoPan treatment on gene expression. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Pelleted RNA was resuspended in nuclease-free water, digested with DNase I to remove contaminating genomic DNA. The integrity of the total RNA was determined using the Agilent Bioanalyzer Lab-on-a-Chip, the RNA sample was converted to cDNA, labeled and fragmented using procedures recommended by Affymetrix. Fragmented labeled cDNA (4.0 μg) was hybridized for 16 hr at 50°C to GeneChip® mouse genome 430 2.0 arrays (Affymetrix). After washing, staining and scanning of the arrays were performed according to the manufacturer’s protocol (Affymetrix), the arrays were analyzed using the GeneChip® Operating Software (GCOS) and global scaling was used to normalize the data from different arrays. Spotfire® DecisionSite® 8.2.1 and the NetAffx™ Analysis Center (Liu et al., 2003) were used to analyze array results. The statistical analyses were from 6 data sets (3 control and 3 HoPan-treated livers) that were independently processed and hybridized.

Statistical analyses

The significance of HoPan treatment was determined using two-tailed, unpaired t-test with the confidence intervals set at 95%. Data with p values less than 0.05 were indicated with an asterisk in the figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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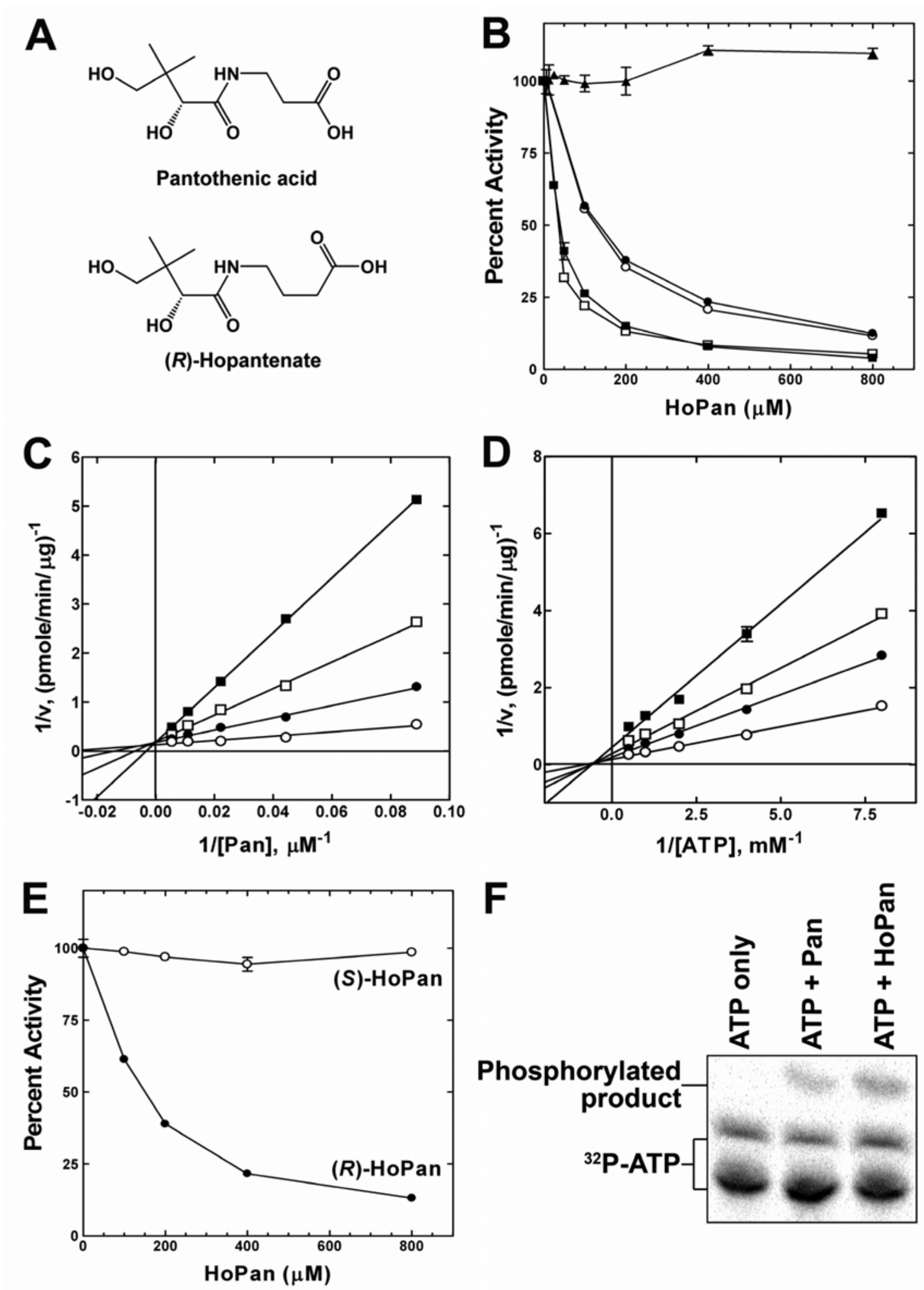

Figure 1. HoPan inhibition of pantothenate kinase

A) In HoPan (hopantenate), the β-alanine moiety of pantothenic acid is substituted with γ-aminobutryic acid, a neurotransmitter, that contains an additional methylene group.

B) HoPan inhibited mouse PanK1α (●), PanK1β (○), mPanK2 (■) and PanK3 (□) with IC_{50} values between 50 and 150 μM. The prokaryotic *E. coli* PanK (▲) was refractory to HoPan inhibition. Data presented are means with standard errors unless otherwise indicated.

C and D) Kinetic analysis of the inhibition of PanK1α by HoPan illustrated that HoPan inhibition was competitive with pantothenate (C), and noncompetitive with respect to ATP (D). The mode of inhibition was determined at several HoPan concentrations, 300 μM (■), 150 μM (□), 75 μM (●) and 0 μM (○). The mode of HoPan inhibition of PanK1β was the same as PanK1α (Figure S1).

E) (*R*)-HoPan (●) inhibited mPanK1α, but (*S*)-HoPan (○) was not active.

F) PanK assays performed using [^{32}P]ATP showed that HoPan was phosphorylated by mPanK1α as well as the authentic pantothenate substrate. Products were detected by thin-layer chromatography as described under "Experimental Procedures."

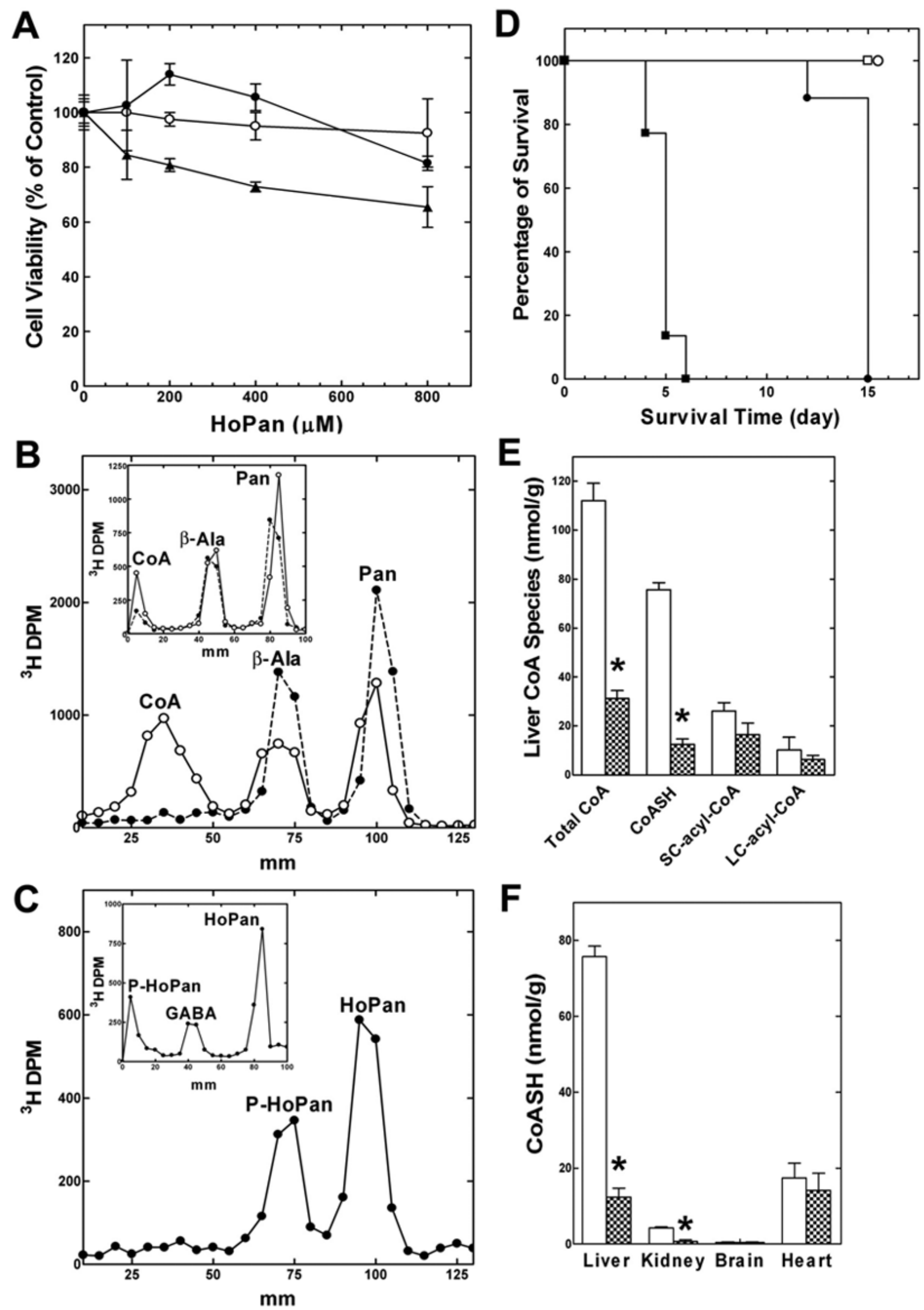

Figure 2. Toxicity of HoPan

A) HoPan was not toxic to cultured cells. HepG2 (●), HEK 293T (○) and PC12 (▲) cells were treated with HoPan at the indicated concentrations (800 μM, 400 μM, 200 μM and 100 μM). Viable cells excluded trypan blue stain and were counted after two doubling times. The number of viable cells is presented as the percentage of the control cells treated with DMSO (vehicle alone).

B) HoPan inhibition of CoA synthesis in isolated hepatocytes. Hepatocytes were isolated from male mouse liver and incubated with [^{3}H]pantothenate in the presence (●) and absence (○) of 5 μM HoPan. Hepatocytes were extracted and fractionated by thin-layer chromatography on

Silica Gel H layers developed in solvent I (butanol:acetic acid:water, 5:2:3, v/v) and solvent II (ethanol:28% ammonium hydroxide, 4:1, v/v) (inset). Pan, pantothenate; β-Ala, β-alanine.
C) HoPan was phosphorylated in vivo. Hepatocytes were labeled with [^{3}H]HoPan and the HoPan-derived intermediates were separated on TLC developed in solvent I and solvent II (inset). P-HoPan, phosphorylated HoPan; GABA, γ-amino-isobutyric acid.
D) Survival of C57BL/6 mice (male (■), n=22, p<0.0001; female (●), n=17, p<0.0001) treated with HoPan (100 μg/g/day) compared to mice treated an equal amount of HoPan plus pantothenate (male (□), n=9; female (○), n=9).
E and F) CoA levels in HoPan-treated (hatched bars) and control (open bars) mouse tissues. (E) shows the distribution of CoA species in male liver in control and HoPan-treated mice. HoPan treatment significantly reduced the levels of total CoA (p<0.0001) and CoASH (p<0.0001) in liver. (F) shows the CoASH levels in four representative mouse tissues. There was a significant drop of free CoA levels in liver and kidney (p < 0.0001), but not in brain or heart of HoPan-treated mice.

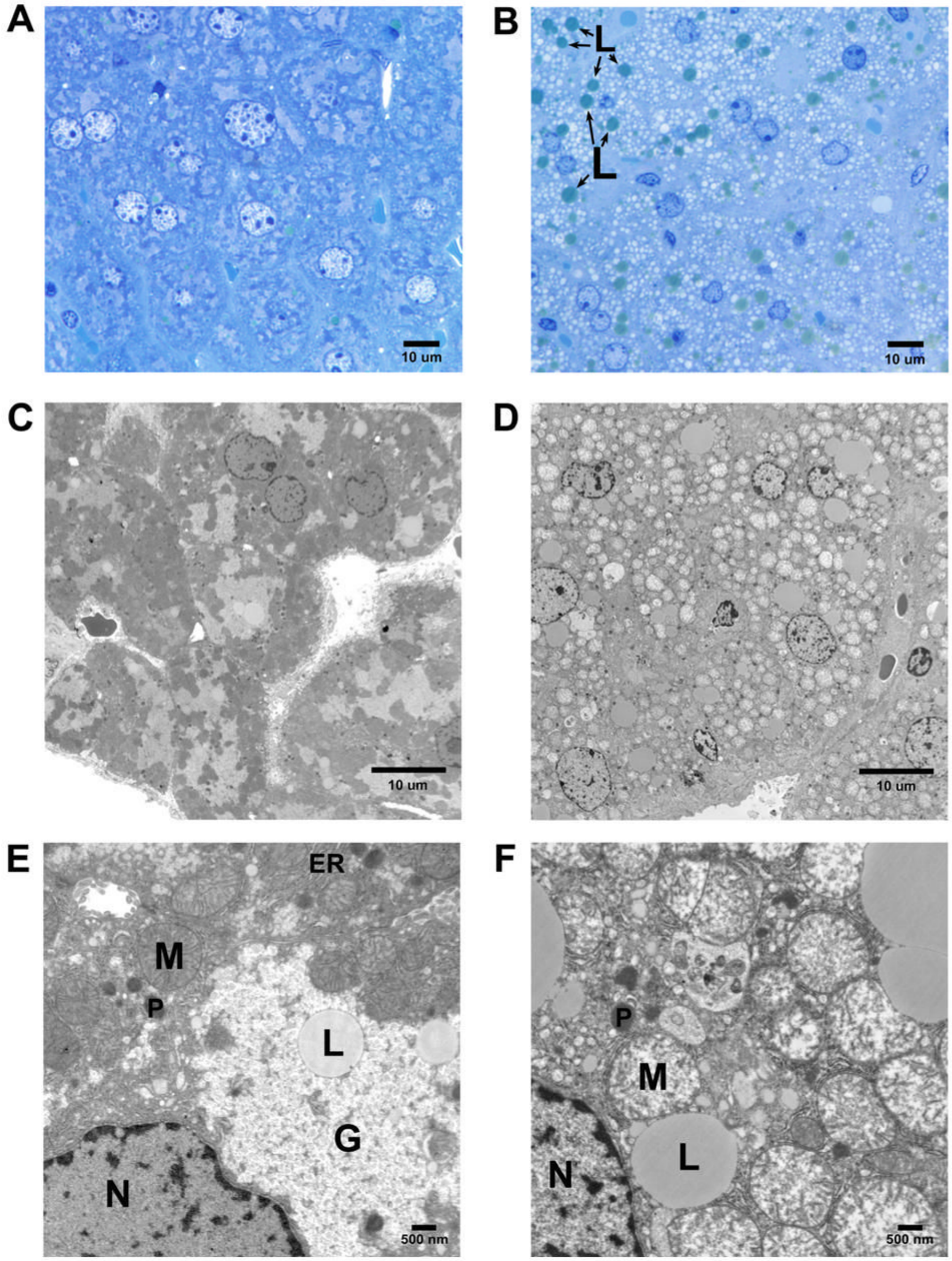

Figure 3. Liver pathology in HoPan-treated mice

Liver sections from a control male mouse (A) and a HoPan-treated mouse (B) stained with toluidine blue. Transmission electron microscopy at two different magnifications of control male mouse liver (C and E) and HoPan-treated liver (D and F). Abbreviations are: L, lipid droplet; ER, endoplasmic reticulum; M, mitochondria; P, peroxisome; N, nucleus; and G, glycogen storage granules.

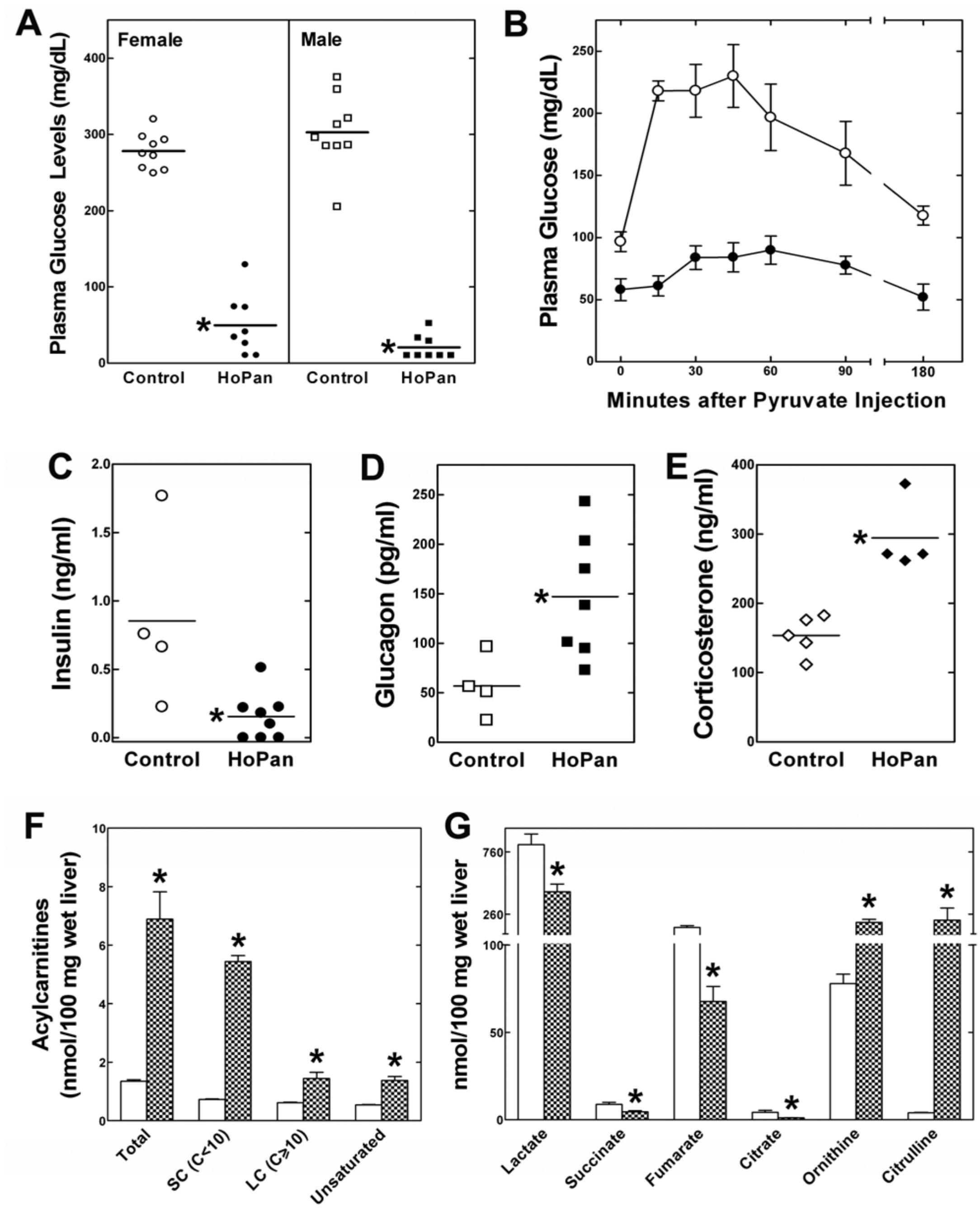

Figure 4. Metabolic imbalances in HoPan-treated mice

A) Plasma glucose levels of HoPan-treated mice (female (●), n=8, p<0.0001; male (■), n=8, p<0.0001) compared to mice treated with an equal amount of HoPan plus pantothenate (female (○), n=9; male (□), n=9). Lines represent the means of each treatment group.

B) Pyruvate tolerance test in male mice (n=5 per group) treated with HoPan (●) or water (○). Pyruvate injection significantly increased blood glucose levels in control mice in 15 min (p<0.0001) whereas no significant increase was observed in HoPan-treated mice.

C, D and E) Serum insulin (C), glucagon (D) and corticosterone (E) levels in HoPan-treated male mice. Closed symbols (●, ■, ◆) designate HoPan-treated mice and open symbols (○, □,

◇) designate the controls. The changes of serum insulin (p=0.014), glucagon (p=0.026) and corticosterone (p=0.0013) levels in HoPan-treated mice were statistically significant.
F and G) Alterations of liver acylcarnitines (F) and organic acids (G) in HoPan-treated mice. *Indicates $p < 0.05$. A complete list of the panel of acylcarnitines and organic acids analyzed, their absolute tissue concentrations in the control and treated mice, and the statistical significance of the differences are found in supplemental materials Table S1. Open bars are the controls and hatched bars are the HoPan-treated mice.

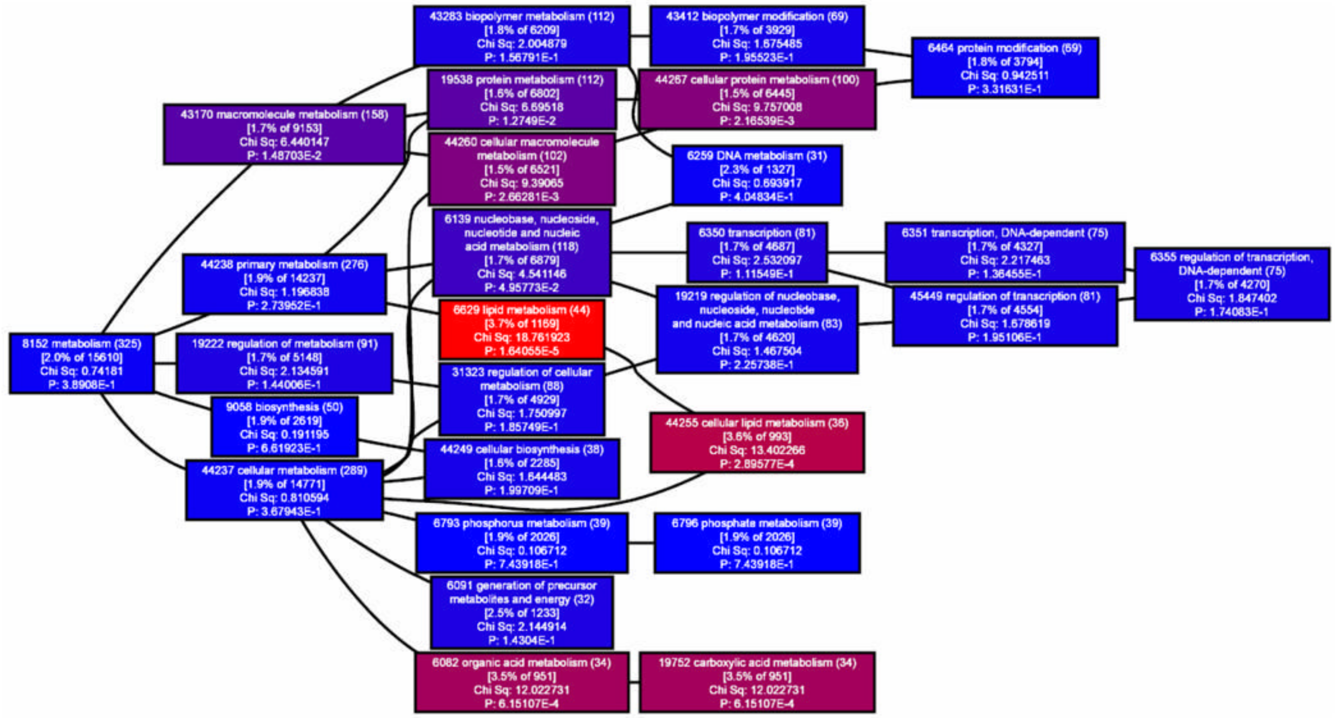

Figure 5. Gene expression alterations in HoPan-treated mice

The biological processes that correlated with the most significant changes in gene expression by HoPan treatment. 772 probe sets that showed greater than 2 fold regulation, p<0.05 and FDR q<0.05 were used in the gene ontology analysis. The graph was obtained using the NetAffx™ Analysis Center. Each box (node) represented one annotated biological process. The probe set count threshold for each node was set at 30. The color of the node was based on Chi square values: red indicates the most significant change, blue the least. A complete list of genes significantly regulated by HoPan treatment is presented in Table S2.

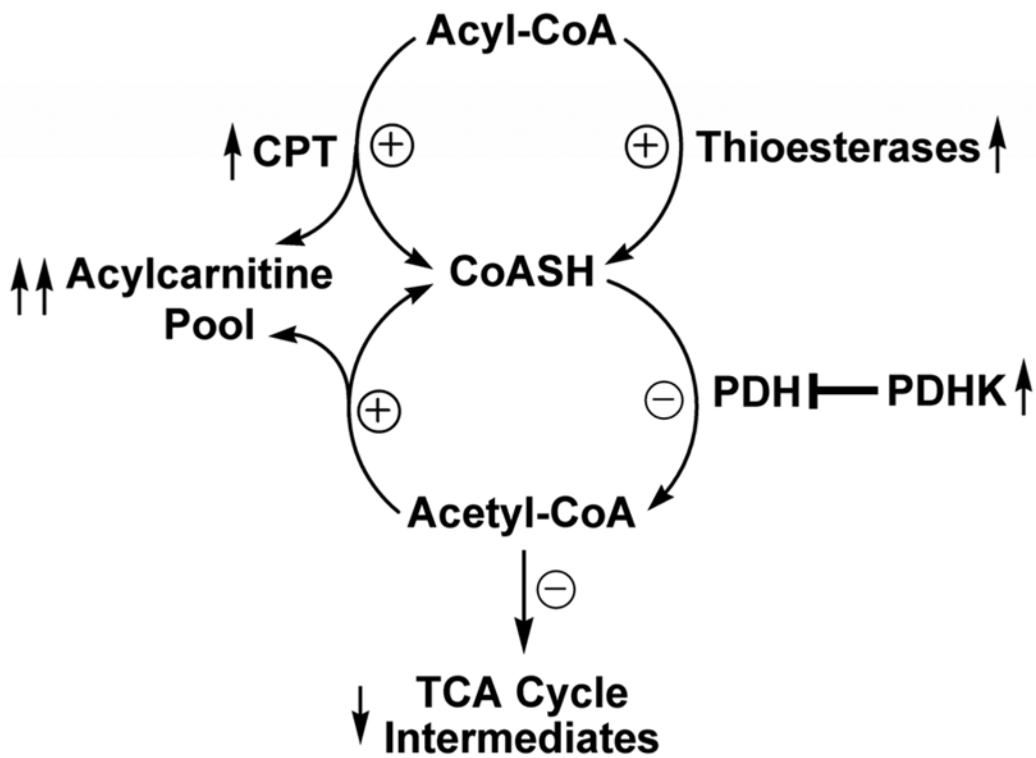

Figure 6. Mechanisms buffering the level of CoASH in liver

Depletion of total hepatic CoA leads to a series of events that combine to spare the CoASH pool. The fall in acetyl-CoA leads to a decrease in mitochondrial energy generation via the TCA cycle reflected by the decrease in the concentration of 4-carbon organic acids participating in the cycle. CoASH conversion to acetyl-CoA is blocked both by the transfer of acetyl groups into the acetylcarnitine pool and the induction of pyruvate dehydrogenase kinase (PDHK) that phosphorylates and inactivates pyruvate dehdrogenase (PDH) shutting off glucose entry into the TCA cycle. Long-chain acyl-CoAs are removed by the elevated expression of thioesterases and carnitine palmitoyltransferase (CPT) that shuttles acyl moieties to the acylcarnitine pool.