Atlas of exercise metabolism reveals time-dependent signatures of metabolic homeostasis

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In brief
Tissue sensitivity and response to exercise vary according to time of day. Sato et al. present an atlas of exercise metabolism, including global metabolomics profiling of multiple tissues, combined with arteriovenous sampling of hindlimb muscle and sampling across the liver to verify net uptake and release of time- and exercise-dependent signaling biochemicals.

Highlights
- Time of exercise defines system-wide activation of energy metabolism
- Exercise timing rewires intra-tissue and inter-tissue metabolite correlations
- Maintenance of inter-tissue metabostasis is specified by exercise time
- Comparative analyses reveal time- and tissue-dependent exerkines
Resource

Atlas of exercise metabolism reveals time-dependent signatures of metabolic homeostasis

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SUMMARY

Tissue sensitivity and response to exercise vary according to the time of day and alignment of circadian clocks, but the optimal exercise time to elicit a desired metabolic outcome is not fully defined. To understand how tissues independently and collectively respond to timed exercise, we applied a systems biology approach. We mapped and compared global metabolite responses of seven different mouse tissues and serum after an acute exercise bout performed at different times of the day. Comparative analyses of intra- and inter-tissue metabolite dynamics, including temporal profiling and blood sampling across liver and hind-limb muscles, uncovered an unbiased view of local and systemic metabolic responses to exercise unique to time of day. This comprehensive atlas of exercise metabolism provides clarity and physiological context regarding the production and distribution of canonical and novel time-dependent exerkine metabolites, such as 2-hydroxybutyrate (2-HB), and reveals insight into the health-promoting benefits of exercise on metabolism.

INTRODUCTION

Circadian clocks orchestrate rhythmic biological processes, including metabolism, hormone production, immunity, and behavior. Temporal gating of physiology is essential for maintaining homeostasis, and chronic disruption of circadian alignment causes metabolic diseases (Masri and Sassone-Corsi, 2018). Light is the main zeitgeber (time giver) communicating external time to the central clock in the hypothalamic suprachiasmatic nucleus (SCN). Rhythmic feeding and locomotor activity, both SCN-controlled processes, entrain peripheral clocks in metabolic tissues independently of light (Damiola et al., 2000; Kemler et al., 2020; Small et al., 2020; Wolff and Esser, 2012). Timing of food intake can rewire temporal coordination of metabolism and gene expression and thereby modify disease progression (Hatori et al., 2012; Hawley et al., 2020; Lundell, 2020).
Figure 1. Multitissue metabolomics upon exercise at the early rest (ZT3) versus early active phase (ZT15)

(A) Study design. Skeletal muscle, serum, liver, heart, hypothalamus (HT), epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), and brown adipose tissue (BAT) were harvested after acute exercise at ZT3 or ZT15 for metabolomic analysis.

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et al., 2020). Circadian control of systemic energy homeostasis and behavioral activity can also be entrained by exercise in a time-of-day-specific manner (Sato et al., 2019; Schroeder et al., 2012). Thus, food restriction, exercise, or energetic stressors that influence the temporal regulation of metabolism are relevant for glycemic control and weight loss in type 2 diabetes and obesity.

Exercise performed at different times of day influences skeletal muscle metabolic pathways and endurance capacity (Ezagouri et al., 2019; Sato et al., 2019). Thus, the timing of exercise may specify close alignment between tissue clocks and promote coherent and efficient temporal gating of metabolic processes. While the major effects of exercise on energy metabolism are well defined (Egan and Zierath, 2013; Hawley et al., 2014), a comprehensive view of how exercise timing determines tissue-specific metabolic adaptations and system-wide tissue coordination is lacking.

Here, we present an atlas of exercise metabolism, including global metabolomics profiling of multiple tissues and serum, and arteriovenous sampling of hindlimb muscle and sampling across the liver to verify net uptake and release of time- and exercise-dependent signaling biochemicals (exerkines). We show how timed exercise rewrites tissue-specific and systemic metabolic and highlight differential tissue production and distribution of exerkines. We also validate 2-hydroxybutyrate (2-HB) as a time-dependent exerkine at the systemic level. Our resource provides physiological context about diurnal production and distribution of a wide range of signaling metabolites linked to sleep, memory, energy homeostasis, endurance capacity, and performance.

RESULTS

Time of exercise determines magnitude and type of metabolic response

To determine how timing of exercise impacts local tissue and systemic metabolism, we collected serum, skeletal muscle (gastrocnemius), liver, heart, hypothalamus (HT), epididymal white adipose tissue (eWAT), inguinal subcutaneous white adipose tissue (iWAT), and interscapular brown adipose tissue (BAT). Exercise or control sham exercise was performed for 1 h on a treadmill at either ZT3 (early light/rest phase) or ZT15 (early dark/active phase), and tissues were collected at ZT4 or 1 h on a treadmill at either ZT3 (early light/rest phase) or ZT15, indicating a greater reliance on fatty acid (FA) oxidation and increased buffering against metabolic stress during exercise performed in the early active phase (Figure 1E). Hypothalamic neurotransmitters serotonin, dopamine, and its catabolite homovanillate (HVA) increased by exercise at ZT15 (Figure 1E). Data were stratified to identify unique metabolites changing according to time of exercise and tissue (Data S4), allowing for the identification of tissue-specific biomarkers.

Systemic activation of metabolic pathways in response to timed exercise

Functional enrichment of metabolites changed by exercise at different times of day

To identify which metabolic pathways were directly impacted by exercise, or in a time- and tissue-specific manner, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis on significantly altered metabolites (Figure 2A). Exercise

(B) Detected biochemicals including named and unnamed biochemicals.
(C) PCA plots of tissue-specific samples. Color of dots refers to time and shape refers to sedentary or exercise conditions.
(D) Volcano plots, Venn diagrams, and bar charts of changed metabolites.
(E) Biological classification of altered metabolites. n = 5–6 per group; altered metabolites determined by two-way ANOVA, p < 0.05. Sed, sedentary; Exe, exercise.
Figure 2. Spatiotemporal impacts of exercise on metabolism

(A) KEGG enrichment analysis identifying time-dependent impact of exercise on local and systemic metabolism. Tissue-specific dotplots show regulation of metabolites mapped to specific pathways. Log2 FC (fold change) refers to exercise-induced metabolite regulation. Size and color of dot indicate significance of regulated (—log10(P)) metabolites. Gray dots refer to metabolites mapped to the pathway but not significantly regulated. Upper white and bottom gray areas indicate metabolites changed by exercise at ZT3 and exercise at ZT15, respectively. Significance of pathway enrichment is displayed on the right dotplot. Color of dots refers to tissue, and size refers to enrichment significance (—log10(p)). Significant (p < 0.001) pathways are shown.

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at ZT15 elicited a robust enrichment of pathways involved in nucleotide metabolism (purine and pyrimidine) and AA metabolism (glycine, serine and threonine, phenylalanine, cysteine and methionine, arginine and proline, beta-alanine, and taurine and hypotaurine). Metabolites related to biosynthesis of unsaturated FAs were increased by exercise at ZT15 in both eWAT and iWAT. The liver showed distinct FA metabolism responses, with FA metabolites increased selectively by ZT3 exercise. This highlights a coregulation of lipid degradation pathways according to exercise time. Carbohydrate metabolism was increased in the liver during exercise at ZT3, indicating preferential utilization of glycogen. AA pathways were enriched in skeletal muscle by exercise at ZT15, including cysteine and methionine, along with purines. Serum exhibited the most striking response to exercise, preferably at ZT15, possibly because serum metabolites are integrative and systemic outcomes of the metabolic responses to exercise in each tissue.

**Glycogenolysis and glycolysis**

Muscle glycogen content was reduced after exercise at ZT15, but not ZT3 (Figure S2A), confirming our earlier findings (Sato et al., 2019). Hepatic glycogen content exhibited robust time-of-day variation. Accordingly, exercise at ZT3 decreased liver glycogen content, whereas at ZT15 glycogen stores were already low. Skeletal muscle and liver were the only tissues to show reduced glucose levels after exercise at ZT15 (Figure S2B). Glycolysis metabolites were also decreased after exercise at ZT15 in muscle but not in liver (Figure S2C), suggesting that during fasted conditions, exercise leads to a greater activation of muscle glycolysis with energy supplied from liver gluconeogenesis but not via hepatic glycogen breakdown. This was supported by gene expression profiles: muscle genes involved in glycolysis were increased after exercise at ZT15 (Figure S2D), whereas liver genes associated with gluconeogenesis were increased upon exercise at ZT3 (Figure S2E). Thus, glucose production from liver through glycolysis and gluconeogenesis may be sufficient during early rest phase exercise at ZT3. Glucose production from the liver may be suppressed during early active phase exercise at ZT15, leading to greater reliance on muscle glycogen.

**AA metabolism**

Since the KEGG enrichment pathway analysis indicated that AA metabolism exhibited a robust time-of-day-dependent impact of exercise, we explored tissue and time dependencies of exercise metabolism. Heatmaps displayed global AA metabolites after exercise, we explored tissue and time dependencies of exercise metabolism exhibited a robust time-of-day-dependent impact of exercise. To understand which metabolic processes might be coupled according to time of exercise, we compared significantly altered metabolites between tissues. Pie charts showed only a marginal overlap (Figure S3A), indicating that exercise-induced metabolic alterations are tissue dependent. However, correlating metabolites commonly changed by exercise suggested more coordinated responses (Figure S3A). Upon exercise at ZT3, liver metabolites were less correlated with other tissues, whereas serum and BAT showed greater correlations with other tissues after exercise at ZT3. Muscle and liver metabolites were highly correlated upon exercise at ZT15 but attenuated after exercise at ZT3. The combination of eWAT and other tissues also exhibited a higher correlation coefficient at ZT15, pointing to a distinct metabolic response of eWAT according to exercise time. Metabolites commonly responding to exercise at ZT3.
Figure 3. Exercise rewires intra- and inter-tissue metabolic correlations

(A) Summary of inter-tissue metabolite correlations. Colored circles refer to tissue and metabolite classes. Ribbons connecting metabolite inter-tissue classes refer to significant correlations between metabolites. Ribbon color refers to correlation coefficient sign (red, positive; blue, negative). Ribbon thickness refers to number of significantly correlated metabolites. The clock icon indicates the timing of exercise (filled triangles) and tissue collection (opened triangles).

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were more enriched with AAs than with lipids, while exercise at ZT15 increased enrichment of AAs and lipids. This highlights greater systemic coordination of AA and lipid metabolism during ZT15 exercise. Carbohydrates were highly enriched within correlated metabolites between muscle and liver after ZT15 exercise. Thus, metabolic pathways are activated in a time- and tissue-dependent manner.

We then focused on metabolites changed within specific tissues or combinations of tissues (Figures S3B and S3C). After exercise at ZT3, the concentration of 14 metabolites (mostly lipids) was changed only in the liver and serum, while 6 AAs unique to serum and heart were changed. The correlation of metabolites altered within serum and liver and within serum and heart was significant (Figure S3D). In response to exercise at ZT15, several metabolites were altered that were unique to serum, liver, or muscle (Figure S3C), with a group of carbohydrates enriched the most. Distinct clusters related to glycogen and glucose metabolism were among metabolites changed between muscle and liver after exercise at ZT15 (Figure S3E), pointing to the activation of glycogenolysis and glycolysis as a common metabolic hallmark. Metabolites commonly changed in serum and tissues were predominately lipids and AAs (Figure S3C), including a common group of lipids in serum and iWAT after ZT15 exercise (Figure S3E). We found systemic and more coordinated activation of lipid and AA metabolism, as well as local activation of carbohydrate metabolism specific to muscle and liver after exercise in the fasted state. 

Exercise rewires intra-tissue and inter-tissue metabolite correlations

Mapping all pair-wise metabolite correlations (Figure 3A), exercise mainly enhanced interactions among serum, heart, liver, and muscle. These differences reflect broad exercise-dependent shifts in tissue coordination in support of energy substrate supply and demand. To reveal underlying metabolic relationships, we combined all tissues and constructed undirected condition-specific correlation networks (Figure 3B). Sedentary and exercise groups shared a similar number of nodes (~3,000 metabolites correlated across tissues) and edges (~9,000 correlations) (Figure 3B). More cross-tissue correlations were found after exercise (Figure 3C). Examining intra-tissue correlations, ~50%–60% nodes were common to sedentary and exercise groups (eWAT as the exception), while only ~20% were specific to either condition (Figures 3D and S4A–S4D; Data S5). Edges reflected exercise-induced metabolic rewiring, with few shared connections between sedentary and exercise conditions. Exercise reprogrammed interactions and reduced overall connectivity within BAT and HT, whereas muscle, heart, iWAT, and serum retained connectivity. Metabolite connectivity was increased within the liver and eWAT, underscoring its prominent role as a metabolic hub; liver node “degree distribution” (edges per node) and “closeness distribution” (centrality of nodes corresponding to the whole network) were increased by exercise (Figure 3E). Integrating networks into a metabolite “correlation universe” revealed how networks within and among tissues are rewired by exercise. Nodes with the highest connectivity were major points of diversions among conditions (Figures 3F and S4D). Inspection of the top commonly reprogrammed nodes revealed that exercise increased connectivity among metabolites involved in AA and lipid metabolism. These include N-acetylleucine, 2-HB/2-hydroxyisobutyrate, ketone body BHB, carnitine synthesis metabolite hydroxy-N6,N6,N6-trimethyllysine, the uremic toxin phenol sulfate, and the purine nucleotide guanine. Exercise reduced connectivity of the tryptophan catabolite and neurotoxin quinolinolate, as well as N-acetylglycine, and a variety of FAs and glycerophospholipids (Figures S4E and S4F).

Exercise at ZT15 selectively strengthens skeletal muscle-liver 24-h tissue correlations

To investigate how time and exercise interact to determine intra-tissue and inter-tissue metabolite temporal correlations, we focused on muscle and the liver, major organs involved in energy homeostasis during exercise and food restriction. We calculated pair-wise metabolite temporal correlations within and between tissues collected at 4-h intervals across 24 h after an exercise bout performed at ZT3 or ZT15 versus sedentary controls (Figures S5A and S5B). Exercise timing determined the number (Figures S5C–S5F), amplitude (Figure S5G), phase (Figures S5H and S5I), and class (Figures S5J and S5K) of 24-h oscillating liver metabolites (Data S2 and S6). Comparing 24-h metabolomes further revealed tissue-, time-, and exercise-dependent alterations (Figure S5L).

The liver and muscle shared fewer correlations under sedentary conditions, with ZT3 sharing the fewest (Figures 4A and 4B; Data S6). Exercise increased temporal correlations between lipids, AAs, nucleotides, and carbohydrates, but the effect was greater at ZT15. Constructing 24-h correlation networks for each condition (Figure 4B), we visualized how exercise increased overall metabolite connectivity between tissues in a time-dependent manner. We detected a similar range of ~600–700 correlated metabolites under sedentary and exercise conditions. Edges were highly context dependent, with 1,411 (ZT3) and 1,583 (ZT15) temporal correlations detected under sedentary conditions, and 2,077 (ZT3) and 3,019 (ZT15) detected after exercise. Most nodes were common between conditions, and yet, the edges were highly context dependent (Figure 4C).
Figure 4. Exercise time determines muscle-liver 24-h temporal correlation

(A) Muscle-liver metabolite correlations according to condition and time. Colored circles refer to tissue and metabolite class. Ribbons refer to correlations between metabolites. Clock icons indicate the time of exercise (filled triangles) and tissue collection (opened triangles) during ZT3 and ZT15.

(B) Networks of muscle-liver correlated metabolites. Each node refers to a metabolite, shape indicates tissue, and color refers to metabolite class. Edges are drawn for each intra-tissue or inter-tissue correlation, and color refers to correlation coefficient sign (red, positive; blue, negative). Table shows total number of nodes (metabolites) and edges (significant correlations) in muscle and liver according to condition and time.

(E) Rewired Muscle-Liver metabolic network

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Node degree and closeness were increased by exercise at ZT15 (Figure 4D), highlighting distinct timing differences in metabolite response and network wiring (Figure 4E). Our data reveal how intra-tissue and inter-tissue metabolism are differentially poised to both react and respond to exercise at different times of the day.

**Time and tissue dependency of exercise-induced metabolites**

Our resource allows for comparison of metabolite exerkines and provides context about their production and distribution in a time- and tissue-dependent manner. We noted distinct baseline, exercise time-, and tissue-dependent differences among established exerkines, including lactate, gamma-aminobutyrate (GABA), 3-aminoisobutyrate (BAIBA), kynurenine, kynurenate, 5-aminomimidazole-4-carboxamide ribonucleotide (AICAR), and α-ketoglutarate (α-KG) (Figure 5A), with greater responses at ZT15. Lactate was reduced, whereas α-KG and kynurene increased in muscle and the liver at ZT15. Kynurene was increased by exercise only in liver at ZT15. BAIBA was increased by exercise in serum, liver, and HT at ZT15. GABA was detected in muscle and HT but was increased by exercise only in muscle. AICAR was increased more by exercise at ZT3 in the heart, as was serum α-KG.

Exercise at ZT15 appeared to preferentially direct skeletal muscle S-adenosylmethionine (SAM) metabolism toward adenine nucleotide salvage, transsulfuration pathways linked to glutathione (GSH) production, and maintenance of cytosolic NAD+/NADH (Figures 5B and 5C). Exercise at ZT15 reduced SAM levels in muscle, liver, and heart. In muscle, exercise reduced downstream SAM metabolites S-adenosylhomocysteine (SAH), 5-methylthioadenosine (MTA), adenosine, and GSH, while the ratio of oxidized to reduced glutathione (GSSG/GSH) was increased at ZT15. Muscle adenine and 5′-AMP were increased by exercise only at ZT15. Adenine conversion to AMP can be increased by availability of 5-phosphoribosyl-1-pyrophosphate (PRPP), which at ZT15 was increased in muscle under sedentary conditions and reduced by exercise. We confirmed the specificity of this metabolic signal by immunoblot analysis of activated AMPK (Figure 5D). Skeletal muscle contains a large pool of adenine nucleotides and maintaining sufficient ATP levels is important to sustain contractions. To avoid metabolic stress and fatigue, adenine nucleotide degradation must be balanced by increased salvage and de novo synthesis (Braught and Terjung, 2001). Our results suggest exercise-induced adenine nucleotide salvage is regulated locally within the muscle in a time-dependent manner.

To further clarify the time-dependent activation of skeletal muscle AMPK upon exercise, we determined how the nutritional state influences adenine nucleotide metabolism. To mimic exercise under different nutritional states, myotubes were subjected to electrical pulse stimulation (EPS) in the absence of glucose. We measured adenine nucleotides and adenosine using liquid chromatography-mass spectrometry (LC-MS) (Figure 5E). AMP production was elevated by EPS in glucose-depleted conditions, consistent with our finding that exercise at ZT15 increased muscle AMP levels and AMPK activation (Figures 5C and 5D). ATP regeneration was attenuated by EPS in glucose-depleted conditions.

Liver and skeletal muscle are producers and net exporters of circulating GSH (Kretzschmar and Muller, 1993; Sen, 1998). GSH production depends on cysteine generated from transsulfuration of homocysteine, with increased flux shown to delay ageing and increase lifespan (Sbodio et al., 2019). SAH produced from SAM-mediated methylation reactions is rapidly converted to homocysteine, which, in the presence of sufficient methionine and vitamin B6, proceeds via cystathionine toward the formation of 2-ketobutyrate (2 KB; also known as 2-oxobutyrate), cysteine, and ammonia (Figures 5B and 5C). As a structural analog of pyruvate, 2-KB is readily reduced to 2-HB by lactate dehydrogenase, causing regeneration of NAD+ since this is a near-equilibrium reaction (Rogatzi et al., 2015), exercise-induced accumulation of 2-HB likely reflects a combination of factors, including cytosolic redox imbalance and energy stress. 2-HB was proposed as a specific in vivo biomarker for NAD+/NADH ratio in liver (Goodman et al., 2020), but since it showed time-dependent accumulation in all tissues studied, it may be a general indicator of systemic NAD+/NADH.

**Time-of-day- and exercise-dependent arteriovenous balance of metabolites**

To gain insight into dynamic metabolite exchange between tissues, we exercised mice at different times of the day and profiled arteriovenous differences in metabolites across hindlimb muscles and the liver (portal to hepatic vein). We collected (1) serum from the portal vein and vena cava (mainly liver veins) and liver and (2) arterial and venous serum and skeletal muscle (Figure 6A).

We detected hundreds of metabolites and related pathways with time- and exercise-dependent arteriovenous (A/V) differences in each tissue (Figures 6B–6D, S6A, and S6B; Data S2). At ZT3, the liver was a net exporter of AAs, lipids, nucleotides, carbohydrates, TCA (tricarboxylic acid) cycle intermediates, and a variety of vitamins and cofactors, and an importer of lipids, AAs, and nucleotides (Figure 6B). Liver uptake and release were increased by exercise at ZT3 but reduced at ZT15. Muscle was a net importer of AAs, lipids, carbohydrates, nucleotides, and other energy metabolites while releasing ~20%–50% fewer metabolites than consumed. Net changes in muscle metabolite balance were minimal under sedentary conditions at ZT15, and yet, with exercise, the net metabolite uptake increased to ZT3 levels. Exercise increased net muscle metabolite release at both time points. This likely reflects time- and exercise-associated changes in blood flow (Bartman et al., 2021) and highlights...
Figure 5. Tissue- and time-dependent links between SAM metabolism, adenine salvage, GSH production, and maintenance of cytosolic NAD+/NADH

(A) Exerkine metabolite plots. Individual data points are colored according to tissue and represented as mean ± SEM (n = 5–6) and analyzed by two-way ANOVA (sedentary versus exercise at corresponding time), *p < 0.05, **p < 0.01, and ***p < 0.001. GABA, gamma-aminobutyrate; BAIBA, 3-aminoisobutyrate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; ω-KG, ω-ketoglutarate.

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the importance of physical activity in the production and release of muscle-derived biomolecules.

Visualizing all A/V muscle and liver metabolites with correlations derived from tissue metabolite profiling (Figure 4A) revealed the direction of metabolite flow between tissue in relation to enzymes that direct metabolic pathways at different time points. While liver release was significant under exercise and sedentary conditions, muscle release was significant only after exercise. Net liver release of spermidine was significant only under sedentary conditions, whereas net muscle release was significant only after exercise.

A/V data also revealed regional differences in circulating metabolite concentrations, with relatively high concentrations of portal vein short-chain FAs like butyrate/isobutyrate, which are produced mainly in the colon by bacterial fermentation. The highest butyrate/isobutyrate levels were detected in the portal vein at ZT15 in sedentary mice (Figure 6I), with low net export from the liver under all conditions. Muscle was a net exporter of butyrate/isobutyrate after exercise at ZT15, although relative concentrations were much lower than that in the portal vein.

Time-of-day-dependent role of 2-HB in systemic and local tissue metabolism

We selected 2-HB/2-hydroxyisobutyrate for further investigation since this metabolite emerged as a central systemic metabolic hub, connecting metabolites in a time- and exercise-dependent fashion (Figure S4E). 2-HB and 2-hydroxyisobutyrate (2-HIB) are isobaric isomers. To clarify this signal and to quantitatively validate additional hits, we used targeted gas chromatography-mass spectrometry (GC-MS) with spiked internal standards. Endogenous 2-HB was elevated in the liver and muscle following exercise at ZT15 (Figure 7A). The liver showed higher levels (±0.01–0.2 μmol/g) than muscle (±0.002–0.03 μmol/g). These concentrations and dynamics resembled AAs, including BCAAs and threonine. The ketone body BHBI showed similar dynamics, albeit at 10× higher levels. 2-HIB was unaltered in response to time or exercise, and concentrations remained near the limit of detection (LOD) and ~20× lower than 2-HB. Thus, exercise increased the production of 2-HB in a time-dependent manner. Applying unsupervised clustering of targeted metabolomics data (Figure 7B), 2-HB was highly correlated with other hydroxybutyric acids, and biomarkers for diabetes (Newgard et al., 2009; Nilsen et al., 2020; Wang et al., 2013), yet inversely correlated with hexoses.

To understand how temporal 2-HB dynamics are impacted by exercise (Figure 7C), we compared baseline data from sedentary conditions except for exercise at ZT15, whereas muscle showed net lactate release only after exercise at ZT15 (Figure 6H). TCA cycle intermediates showed heterogeneous responses (Figure S6E). α-KG showed net uptake by the liver under all conditions and time points. While α-KG trended toward net muscle release, this was significant only at ZT3. Succinate showed net liver release under all conditions, and yet, the only significant change in muscle was the net uptake after exercise at ZT15. Fumarate and malate showed similar trends of net release by the liver, but release by the muscle was only significant after exercise at ZT15. These changes reflect a flexible, dynamic, and context-specific flux into central metabolic pathways at different time points.

We noted time- and exercise-dependent A/V differences for metabolites associated with healthy aging (Figure S6F). Liver and muscle were both net exporters of the NAD+ precursor nicotinate riboside. While liver release was significant under exercise and sedentary conditions, muscle release was significant only after exercise. Net liver release of spermidine was significant only under sedentary conditions, whereas net muscle release was significant only after exercise.

To understand how temporal 2-HB dynamics are impacted by exercise (Figure 7C), we compared baseline data from sedentary conditions except for exercise at ZT15, whereas muscle showed net lactate release only after exercise at ZT15 (Figure 6H). TCA cycle intermediates showed heterogeneous responses (Figure S6E). α-KG showed net uptake by the liver under all conditions and time points. While α-KG trended toward net muscle release, this was significant only at ZT3. Succinate showed net liver release under all conditions, and yet, the only significant change in muscle was the net uptake after exercise at ZT15. Fumarate and malate showed similar trends of net release by the liver, but release by the muscle was only significant after exercise at ZT15. These changes reflect a flexible, dynamic, and context-specific flux into central metabolic pathways at different time points.
Figure 6. Arteriovenous (A/V) differences of liver and muscle in response to timed exercise
(A) Study design. Tissues were collected immediately after exercise at ZT3 or ZT15. For sampling across the liver, serum was collected from the portal vein and caval vein. For hindlimb muscle A/V sampling, serum was collected from the abdominal aorta (abd. aorta) and iliac vein. Figure elements modified from SMART (Servier Medical Art), licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.
(B) Number and class of altered A/V metabolites (adjusted p < 0.05, paired t test).

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mice (Dyar et al., 2018b) to our data after timed exercise. 2-HB levels in serum, liver, and muscle oscillate together over 24 h, with lower levels during the light phase and ~2- to 4-fold higher levels during the dark phase. Exercise and sham exercise elevated 2-HB levels in liver and muscle during trough levels at ZT4, but the increase was ~2 x greater with exercise. Exercise at ZT15 increased 2-HB concentration ~3–5 x beyond the normal peak, but levels returned to baseline within 4 h. To gain insight into relative tissue 2-HB uptake and release, we examined our A/V data. Net release by liver remained significant under all conditions, except for ZT15 exercise, when systemic 2-HB levels were highest (Figure 7D). Muscle net uptake of 2-HB was significant only under ZT3 sedentary conditions, when systemic levels were lowest.

To investigate the role of 2-HB as a signaling molecule, we administered deuterated 2-HB to mice at different doses and times of day (Figure 7E). We collected tissues and monitored 2-HB biodistribution and its impact on metabolism using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) imaging (Kunzke et al., 2020; Prade et al., 2020). Exogenous 2-HB ([H]106.0589) was detected in liver and muscle in a dose- and time-dependent manner (Figures 7F–7I, S7A, and S7B). Liver and muscle showed similar uptake of 2-HB regardless of the time of day, but exogenous 2-HB remained elevated 2 h after injection only at ZT15. We identified significantly associated metabolites that correlated with exogenous 2-HB (Figures S7C and S7D; Data S7), including 106 liver and 127 muscle metabolites mostly involved in AA, sugar, and nucleotide metabolism.

To investigate a role for 2-HB in systemic metabolism, we administered unlabeled 2-HB to mice at monitored energy expenditure (EE) and respiratory exchange ratio (RER) by indirect calorimetry (Figure 7J). The higher dose (10 mmol/kg) acutely and transiently suppressed EE ~30 min after injection (Figure 7K). This effect was greater during the dark phase when circadian EE is at its highest (Dyar et al., 2018a). RER dropped acutely at night (Figure 7L) and remained low for several hours, suggesting an abrupt and persistent shift from glucose oxidation toward lipid utilization, as occurs during the light phase when mice are fasting. In support of our interpretation that 2-HB suppresses systemic glucose utilization, blood glucose was elevated 60 min after injection at ZT3 and ZT15 (Figure 7M). Thus, time- and exercise-dependent metabolites like 2-HB might communicate energy status under conditions of energy stress and impact tissue metabolism and systemic energy homeostasis.

**DISCUSSION**

This resource adds spatiotemporal insights about the relative effects of exercise on tissue and systemic metabolism and temporal perspectives about the relative impact of performing exercise at different times of the day. The cross-tissue correlations highlight robust metabolic crosstalk between muscle and liver upon exercise in a time-dependent manner, with early active phase exercise at ZT15 having a greater impact on muscle-liver coordination. The liver plays an essential role as an energy producer during fasting and exercise, with major roles in glycogenolysis, gluconeogenesis, lipolysis, and ketogenesis (Rui, 2014; Warner et al., 2020). During early rest phase exercise at ZT3, when hepatic energy stores are enriched, the liver is primarily committed to energy metabolite production via glycogen and lipid degradation for skeletal muscle. Conversely, during early active phase exercise at ZT15, when hepatic glycogen content is reduced, skeletal muscle requires alternative energy sources. We observed reduced glucose in muscle and liver, lipolysis in WAT, and systemic activation of ketone body metabolism and AA breakdown upon exercise at ZT15. This local energy demand in skeletal muscle, coupled with temporal variations of hepatic glycogen storage and feeding-fasting cycles, appears to result in a tighter metabolic coordination between tissues. Future studies examining synergistic effects between nutritional state and exercise are warranted to ascertain which factors are more prominent for the time-of-day-dependent impact of exercise: fed/fasted cycles or circadian cycles.

Several metabolites act as stimuli for cellular energy sensors and signaling molecules, thus causing adaptive rewiring of signaling pathways and transcriptional networks (Dai et al., 2020). Metabolites and energy sensors can directly regulate circadian clock function (Nakahata et al., 2009; Ramsey et al., 2009). Activation of the energy sensor AMPK directly phosphorylates and destabilizes circadian transcriptional repressors CRY1/2, leading to circadian and gluconeogenic gene reprogramming (Lamia et al., 2009). Our study revealed a time- and muscle-specific impact of exercise on the accumulation of 5‘-AMP, simultaneously leading to muscle-specific activation of AMPK. Additional exercise-stimulated molecules transduce metabolic information and mediate circadian reprogramming, including histone modifiers (SIRT1, HDACs) and transcription factors (HIF1α, GR, PPARs) (Adamovich et al., 2017; Guan et al., 2018; Peek et al., 2017; Quagliarini et al., 2019). Treadmill exercise in mice and electrical stimulation of cultured muscle cells can differentially advance or delay the circadian clock phase depending on the time of intervention (Kemler et al., 2020). Thus, exercise may reset misaligned muscle clocks if timed appropriately, for example, under fasted conditions. Our resource can be further mined for additional information about potential clock modifiers and can serve as a first step toward identifying the full metabolic responses.
Figure 7. Validation of 2-hydroxybutyrate (2-HB) as a time-dependent exerkine

(A) Quantification of selected metabolites by targeted gas chromatography mass spectrometry using spiked internal standards (n = 6; n.s., not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, unpaired two-tailed t test).

(B) Heatmap of targeted metabolites showing top 25 (liver) and 10 (muscle) most significant features detected by PCA plot.

(C) Diurnal variations of 2-hydroxybutyrate (2-HB) (mean ± SEM; n = 5–6; baseline data derived from Dyar et al. (2018b).)

(D) Boxplots and relative A/V differences of 2-HB (n = 6; adjusted *p < 0.05, **p < 0.01, and ***p < 0.001, paired t test; horizontal lines of boxes indicate median, size of boxes reflects upper and lower quartiles, whiskers are minimum and maximum values, and dashed lines connect A/V data from individual mice).

(E) Study design for MALDI-MS imaging validation using deuterated 2-HB. s.c., subcutaneous.

(F) Quantification of exogenous 2-HB (mean pixel intensity ± SEM).

(G) Liver section showing exogenous 2-HB 30 min after injection (10 mmol/kg) at ZT15.

(legend continued on next page)
complement of tissue and systemic metabolite messengers mediating clock entrainment.

By examining tissues in a physiological context, our multisite metabolomics atlas can shed light on the time and tissue dependence of exerkines (Murphy et al., 2020). A limitation of our study is the timing of sample collection, as peak exerkine production and tissue distribution may be outside of the time intervals we explored. Future studies implementing different modes of exercise, higher temporal resolution, or additional arteriovenous sampling of exerkine-producing and target tissues will provide further physiological context. Nevertheless, we found exerkines present in different tissues in a time- and context-dependent manner. Additional work is needed to clarify the relative physiological importance of these exerkines, as well as tissue specificity, and how they communicate metabolic state between tissues. Our resource can serve as a starting point by indicating where exerkines may first arise and when their presence may be most physiologically relevant. As new metabolite exerkines are identified, our dataset can be revisited to test and explore possible interactions.

We identified 2-HB as an exercise-induced metabolite that may reflect the cytosolic redox state and energy stress at a systemic level. 2-HB levels were elevated in all tissues but only after early active phase exercise at ZT15. Selective accumulation of 2-HB indicates exercise at ZT15 evoked systemic metabolic stress. Normally considered a byproduct of GSH production or threonine catabolism, 2-HB also accumulates when mitochondrial metabolism of its precursor 2-KB is impaired (Miyazaki et al., 2015) or in response to an elevated NADH/NAD⁺ ratio due to increased lipid oxidation. In this context, 2-KB can serve as an electron acceptor, forming 2-HB and regenerating NAD⁺ through oxidation of cytosolic NADH by LDH (Gall et al., 2010; Landas, 1975; Lord and Bralley, 2008; Miyazaki et al., 2015). 2-HB is a biomarker of cardiometabolic traits, mitochondrial dysfunction, lactic acidosis, ketoacidosis, prolonged fasting, and the maintenance of redox homeostasis and cell survival under stressed conditions (Gall et al., 2010; Goodman et al., 2020; Hsu et al., 2020; Liu et al., 2018; Thompson Leguait et al., 2015). We previously identified 2-HB as a 24 h oscillating metabolite in skeletal muscle, liver, and serum (Dyar et al., 2018b). We report that circulating (exogenous) 2-HB transiently reduced EE and altered fuel use in a time-dependent manner. This resulted in increased glycemia and changes in liver and muscle metabolism. Thus, 2-HB may limit glucose utilization during energy stress and possibly clarify its strong association with type 2 diabetes risk.

In conclusion, this resource adds essential perspectives regarding the time-of-day-specific effects of exercise on systemic metabolism and tissue crosstalk. The production and consumption of skeletal muscle and liver metabolites during exercise changes according to the time of day, with 2-HB contributing as a systemic “exerkine” during exercise. This atlas will serve to deepen the understanding of the pleiotropic effects of exercise and uncover mechanistic insights to maximize the benefits of exercise on metabolic health.

**Limitations of study**
In this study, we did not specifically consider the impact of sex, age, or metabolic disease in assessing the effects of exercise on systemic metabolism and tissue crosstalk. Our study was limited to a mouse model subjected to treadmill running, and results in humans, as well as the response to different exercise modalities, may vary. Although we assessed co-incident changes in metabolite levels in seven tissues and serum, as well as A/V differences in the liver and hindlimb muscle in response to acute exercise, future studies using tracer approaches will be useful to fully resolve the dynamic “metabolic circuitry” and “tissue crosstalk” in the whole organism.

**STAR★METHODS**
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**SUPPLEMENTAL INFORMATION**
Supplemental information can be found online at https://doi.org/10.1016/j.cmet.2021.12.016.
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AUTOR CONTRIBUTIONS

S.S., K.A.D., J.T.T., A.L.B., M. Schönke, P.B., R.B., A.W., T.M., J.J.H., D.L., J.R.Z., and P.-S.-C. designed experiments. S.S., K.A.D., S.P.A., A.M.E., S.L.J., K.T., T.K., V.M.P., L.S., A.L.B., and M. Schönke performed experiments. S.S., K.A.D., S.C., M. Samad, P.B., and D.L. analyzed data. S.S., K.A.D., J.T.T., D.L., and J.R.Z. prepared figures and wrote the manuscript. All authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

J.R.Z. is an Advisory Board member for Cell Metabolism. The authors declare no competing interests.

REFERENCES

Adamovich, Y., Ladeuix, B., Golik, M., Koeners, M.P., and Asher, G. (2017). Rhythmic oxygen levels reset circadian clocks through HIF1alpha. Cell Metab 25, 93–101.
Agostinelli, F., Ceglia, N., Shahbaba, B., Sassone-Corsi, P., and Baldi, P. (2018). What time is it? Deep learning approaches for circadian rhythms. Bioinformatics 34, 18–117.
Achier, M., Borgmann, D., Krumsiek, J., Buck, A., MacDonald, P.E., Fox, J.E.M., Lyon, J., Light, P.E., Keipert, S., Jastroch, M., et al. (2017). N-acetyl tau-rines and acylcarnitines cause an imbalance in insulin synthesis and secretion provoking beta cell dysfunction in type 2 diabetes. Cell Metab 25, 1334–1347.e4.
Bartman, C.R., TeSlaa, T., and Rabinowitz, J.D. (2021). Quantitative flux analysis in mammals. Nat. Metab. 3, 896–908.
Brandauer, J., Andersen, M.A., Kellezi, H., Risius, S., Frosig, C., Vienberg, S.G., and Treebak, J.T. (2015). AMP-activated protein kinase controls exercise training- and AICAR-induced increases in SIRT3 and MnSOD. Front. Physiol. 6, 85.
Braut, J.J., and Terjung, R.L. (2001). Purine salvage to adenine nucleotides in different skeletal muscle fiber types. J. Appl. Physiol. 91 (1985), 231–238.
Ceglia, N., Liu, Y., Chen, S., Agostinelli, F., Ecker-Mahan, K., Sassone-Corsi, P., and Baldi, P. (2018). CirdaciOmins: circadian omic web portal. Nucleic Acids Res 46, W157–W162.
Chong, J., Wishart, D.S., and Xia, J. (2019). Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. Curr. Protoc. Bioinformatics 68, e86.
Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 28, 27–30.

Kemler, D., Wolff, C.A., and Esser, K.A. (2020). Time-of-day dependent effects of contractile activity on the phase of the skeletal muscle clock. J. Physiol. 598, 3631–3644.

Kretzschmar, M., and Müller, D. (1993). Aging, training and exercise. A review of effects on plasma glutathione and lipid peroxides. Sports Med 15, 196–209.

Kunzke, T., Buck, A., Prade, V.M., Feuchtunger, A., Prokopchuk, O., Martignon, M.E., Heisz, S., Hauner, H., Janssen, K.P., Walch, A., et al. (2020). Derangements of amino acids in cachectic skeletal muscle are caused by mitochondrial dysfunction. J. Cachexia Sarcopenia Muscle 11, 226–240.

Lamia, K.A., Sachdeva, U.M., DiTacchio, L., Williams, E.C., Alvarez, J.G., Egan, D.F., Vasquez, D.S., Juguilon, H., Panda, S., Shaw, R.J., et al. (2009). AMPK regulates the circadian clock by chromophore phosphorylation and degradation. Science 326, 437–440.

Landas, S. (1975). The formation of 2-hydroxybutyric acid in experimental animals. Clin. Chim. Acta 58, 23–32.

Liu, Y., Guo, J.Z., Liu, Y., Wang, K., Ding, W., Wang, H., Liu, X., Zhou, S., Lu, X.C., Yang, H.B., et al. (2018). Nuclear lactate dehydrogenase A senses ROS to produce alpha-hydroxybutyrate for HPV-induced cervical tumor growth. Nat. Commun. 9, 4429.

Lord, R.S., and Bralley, J.A. (2008). Clinical applications of urinary organic acids. Part I: detoxification markers. Altern. Med. Rev. 13, 205–215.

Lundell, L.S., Parr, E.B., Devlin, B.L., Ingerslev, L.R., Attntaq, A., Sato, S., Sassone-Corsi, P., Barrès, R., Zierath, J.R., and Hawley, J.A. (2020). Time-restricted feeding alters lipid and amino acid metabolism rhythmicity without perturbing clock gene expression. Nat. Commun. 11, 4643.

Luukkonen, P.K., Zhou, Y., Nichina Haridas, P.A., Dwivedi, O.P., Hyötyläinen, T., Ali, A., Juuti, A., Leivonen, M., Tukiainen, T., Ahonen, L., et al. (2017). Impaired hepatic lipid synthesis from polyunsaturated fatty acids in TMB6F2 E167K variant carriers with NAFLD. J. Hepatol. 67, 128–136.

Masri, S., and Sassone-Corsi, P. (2018). The emerging link between cancer metabolism, and circadian rhythms. Nat. Med. 24, 1795–1803.

Miyazaki, T., Honda, A., Ikegami, T., Iwamoto, J., Monma, T., Hirayama, T., Saito, Y., Yamashita, K., and Matsuzaki, Y. (2015). Simultaneous quantification of salivary 3-hydroxybutyrate, 3-hydroxyisobutyrate, 3-hydroxy-3-methylbutyrate, and 2-hydroxybutyrate as possible markers of amino acid and fatty acid catabolic pathways by LC-ESI-MS/MS. Springerplus 4, 494.

Morville, T., Sahl, R.E., Moritz, T., Helge, J.W., and Clemmensen, C. (2020). Plasma metabolome profiling of resistance exercise and endurance exercise in lean humans and contributes to insulin resistance. And obesity that modulates white and brown adipocyte metabolism. Diabetes 69, 1903–1916.

Patel, V.R., Eckel-Mahan, K., Sassone-Corsi, P., and Baldi, P. (2012). Circadianomics: integrating circadian genomics, transcriptomics, proteomics and metabolomics. Nat. Methods 9, 772–773.

Peek, C.B., Levine, D.C., Cedernaes, J., Taguchi, A., Kobayashi, Y., Tsai, S.J., Bonar, N.A., McNulty, M.R., Ramsey, K.M., and Bass, J. (2017). Circadian clock interaction with HIF1alpha mediates oxygen metabolism and anaerobic glycolysis in skeletal muscle. Cell Metab 25, 86–92.

Prade, V.M., Kunzke, T., Feuchtunger, A., Rohm, L., Luber, B., Lordick, F., Buck, A., and Walch, A. (2020). De novo discovery of metabolic heterogeneity with immunophenotype-guided imaging mass spectrometry. Mol. Metab. 36, 100953.

Quagliranni, F., Mir, A.A., Balazs, K., Wierer, M., Dyrar, K.A., Jouffe, C., Makris, K., Hawe, J., Henig, M., Filipp, F.V., et al. (2019). Cistrome reprogramming of the diurnal glucocorticoid hormone response by high-fat diet. Mol. Cell 76, 531–545.e5.

Ramsey, K.M., Yoshino, J., Brace, C.S., Abrassart, D., Kobayashi, Y., Marcheva, B., Wong, H.K., Chong, J.J.L., Buhr, E.D., Lee, C., et al. (2009). Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. Science 324, 651–654.

Rogatzi, M.J., Ferguson, B.S., Goodwin, M.L., and Gladden, L.B. (2015). Lactate is always the end product of glycolysis. Front. Neurosci. 9, 22.

Rui, L. (2014). Energy metabolism in the liver. Compr. Physiol. 4, 177–197.

Sato, S., Basse, A.L., Schöne, M., Chen, S, Samad, M., Attntaq, A., Laker, R.C., Dalbrom, E., Barrès, R., Baldi, P., et al. (2019). Time of exercise specifies the impact on muscle metabolic pathways and systemic energy homeostasis. Cell Metab 30, 92–110.e4.

Sbodio, J.I., Snyder, S.H., and Paul, B.D. (2019). Regulators of the transsulfa- tion pathway. Br. J. Pharmacol. 176, 583–593.

Schoeders, A.M., Truong, D., Loh, D.H., Jordan, M.C., Roos, K.P., and Colwell, C.S. (2012). Voluntary scheduled exercise alters diurnal rhythms of behaviour, physiology and gene expression in wild-type and vasoactive intestinal peptide-deficient mice. J. Physiol. 590, 6213–6226.

Sen, C.K. (1998). Glutathione: A key role in skeletal muscle metabolism. In Oxidative stress in skeletal muscle, A.Z. Reznick, L. Packer, C.K. Sen, J.O. Holloszy, and M.J. Jackson, eds. (Birkhäuser), pp. 127–139.

Small, L., Attntaq, A., Laker, R.C., Ehrlich, A., Pattamaprapanont, P., Villarroel, J., Pillon, N.J., Zierath, J.R., and Barrès, R. (2020). Contraction influences Per2 gene expression in skeletal muscle through a calcium-dependent pathway. J. Physiol. 598, 5739–5752.

Thompson Legault, J., Strittmatter, L., Tardif, J., Sharma, R., Tremblay-Valliant, V., Aubut, C., Boucher, G., Clish, C.B., Cyr, D., Daneault, C., et al. (2015). A metabolic signature of mitochondrial dysfunction revealed through a monogenic form of Leigh syndrome. Cell Rep 13, 981–989.

Treejak, J., Pehmoller, C., Kristensen, J.M., Kjobsted, R., Birk, J.B., Schjerling, P., Richter, E.A., Goodyear, L.J., and Wojtaszewski, J.F. (2014). Acute exercise and physiological insulin induce distinct phosphorylation signatures on TBC1D1 and TBC1D4 proteins in human skeletal muscle. J. Physiol. 592, 351–375.

Wang, T.J., Ngo, D., Psychogios, N., Dejam, A., Larson, M.G., Vasan, R.S., Gharbani, A., O’Sullivan, J., Cheng, S., Rhee, E.P., et al. (2013). 2-Aminoadipic acid is a biomarker for diabetes risk. J. Clin. Invest. 123, 4309–4317.

Warner, S.O., Yao, M.V., Cason, R.L., and Winnick, J.J. (2020). Exercise-induced improvements to whole body glucose metabolism in type 2 diabetes: the essential role of the liver. Front. Endocrinol. (Lausanne) 11, 567.

Wolff, G., and Esser, K.A. (2012). Scheduled exercise phase shifts the circadian clock in skeletal muscle. Med. Sci. Sports Exerc. 44, 1663–1670.
# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-phospho-AMPKα  | Cell Signaling Technology | Cat# 2535; RRID: AB_331250 |
| Anti-AMPKα          | Cell Signaling Technology | Cat# 2603; RRID: AB_490795 |
| Anti-p84            | Genetex | Cat# GTX70220; RRID: AB_372637 |
| Anti-Mouse IgG, HRP conjugate | EMD Millipore | Cat# AP160P; RRID: AB_92531 |
| Anti-Rabbit IgG, HRP-linked | Cell Signaling Technology | Cat# 7074; RRID: AB_2099233 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Methanol; LC-MS Ultra CHROMASOLV | Honeywell | 14262-2L |
| Hexane; GC-MS SupraSolv | Supelco | 1007951000 |
| D-3-Hydroxybutyrate; Sodium (13C4) | Cambridge Isotope | CLM-3853-PK |
| L-Valine-d8 | Sigma-Aldrich | 486027-1G |
| Palmitic acid-d31 | Cayman chemicals | 1-800-364-9897 |
| Succinic acid-2,2,3,3-d4 | Sigma-Aldrich | 293075-1G |
| L-Glutamic acid-13C15N | Sigma-Aldrich | 607851-250MG |
| 4,4-Dibromoocotfluorobiphenyl | Sigma-Aldrich | 101990-1G |
| 2-Hydroxybutyric acid; Sodium | Sigma-Aldrich | 220116-5G |
| 2-Hydroxyisobutyric acid | Sigma-Aldrich | 323594-25G |
| 3-Hydroxybutyric acid | Sigma-Aldrich | 166898-1G |
| 3-Hydroxyisobutyric acid; Sodium | Sigma-Aldrich | 11161-100MG |
| L-Leucine | Sigma-Aldrich | AAS18-5ML |
| L-Isoleucine | Sigma-Aldrich | AAS18-5ML |
| L-Valine | Sigma-Aldrich | AAS18-5ML |
| L-Glutamic acid | Sigma-Aldrich | AAS18-5ML |
| L-Aspartic acid | Sigma-Aldrich | AAS18-5ML |
| L-Proline | Sigma-Aldrich | AAS18-5ML |
| L-Alanine | Sigma-Aldrich | AAS18-5ML |
| Oleic acid | Cayman Chemicals | 90260 |
| Stearic acid | Cayman Chemicals | 10011298 |
| Malic acid | Sigma-Aldrich | 240176-50G |
| Lactic acid | Sigma-Aldrich | L6402-1G |
| Methoxamine (MOX) | Thermo Scientific | TS-45950 |
| N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% Trimethylchlorosilane (BSTFA + 1%TMCS) | Thermo Scientific | TS-38831 |
| (+/-)-2-Hydroxybutyric acid-2,3,3-d3; Sodium | CDN isotopes | D-7002(lotAB-224) |
| Indium tin oxide-coated conductive slides | Bruker | 8237001 |
| Poly-l-lysine | Sigma-Aldrich | P8920-100ML |
| Nonidet P-40 | Sigma-Aldrich | 21-3277 |
| 1,5-diaminonaphthalene (DAN) | Sigma-Aldrich | 56451-250MG |
| Hematoxylin | Leica | 3801698A |
| Eosin | Leica | 3801698D |
| Acetonitrile | Sigma-Aldrich | 34851-2.5L |
| TRIZol | ThermoFisher Scientific | Cat# 15596026 |
| Maxima H Minus Mastermix | ThermoFisher Scientific | Cat# M1662 |
| PowerUp SYBR Master Mix | ThermoFisher Scientific | Cat# A25918 |
| Protein Assay SYBR Reagent | Bio-Rad Laboratories | Cat# 500-0006 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Nitrocellulose Membrane, 0.45 μm | Bio-Rad Laboratories | Cat# 1620115 |
| Immobilon Western Chemiluminescent HRP substrate | EMD Millipore | Cat# WBKLS0500 |
| cOmplete, EDTA-free Protease Inhibitor Cocktail | Roche | Cat# 11873580001 |
| Perchloric acid | Sigma-Aldrich | 244252-100ML |
| D-Glucose | Sigma-Aldrich | G8270-1KG |
| D-Mannitol | Sigma-Aldrich | M4125-500G |
| DMEM, high glucose | ThermoFisher Scientific | 11965084 |
| Penicillin-Streptomycin | ThermoFisher Scientific | 15140122 |
| Horse Serum | Sigma-Aldrich | H1270-1L |
| Fetal Bovine Serum | ThermoFisher Scientific | 16000 |

**Deposited data**

| MOUSE EXERCISE METABOLOME | MOUSE HEPATIC MORNING-SEDENTARY | MOUSE HEPATIC MORNING-EXERCISE | MOUSE HEPATIC EVENING-SEDENTARY | MOUSE HEPATIC EVENING-EXERCISE |
|-----------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Mouse skeletal muscle, sedentary, ZT3 | http://circadiomics.igb.uci.edu/ | MOUSE EXERCISE METABOLOME MORNING-SEDENTARY | MOUSE EXERCISE METABOLOME MORNING-EXERCISE | MOUSE HEPATIC MORNING-SEDENTARY |
| Mouse skeletal muscle, exercise, ZT3 | http://circadiomics.igb.uci.edu/ | MOUSE EXERCISE METABOLOME EVENING-SEDENTARY | MOUSE EXERCISE METABOLOME EVENING-EXERCISE | MOUSE HEPATIC EVENING-SEDENTARY |
| Mouse skeletal muscle, sedentary, ZT15 | http://circadiomics.igb.uci.edu/ | MOUSE HEPATIC EVENING-SEDENTARY | MOUSE HEPATIC EVENING-EXERCISE | MOUSE HEPATIC MORNING-SEDENTARY |
| Mouse skeletal muscle, exercise, ZT15 | http://circadiomics.igb.uci.edu/ | MOUSE HEPATIC EVENING-SEDENTARY | MOUSE HEPATIC EVENING-EXERCISE | MOUSE HEPATIC MORNING-SEDENTARY |
| Mouse liver, sedentary, ZT3 | http://circadiomics.igb.uci.edu/ | TissueProfiling | TissueProfiling | TissueProfiling |
| Mouse liver, exercise, ZT3 | http://circadiomics.igb.uci.edu/ | TissueProfiling | TissueProfiling | TissueProfiling |
| Mouse liver, sedentary, ZT15 | http://circadiomics.igb.uci.edu/ | TissueProfiling | TissueProfiling | TissueProfiling |
| Mouse liver, exercise, ZT15 | http://circadiomics.igb.uci.edu/ | TissueProfiling | TissueProfiling | TissueProfiling |
| Mouse muscle 24-hr metabolome, sedentary & exercise at ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | 24h_LiverMuscle | 24h_LiverMuscle | 24h_LiverMuscle |
| Mouse liver 24-hr metabolome, sedentary & exercise at ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | 24h_LiverMuscle | 24h_LiverMuscle | 24h_LiverMuscle |
| Mouse serum metabolome, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | TissueProfiling | TissueProfiling | TissueProfiling |
| Mouse heart metabolome, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | TissueProfiling | TissueProfiling | TissueProfiling |
| Mouse hypothalamus metabolome, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | TissueProfiling | TissueProfiling | TissueProfiling |
| Mouse epididymal & inguinal white adipose tissue metabolomes, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | TissueProfiling | TissueProfiling | TissueProfiling |
| Mouse brown adipose tissue metabolome, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | TissueProfiling | TissueProfiling | TissueProfiling |
| A/V metabolome, skeletal muscle, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | A/V_LiverMuscle | A/V_LiverMuscle | A/V_LiverMuscle |
| A/V metabolome, hindlimb arterial blood, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | A/V_LiverMuscle | A/V_LiverMuscle | A/V_LiverMuscle |
| A/V metabolome, hindlimb venous blood, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | A/V_LiverMuscle | A/V_LiverMuscle | A/V_LiverMuscle |
| A/V metabolome, liver, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | A/V_LiverMuscle | A/V_LiverMuscle | A/V_LiverMuscle |
| A/V metabolome, portal vein, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | A/V_LiverMuscle | A/V_LiverMuscle | A/V_LiverMuscle |
| A/V metabolome, vena cava, sedentary & exercise, ZT3 & ZT15 | https://doi.org/10.17632/6x5vd4d5rd.1 | A/V_LiverMuscle | A/V_LiverMuscle | A/V_LiverMuscle |
| Original Excel data sheets + WB pictures for figures | N/A | This paper (Data S1) |
| Transcriptomics data | (Sato et al., 2019) | GEO: GSE126962 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Juleen R. Zierath (Juleen.Zierath@ki.se).

Materials Availability
This study did not generate new unique materials.

Data and Code Availability
24h Metabolomics data from skeletal muscle and liver are available from CircadiOmics (Ceglia et al., 2018; Patel et al., 2012). Excel datasheets used to create all graphs in the paper and uncropped scans of all the Western blot images presented in the paper are found in Data S1. Original and scaled metabolomics data can be found in Data S2. Raw and processed metabolomics data have also been deposited into Mendeley Data (https://doi.org/10.17632/6x5vd4d5rd.1).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal experiments complied with the European directive 2010/63/EU of the European Parliament and were approved by the Danish Animal Experiments Inspectorate (2012-15-2934-26 and 2015-15-0201-796). 2-HB experiments were approved by the local animal welfare authority in Germany (District government of upper Bavaria No.55.2-2532.Vet_2-17-125). For exercise experiments, specific Pathogen Free (SPF) male C57BL6/JBomTac mice were purchased from Taconic Biosciences and maintained at the animal facilities of the University of Copenhagen, Denmark. For 2-HB validation studies, SPF male C57BL6/J mice were purchased from Janvier and maintained at the animal facilities of the Helmholtz Zentrum München.

METHOD DETAILS

Animals, Exercise, and Sample Collection
Mice were reared at 22±1°C with 12h light/dark cycle (light on at 6AM (ZT0) and light off at 6PM (ZT12)) and fed a standard rodent laboratory diet (#1310, Altromin, Germany) ad libitum. At 10-11 weeks of age, the mice were divided into two different groups of exercise or sham-exercise treatment either during the early rest phase group (ZT3) or the early active phase group (ZT15). Exercised mice in the early rest phase group were subjected to a single bout of acute exercise for 1h at ZT3, whereas exercised mice in the early active phase group were subjected to the exercise for 1h at ZT15. Sedentary control mice for corresponding exercised mice at the different daily times were placed on a stationary treadmill for 1h (sham-exercise). The exercise protocol has been described...
previously (Brandauer et al., 2015; Sato et al., 2019; Treebak et al., 2014). Briefly, the mice were subjected to a single-bout of acute treadmill running (Columbus Instruments, OH) following a 4-day acclimatization protocol as described below:

Day 1 (15 min total; 5% incline)
For the first 5 min, mice were placed on the stationary treadmill.
Start running at 6 m/min and accelerate by 2 m/min every 2 or 3 min up to 12 m/min

Day 2 (15 min total; 5% incline)
Start running at 6 m/min and accelerate by 2 m/min every 3 min up to 14 m/min

Day 3 (15 min total; 5% incline)
Start running at 6 m/min and accelerate by 2 m/min every 2 or 3 min up to 16 m/min

Day 4
Rest

Day 5 (60 min total; 5% incline)
Start running at 6 m/min and accelerate by 2 m/min every 2 min up to 16 m/min
Electrical grids forced mice to keep running for the corresponding duration of exercise.

Under isoflurane anesthesia, skeletal muscle and liver were collected 0, 4, 8, 12, 16, and 20h after 1hr of exercise or sham-exercise performed during the early rest (ZT3) or active phase (ZT15). Serum, heart, hypothalamus (HT), epididymal white adipose tissue (eWAT), inguinal WAT (iWAT), and brown adipose tissue (BAT) were collected immediately after 1hr of exercise or sham-exercise performed during the early rest (ZT3) or active phase (ZT15). All tissues were immediately snap frozen in liquid nitrogen and stored at -80°C for subsequent use. For blood sampling across hindlimb skeletal muscles and liver, separate cohorts of 10-12-week-old SPF male C57B6/JBomTac mice were subjected to the same acute exercise or sham-exercise protocol either at ZT3 or ZT15 and then anesthetized.

**Arteriovenous sampling across hindlimb skeletal muscles**
Collection of venous blood from the skeletal muscle was drawn from the right common iliac vein using a 1 ml syringe and a 27 G needle. For arterial blood sampling the blood was drawn from the abdominal aorta using a catheter (BD Insyte Autoguard, 24 GA 0.75 IN, 0.7 x 19 mm, BD, Denmark). After the blood sampling, the gastrocnemius muscle was dissected and immediately snap frozen in liquid nitrogen before storage at -80°C.

**Blood sampling across the liver**
The blood flow from the lower extremities and other organs that feed into the vena cava, apart from the liver, was occluded by a ligature placed around the vena cava inferior and aorta above the renal veins. Blood samples were then collected from the portal vein using a 1 ml syringe and 27 G needle, and blood from liver was drawn, after opening of the thorax, through a catheter similar to the catheter used for arterial blood sampling, inserted into the inferior caval vein, via the heart, and advanced to the liver. To avoid blood flow from the upper extremities a ligature was placed around the catheter in the vena cava. The liver was dissected immediately, and the samples were snap frozen in liquid nitrogen and stored at -80°C for subsequent use.

**Global Metabolomics Profiling**
Metabolomic analysis was performed by Metabolon (Durham, NC).

**Sample Preparation**
Samples were prepared using the automated MicroLab STAR system (Hamilton, NV). To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to remove chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (GenoGrinder, 2000, Glen Mills, NJ) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESL, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and once sample was reserved for backup. Samples were placed briefly on a Zymark TurboVap (Caliper Life Sciences, CA) to remove the organic solvent.

**UPLC-MS/MS**
All methods utilized a Waters ACQUITY ultra-performance liquid chromatography and a Q-Exactive high resolution/accurate mass spectrometer (ThermoFisher Scientific) interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18 2.1x100 mm, 1.7 μm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid. Another aliquot was also analyzed using acidic positive ion conditions, however it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same aforementioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% formic acid and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5 mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1 x 150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM Ammonium Formate, pH 10.8.
Data Extraction, Compound Identification, Metabolite Quantification, and Data Normalization

Raw data were extracted, peak-identified and quality control processed using Metabolon’s proprietary hardware and software (Evans et al., 2009; Ford et al., 2020). Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Biochemical identifications are based on three criteria: retention index within a narrow retention time/index window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. Additional mass spectral entries have been created for structurally unnamed biochemicals, which have been identified by virtue of their recurrent nature. Peaks were quantified using area-under-the-curve. For measurements spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. All the samples in a particular tissue type were run in the same batch using the same instrument, so minimal instrument technical variation is present. Samples were run in a balanced manner using the group ID. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately. Following additional normalization (to mass extracted for BAT and hypothalamus, and to volume for serum), log transformation and imputation of missing values with the minimum observed value for each compound, Mixed Model Contrasts were used to identify biochemicals that differed significantly between groups. Imputation is applied within each sample type (muscle, liver, serum, etc.) regardless of the sampling timing and exercise condition. Imputation is applied to all the metabolites that are not detected (due to metabolites being under the threshold of detection (LOD)) in a per sample basis. If a metabolite is detected it is quantified (raw area counts) automatically. If there was only one value, it was still imputed with the minimum (this single value), but because the values would all be the same post-imputation, significance testing would not be performed on those metabolites (Missing values: 6.4% Muscle, 4.1% Serum, 4.0% Liver, 3.9% Heart, 5.0% HT, 13.1% eWAT, 5.8% iWAT, and 4.7% BAT).

Exclusion of metabolites related to xenobiotics

For differential analyses of metabolites identified and changed under experimental conditions (Figures 1B–1D, S1A–S1D, and 6D), all metabolites named and categorized according to Metabolon superpathways – amino acids, carbohydrates, energy, peptide, lipid, nucleotide, cofactors and vitamins, and xenobiotics –, and unnamed metabolites were included. For subsequent metabolite classification, pathway enrichment, and metabolite correlation analyses, metabolites categorized as xenobiotics and unnamed metabolites were excluded.

Targeted Gas Chromatography Mass Spectrometry (GC-MS)

Skeletal muscle (quadriceps) and liver samples collected after single-bout acute exercise or sham-exercise were used for further identification and quantification of polar metabolome biomarkers, including 2-hydroxybutyrate (2-HB) and 2-hydroxyisobutyrate (2-HIB), by targeted GC-MS. Preparation of liver and muscle tissue is based on prior methods, modified to suit the experimental design and analytical instruments (Luukkonen et al., 2017). Each tissue sample was transferred from -80 °C into a Covaris tissueTUBE (S20140), immersed in liquid nitrogen, and crushed with Covaris CryoPrep impactor CP02. Around 10 to 20 mg of powdered tissue were then transferred into 1.5 ml Eppendorf tubes and used for extraction. Exact mass was measured and written down for post-processing and normalization. Cryogenically frozen tissue powder was mixed with 60 μl of methanol containing internal standards: L-Valine-d8; 20 mg/l, Succinic acid-2,2,3,3-d4; 20 mg/l, L-Glutamic acid-13C5; 100 mg/l, D-3-hydroxybutyrate (13C4); 10 mg/l, Palmitic acid-d31; 100 mg/l, Additional 340 μl of methanol was added to the mixture. The suspension was vortexed and sonicated in ice-cold ultrasound bath for 15 min to facilitate metabolite extraction. After 15 min-incubation at 4 °C for protein precipitation, the suspension was centrifuged for 3 min on 10,000 rpm at 4 °C and 200 μl of extract was aliquoted. Additional 50 μl was taken to create a pooled sample dedicated for quality control. Extract was dried under a stream of nitrogen with 6 l/min flow for 45 min. Native standards of 2-HB and 2-HIB were dissolved in methanol and diluted into calibration curve series, which was used for metabolite identification and quantification. Automatic derivatization was made using Gerstel MPS robot (Hartonen et al., 2013). Mox derivatizing agent (25 μl) was added to dried extract followed by 1h derivatization on 45 °C. In a second step, 25 μl of BSTFA containing 1% TMCS was added and the same reaction conditions were used. Before the injection, 50 μl of hexane containing 10 mg/l of 4,4-di-bromooctafluorobiphenyl was added. Compound was used to control the precision of 1 μl injection. Samples were analyzed using a gas chromatograph coupled with time of flight mass spectrometer (Leco Pegasus BT). Metabolites were separated on Restek Rxiv 5ms column (pn:13423-6850) with Helium flow of 1.2 ml/min and 270 °C inlet temperature. Temperature gradient started with 40 °C where it was stable for a minute. After that, temperature was increased with a rate of 20 °C/min until it reached 340 °C, where it was stable for additional 3 min. Detector acquired mass spectra in scan mode, in the range from 50 to 750 Da with 10 Hz speed. To avoid solvent peak, acquisition started after 320s. MS was equipped with an EI source with standard 70eV fragmentation.

In Vivo Administration of 2-Hydroxybutyrate (2-HB)

All experiments were performed on 3-month-old male C57BL/6J mice from Janvier housed at room temperature (23 °C) under a 12h light:12h dark cycle with standard chow diet (#1310, Altromin, Germany) and water provided ad libitum. Labeled 2-HB (2,3,3-d3), unlabeled 2-HB, or vehicle (saline) were administered by subcutaneous injection at the indicated doses and times. Tissues were collected at the indicated times immediately after cervical dislocation, snap frozen in liquid nitrogen and stored at -80 °C for subsequent use. Serum was collected from trunk blood immediately chilled on ice, centrifuged at 1500 g and 4 °C, and stored at -80 °C. Serum glucose was determined by AU480 Chemistry Analyzer (Beckman Coulter). Energy expenditure and respiratory exchange ratio were assessed using a combined indirect calorimetry system (TSE Systems).
Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) Imaging

Frozen tissue samples were cryosectioned into 12 μm sections using Microm560 (Microm International, Walldorf, Germany) and thaw mounted onto indium tin oxide-coated conductive slides (Bruker Daltonics, Bremen, Germany). The slides were pre-treated with 1:1 poly-l-lysine (Sigma-Aldrich, Munich, Germany) and 0.1% Nonidet P-40 (Sigma) before mounting. Measurements were conducted, as described (Aichier et al., 2017; Kunzke et al., 2020). Briefly, the samples were covered with 10 mg/ml 1,5-diaminonaphthalene (DAN) matrix (Sigma-Aldrich) in 70% acetonitrile, using a SunCollect sprayer (Sunchrom, Friedrichsdorf, Germany). Data were acquired in negative ion mode using a Bruker Solarix 7.0 T Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonics) over a mass range of 50–650 m/z and at a lateral resolution of 60 μm. SCiLS Lab (v. 2021b Pro) was used for peak picking and exported imzML files were processed with SPACiAL (Prade et al., 2020). After acquisition of the mass spectrometry data, the matrix was removed with 70% ethanol, and the tissue sections were stained with haematoxylin and eosin, coverslipped, scanned with a Mirax desk slide scanner (Zeiss, Göttingen, Germany) using a 20× magnification objective, and co-registered with the respective mass spectrometry imaging data. Metabolites were annotated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) and Compass IsotopePattern (Bruker), allowing a mass error of 4 ppm, M+H adducts, M+H2O-H adducts, M+Na-2H adducts, M+K-2H adducts, and M+Cl adducts.

Real-Time Quantitative Polymerase Chain Reaction (Real-Time qPCR)

Using TRIzol reagent (ThermoFisher Scientific), total RNA was extracted from liver collected immediately after exercise or sham-exercise at each ZT3 and ZT15. One μg of total RNA was reverse transcribed to cDNA using Maxima H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific). cDNA was applied to real-time qPCR using PowerUp SYBR Master Mix (ThermoFisher Scientific). Gene expression was normalized to 18S rRNA. Primers used for qPCR are listed below:

- **mGys2** For 5'-ggagctctggatacctaaagc-3', Rev 5'-gggcttgcttgcttgcttgc-3'
- **mPygf** For 5'-ccagagatcctaccccataaatc-3', Rev 5'-ccaccaacaagactctctgtttc-3'
- **mG6pc** For 5'-ttggtccgcctgtccttgc-3', Rev 5'-ggagctttacacagccagca-3'
- **mPck1** For 5'-gttgtggctgtcctctgtc-3', Rev 5'-gggaactcttgcttgtaaatc-3'
- **mHk2** For 5'-aaactcggcttgggagac-3', Rev 5'-cacaaggggtttgggagac-3'
- **mPdk4** For 5'-gagctgttattccacatcgctgat-3', Rev 5'-cgagctctcagctctgtct-3'
- **mPpara** For 5'-caagaggccagctgtgatcc-3', Rev 5'-ccgcaagaagcccttcagc-3'
- **mPpard** For 5'-gtgctgacgctggctctgatc-3', Rev 5'-ctctttctctctctctct-3'
- **mAss1** For 5'-ccacaacaagatgcctaaagta-3', Rev 5'-atctgtggtggtgggagac-3'
- **mSltf6a1** For 5'-ggtatactatatataatttgctgttc-3', Rev 5'-gcttgccggattttgctaccaaa-3'
- **mPnpla2** For 5'-gtcgatcgctggctgccttcgatcc-3', Rev 5'-tctctttctctttttgcttc-3'
- **mPlin4** For 5'-ggactataacacagacacacagac-3', Rev 5'-tcgatggtggtgggtaccc-3'

18S *rRNA* For 5'-cgcggctagaggttaattc-3', Rev 5'-cgaacctcggactctgc-3'.

Western Blot

Whole cell lysates were extracted from skeletal muscle, liver, and heart using the buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 5 mM Na₂HPO₄, 10 mM NaF, 1% Triton X-100, 100 μM glycerol, 20 mM Tris pH7.8, 1 mM EDTA, 0.2 mM PMSF, 0.5 mM Na₃VO₄, 10 mM NAM, 0.33 mM TAM, 1X protein inhibitor cocktail (Roche). Whole cell lysates were extracted from eWAT as described (Diaz Marin et al., 2019). Twenty μg of lysate per sample was applied to SDS-PAGE. Anti-phospho AMPKα (Cell Signaling Technology, 2535), anti-AMPKα (Cell Signaling Technology, 2603), and anti-p84 (Genetex, GTX70220) antibodies were used for Western blot analyses.

Myotube Contraction and Targeted Liquid Chromatography Mass Spectrometry (LC-MS)

C2C12 myoblasts (#CRL-1772; American Type Culture Collection) were seeded into 35 mm at a density of 10⁵ cells/well, cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, 25 mM glucose) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin at 37°C, 5% CO₂. After reaching ~90% confluence, differentiation was induced by switching from 10% FBS to 2% horse serum. Differentiation medium was changed every 2nd day until fully differentiated myotubes could be visualized under a light microscope (5–6 days). On day 6 of differentiation, cells were washed in warm PBS and the media was changed to a standard Krebs Ringer Phosphate buffer supplemented with 0.2% BSA and 20 mM glucose or 20 mM mannitol (used to prevent osmotic stress) for a total of 6h. Electrical pulse stimulation (EPS) was applied to the cells in culture to induce contraction in the last 3 hours of incubation using the ion-optics system C-dish electrode in a six-well format. The EPS protocol consisted of 3h with 1 Hz frequency, 2 ms in duration and 30 V. Immediately after the end of the contraction protocol the cells were transferred to ice, washed with cold PBS and metabolites were extracted and the cells scraped in 150 μl of ice-cold 0.5 M perchloric acid. Samples were vortexed for 20s and then centrifuged at 14,000 g for 3 min at 4°C and then 100 μl of supernatant was neutralized with the addition of 25 μl of 2.3M KHCO₃ centrifuged again at 14,000 g for 3 min at 4°C, and then 25 μl of the supernatant was transferred to an HPLC tube containing 25 μl of HPLC-grade methanol. LC-MS was performed on an ultra-performance liquid chromatography-tandem mass spectrometer (UPLC-MS-MS, ACQUITY UPLC coupled with Xevo TQ-XS, Waters Corporation, Milford, Massachusetts). The...
auto-sampler was set at 6°C and the column ACQUITY premier BEH Amide 1.7 μm VanGuard FIT 2.1 x 150 mm (Waters Corporation, Milford) temperature was maintained at 40°C. The total run time was 19 min at a flow rate of 200 μL/min. Initially, the gradient started with a 1.5 min at 90% mobile phase B (Acetonitrile/H2O, 90/10 (v/v), 10 mM ammonium acetate, pH at 9), and then rising to 50% mobile phase A (H2O, 10 mM ammonium acetate, pH at 9) over the next 3.5 min. Mobile phase A was quickly increased to 65% in 0.5 min and maintained for 4 min and finally equilibrated to the initial conditions for 4 min. Both mobile phases were spiked with 5 μM methylenediphosphonic acid as a deactivator additive. An injection volume of 1 μL of sample extract was used. MRM transitions 268.1 > 136 and 348 > 136 were used for quantification for Adenosine and AMP respectively. Calibration curves of Adenosine (0.05 nM to 1 μM) and AMP (0.5 nM to 10 μM) were carried out for quantification. Limits of determination (LOD) were 5 nM for both compounds, defined by signal to noise ratio (S/N) > 3. The relative standard deviation (RSD) for both compounds was below 10%. C2C12 experiments were performed twice on separate days and n number indicates individual wells.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical Analysis**
For the statistical analysis of metabolite levels among different experimental conditions, two-way ANOVA (serum, heart, hypothalamus, eWAT, iWAT, and BAT) and three-way ANOVA (muscle and liver) Model Contrasts were used to identify biochemicals that differed significantly between experimental groups, following normalization, log transformation and imputation of missing values, if any, with the minimum observed value for each compound. Differences between two groups were considered statistically significant for p < 0.05. Data are presented as mean ± SEM. Storey’s q value method for estimating the False Discovery Rate (FDR) was used to correct for multiple testing. For the statistical analyses of gene expression and glycogen content, data are presented as mean ± SEM and were analyzed by Two-way ANOVA with Sidak’s post hoc testing where applicable (Prism 7.0). For the analysis of rhythmic metabolites in liver, the nonparametric test Bio Cycle was used incorporating a window of 20-28 h for the determination of circadian periodicity (Agostinelli et al., 2016), including amplitude and phase analysis. A gene or metabolite was considered circadian based on a p value cutoff of 0.05. For single group comparisons, an unpaired two-tailed t test with a p value cutoff of 0.05 was used.

**MetaboAnalyst**
Metabolites positively or negatively associated with 2-HB were determined according to Spearman correlation adjusted p < 0.05 after multiple testing by Benjamini-Hochberg. Enrichment analysis of 2-HB associated metabolites was performed using MetaboAnalyst (Chong et al., 2019) with default parameters and Kyoto Encyclopedia of Genes and Genomes (KEGG) IDs. Quantitative Enrichment Analysis was performed via the Enrichment Analysis module using the pathway based KEGG metabolite set library (84 metabolite sets based on human metabolic pathways) or the main-class chemical structures library (464 main chemical class metabolite sets).

**Metabolite Correlation and Network Statistics**
Metabolite data were auto scaled before correlation analysis. Pearson’s correlation coefficient was calculated on log normalized data to estimate inter- and intra-tissue metabolite pair correlations. Networks are based on metabolite-pair correlations with an estimated p value < 0.0001. Undirected networks were analyzed and visualized using the Compound Spring Embedder (CoSE) layout of Cytoscape (Dogrusoz et al., 2009). Node degree refers to the number of connected neighbor nodes/metabolites. Node closeness was calculated using MATLAB 2020a centrality function as the reciprocal of the sum of the length of the shortest paths between the node and all other nodes in the graph.

**Arterial and Venous (A/V) Differences**
Peak area data were batch normalized. Serum data were normalized to volume extracted. Metabolite values greater than 4 times the standard deviation were considered as outliers and removed. Metabolites with more that 50% missing data were removed from the data. Data were log normalized and missing values were imputed using k-nearest-neighbor. Samples were auto-scaled. Significant tissue uptake or release was estimated using a paired t test for serum A/V data. Log-Fold change of tissue uptake/release was calculated based on replicate median values.

**KEGG Enrichment Analysis**
Metabolites for enrichment were selected based on ANOVA test for differences between groups (exercise vs sedentary, tissue profiling) or paired t test for significant uptake/release (A/V profiling), p values < 0.05. Metabolites were annotated based on Metabolon KEGG annotation. KEGG pathway definitions were downloaded from https://www.genome.jp, release 98.0. KEGG pathway significance was estimated using a hypergeometrical distribution test.

**Data Processing and Quantification for Targeted GC-MS**
Raw files were converted into centroid mode and exported as netCDF files. Metabolites without available standard were identified based on the spectral similarity and retention index criteria using in-house and NIST 17 spectral libraries. 0.85 of similarity score and less than 30 RI difference was used for successful annotation. Sugars with multiple hits were assigned to their structural group. Using spectral similarity of injected standards and putatively identified metabolites, we determined characteristic quantifier ions
and retention time. Characteristic peaks were extracted and integrated using in-house script developed by Swedish Metabolome Center. Extraction was performed in targeted manner. Metabolites with calibration curves were normalized with group specific internal standards: L-Valine-d8, Succinic acid-2,2,3,3-d4, L-Glutamic acid-13C5 15N, D-3-hydroxybutyrate (13C4) and Palmitic acid-d31, and compared with dilution series of native standards. Putatively identified metabolites were normalized to injection standard: 4,4-Dibromoocstfluorobiphenyl. At the end, concentrations and peak areas were normalized to the initial amount of tissue. Results were expressed as µmol of compound in g of tissue for calibration curve covered metabolites and as normalized areas for putatively identified metabolites.