SUPPLEMENTAL MATERIAL

EXPANDED METHODS

Morphological assessment

Liver Fat Content Assessment. The NAFLD activity score (NAS) was used on digitally recorded images at 40x magnification from the label but blinded liver slides stained with H&E.[1-5] Five pictures from each animal in each group were graded for individual criteria, and the aggregated scores were saved for data analyses. Criteria used for the NAS include macro-vesicular steatosis, micro-vesicular steatosis, inflammatory cell infiltrate, and cellular hypertrophy.
Steatosis was graded whether the fat vacuoles displace the nucleus (macro) or not (micro) as *0*=< 5%, *1*=5-33%, *2*=34-66%, and *3*=>66%. Inflammatory foci were defined as an aggregate ofmore than 5 inflammatory cells as a cluster and it was scored as *0* (< 0.5 foci), *1* (0.5-1.0 foci), *2*(1.0-2.0 foci), and *3* (>2.0 foci). Hepatocellular hypertrophy was defined as cellular enlargementmore than 1.5 times the normal hepatocyte diameter. The number of cells and their size were determined using ImageJ1.51u software (NIH, MD).

Liver Collagen Deposition Assessment. Masson´s Trichrome staining was performed following a standardized protocol to assess fibrosis development. [6-8] Stained liver slides were graded for fibrosis using the following scale: *0*: None; *1*: Enlarged, fibrotic portal tracts; *2*: peri-portal or portal-portal septa, but intact architecture; *3*: Fibrosis with architectural distortion, but no evident cirrhosis; *4*: probable or definitive cirrhosis with bridging fibrosis. Five pictures from each animalin each group were graded for individual criteria, and the aggregated scores were saved for data analyses. Digitally recorded images at X40 magnification from labeled but blinded liver slides were evaluated, and scores were recorded for data comparison.

Liver Cell Senescence Activity Assessment. The SA-β-gal activity was detected using the Senescence Associated β-Galactosidase Staining kit (Cell signaling Technology #9860, MA) on fresh snap frozen on liquid nitrogen liver tissue embedded on OCT. Briefly, 6µm liver sections were fixed in kit fixative solution for 2min at room temperature, then washed in PBS twice. Slides were stained overnight in SA- β-gal staining solution at 37°C and pH5.5. In the morning, slides were rinsed with PBSx2, counter-stained with Nuclear Fast Red solution for 3-5min to have a final PBS wash. Sections were dehydrated using an increasing concentration of alcohol,cleared in xylene, and mounted with permount. Five pictures from each animal in each group were graded for individual criteria, and the aggregated scores were saved for data analyses. The percentage of cells with SA-β-gal activity (blue-stained/over total counted cells) was evaluated on the same day. Digitally recorded images at X40 magnification from labeled butblinded liver slides were saved using ImageJ1.51u software.

Liver Cell Apoptosis Activity Assessment. Apoptosis activity was detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method on digital records from blinded liver slides (Click-iT Plus TUNEL assay kit, Invitrogen by Thermo Fisher Scientific, MA). Five pictures from each animal in each group were graded for individual criteria, and the aggregated scores were saved for data analyses. Images at x40 magnification were analyzed using ImageJ1.51u software, and data was generated by comparing the percentage of positive counted cells on DAPI/GFP superimposed images. A Faculty Pathologist supervised morphological assessment of all liver slides at Marshall University.

Metabolomics: Plasma Treatment and Mass Spectrometry

Materials and reagents. General chemicals, as well as glutathione (GSH) and glutathione disulfide (GS:SG) were from Sigma-Aldrich (St. Louis, MO). Homo-glutathione was from Chem-Impex International (Wood Dale, IL). Ophthalmic acid was from Bachem (Torrance, CA). Acetonitrile was procured from Fisher Scientific (Pittsburgh, PA). Octanoate was purchased

from Sigma (Milwaukee, WI).

Plasma Treatment. Heparinized blood, collected in glass tubes, was cooled by gentle repeated inversions in an ice water slurry for 1min and centrifuged (3,000g) at 4°C for 10min. To prevent its oxidation, GSH was immediately converted to a stable thioether by treating 100µl of blood with 100µl of 50mM iodoacetate in 10mM ammonium bicarbonate, pH=10, adjusted with concentrated ammonia hydroxide. After the buffy layer and the red blood cell pellet were removed, aliquots of plasma were treated with 50µl of iodoacetate buffer (vol:vol, 1:1), collectedin pre-labeled micro-tubes and quick-frozen to be stored at -80°C until analysis.

Liquid Chromatography-Mass Spectrometry (LC-MS) for Glutathione sp. Reduced and oxidized glutathione (GSH and GSSG) and OA in plasma were analyzed using methods validated previously with minor modifications. [9-12] In brief, plasma samples were first treated with iodoacetate to derivatize GSH as GS-carboxymethyl, then GSSG in plasma were converted to GS-cyanomethyl using iodoacetonitrile after dithiothreitol reduction. The internal standard homo-glutathione was spiked at the beginning of the process. OA determination was done separately by protein precipitation. All calibration curves consisted of two blanks and seven calibration points. The curve ranges were as follows: GSH, 0.78 – 200µM; GSSG, 0.157 – 40µM; ophthalmate,0.156 – 20µM. A weighting factor of 1/x2 was applied over the calibration curves. The resulting peak area ratios of analyte/internal standard were plotted against the concentrations. Electrospray-ionization mass spectrometry of thioethers was performed on a Thermo Scientific TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, Waltham, MA), equipped with a heated electrospray ion source (HESI-II), coupled to an Agilent 1200 HPLC. The chromatography was done with a reversed-phase C18 column (Synergi 4µ Hydro-RP, 50x2.0mm, Phenomenex). The compounds of interest were separated from mouse plasma endogenous components using 1% acetonitrile containing 0.1% formic acid at 0.1ml/min isocratic. The mass spectrometry was operated under positive ionization mode with the ion spray voltage at 4000V. The ion transfer tube temperature was maintained at 400°C, and the vaporizer temperature was 40°C. The gas setting for sheath, aux, and ion sweep were 50, 2, and 0 arb, respectively. Argon gas pressure was 1.5mTorr used as the collision gas in Q2. Thecollision energy was 15V for monitored transitions. Peak width for Q1 and Q3 was set at 0.7FWHM. Xcalibur software (version2.1.0, Thermo) was used for data registration. The area under the curve of the spectra was recorded and captured on a database for concentration calculations and data analyses.

Liquid Chromatography-Mass Spectrometry (LC-MS) for Non-Targeted Metabolon. Solvent extraction from thaw samples was performed with MAA (methanol: acetonitrile: acetone; 1:1:1) and internal standards were reconstituted with methanol: H_2O; 2:98. LC-MS analyses were performed on a 1290 Infinity Binary LC system from Agilent used for chromatographic separation in conjunction with a Waters Acquity UPLC HSS T3 1.8µm 2.1x100mm column in connection with a Water Acquity UPLC HSS T# 1.8µm pre-column. The column temperature was set up at 55°C at a flow rate of 0.45ml/min with time intervals for system equilibration (7min) and data acquisition (27min) for a total run time of 34min. Mobile phase-A was 0.1% formic acid in the water, and mobile phase-B was 0.1% formic acid in methanol. For elution, mobile phase-A and B were initially held at 98%:2% for 20min. Then, from 20.1min, the mobilephase was brought to 25%-A: 75%-B and held there for 2 min. Then, from 22.1min, the eluent was brought to 2%-A and held there until 30min. Finally, from 30.1 to 37min, the eluent was bought back for re-equilibration to 98%-A. Positive and Negative mass spectra curves were acquired in scan mode with a mass range of 50 to 1000m/z. Inline calibration was performed using debrisoquine sulfate (m/z 176.1182) and HP-0921 from Agilent (m/z 922.0098) in the positive mode, and 4-NBA (m/z 166.0146) and HP-0921 from Agilent (m/z 966.0007 formate adduct) in the negative mode. Mass spectrometer was set up as follows: gas temperature of theion source at 325°C with drying gas flow at 10 l/ml; the nebulizer pressure was 45psi with a sheath gas at 400°C, a sheath flow of 12 l/ml and capillary voltage of 4000V, fragmentor voltageat 140V, and skimmer voltage at 65V. Raw

data were deconvoluted with the National Institute ofStandards and Technology (NIST) Automated Mass Spectral Deconvolution and Identification Software (AMDIS). After spectral analysis and data processing of ≈ 800 signals, 94 signals could be identified in 89% of all samples. Identified signals were confirmed by our metabolomic library and the Fiehn library (Agilent Technologies Inc, Santa Clara, CA). For further quantification, the data was exported to the University of Michigan Core Metabolomic Server.
The concentration of each metabolite was expressed as its relative peak area (divided by the area of the corresponding internal standard in the same chromatogram). Some small compounds, although they were specifically targeted, were not found or identified with certaintyin the present model. They included glycerol, pyruvate, and aceto-acetone. All 94 identified metabolites were included in the statistical analyses.

Statistical analyses of non-targeted metabolites.

Statistical modeling was performed using a linear mixed-effect model of analysis of variance (mixed two-way ANOVA), fitted univariately to each variable (single metabolite concentration). For statistical inference, we used empirical Bayes methods and posterior estimators derived from them (moderated F-, t-, and B statistics) that have proven to result in higher statistical power and to be useful for ranking variables in terms of evidence for differential expression.[13-17]Information was borrowed by constraining the within-block correlations to be equal between variables and using empirical Bayes methods to moderate the standard deviations between them. These methods are particularly appropriate when only a few samples are available, as is always the case in high throughput datasets. [13, 18] Besides, the transformed $\log^{10}$data was interrogated by comparing the HFD group to the NMC group and the intervention groups (pNaK and exercise) to generated heat maps using the R software V5.1 (licensed to the University). Each metabolite from each animal in each group was compared at weeks 12, 16, 20 and 24.

Protein Expression and Activity

Western Blots (WB). Protein gene expressions of FOX01, and Src were evaluated by Western blots. 80µg ofhomogenized liver tissue were prepared to be loaded on nitrocellulose membranes, which were subsequently exposed to protein-specific monoclonal antibody (FOXO1 #2880S from Cell Signaling, pSrc #44-660G from Thermo Fisher Scientific, and c-Src sc-8056 from Santa Cruz Biotech) and developed according to protocols for the FluorChem M System (San Jose, CA). In addition, WB was used to detect thepresence/absence of pNaK in liver tissue in treated/non-treated animal groups and to quantitate the expression of the α1-subunit of the Na/K-ATPase. Our laboratory developed a polyclonal rabbit antibody against pNaK. Polyclonal rabbit antibody against the α1-subunit was purchased from Millipore Sigma (www.emdmillipore.com). The integrated density of the bands in the spectra was measured using ImageJ1.51u software, and spectra/data was saved on laboratory books and GraphPad Prism software, respectively (Loyola, CA, V7.04 licensed the University). For the Src expression, phosphorylated (pSrc) and complete (cSrc) varieties were run on liver samples, and integrated densities were measured. The ratio pSrc/ pSrc+cSrc was displayed.

Real-Time Poly-Chain Reaction (RT-PCR). The effect on lipid metabolism by diet ± interventions was assessed by the expression of the Peroxisome Proliferator-Activated Receptors (PPARs) and its transcriptional coactivator PGC1α using RT-PCR methods. Expression of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was run as a housekeeping gene. Total RNA was extracted from flash-frozen liver tissue using RNeasy Protect Mini kit (QIAGEN, MD) according to the manufacturer's instructions. Total RNA (1µg) was transcribed into cDNA using SuperScript III (Invitrogen, CA) reverse transcription reagents. A quantitative real-time polymerase chain reaction analyzed total RNA. RT-PCR was performed using LightCycler 480 SYBR Green I Master (Roche Life Science) reagents on a Roche LightCycler 480 Instrument II (Roche Life Science, Indianapolis, IN). Specific primers for PPARγ, PGC1α,and GAPDH were used (PPARγ forward TTGAAAGAAGCCAACACTAAACCAC, and PPARγ reverse AATGGCATCTCTGTGTC AACCAT; PGC1α forward GGAGACGTGACCACTGACAATGA, and PGC1α reverse TGTTGG

CTGGTGCCAGTAAGAG; GAPDH forward GCACCGTCAAGGCTGAGAAC, and GAPDH reverse TGGTGAAGACGCCAGTGGA). Each reaction was performed in triplicate, and all experimental samples were normalized using GAPDH as an internal control. The comparative threshold cycle (Ct) method was used to calculate the fold amplification as specified by the manufacturer. Total RNA was analyzed as well to assess the expression of the α1-subunit of the Na/K-ATPase.

Na/K-ATPase Activity Assay. The Na/K-ATPase activity was determined as previously described.[19] Briefly, 100mg of wet weight liver tissue was minced and homogenized in 2ml of buffer solution (0.25M sucrose, 1.25mM EGTA, and 10mM Tris-HCl, pH 7.0) in Glass-cold high-speed homogenizer at 2^0C to yield a 1:2dilution; after filtration through a gauze, further dilutionsof the homogenates (1:4 and 1:8) was made by adding homogenizing buffer. Diluted samples were warmed to 37^0C for 5mins followed by the mixing of diluted sample with reactive medium (vol:vol, 1:9) for a final concentrations of ATP (5.0mM), Mg^{++} (5.0mM), Na^+ (120mM), K^+ (12.5mM), Tris (25.0mM), Cl^- (137.5mM) and azide (5.0mM) at a pH=7.40 and constant T=37^0C. The reaction was terminated by adding cold TCA at 5min of reaction time. The released inorganic phosphate (P_i) was detected by the use of BioMol Green reagent (Enzo Life Sciences,NY) at room temperature. Samples, controls, and standard phosphate solutions were incubated with BioMol green for 25min in the dark, followed by optical density read at 620nm. For each run, a standard curve was generated to calculate the consumption of Pi in μmoles/mg protein/hr.The protein content was determined by the Lowry method, and estimates of Na/K-ATPase activity for a negative control parallel run (10mM of Ouabain added to sample) were performed.

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Supplemental Material

Table 1

List of amino-acids, carbohydrates, and lipids from plasma of rodents exposed to normal mouse chow (NMC) or high fat diet (HFD). Animals with established NASH were provided with no intervention or an intervention, pNaKtide or exercise protocol.

		Experimental Groups						
METABOLITE NAME	Kind	NMC	HFD		HFD	pNaKtide	Exercise	
		24Week	24Week	p-value*	24Week	24Week	24Week	p-value*
3-UREIDOPROPIONIC ACID	Peptide	13633±4566	15533±10936	NS	15533±10936	36844±15500	25387±6649	<0.05
4-COUMARIC ACID	Peptide	31824±19126	13121±6363	<.0.05	13121±6363	10785±9725	12726±5232	NS
4-HYDROXYBENZOIC ACID	Peptide	9132±1185	5225±ND	*NP	5225±ND	ND	8483±ND	NP
2-OXOPENTANOIC ACIDS	Peptide	1713633±334229	1278205±320809	<0.05	1278205±320809	1283838±347822	1541819±389056	NS
GLUTAMINE	Peptide	22951±9624	20569±9510	NS	20569±9510	28264±1254	31441±7436	NS
GLUTATHIONE (OXIDIZED)	Peptide	21631±9770	58391±28237	<0.05	58391±28237	32876±7608	24511±6561	<0.05
HIPPURIC ACID	Peptide	22131742±1988863	22456867±2210184	NS	22456867±2210184	16549282±9429530	18779494±2949507	NS
ISOLEUCINE	Peptide	212672±45422	186668±24327	NS	186668±24327	199393±71768	228445±86743	NS
KYNURENIC ACID	Peptide	15956±5823	9186±4342	NS	9186±4342	7900±1479	7803±1002	NS
L-HISTIDINE	Peptide	8280±3336	7551±3216	NS	7551±3216	10549±2406	15440±5078	NS
L-TRYPTOPHAN	Peptide	526582±58182	511388±52165	NS	511388±52165	455774±98196	470123±181366	NS
LYSINE	Peptide	8474±2194	10975±922	<0.05	10975±922	14163±1867	19853±4068	<0.05
N-ACETYLGLYCINE	Peptide	19086±16253	6539±ND	NP	6539±ND	11632±ND	9306±ND	NP
N-ACETYL-L-ALANINE	Peptide	2428±ND	20516±10736	NP	20516±10736	29284±ND	21585±10641	NP
N-ACETYL-L-LEUCINE	Peptide	130301±44499	105017±29922	<0.05	105017±29922	119675±79799	109605±39073	NS
N-ACETYL-L-PHENYLALANINE	Peptide	61046±11829	38194±6372	<0.05	38194±6372	30019±14979	39666±16066	NS
PHENYLALANINE	Peptide	341328±50115	364373±46304	NS	364373±46304	361671±92631	347017±73207	NS
PIMELIC ACID	Peptide	7642±1255	8244±360	NS	8244±360	11154±3274	7105±ND	NP
THYMIDINE	Peptide	147065±30243	136643±23843	NS	136643±23843	157329±28806	125731±22723	NS
THYMINE	Peptide	263379±10027	245320±22888	NS	245320±22888	242683±47507	237349±53987	NS
TYROSINE	Peptide	223150±20045	184140±63255	NS	184140±63255	215290±111067	302818±70368	<0.05
URIC ACID	Peptide	226627±245663	597501±781901	NS	597501±781901	346352±446629	391535±464285	NS
URIDINE	Peptide	172167±85947	398329±251138	<0.05	398329±251138	347011±174990	239531±95502	NS
ZEATIN	Peptide	167655±24268	150091±10944	<0.05	150091±10944	135863±29654	135411±18393	<0.05

*If n<5 for a metabolite in a group, the statistical analysis was not performed (NP).

METABOLITE NAME	Kind	Experimental Groups						
		NMC	HFD		HFD	pNaKtide	Exercise	
		24Week	24Week	p-value*	24Week	24Week	24Week	p-value*
BUTYRIC ACID	Carbohydrate	136900±13436	238877±40419	<0.05	238877±40419	342006±144902	400409±127502	<0.05
BENZOIC ACID	Carbohydrate	52510±18477	96710±6365	<0.05	96710±6365	58182±28555	70640±17209	<0.05
CITRAMALIC ACID	Carbohydrate	1700753±578074	1864369±856907	NS	1864369±856907	2465687±1100861	2742937±1038740	<0.05
CITRIC ACID	Carbohydrate	2708109±201367	3746763±46031	<0.05	3746763±46031	3282571±877303	2893585±359716	<0.05
DEOXYURIDINE	Carbohydrate	159485±28692	113325±57977	NS	113325±57977	118369±63345	110416±54091	NS
D-GLUCOSAMINE 6-SULFATE	Carbohydrate	18374±8325	17577±1312	*NP	17577±1312	9953±32	5867±3090	NP
GLUCOSE	Carbohydrate	210782±227566	345560±150718	<0.05	345560±150718	180332±210096	187387±173998	<0.05
GLUTARIC ACID	Carbohydrate	55036±20932	150603±61610	<0.05	150603±61610	219213±213481	198069±129484	NS
HOMOVANILLIC ACID	carbohydrate	13689±1028	10045±3139	<0.05	10045±3139	7951±1041	7179±939	<0.05
INDOLE-3-PYRUVIC ACID	carbohydrate	5357±3622	4785±2582	NS	4785±2582	14389±7618	12504±11303	<0.05
INOSINE's	Carbohydrate	104312±26218	163697±66970	<0.05	163697±66970	161328±43310	131891±46443	NS
ISOCITRIC ACID	carbohydrate	3562142±250803	4110980±995231	<0.05	4110980±995231	4003415±820203	3761590±403912	NS
LACTIC ACID	carbohydrate	233017±53067	269941±120642	NS	269941±120642	224862±85464	281236±73993	NS
MALEIC ACID	carbohydrate	42927±15026	73767±24525	<0.05	73767±24525	79419±57489	81615±32297	NS
MALIC ACID	carbohydrate	46118±11016	57993±16328	<0.05	57993±16328	112550±121282	98393±49210	<0.05
METHYL BETA-D-GALACTOSIDE	carbohydrate	139948±15875	15084±9067	<0.05	15084±9067	21798±6509	10411±2838	<0.05
METHYLMALONIC ACID	carbohydrate	18467±6095	21842±1848	NS	21842±1848	20232±4480	18512±3916	NS
OXALOACETIC ACID	carbohydrate	412772±48365	388334±39291	NS	388334±39291	399300±75048	387790±30313	NS
PHTHALIC ACID	carbohydrate	22434±0	11568±2642	NP	11568±2642	11294±1624	8818±0	NP
SUCCINIC ACID	carbohydrate	209834±153853	551798±356372	<0.05	551798±356372	644551±876855	279416±130807	NS
THYMIDINE	carbohydrate	147065±30243	136643±23843	NS	136643±23843	136260±48226	125731±22723	NS
TRANS-ACONITIC ACID	carbohydrate	234201±210269	313096±221031	NS	313096±221031	302900±193617	238550±114493	NS
XANTHINE	carbohydrate	32567±0	63862±58166	NP	63862±58166	63862±58166	152911±113380	NS
XANTHOSINE	carbohydrate	15451±0	23618±13508	NP	23618±13508	64524±0	0±0	NP

*If n<5 for a metabolite in a group, the statistical analysis was not performed (NP).

METABOLITE NAME	Kind	Experimental Groups						
		NMC	HFD		HFD	pNaKtide	Exercise	
		24Week	24Week	p-value*	24Week	24Week	24Week	p-value*
ADIPIC ACID	Lipid	9023±2135	14024±5700	<0.05	14024±5700	17227±4781	13639±5015	NS
ARACHIDIC ACID	Lipid	17808±7593	16941±10347	NS	16941±10347	18081±12009	18229±9083	NS
ARACHIDONIC ACID	Lipid	56224±20232	187550±89136	<0.05	187550±89136	96568±63643	88434±46903	<0.05
BEHENIC ACID	Lipid	17921±2014	14981±3108	NS	14981±3108	18276±7055	16349±3991	NS
CAPRYLIC ACID	Lipid	8235±0	14695±5273	NP	14695±5273	51796±23068	87502±50693	<0.05
CIS-11-EICOSENOIC ACID	Lipid	6653±1896	7079±2194	NS	7079±2194	16156±12193	7895±1027	NS
CORTICOSTERONE	Lipid	51373±5709	85029±11330	<0.05	85029±11330	77224±20347	57310±18100	<0.05
DOCOSAHEXAENOIC ACID	Lipid	140460±91921	125748±111514	NS	125748±111514	55801±60722	47702±31259	NS
EPIBRASSINOLIDE	Lipid	430639±36260	392399±39397	NS	392399±39397	379010±53178	378321±74660	NS
ERUCIC ACID	Lipid	6393±3349	7132±3663	NS	7132±3663	6543±4767	6600±3463	NS
HEPTADECANOIC ACID	Lipid	3545±722	4146±1220	NS	4146±1220	7261±2387	4673±976	<0.05
LIGNOCERIC ACID	Lipid	2016±669	1951±750	NS	1951±750	3226±876	2165±723	<0.05
LINOLEIC ACID	Lipid	102817±66692	114235±34869	NS	114235±34869	166039±49043	92415±36615	<0.05
MYRISTIC ACID	Lipid	4038±1518	3553±995	NS	3553±995	5750±3056	3231±1067	NS
MYRISTOLEIC ACID	Lipid	8435±4072	10032±2060	NS	10032±2060	12153±6768	7023±2555	NS
NERVONIC ACID	Lipid	1959±482	1798±334	NS	1798±334	2468±1169	1362±419	<0.05
OLEIC ACID	Lipid	177669±100039	236903±51510	NS	236903±51510	380254±138107	215827±33938	<0.05
PALMITIC ACID	Lipid	56987±32515	63557±8874	NS	63557±8874	130281±83954	68882±19078	<0.05
PALMITOLEIC ACID	Lipid	20565±16069	27060±7841	NS	27060±7841	39032±10464	24501±10357	<0.05
SEBACIC ACID	Lipid	28480±8595	75404±0	NP	0±0	4752±0	4618±0	N.P.
STEARIC ACID	Lipid	57709±20613	75404±15206	NS	75404±15206	122990±68209	83680±24176	NS

*If n<5 for a metabolite in a group, the statistical analysis was not performed (NP).