

AMP-activated protein kinase regulates nicotinamide phosphoribosyl transferase expression in skeletal muscle

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Key points

- NAD is a substrate for sirtuins (SIRT6), which regulate gene transcription in response to specific metabolic stresses.
- Nicotinamide phosphoribosyl transferase (Nampt) is the rate-limiting enzyme in the NAD salvage pathway.
- Using transgenic mouse models, we tested the hypothesis that skeletal muscle Nampt protein abundance would increase in response to metabolic stress in a manner dependent on the cellular nucleotide sensor, AMP-activated protein kinase (AMPK).
- Exercise training, as well as repeated pharmacological activation of AMPK by 5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide (AICAR), increased Nampt protein abundance. However, only the AICAR-mediated increase in Nampt protein abundance was dependent on AMPK.
- Our results suggest that cellular energy charge and nutrient sensing by SIRT6 may be mechanistically related, and that Nampt may play a key role for cellular adaptation to metabolic stress.

Abstract Deacetylases such as sirtuins (SIRT6) convert NAD to nicotinamide (NAM). Nicotinamide phosphoribosyl transferase (Nampt) is the rate-limiting enzyme in the NAD salvage pathway responsible for converting NAM to NAD to maintain cellular redox state. Activation of AMP-activated protein kinase (AMPK) increases SIRT6 activity by elevating NAD levels. As NAM directly inhibits SIRT6, increased Nampt activation or expression could be a metabolic stress response. Evidence suggests that AMPK regulates Nampt mRNA content, but whether repeated AMPK activation is necessary for increasing Nampt protein levels is unknown. To this end, we assessed whether exercise training- or 5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide (AICAR)-mediated increases in skeletal muscle Nampt abundance are AMPK dependent. One-legged knee-extensor exercise training in humans increased Nampt protein by 16% ($P < 0.05$) in the trained, but not the untrained leg. Moreover, increases in Nampt mRNA

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following acute exercise or AICAR treatment ($P < 0.05$ for both) were maintained in mouse skeletal muscle lacking a functional AMPK $\alpha 2$ subunit. Nampt protein was reduced in skeletal muscle of sedentary AMPK $\alpha 2$ kinase dead (KD), but 6.5 weeks of endurance exercise training increased skeletal muscle Nampt protein to a similar extent in both wild-type (WT) (24%) and AMPK $\alpha 2$ KD (18%) mice. In contrast, 4 weeks of daily AICAR treatment increased Nampt protein in skeletal muscle in WT mice (27%), but this effect did not occur in AMPK $\alpha 2$ KD mice. In conclusion, functional $\alpha 2$ -containing AMPK heterotrimers are required for elevation of skeletal muscle Nampt protein, but not mRNA induction. These findings suggest AMPK plays a post-translational role in the regulation of skeletal muscle Nampt protein abundance, and further indicate that the regulation of cellular energy charge and nutrient sensing is mechanistically related.

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Abbreviations $\alpha 2i$, catalytically inactive alpha 2 subunit; $\gamma 1$ TG, transgenic $\gamma 1$ subunit; AICAR, 5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide; AMPK, AMP-activated protein kinase; A.U., arbitrary units; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KD, kinase dead; KO, knockout; NAM, nicotinamide; Nampt, nicotinamide phosphoribosyl transferase; PGC-1 α , peroxisome proliferator-activated receptor γ -coactivator-1 α ; P/S, penicillin streptomycin; qPCR, quantitative polymerase chain reaction; sh, short hairpin; SIRT, sirtuin; TBP, tata box-binding protein; TG, transgenic; WT, wild-type; ZMP, 5-aminoimidazole-4-carboxamide ribotide.

Introduction

Mitochondrial oxidative ATP synthesis is tightly coupled to the cycling of NAD between oxidised (NAD) and reduced (NADH) forms. The contribution of NAD to other cellular processes has long been assumed (Rechsteiner *et al.* 1976), and the discovery that NAD acts as a required substrate in signalling pathways critical in maintaining cellular metabolic homeostasis (Cantó *et al.* 2009) has heightened interest in NAD metabolism.

Sirtuins (SIRT) were first recognised for their potential role in promoting longevity in response to caloric restriction by a mechanism that involves modulation of mitochondrial respiration capacity (Lin *et al.* 2000, 2002; Dali-Youcef *et al.* 2007). NAD acts as a substrate for SIRT (designated in mammals as SIRT1–SIRT7), resulting in SIRT-dependent histone deacetylation and modulation of other proteins. During this reaction, NAD is converted to nicotinamide (NAM). Because NAM inhibits SIRT activity (Bitterman *et al.* 2002), NAM must be reconverted to NAD to maintain SIRT activity and mitochondrial metabolism. The rate-limiting enzyme in the NAD salvage pathway is nicotinamide phosphoribosyl transferase (Nampt; Revollo *et al.* 2004; Garten *et al.* 2009). Thus, Nampt may influence the cellular response to a variety of metabolic stresses such as caloric restriction or exercise via regulation of NAM biosynthesis.

SIRT1, the most intensively studied SIRT to date, deacetylates non-histone proteins such as peroxisome proliferator-activated receptor γ -coactivator-1 α (PGC-1 α), a key element in the adaptive response to metabolic stress-induced mitochondrial biogenesis (Puigserver *et al.*

1998; Nemoto *et al.* 2005; Rodgers *et al.* 2005), as well as p53 (Luo *et al.* 2001), p300 (Bouras *et al.* 2005) and MyoD (Fulco *et al.* 2008). Although the role of SIRT1 in mediating exercise-induced increases in mitochondrial biogenesis has been challenged (Philp *et al.* 2011), SIRT1-dependent responses to exercise and fasting are compromised in AMP-activated protein kinase (AMPK)-deficient skeletal muscle (Cantó *et al.* 2010). AMPK is a heterotrimeric protein consisting of multiple isoforms of catalytic ($\alpha 1$, $\alpha 2$) and regulatory ($\beta 1$, $\beta 2$ and $\gamma 1$, $\gamma 2$, $\gamma 3$) subunits, which mainly functions as a major sensor of cellular fuel status (Koh *et al.* 2008). In human and rodent skeletal muscle, AMPK trimers containing $\alpha 2$ catalytic subunits are dominant (Wojtaszewski *et al.* 2005; Treebak *et al.* 2009). Thus, a signalling network containing AMPK, Nampt and SIRT1 may interact at the level of PGC-1 α to mediate transcriptional responses.

AMPK activation raises intracellular NAD concentrations and activates SIRT1 (Cantó *et al.* 2009), possibly via augmented Nampt activity or protein abundance. Skeletal muscle Nampt protein abundance is increased with endurance exercise training in humans (Costford *et al.* 2010), but whether these effects are specific to contracting muscle or secondary to improvements in the whole-body metabolic milieu concurrent with training is unclear. Interestingly, exercise- and fasting-induced increases in Nampt mRNA levels are blunted in skeletal muscle of AMPK $\gamma 3$ knockout (KO) mice (Cantó *et al.* 2010). Moreover, Nampt expression is increased during glucose restriction in C2C12 mouse myoblasts and mouse skeletal muscle in an AMPK-dependent manner

(Fulco *et al.* 2008; Wang *et al.* 2012). Collectively, these findings suggest that cellular fuel sensing and downstream alterations in metabolism may be mechanistically connected via AMPK and Nampt.

Here we assessed the effect of one-legged exercise training on skeletal muscle Nampt protein abundance in healthy volunteers. Because of the apparent functional connection between the cellular energy level and SIRT activity with AMPK and Nampt functioning as potentially critical intermediates, we hypothesised that increases in skeletal muscle Nampt protein are dependent on AMPK signalling. To address this, we studied several mouse models of reduced skeletal muscle AMPK activity to determine the effect of exercise and AMPK activators (5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide (AICAR) and metformin) on muscle Nampt protein abundance. Because AMPK is required for the ability of PGC-1 α to function as a transcriptional co-activator (Jäger *et al.* 2007), we also tested the hypothesis that Nampt protein is regulated by PGC-1 α in response to exercise training and repeated AMPK activation using PGC-1 α -deficient mice.

Methods

Ethical approval

All animal experiments were approved by the Danish Animal Experimental Inspectorate, and complied with the European Convention for the protection of Vertebrate Animals used for Experiments and Other Scientific Purposes (Council of Europe 123, Strasbourg, France, 1985). Protocols for experiments conducted at Joslin Diabetes Center were in agreement with guidelines of the Institutional Animal Care and Use Committee of the Joslin Diabetes Center, and the National Institutes of Health. In addition, experiments conformed to the principles of UK regulations as previously described (Drummond, 2009). The number of animals used for each experiment is stated in each specific section. The human training experiment was approved by the local ethics committee and performed in agreement with the Declaration of Helsinki. All subjects provided informed consent before participating in the study.

Generation of cell lines

To assess the validity of Nampt antibodies used in this study, C2C12 mouse myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen # 41965-062) containing 10% foetal bovine serum (FBS, Sigma # F7524) and 0.05 $\mu\text{g mL}^{-1}$ penicillin streptomycin (P/S; Invitrogen # 15070-063) at 37°C, 5% CO₂. For overexpression of FLAG-tagged Nampt, mouse Nampt was cloned into p3xflag-cmv-9-10_G903 vector (Sigma # 4401), and C2C12 myoblasts were trans-

ected with 2 $\mu\text{g well}^{-1}$ (9.6 cm²) using Lipofectamine 2000 (Invitrogen #11668-027) and OptiMem (Invitrogen # 51985-026) according to manufacturer's instructions. Cells were harvested the following day by washing once using ice-cold phosphate-buffered saline and adding lysis buffer (in mM: HEPES, 50, pH 7.4; 10% glycerol; 1% IGEPAL; NaCl, 150; NaF, 10; EDTA, 1; EGTA, 1; sodium pyrophosphate, 20; sodium orthovanadate, 2; protease inhibitors (SigmaFast, Sigma Aldrich) according to manufacturer's instructions). Protein concentration was determined via bicinchoninic acid assay (Thermo Scientific # 23223).

Stable Nampt knockdown C2C12 cells were generated using short hairpin (sh)RNA delivered by lentiviral infection. Human embryonic kidney 293FT cells (60% confluent, 9.6 cm² plate) were co-transfected with pLKO (shRNA-containing plasmid), psPAX2 packaging plasmid and pMD2.G envelope plasmid (ratio: 2 $\mu\text{g pLKO}$; 1.5 $\mu\text{g psPAX2}$; 0.5 $\mu\text{g pMD2.G}$) using Superfect transfection reagent (Qiagen) and OptiMem. Two separate shRNA sequences (Nampt_Sh1, Nampt_Sh2; Open Biosystems # RMM3981–201818874, RMM3981–201824136) and a scrambled control (Sigma # SHC202) were used. The medium was replaced the following morning with DMEM containing 10% FBS and P/S. Forty-eight hours later, virus-containing media was collected and used to infect 40% confluent proliferating C2C12 myoblasts. Cells were changed to media containing 2.5 $\mu\text{g mL}^{-1}$ puromycin (Sigma) 24 h after infection. Cells were maintained in selection media until immediately prior to an experiment.

Exercise training – humans

Skeletal muscle samples from eight young male subjects were obtained from a previous study (Frøsig *et al.* 2004). The training programme consisted of 15 sessions of one-legged knee extensor endurance training over the course of 3 weeks. Subjects performed four sessions in week 1, five sessions in week 2, and six sessions in week 3. The duration of training sessions started at 1 h per session and was gradually and consistently increased to 2 h per session for all subjects. Needle biopsies were obtained before training from the vastus lateralis muscle of the rested and exercised leg under local anaesthesia (2% lidocaine), and again 15 h after the final exercise bout.

Assessment of Nampt protein abundance in non-stimulated mouse skeletal muscle

To assess the importance of AMPK on Nampt protein abundance, we studied three different transgenic mouse strains ($n = 5–11$ per strain) and corresponding wild-type (WT) littermates ($n = 6–9$ per strain). Tibialis anterior muscles from skeletal muscle-specific LKB1 KO mice (LKB1 KO; the major activating kinase of AMPK),

transgenic mice carrying a muscle-specific inactive AMPK $\alpha 2$ isoform (AMPK $\alpha 2i$) and from transgenic mice with elevated muscle AMPK activity due to a muscle-specific AMPK-activating R70Q $\gamma 1$ mutation in the AMPK $\gamma 1$ subunit (AMPK $\gamma 1$ TG) were removed following cervical dislocation and immediately frozen in liquid nitrogen. These models have been described in detail previously (Koh *et al.* 2006; Barré *et al.* 2007; Fujii *et al.* 2008).

Acute exercise – mice

For familiarisation to treadmill running prior to experiments, mice were exercised for approximately 5 min day⁻¹ for three consecutive days at speeds of approximately 5–10 m min⁻¹. Male AMPK $\alpha 2$ KO mice ($n = 32$ –40) and WT littermates ($n = 32$ –40; Viollet *et al.* 2003) were run on a motorised treadmill for 90 min (10 min at 13 m min⁻¹, 80 min at 17 m min⁻¹), as previously described (Jørgensen *et al.* 2005). Mice were killed by cervical dislocation, and quadriceps muscles were removed from sedentary animals and immediately following, 1 h and 3 h following exercise cessation.

Exercise training – mice

Female mice overexpressing a kinase dead (KD) $\alpha 2$ AMPK subunit (Mu *et al.* 2001; $n = 28$) and WT littermates ($n = 28$) underwent 6.5 weeks of endurance exercise training or served as sedentary controls. Mice had free access to wheel cages for voluntary running 7 days week⁻¹. Running distance was recorded using a cycling odometer (BC1009, Sigma, Germany). In addition, mice were exercised 1 h day⁻¹ at 16 m min⁻¹ on a motorised treadmill (Columbus Instruments) on weekdays. During the first week of the training period, treadmill exercise was performed for 10 min on day 1, 20 min on day 2, 30 min on day 3, 40 min on day 4 and 50 min on day 5. The morning following the final exercise bout, mice were anaesthetised by an intraperitoneal injection using Avertin (250 mg tribromoethanol kg⁻¹ body weight). Quadriceps muscles were removed, frozen in liquid nitrogen and stored at -80°C until further analysis. Following a similar combined treadmill and wheel-cage training protocol, PGC-1 α KO and WT mice (Lin *et al.* 2004) were exercised for 5 weeks. Quadriceps muscle samples from this experiment have previously been used for other analyses (Leick *et al.* 2008).

Acute AICAR treatment

Following a 6 h fast, 36 female C57BL/6J mice were injected subcutaneously with either saline or AICAR (500 mg kg⁻¹ body weight) to determine the time course of AICAR-mediated Nampt induction. Mice were killed by cervical dislocation 2, 4 and 8 h after injection,

and quadriceps muscles were removed, frozen in liquid nitrogen and analysed for Nampt mRNA expression. Following this experiment, female AMPK $\alpha 2$ KD and control animals received a single intraperitoneal injection of 500 mg kg⁻¹ body weight AICAR or 0.9% NaCl solution. Eight hours following the injection, mice were killed by cervical dislocation, and quadriceps muscles were removed, frozen in liquid nitrogen and stored at -80°C .

Repeated AICAR treatment

Two genetic mouse models were used to study the effect of repeated AICAR administration on skeletal muscle Nampt expression. Male AMPK $\alpha 2$ KD ($n = 15$) and control mice ($n = 16$) received daily subcutaneous injections of 500 mg kg⁻¹ body weight AICAR or 0.9% NaCl solution for 4 weeks. Mice were anaesthetised by an intraperitoneal injection using Avertin (250 mg kg⁻¹ body weight) 24 h after the last injection, and quadriceps muscles were removed, frozen in liquid nitrogen and stored at -80°C . Samples were also obtained from a previously published study of PGC-1 α KO and WT mice that were treated under the same conditions and as previously described (Leick *et al.* 2010).

Metformin treatment

AMPK $\alpha 2$ KD ($n = 24$) and control mice ($n = 22$) were treated with an oral dosage of 150 mg kg⁻¹ metformin twice per day (i.e. a total dose of 300 mg kg⁻¹ per day) or saline for 2 weeks. Samples were obtained from a previously published study (Kristensen *et al.* 2013). Metformin or saline solutions were administered via oral gavage. The final dose of metformin or saline was administered on the afternoon preceding the experimental day. Mice were anaesthetised by an intraperitoneal injection of pentobarbital (100 mg kg⁻¹ body weight). Gastrocnemius muscles were removed, separated into white and red portions, frozen in liquid nitrogen, and stored at -80°C .

Western blot analysis

Muscle samples were processed in ice-cold lysis buffer (in mM: Hepes, 50, pH 7.4; 10% glycerol; 1% IGEPAL; NaCl, 150; NaF, 10; EDTA, 1; EGTA, 1; sodium pyrophosphate, 20; sodium orthovanadate, 2; protease inhibitors (SigmaFast, Sigma Aldrich) according to manufacturer's instructions), resolved using SDS-PAGE, and transferred as previously described (Frøsig *et al.* 2004). Aliquots were loaded in a balanced manner, with samples from all experimental conditions present on all gels. Following transfer, mouse samples were subjected to immunoblot analysis to detect Nampt protein (Bethyl, A300–372A). Exercise training-induced adaptation in

skeletal muscle was confirmed by immunoblot analysis for hexokinase II protein (Cell Signalling, 2687). Human samples were subjected to immunoblot analysis to detect Nampt protein (Bethyl, A300–779A). Samples from C2C12 cells overexpressing a Nampt-FLAG were subjected to immunoblot analysis using an anti-FLAG antibody (Sigma, 7425). Western blots were visualised using a BioRad ChemiDoc chemiluminescence system, and densitometry analyses were performed using ImageLab software version 3.0 (Bio-Rad, Hercules, CA, USA).

Quantitative polymerase chain reaction (qPCR)

Total RNA from 20–30 mg of mouse muscle or C2C12 samples were extracted using Trizol (Qiagen). RNA (1 μ g) was reverse-transcribed with a high-capacity complementary DNA (cDNA) reverse transcription kit (Applied Biosystems). Realtime PCR was performed, starting with 12.5 ng of cDNA and both sense and antisense oligonucleotides (300 nM each) in a final volume of 10 μ l with the SYBR Green PCR Master Mix (Applied Biosystems). Fluorescence was monitored and analysed in a CFX96 Realtime system (BioRad). The obtained cycle threshold (Ct) values reflecting the initial content of the specific transcript in the samples were converted to an arbitrary amount by using standard curves obtained from a serial dilution of a pooled sample made from all samples. Gene expression was normalised by housekeeping gene (TBP or glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) expression performed in parallel or by unit of input cDNA (Qubit ssDNA assay kit, Invitrogen). Amplification of specific transcripts was confirmed by analysing melting curve profiles at the end of each PCR experiment. Primer sequences for Nampt were: FP: 5'-CTCTTCGCAAGAGACTGCTGG-3'; RP: 5'-GAGCAATTCGCCACAGTATC-3'. TBP primers used were: FP: ACCCTTCACCAATGACTCCTATG; RP: TGACTGCAGCAAATCGCTTGG. A standard commercial GAPDH assay (Applied Biosystems) was used.

Statistics

Data are reported as means \pm SEM. Samples from the human training study (Fig. 2) were analysed using a 2×2 repeated-measures ANOVA. Differences in Nampt protein abundance between mouse models of altered AMPK activity (Fig. 3) were analysed via unpaired, two-tailed *t* tests. The effect of acute exercise on Nampt mRNA (Fig. 4) was analysed using a 2×4 ANOVA (genotype by time point). For exercise training and AICAR studies in mice (Figs 5, 6B and C and 7), data were analysed using a 2×2 ANOVA (genotype by time point). The effect of acute AICAR on Nampt mRNA (Fig. 6A) was analysed via 2×3 (treatment by time point) ANOVA. For metformin studies (Fig. 8), data were analysed using

a $2 \times 2 \times 2$ ANOVA (genotype by time point by tissue). Statistical significance was set at $P < 0.05$.

Results

Test of antibody specificity

The validity of the Nampt antibodies (Bethyl, A300–372A (mouse) and A300–779A (human)) used throughout this study was tested in C2C12 myoblast cells after silencing or overexpressing Nampt protein. Nampt was silenced using a shRNA lentiviral approach and transiently overexpressed using FLAG-tagged Nampt in mouse C2C12 myoblast cells. qPCR experiments showed a consistent $\approx 90\%$ reduction in Nampt mRNA levels (Fig. 1A). To confirm the specificity of the Nampt signal, lysates from cells overexpressing Nampt-FLAG were resolved using SDS-PAGE together with control C2C12 and Nampt knockdown cells (Fig. 1B). A 'split blot' analysis was performed where the same sample from a FLAG-Nampt-overexpressing cell was resolved in three adjacent wells. After transfer to polyvinylidene difluoride, the membrane was cut through the centre well and the membrane halves were probed with anti-FLAG and the A300–372A anti-Nampt antibodies, respectively. Complete alignment of the bands was confirmed (Fig. 1C). Finally, another split blot analysis was performed using lysates from mouse and human skeletal muscle and mouse liver to compare the signal from the A300–372A antibody with the signal from the A300–779A antibody (Fig. 1D). The bands detected in human skeletal muscle using the two different antibodies ran at the expected molecular mass (≈ 52 kDa). Collectively, these experiments confirm that the Nampt antibody A300–372A specifically detects Nampt in mouse C2C12 cultured cells and is suitable for the detection of Nampt in mouse skeletal muscle. The antibody A300–779A also detects Nampt in human skeletal muscle, but it does not appear to cross-react with mouse Nampt protein.

Endurance exercise training in humans increases skeletal muscle Nampt protein

A longitudinal study reveals that exercise training increases Nampt expression in human vastus lateralis muscle (Costford *et al.* 2010). We employed a 3 week one-legged knee extensor training programme to determine whether exercise training increases Nampt protein directly in the exercised muscle or secondarily to improvements or alterations in whole-body metabolism. This is a well-controlled exercise modality that results in specific activation of the quadriceps femoris (Andersen *et al.* 1985). One-legged endurance exercise training increased Nampt protein abundance in the trained, but not the untrained, leg (Fig. 2; $P < 0.05$).

AMPK affects basal Nampt protein abundance

AMPK is an important mediator of muscular adaptations to exercise (Jørgensen *et al.* 2006; Egan & Zierath, 2013). The relationship between AMPK and Nampt, as well as effects on SIRT biology have been documented (Fulco *et al.* 2008). We hypothesised that Nampt concentrations in sedentary muscle depend on functional AMPK $\alpha 2$ signalling. Our findings show Nampt protein level was reduced in skeletal muscles in which LKB1 or AMPK activity is ablated (Fig. 3A and B). Conversely, Nampt protein abundance was increased in skeletal muscle from a mouse model of increased AMPK activity (AMPK $\gamma 1$ R70Q transgenic mice; Barré *et al.* 2007), compared with WT controls (Fig. 3C).

Acute exercise increases Nampt mRNA independent of AMPK $\alpha 2$

Swimming exercise increases Nampt mRNA in mouse skeletal muscle in an AMPK $\gamma 3$ -dependent manner (Cantó *et al.* 2010). To test whether AMPK $\alpha 2$ -containing

heterotrimers are involved in exercise-mediated Nampt mRNA induction, we assessed the effect of a single acute bout of treadmill running on Nampt mRNA in WT and AMPK $\alpha 2$ KO mice. Interestingly, Nampt mRNA expression was increased 3 h after exercise cessation in an AMPK-independent manner (Fig. 4).

Endurance training increases skeletal muscle Nampt protein in an AMPK- and PGC-1 α -independent manner

Given that: (1) basal Nampt protein abundance partly depends on AMPK $\alpha 2$; and (2) endurance exercise training increases Nampt concentrations, we asked whether exercise training-induced increases in muscle Nampt protein depend on AMPK $\alpha 2$. WT and $\alpha 2$ AMPK KD mice completed an endurance exercise training protocol in which voluntary wheel running was combined with forced exercise on a motorised treadmill for 6.5 weeks. Endurance training elevated Nampt protein levels relative to sedentary controls ($P < 0.05$; Fig. 5A). This training effect was independent of AMPK activity, as the training response in

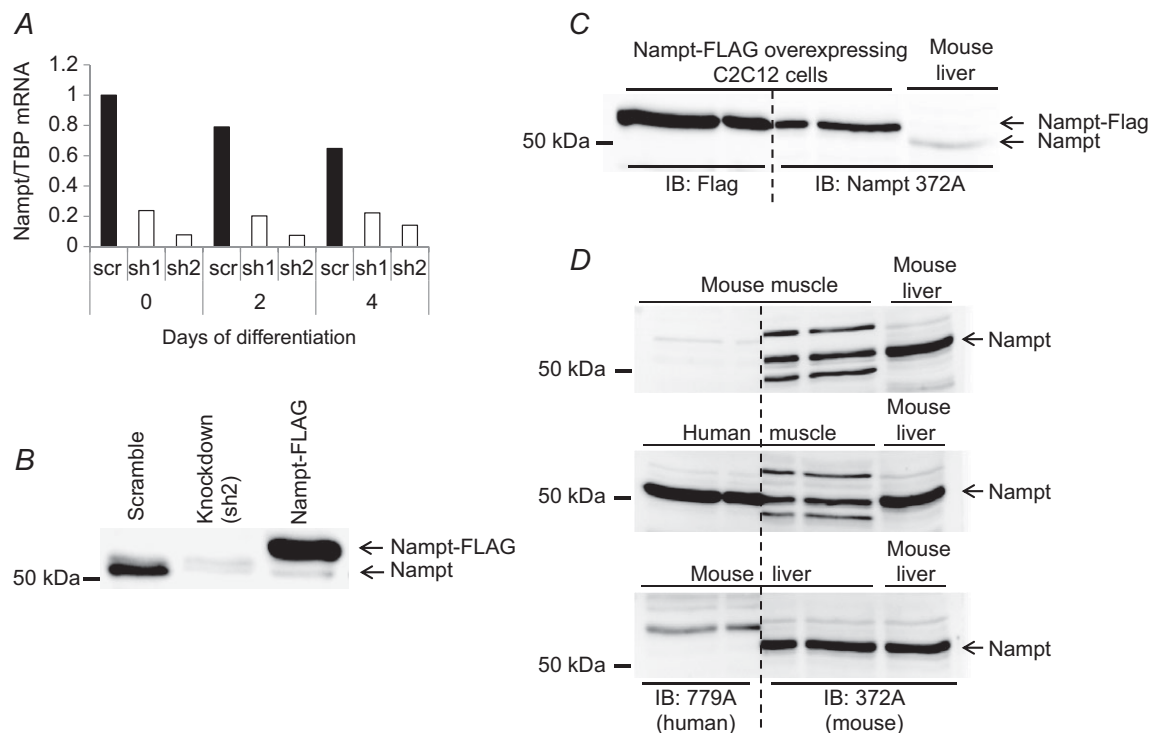


Figure 1. Nampt antibody specificity and validity

Nampt knockdown reduces (A) Nampt mRNA levels and (B) Nampt protein expression in C2C12 myoblasts. C, three identical C2C12 lysate aliquots from cells overexpressing FLAG-tagged Nampt were resolved alongside a mouse liver sample (right-hand lane). After protein transfer to polyvinylidene difluoride, the membrane was cut in two pieces as indicated by the dashed line, incubated with antibodies specific to FLAG (left side) and Nampt (right side), and re-assembled before visualisation. D, antibody specificity in human and mouse tissues. In separate gels, mouse muscle, human muscle and mouse liver lysates were resolved alongside a mouse liver sample (right-hand lane). After protein transfer to polyvinylidene difluoride, the membrane was then cut in two pieces as indicated by the dashed line, incubated with human Nampt (left side) or mouse Nampt antibody (right side), and re-assembled before visualisation. sh1, sh2, short hairpin clone 1, 2.

Nampt protein abundance was not significantly different between WT and AMPK $\alpha 2$ KD mice. WT and KD mice performed similar amounts of voluntary running (WT: 150 ± 20.1 km vs. KD: 150 ± 21.1 km). A similar training-induced adaptation in hexokinase II protein abundance between WT and AMPK $\alpha 2$ KD mice further reinforced a comparable response to exercise training (Fig. 5B). Moreover, Nampt mRNA levels in these samples were similar between WT and AMPK $\alpha 2$ KD muscle,

and did not differ between control and trained animals (Fig. 5C).

AMPK phosphorylates PGC-1 α on at least two residues that appear to be necessary for SIRT1-mediated deacetylation/activation of PGC-1 α (Jäger *et al.* 2007; Cantó *et al.* 2009). Based on our evidence that AMPK is necessary for full Nampt expression in skeletal muscle (Figs 3 and 5A), Nampt expression is possibly regulated by the AMPK-PGC-1 α signalling axis. To test this hypothesis, we measured Nampt protein abundance in PGC-1 α KO and WT mice in the untrained state and in response to endurance exercise training (Leick *et al.* 2008). Nampt protein abundance was similar between both genotypes in

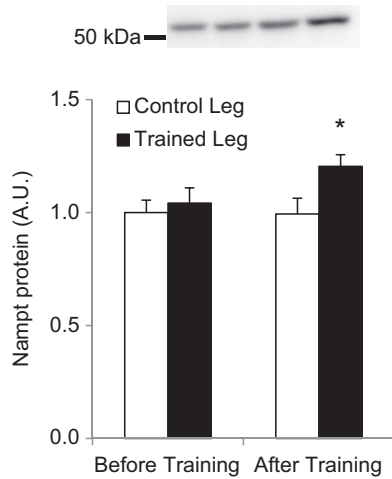


Figure 2. Three weeks of one-legged knee extensor exercise training in humans increases Nampt protein in trained, but not untrained, skeletal muscle

Human volunteers performed 15 sessions of one-legged knee extensor endurance training over the course of 3 weeks. Skeletal muscle biopsies were obtained from both vastus lateralis muscles before and after training ($n = 8$). * Indicates treatment \times time interaction effect ($P < 0.05$).

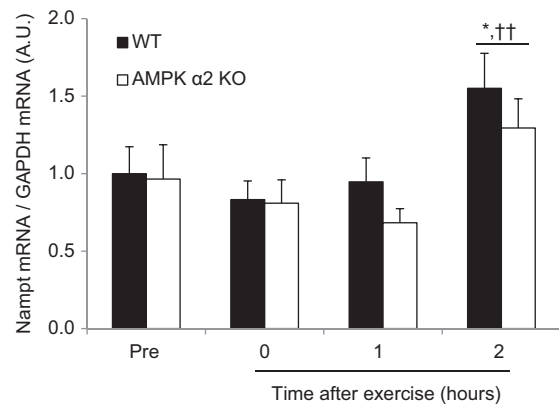


Figure 4. Acute exercise increases mouse skeletal muscle Nampt mRNA in an AMPK $\alpha 2$ -independent manner

Nampt mRNA was measured in AMPK $\alpha 2$ WT and KO mouse muscle 3 h after completion of a 90 min treadmill exercise bout ($n = 6-13$). * Indicates vs. Pre ($P < 0.05$); †† indicates vs. 0, 1 h ($P < 0.01$).

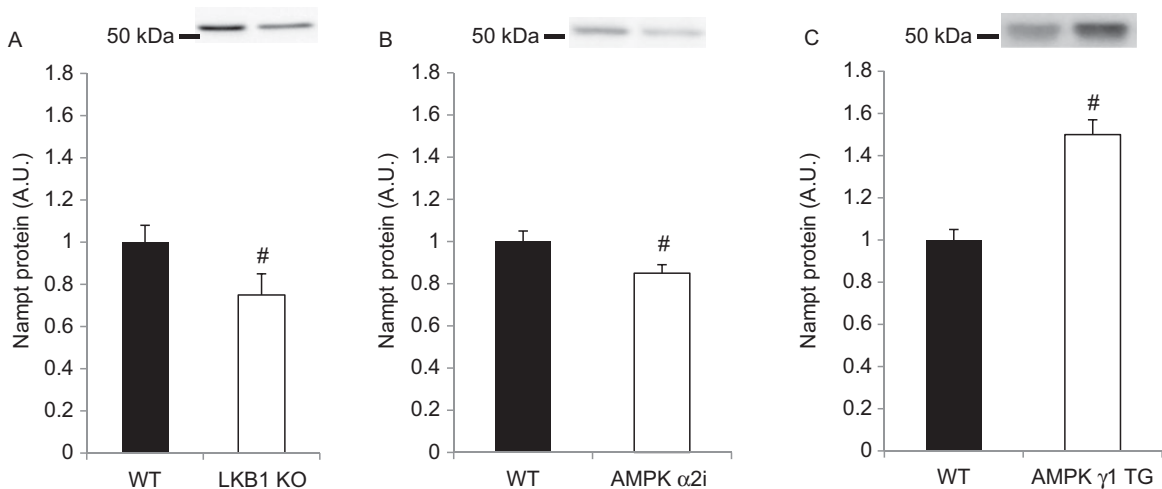


Figure 3. Nampt protein levels are related to AMPK activity in mouse skeletal muscle

Mouse skeletal muscle Nampt protein was measured in tibialis anterior muscle of mouse models with reduced AMPK activity, such as (A) LKB1 KO ($n = 9-11$), (B) AMPK $\alpha 2i$ ($n = 6$) or (C) AMPK $\gamma 1$ transgenic mice, which show chronically elevated AMPK activity in skeletal muscle ($n = 5-6$). # Indicates vs. WT ($P < 0.05$).

the untrained and trained state (Fig. 5D). These findings suggest either that regulation of Nampt protein levels is independent of PGC-1 α , or that redundant signalling mechanisms may compensate for a lack of PGC-1 α .

Pharmacological activation of AMPK by AICAR, but not metformin, increases Nampt

Our results from the exercise studies suggest that a functional AMPK α 2 subunit is not required for the exercise-induced increases in muscle Nampt. Because exercise causes metabolic adaptations in skeletal muscle via AMPK and several other complementary mechanisms

(Jørgensen *et al.* 2006; Egan & Zierath, 2013), we treated mice with the AMPK activators, AICAR and metformin, to assess the contribution of AMPK in the regulation of Nampt more directly.

AICAR is a cell-permeable nucleotide that can be converted to 5-aminoimidazole-4-carboxamide ribotide (ZMP) in the cell. ZMP shares some structural homologies with AMP and mimics the activating effects of AMP on AMPK (Corton *et al.* 1995). We tested the hypothesis that a single subcutaneous injection of AICAR would increase Nampt mRNA expression in skeletal muscle in an AMPK-dependent manner. A time course experiment to optimise the timing of the AICAR treatment indicated

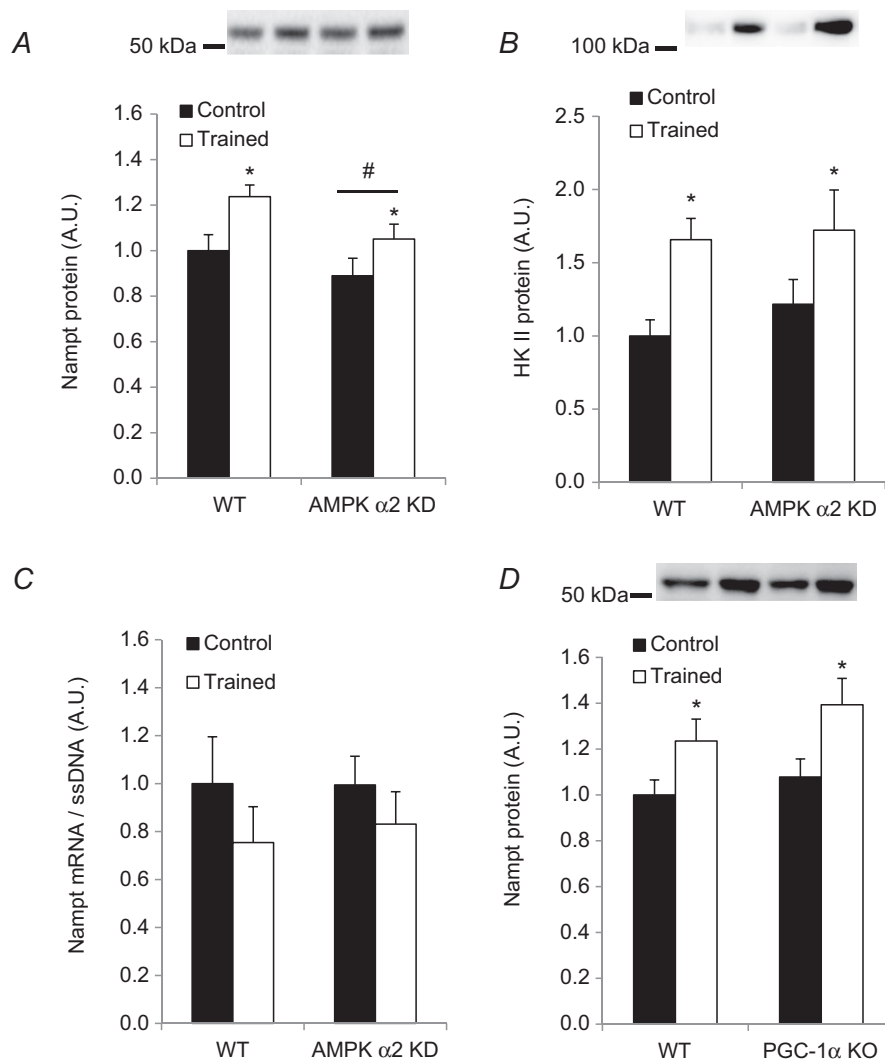


Figure 5. Combined wheel-cage and treadmill training increases Nampt protein in mouse skeletal muscle in an AMPK α 2- and PGC-1 α -independent manner

Quadriceps muscles of sedentary or trained (6.5 weeks of combined voluntary wheel-cage and forced exercise training) WT and AMPK α 2 KD mice ($n = 12-16$) were removed the morning following the final exercise bout, and (A) Nampt protein, (B) hexokinase II protein and (C) Nampt mRNA levels were measured. D, Nampt protein abundance was measured in WT and PGC-1 α KO mice that underwent 5 weeks of combined voluntary wheel-cage and forced endurance training, or served as sedentary controls ($n = 16$). * Indicates vs. control ($P < 0.05$); ** indicates vs. control ($P < 0.01$); # indicates vs. WT ($P < 0.05$).

Nampt mRNA induction 8 h after AICAR treatment in C57BL/6J mice relative to saline-treated animals ($P < 0.05$; Fig. 6A). Subsequently, WT and AMPK $\alpha 2$ KD mice were injected with AICAR, and Nampt mRNA was evaluated after 8 h. Basal Nampt mRNA levels and AICAR-induced increases in Nampt mRNA were similar in AMPK $\alpha 2$ KD mice and control mice (Fig. 6B). Acute AICAR treatment did not alter Nampt protein abundance (Fig. 6C).

Although AICAR-induced Nampt mRNA induction occurred via an AMPK-independent mechanism, Nampt protein abundance was reduced in mice lacking a functional AMPK $\alpha 2$ subunit (Figs 3B, 5A and 6C). This may indicate that AMPK regulates Nampt protein by a post-transcriptional or -translational mechanism. We therefore determined whether repeated AICAR treatment increases Nampt protein in an AMPK-dependent manner. Four weeks of daily subcutaneous AICAR injections increased Nampt abundance in WT, but not AMPK $\alpha 2$ KD, mice ($P < 0.05$; Fig. 7A). Similarly, repeated AICAR treatment increased hexokinase II abundance in skeletal muscle of WT but not AMPK $\alpha 2$ KD mice (Fig. 7B). Supporting our finding that AICAR increases Nampt mRNA independent of AMPK (Fig. 6B), we found that Nampt mRNA levels after repeated AICAR treatment were significantly elevated independent of AMPK $\alpha 2$ ($P < 0.01$; Fig. 7C). Finally, AICAR increased Nampt protein abundance in the quadriceps muscle by a PGC-1 α -independent mechanism ($P < 0.01$; Fig. 7D). These data indicate that pharmacological activation of AMPK can increase Nampt protein abundance in an AMPK $\alpha 2$ -dependent manner that does not require the transcriptional co-activator PGC-1 α .

Metformin is a potent anti-diabetic drug that has a major effect on the suppression of hepatic glucose production. However, metformin activates AMPK in skeletal muscle (Musi *et al.* 2002) and increases glucose uptake (Zhou *et al.* 2001) by both AMPK-dependent and -independent mechanisms (Turban *et al.* 2012). Therefore, we tested the hypothesis that metformin would increase Nampt protein levels in an AMPK-dependent manner. Although we have found that a single oral dose of metformin significantly increases AMPK phosphorylation in skeletal muscle in the hours after administration (J. M. Kristensen, J. T. Trebak and J. F. P. Wojtaszewski, unpublished observation), Nampt protein levels were unaltered overall in the gastrocnemius muscle of WT or AMPK $\alpha 2$ KD mice after 2 weeks of oral metformin administration (Fig. 8). However, Nampt protein levels were consistently lower in white relative to red gastrocnemius muscle ($P < 0.01$). When white gastrocnemius samples were analysed separately, we detected a borderline significant increase in Nampt following metformin treatment (main effect, $P = 0.06$; observed power = 0.39), with a greater relative response to metformin in KD muscle (25%) than WT muscle (8%).

Discussion

Activation of AMPK raises intracellular NAD concentrations and activates SIRT1, whereas AMPK deficiency compromises SIRT1-dependent responses to exercise and fasting (Cantó *et al.* 2009). A putative adaptive response to an accelerated NAM turnover caused by augmentations in SIRT activity may involve

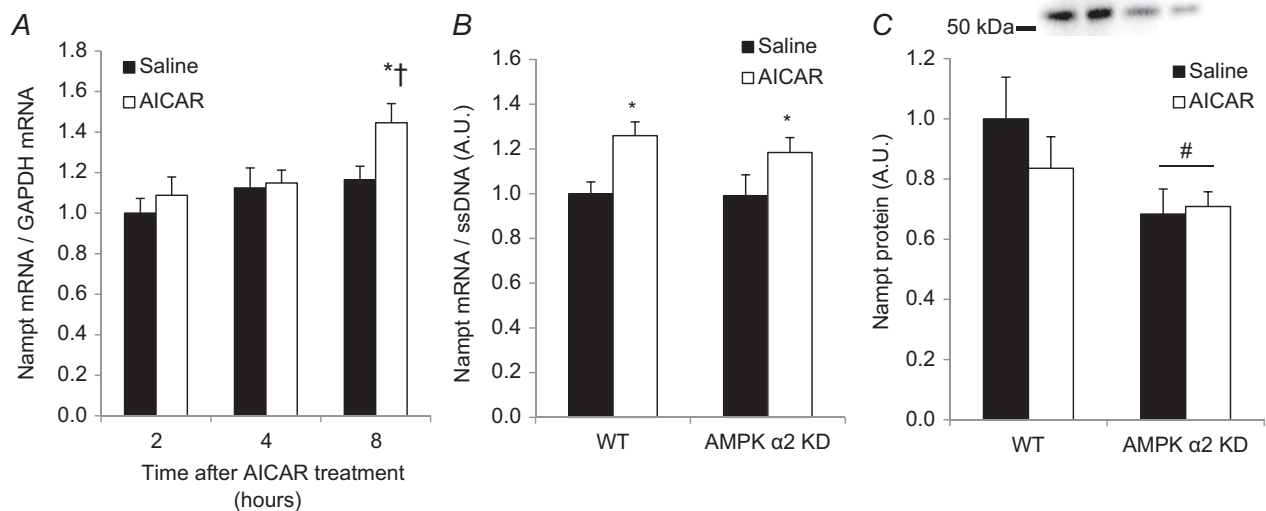


Figure 6. Acute AICAR treatment increases Nampt mRNA independent of AMPK $\alpha 2$

A, Nampt mRNA was measured in C57BL/6J mouse quadriceps muscle 2, 4 and 8 h after AICAR injection (500 mg kg^{-1} body weight; $n = 6$). B, Nampt mRNA concentrations and C) Nampt protein abundance were assessed 8 h after AICAR treatment (500 mg kg^{-1} body weight; $n = 10\text{--}13$). * Indicates vs. saline ($P < 0.05$); † indicates vs. 2 and 4 h ($P < 0.05$); # indicates vs. WT ($P < 0.05$).

an increase in Nampt expression or activity. Several lines of evidence suggest that Nampt gene expression is dependent on a functional AMPK signalling cascade (Fulco *et al.* 2008). However, direct evidence to suggest that AMPK is necessary for maintaining Nampt protein abundance is lacking.

Here we demonstrate that skeletal muscle Nampt expression is partly dependent on AMPK heterotrimers containing a functional $\alpha 2$ catalytic subunit. Nampt protein abundance is consistently reduced in skeletal muscle of mouse models with ablated AMPK activity, and increased in a model of chronically increased AMPK activity. Moreover, repeated AICAR injections increased skeletal muscle Nampt protein abundance in WT mice,

but not in AMPK $\alpha 2$ KD mice, implicating AMPK signalling in regulating Nampt protein levels. Together, these results suggest that Nampt protein abundance is partly determined by cellular energy status via AMPK $\alpha 2$ -containing complexes in skeletal muscle, where deficiency or sustained activation of AMPK results in reduced or increased protein levels of Nampt, respectively.

We provide evidence that acute exercise increases Nampt mRNA induction in both WT and AMPK $\alpha 2$ KO mice. How these data agree with previous findings of a blunted Nampt mRNA induction in the quadriceps muscle of AMPK $\gamma 3$ KO mice following 2 h of acute swimming is not immediately apparent (Cantó *et al.* 2010). The difference between these studies may be

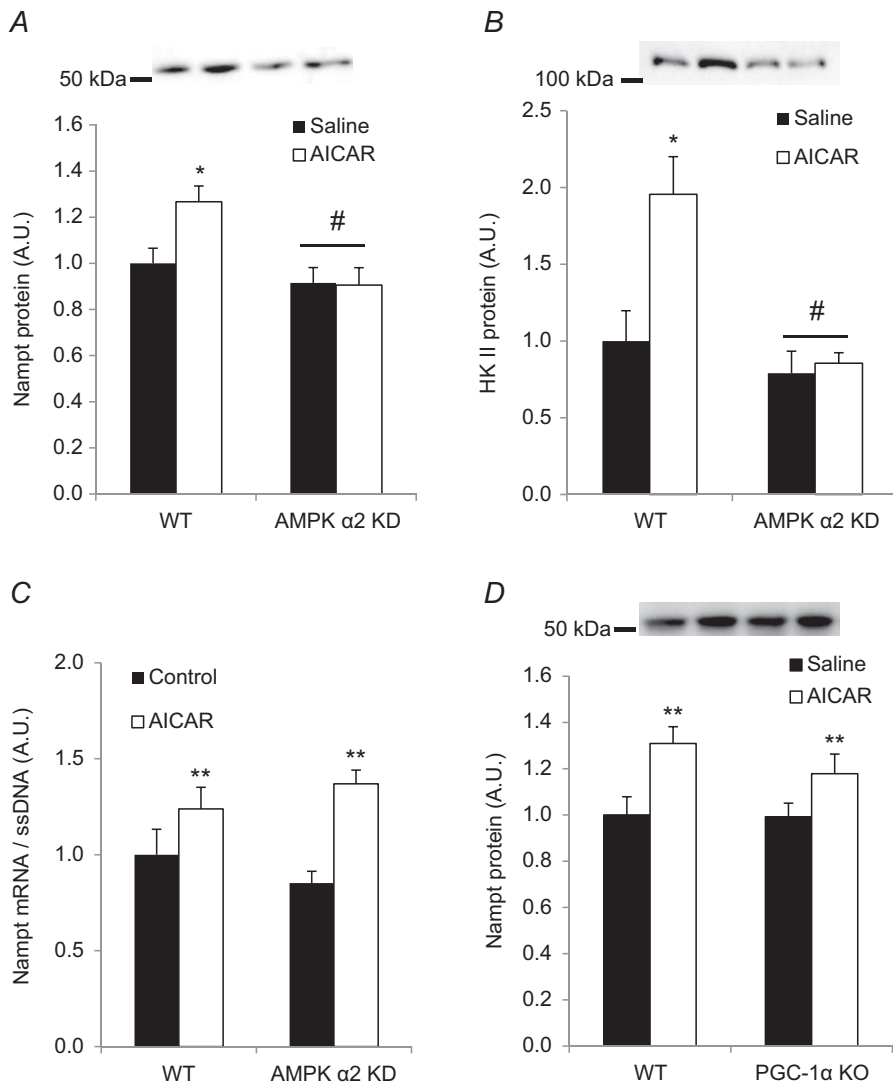


Figure 7. Repeated AICAR administration increases skeletal muscle Nampt in an AMPK-dependent but PGC1 α -independent manner

A, Nampt protein; B, hexokinase II protein and C, Nampt mRNA levels were measured in quadriceps of WT or AMPK $\alpha 2$ KD animals following 4 weeks of daily treatment with AICAR (500 mg kg⁻¹ body weight) or saline ($n = 7-8$). D, Nampt protein levels were measured in both WT and PGC-1 α KO mice following 4 weeks of daily treatment with AICAR or saline ($n = 8$). * Indicates vs. saline ($P < 0.05$); # indicates vs. WT ($P < 0.05$); ** indicates vs. saline ($P < 0.01$).

related to the alternative modes of exercise studied (90 min of treadmill running *vs.* four 30 min bouts of swimming separated by 5 min recovery). These exercise modalities may differentially affect muscle bioenergetics, and consequently influence the role of AMPK in the exercise-induced upregulation of Nampt mRNA. Skeletal muscle from AMPK $\gamma 3$ KO mice rapidly fatigues during acute intensive exercise (Barnes *et al.* 2005) and shows reduced glycogen re-synthesis during recovery (Barnes *et al.* 2004), indicating a key role of the AMPK $\gamma 3$ subunit in supporting muscle bioenergetics in response to exercise. Our treadmill exercise experiments were performed in fed mice, whereas the AMPK $\gamma 3$ KO mice were fasted prior to swimming exercise (Cantó *et al.* 2010). Considering the impaired glycogen re-synthesis in AMPK $\gamma 3$ KO mice and a compromised effect of caloric restriction on skeletal muscle Nampt protein abundance in AMPK $\alpha 2$ KO mice (Wang *et al.* 2012), nutritional status or cellular energy charge before the start of exercise may influence the role of AMPK in determining an exercise-induced increase in Nampt mRNA. Alternatively, other AMPK subunits, such as the $\alpha 1$ subunit that is upregulated in the AMPK $\alpha 2$ KO mice (Jørgensen *et al.* 2007), may play yet unidentified specialised roles in mediating the acute effects of exercise on Nampt mRNA induction.

Increases in Nampt protein abundance following exercise training, but not repeated AICAR administration, are preserved in AMPK $\alpha 2$ KD mice. These

data are consistent with earlier evidence suggesting exercise-induced protein synthesis in skeletal muscle does not depend solely on AMPK signalling, while AICAR treatment requires functional AMPK signalling. For example, exercise training but not AICAR-induced metabolic adaptations in skeletal muscle are maintained in AMPK $\alpha 2$ KO mice (Jørgensen *et al.* 2005, 2007). Thus, redundant pathways such as calcium-calmodulin signalling may mediate the synthesis of specific proteins in response to exercise (Rose *et al.* 2009).

Our data on mRNA levels following exercise training and repeated AICAR are consistent with mRNA data from our acute exercise and AICAR treatment studies in that an effect of AMPK $\alpha 2$ on Nampt mRNA was not detected. Nampt mRNA was significantly elevated in the quadriceps muscle following 4 weeks of AICAR treatment, similar to the response observed after acute AICAR treatment. In contrast, Nampt mRNA was not increased after exercise training. Thus, we speculate that the metabolic effects of exercise on Nampt mRNA induction may be more transient than the effect of AICAR. Exercise-induced increases in AMP levels are relatively transient, and while skeletal muscle ZMP levels return to near baseline values within an hour after AICAR infusion (Sabina *et al.* 1982), a single dose of AICAR, comparable to the dose given in this study, elevates intracellular ZMP for hours in skeletal muscle as well as other tissues (Holmes *et al.* 1999; Bumpus & Johnson, 2011). This prolonged perturbation of cellular energy charge in response to AICAR treatment may account for the differential effect of exercise training and repeated AICAR treatment on Nampt mRNA expression and protein abundance.

A pool of AMPK $\alpha 2$ is thought to translocate to the nucleus upon activation (McGee *et al.* 2003), where it phosphorylates PGC-1 α that is subsequently deacetylated by SIRT1 (Jäger *et al.* 2007; Cantó *et al.* 2009). However, PGC-1 α KO was without effect on Nampt protein abundance in sedentary or trained skeletal muscle. In AMPK $\alpha 2$ KD mice, Nampt mRNA expression was similar between WT and AMPK $\alpha 2$ KD mice in basal, as well as AICAR-stimulated muscle, although Nampt protein abundance partly depends on AMPK. Collectively, these data are consistent with a post-transcriptional or -translational regulation of Nampt by AMPK. Interestingly, AMPK activation suppresses endothelial cell expression of angiotensin-converting enzyme post-translationally via phosphorylation of p53 and upregulation of miR 143/145 (Kohlstedt *et al.* 2013). These data suggest that AMPK can regulate protein abundance via post-translational mechanisms. Whether a similar mechanism can account for the ability of AMPK to regulate Nampt protein abundance remains to be determined.

Metformin is a biguanide that primarily acts by activating hepatic AMPK, with modest effects on skeletal muscle AMPK (Zhou *et al.* 2001; Musi *et al.* 2002).

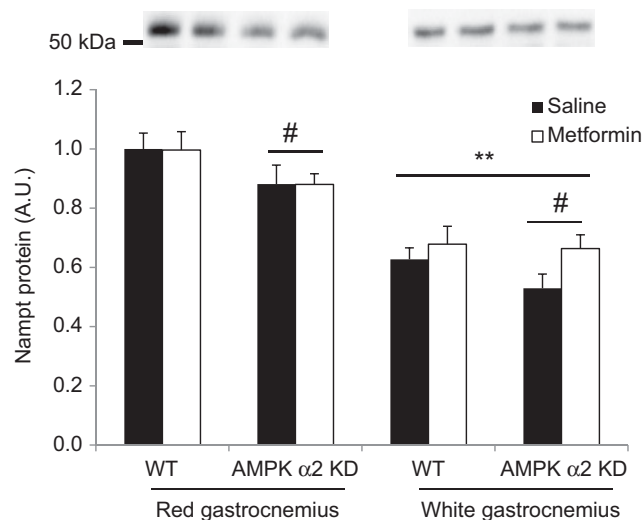


Figure 8. Effect of repeated metformin treatment on skeletal muscle Nampt concentrations

Nampt concentrations were measured in white and red gastrocnemius muscle of WT and AMPK $\alpha 2$ KD that were treated with 2 weeks of oral metformin treatment (300 mg kg⁻¹ body weight) or saline. # Indicates *vs.* WT ($P < 0.05$); ** indicates *vs.* red gastrocnemius ($P < 0.01$); $n = 10$ –12. Metformin treatment increased Nampt protein nearly significantly in white gastrocnemius (two-way ANOVA; main metformin treatment effect, $P = 0.06$; observed power = 0.39).

We are aware of only one other report concerning the effects of repeated metformin treatment on Nampt protein abundance (Caton *et al.* 2011). However, Nampt abundance was evaluated in adipose tissue, rather than skeletal muscle as studied here. Using a similar dose of metformin (250 mg kg⁻¹ day⁻¹ for 7 days *vs.* 300 mg kg⁻¹ day⁻¹ in this study), metformin treatment increased Nampt protein abundance in adipose tissue of db/db mice. Here we find that metformin did not consistently alter skeletal muscle Nampt protein content, despite the fact that we chose a metformin dosage that was intended to mimic pharmacologically active circulating metformin concentrations in humans (Bailey & Puah, 1986; Cusi & DeFronzo, 1998). Metformin treatment was shown to ameliorate defects in mitochondrial respiration in predominantly glycolytic skeletal muscle from AMPK $\alpha 2$ KD mice (Kristensen *et al.* 2013). We detected borderline significant increases of Nampt protein in white (also predominantly glycolytic) gastrocnemius muscle with metformin, and we speculate that the effects of metformin on mitochondrial function and Nampt abundance may be particularly evident in glycolytic muscle fibres.

In conclusion, endurance exercise training increases Nampt protein abundance directly in exercise-trained muscle in humans. Thus, intrinsic changes in skeletal muscle, rather than systemic factors, contribute to the regulation of Nampt protein in response to exercise training. Moreover, AICAR- but not exercise-induced increases in Nampt protein abundance in mouse skeletal muscle depend on AMPK $\alpha 2$. In contrast, AMPK $\alpha 2$ -containing heterotrimers are not required for regulating Nampt mRNA expression in response to either AICAR or treadmill exercise. Thus, AMPK-independent mechanisms may control Nampt-mediated gene transcription. Our study establishes a clear connection between AMPK activation and recycling of NAD by Nampt. Future studies are warranted to identify the exact mechanism by which AMPK regulates Nampt protein abundance, as well as other regulatory signals that determine Nampt expression.

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Additional information

Competing interests

The authors report no conflict of interest.

Author contributions

The experiments in the present manuscript were conducted in Section of Integrated Physiology at the Novo Nordisk

Foundation Center for Basic Metabolic Research and at Joslin Diabetes Center. Conception and design of the experiments: J.B., J.T.T., L.J.G; collection, analysis and interpretation of data: J.B., J.T.T., S.G.V., M.A.A., S.R., S.R., P.S.L., J.M.K., C.F., L.L., J.F., S.J., B.K., J.F.P.W., E.A.R., L.J.G., H.P; drafting the article or revising it critically for important intellectual content: J.B., J.T.T., S.G.V., M.A.A., J.F.P.W., E.A.R., J.R.Z., L.J.G., H.P.

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