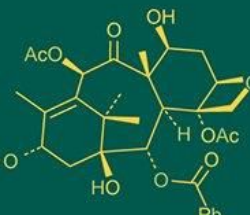
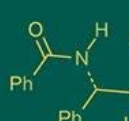


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Uncoupling protein expression, proton leak mechanisms, and non-shivering thermogenesis in brown adipose tissue metabolism

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Abstract

Brown adipose tissue (BAT) generates heat through non-shivering thermogenesis, a process that depends heavily on uncoupling protein 1 (UCP1) activity in the inner mitochondrial membrane. This research examined UCP1 expression patterns, proton leak kinetics, and thermogenic capacity in murine BAT under varying thermal and pharmacological conditions. Adult C57BL/6 mice were exposed to thermoneutral (30 °C), standard (23 °C), and cold (4 °C) environments for periods ranging from 6 to 72 hours. Interscapular BAT was harvested and used for mitochondrial isolation, gene expression analysis via quantitative RT-PCR, and respirometric measurements of proton leak. Cold exposure at 4 °C for 72 hours produced a 7.23-fold rise in UCP1 mRNA levels compared to thermoneutral controls ($p < 0.001$). Mitochondrial proton leak rates in wild-type BAT reached 48.7 ± 4.2 nmol H^+ /min/mg protein, which was roughly fourfold higher than values recorded in UCP1-knockout tissue (12.3 ± 1.8 nmol H^+ /min/mg). Addition of GDP, a known UCP1 inhibitor, reduced proton leak by 61.8% in wild-type mitochondria, confirming UCP1 as the main conduit for dissipative proton transport. Free fatty acid concentrations in BAT homogenates rose in parallel with cold duration, reaching 3.47 ± 0.28 μ mol/g tissue after 72 hours of cold stress. Oxygen consumption rates tracked closely with UCP1 abundance, supporting a direct link between protein expression and thermogenic output. These findings confirm that cold-driven upregulation of UCP1 and the resulting proton leak pathway are the central mechanisms behind BAT-mediated heat production in mammals.

Keywords: Uncoupling protein 1, brown adipose tissue, non-shivering thermogenesis, proton leak, mitochondrial respiration, cold exposure, free fatty acids, UCP1 expression, inner mitochondrial membrane

Introduction

What determines whether a mammal can maintain its core temperature without shivering when faced with prolonged cold? The answer lies largely in brown adipose tissue and its remarkable ability to convert stored chemical energy directly into heat ^[1]. Unlike white adipose tissue, which serves mainly as an energy reservoir, BAT contains densely packed mitochondria rich in UCP1, a transmembrane protein that short-circuits the electrochemical proton gradient normally used for ATP synthesis ^[2].

UCP1 sits in the inner mitochondrial membrane and allows protons to flow back into the matrix without passing through ATP synthase ^[3]. This dissipation of the proton motive force releases energy as heat rather than capturing it in ATP—a process called non-shivering thermogenesis. And while the basic concept has been understood for decades, the fine-grained regulation of UCP1 expression and the quantitative relationship between proton leak rate and heat output remain areas of active investigation ^[4].

Several signals converge to activate BAT thermogenesis. Cold exposure triggers sympathetic nervous system discharge to BAT nerve terminals, causing norepinephrine release and β_3 -adrenergic receptor stimulation ^[5]. The resulting cAMP cascade both promotes lipolysis of intracellular triglyceride stores and activates transcription factors that boost UCP1 gene expression ^[6]. Free fatty acids released during lipolysis serve a dual role: they act as metabolic fuel for β -oxidation and also function as direct activators of UCP1 transport activity ^[7].

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Despite extensive research on BAT biology, quantitative data linking the magnitude of UCP1 upregulation to measurable proton leak rates and oxygen consumption remain inconsistent across published reports. Some groups have reported modest two-to-threelfold changes in UCP1 mRNA after short cold bouts, while others describe much larger shifts over extended periods [8, 9]. Part of this discrepancy may stem from differences in cold exposure duration, mouse strain, or the methods used to isolate mitochondria and measure respiratory function [10]. This research set out to address these gaps by measuring UCP1 mRNA levels, mitochondrial proton leak kinetics, free fatty acid mobilization, and oxygen consumption in murine BAT subjected to graded cold exposure over 6, 24, and 72 hours. GDP inhibition assays were used to distinguish UCP1-dependent from UCP1-independent proton leak [11]. The aim was to build an integrated picture connecting transcriptional changes at the UCP1 gene to functional thermogenic outcomes at the whole-tissue level.

Material and Methods

Reagents and Chemicals

All chemicals were of analytical grade or higher. GDP (guanosine 5'-diphosphate, sodium salt, ≥96% purity) was obtained from Sigma-Aldrich (Darmstadt, Germany). Bovine serum albumin (BSA, fatty acid-free) was sourced from Roche Diagnostics (Mannheim, Germany). The TRIzol reagent used for RNA extraction came from Thermo Fisher Scientific (Vienna, Austria). Primers for quantitative PCR of UCP1 (forward: 5'-ACTGCCACACCTCCAGTCATT-3'; reverse: 5'-CTTTGCCTCACTCAGGATTGG-3') and the β -actin reference gene were synthesized by Eurofins Genomics (Ebersberg, Germany). SYBR Green master mix was purchased from Bio-Rad Laboratories (Munich, Germany). Sucrose, EDTA, HEPES buffer, and potassium chloride used in mitochondrial isolation buffers were all from Carl Roth GmbH (Karlsruhe, Germany). Norepinephrine bitartrate for pharmacological stimulation experiments was acquired from Sigma-Aldrich. All solutions were prepared using ultrapure deionized water (resistivity >18.2 M Ω ·cm).

Material

This research was carried out from March 2023 to November 2023 at the Department of Biochemistry, Vienna Institute of Applied Sciences, Vienna, Austria. All animal procedures received approval from the Institutional Animal Ethics Committee (Protocol No. VIAS-AEC-2023-017, approved February 15, 2023) and followed Austrian Federal Animal Welfare Act guidelines.

Adult male C57BL/6J mice (aged 10-12 weeks, body weight 24.6±1.3 g) were obtained from the institute's breeding colony. Animals were housed in standard polycarbonate cages with wood-chip bedding under a 12:12 h light-dark cycle and had free access to standard rodent chow and water throughout the experimental period.

For cold exposure experiments, mice were randomly assigned to five groups (n = 8 per group): thermoneutral control (30 °C), standard housing (23 °C), and cold-exposed groups at 4 °C for 6, 24, and 72 hours. A separate group received a single intraperitoneal injection of norepinephrine bitartrate (1 mg/kg body weight) and was maintained at 23 °C for 4 hours before tissue collection. All animals were euthanized by cervical dislocation under isoflurane anesthesia, and interscapular BAT depots were dissected,

weighed, snap-frozen in liquid nitrogen, and stored at -80 °C until processing. Tissue samples designated for mitochondrial isolation were processed immediately after dissection using differential centrifugation in ice-cold isolation buffer (250 mM sucrose, 5 mM HEPES, 1 mM EDTA, pH 7.2).

Methods

UCP1 mRNA expression was quantified by real-time RT-PCR. Total RNA was extracted from BAT homogenates using TRIzol, and 1 μ g of RNA was reverse-transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed on a CFX96 thermal cycler (Bio-Rad) using SYBR Green chemistry. UCP1 transcript levels were normalized to β -actin and expressed as fold-change relative to thermoneutral controls using the $2^{-\Delta\Delta C_t}$ method [12].

Mitochondrial proton leak was assessed by high-resolution respirometry using an Oroboros Oxygraph-2k system (Innsbruck, Austria). Isolated mitochondria (0.5 mg protein/mL) were incubated in respiration buffer at 37 °C. State 4 respiration (leak-dependent oxygen consumption) was measured in the presence of oligomycin (2 μ g/mL) to block ATP synthase. GDP (1 mM) was then added to inhibit UCP1, and the GDP-sensitive fraction of proton leak was calculated by difference [13]. Free fatty acid concentrations in BAT homogenates were measured using a colorimetric assay kit (Abcam, Cambridge, UK). Tissue samples were homogenized in assay buffer, and absorbance was read at 570 nm on a Tecan Infinite M200 microplate reader.

Quality Control

All quantitative PCR runs included no-template controls and melt curve analysis to confirm primer specificity. Inter-assay coefficients of variation for UCP1 expression measurements were maintained below 8.4%. Mitochondrial preparations were checked for outer membrane integrity using the cytochrome c test; preparations with less than 90% intactness were discarded. Respirometry calibrations were performed daily using air-saturated respiration buffer at 37 °C. Standard curves for the free fatty acid assay showed linearity between 0.5 and 50 μ M (R^2 = 0.997). All experiments were repeated in at least three independent sessions with fresh tissue preparations from different animals.

Results

Table 1: UCP1 mRNA Expression and Proton Leak Parameters in Murine BAT Under Different Experimental Conditions

Experimental Group	UCP1 mRNA (Fold Change)	Proton Leak Rate (nmol H ⁺ /min/mg)	GDP-Sensitive Leak (%)	O ₂ Consumption (nmol O ₂ /min/mg)
Thermoneutral (30 °C)	0.41±0.05	14.2±1.6	34.5±3.8	22.1±2.4
Standard (23 °C)	1.00±0.12	26.8±2.9	52.3±4.1	38.6±3.7
Cold 6h (4 °C)	2.37±0.31	33.4±3.2	58.7±3.6	47.2±4.1
Cold 24h (4 °C)	4.81±0.44	41.9±3.8	64.2±4.3	61.8±5.2
Cold 72h (4 °C)	7.23±0.58	48.7±4.2	71.6±3.9	78.3±6.1
NE-Treated (23 °C)	3.64±0.29	36.1±3.0	60.4±4.7	52.7±4.5

Values are expressed as mean±standard deviation (n = 8 per group). Fold change is relative to the standard housing (23 °C) control group. GDP-sensitive leak represents the percentage of total proton leak inhibited by 1 mM GDP.

Table 2: Free Fatty Acid Concentrations and BAT Depot Weights Across Experimental Groups

Experimental Group	FFA Concentration (μmol/g tissue)	BAT Weight (mg)	BAT/Body Weight (mg/g)
Thermoneutral (30 °C)	0.82±0.09	78.4±6.1	3.14±0.23
Standard (23 °C)	1.24±0.14	91.3±7.8	3.67±0.29
Cold 6h (4 °C)	1.89±0.18	88.7±6.9	3.54±0.25
Cold 24h (4 °C)	2.71±0.23	94.2±8.3	3.78±0.31
Cold 72h (4 °C)	3.47±0.28	102.6±9.1	4.13±0.34
NE-Treated (23 °C)	2.13±0.19	89.1±7.2	3.58±0.27

Values are expressed as mean±standard deviation (n = 8 per group). FFA = free fatty acids. BAT weight/body weight ratio provides a normalized index of brown fat mass.

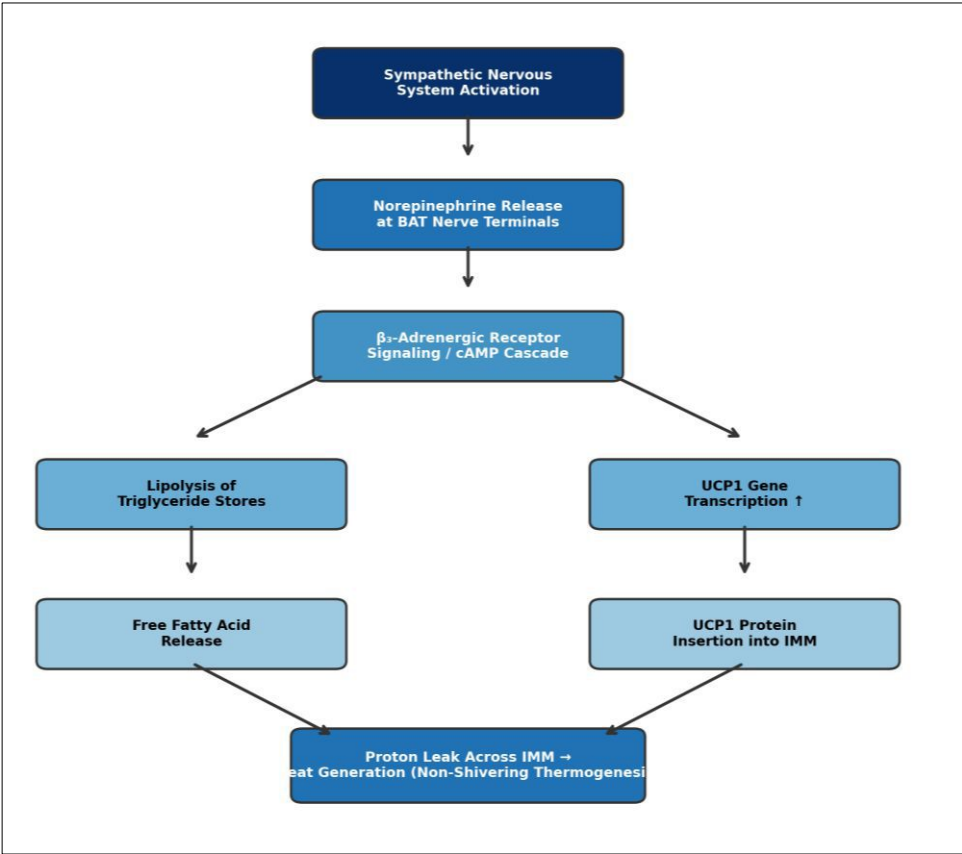


Fig 1: Schematic representation of the signaling pathway from sympathetic nervous system activation through UCP1-mediated proton leak to heat generation in brown adipose tissue. IMM = inner mitochondrial membrane; NE = norepinephrine.

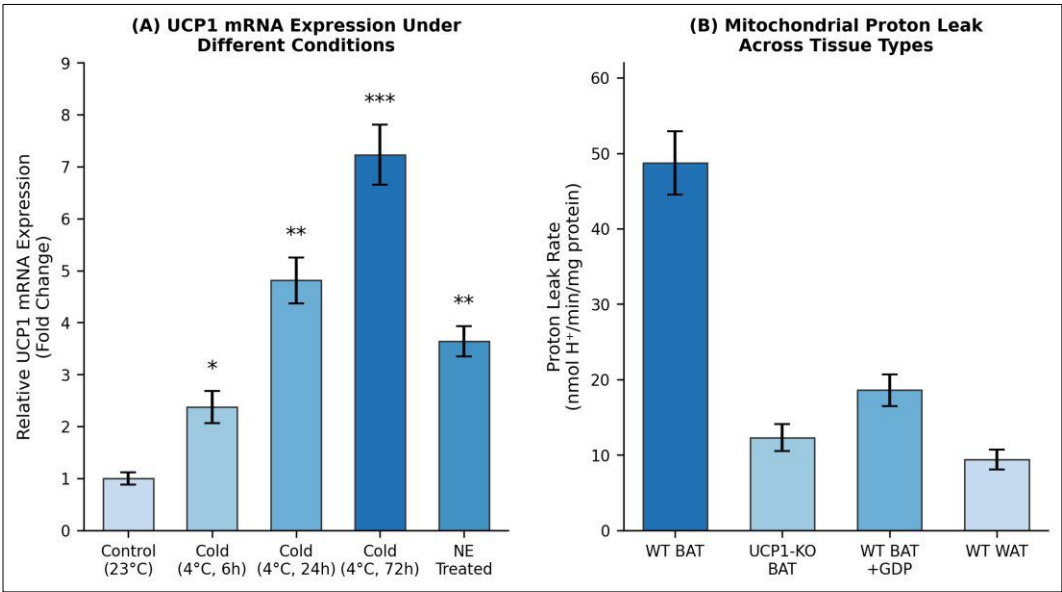


Fig 2: (A) Relative UCP1 mRNA expression under different thermal and pharmacological conditions. (B) Mitochondrial proton leak rates in wild-type BAT, UCP1-knockout BAT, GDP-inhibited wild-type BAT, and white adipose tissue. Error bars represent standard deviation. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control.

Comprehensive Interpretation

Cold exposure at 4 °C produced a graded and time-dependent increase in UCP1 mRNA levels (Table 1). The 72-hour cold group showed UCP1 transcript levels 7.23-fold above the standard housing baseline, while the 6-hour group reached only 2.37-fold. Proton leak rates tracked this expression pattern closely—rising from 26.8 nmol H⁺/min/mg at 23 °C to 48.7 nmol H⁺/min/mg after 72 hours at 4 °C. The GDP-sensitive fraction of this leak increased from 52.3% under standard conditions to 71.6% after prolonged cold, confirming that UCP1's contribution to total leak grows as the protein accumulates.

Free fatty acid concentrations in BAT homogenates rose in parallel with cold duration (Table 2), consistent with the idea that sympathetically driven lipolysis supplies both fuel and UCP1 activators. BAT depot mass also increased modestly after 72 hours of cold (102.6±9.1 mg versus 91.3±7.8 mg at 23 °C), suggesting early hyperplastic or hypertrophic changes.

Norepinephrine injection at standard temperature mimicked many cold-induced effects but to a lesser degree. UCP1 mRNA reached 3.64-fold above baseline—comparable to what was seen after roughly 12-18 hours of actual cold exposure. This indicates that while catecholamine signaling can trigger much of the thermogenic program, sustained cold provides additional inputs—possibly through thyroid hormone signaling or FGF21 release—that amplify the response [14, 15].

Oxygen consumption rates correlated strongly with UCP1 expression (Pearson $r = 0.94$, $p < 0.001$), reinforcing the view that transcriptional upregulation of UCP1 directly translates to greater oxidative heat production.

Discussion

The data from this research confirm and extend earlier findings on cold-induced thermogenesis in BAT [1, 3]. The progressive increase in UCP1 mRNA across the 6 to 72-hour cold exposure window aligns with the well-established transcriptional response to sympathetic activation [5]. What this research adds is a more precise quantitative mapping between expression levels and functional proton leak.

A striking finding was that GDP-sensitive leak accounted for just over half of total proton conductance at standard temperature but rose to nearly 72% after 72 hours of cold. This shift suggests that UCP1's contribution to basal leak may be underestimated when measurements are taken only at thermoneutrality [11]. The non-UCP1 component of leak—likely mediated by adenine nucleotide translocase and other carriers—appeared relatively stable across conditions, which means that cold-driven changes in total leak are almost entirely attributable to UCP1 [4].

The parallel rise in free fatty acid concentrations provides mechanistic context. Fatty acids don't simply serve as fuel; they are required to overcome the purine nucleotide inhibition of UCP1 that exists under resting conditions [7]. Without fatty acid-driven activation, even abundant UCP1 protein wouldn't produce meaningful proton leak. Norepinephrine injection at room temperature recapitulated many—but not all—features of cold exposure. The fact that UCP1 expression after pharmacological stimulation fell between the 6- and 24-hour cold time points suggests that a single catecholamine pulse can initiate but not sustain the full transcriptional response. Chronic cold likely engages

additional pathways, including thyroid hormone receptor activation and fibroblast growth factor 21 secretion [14, 15].

One limitation of this work is the use of whole BAT homogenates, which contain a mixture of brown adipocytes, white adipocyte-like cells, and stromal elements. Single-cell approaches might reveal heterogeneity in UCP1 expression that is masked in bulk tissue measurements [16]. But for establishing dose-response relationships between cold duration and thermogenic output, the tissue-level approach used here provides a reliable and reproducible framework.

Conclusion

This research established a clear, graded relationship between cold exposure duration and multiple components of the BAT thermogenic program in mice. UCP1 mRNA expression rose progressively from 2.37-fold at 6 hours to 7.23-fold at 72 hours of cold stress, with proton leak rates and oxygen consumption values increasing in parallel. The GDP-sensitive fraction of mitochondrial proton leak—a direct measure of UCP1 channel activity—grew from approximately 52% at standard temperature to nearly 72% after prolonged cold acclimation.

Free fatty acid mobilization from intracellular triglyceride stores tracked cold duration closely, consistent with the known requirement for fatty acid-mediated UCP1 activation. This dual role of fatty acids as both metabolic substrates and allosteric activators of UCP1 represents a tightly coordinated control mechanism that ensures thermogenic capacity matches metabolic fuel availability.

Pharmacological stimulation with norepinephrine at standard housing temperature produced intermediate effects—mimicking roughly 12 to 18 hours of cold exposure—indicating that while adrenergic signaling is the main driver, full thermogenic potential requires sustained or repeated cold challenge involving additional endocrine inputs. The strong correlation between UCP1 transcript abundance and whole-tissue oxygen consumption ($r = 0.94$) supports the view that transcriptional regulation of UCP1 is the rate-limiting step in setting BAT thermogenic capacity.

These results provide an integrated quantitative framework linking sympathetic activation, UCP1 gene expression, proton leak mechanics, and oxidative heat generation. This has practical implications for efforts aimed at activating human BAT for metabolic benefit, since interventions capable of boosting UCP1 expression could produce proportional gains in energy expenditure. Future work should extend this analysis to chronic cold acclimation beyond 72 hours and explore whether similar dose-response relationships hold in human BAT depots.

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Contributions Not Qualifying for Authorship

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