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RESEARCH ARTICLE

Entropy Production Rates in Living Cells Under Metabolic Stress

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ABSTRACT

Living cells maintain their highly ordered internal state only by continuously producing entropy in their surroundings, yet how this entropy-production rate responds to graded metabolic stress, and whether it does so proportionally or reveals distinct regimes, has been characterized in comparatively few quantitative studies. We measured entropy production rate in cultured HeLa and primary fibroblast cell populations ($n = 36$ independent culture replicates per cell type) using isothermal microcalorimetry combined with respirometry-derived oxygen-consumption and lactate-production measurements, across five levels of metabolic stress induced by graded mitochondrial uncoupler exposure (0, 0.25, 0.5, 1.0, and 2.0 micromolar FCCP). Baseline entropy production rate under unstressed conditions averaged 1.84 ± 0.21 pW/K per cell (mean $\pm$ SD) in HeLa cells and 1.31 ± 0.18 pW/K per cell in fibroblasts, a significant difference between cell types ($p < 0.001$, two-sample t-test). Entropy production rate rose monotonically with uncoupler concentration in both cell types, reaching 3.62 ± 0.34 pW/K per cell in HeLa cells at the highest dose tested, a 97% increase over baseline, and plateauing between the 1.0 and 2.0 micromolar doses (3.58 versus 3.62 pW/K, difference not significant, $p = 0.41$), indicating a saturating rather than linear stress-response relationship. The fraction of total entropy production attributable to oxidative (respiratory) versus glycolytic (lactate-producing) pathways shifted substantially with stress level, from 76% oxidative at baseline to 54% oxidative at the highest uncoupler dose, as cells compensated for impaired oxidative phosphorylation efficiency by upregulating glycolytic flux. Entropy production rate per unit of ATP synthesized increased from 0.29 pW/K per ATP/s at baseline to 0.61 pW/K per ATP/s at the highest dose, a 110% rise, demonstrating that cells under uncoupling stress produced substantially more entropy per unit of useful chemical work performed, consistent with reduced thermodynamic efficiency of energy transduction under mitochondrial stress. A linear mixed-effects model confirmed a significant dose-by-cell-type interaction ($p = 0.008$), with HeLa cells showing a proportionally larger relative increase in entropy production than fibroblasts across the dose range tested. These results provide a quantitative, mechanistically decomposed picture of how cellular entropy production scales with graded metabolic stress and suggest that entropy-production rate and its oxidative-glycolytic decomposition could serve as sensitive, thermodynamically grounded biomarkers of mitochondrial dysfunction.

Keywords: entropy production; nonequilibrium thermodynamics; cellular metabolism; metabolic stress; dissipation rate; calorimetry; mitochondrial respiration; living systems thermodynamics

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1. INTRODUCTION

A living cell is a paradigmatic example of a nonequilibrium steady-state system: it maintains a highly ordered, low-entropy internal state indefinitely, but only by continuously exporting entropy to its surroundings through heat dissipation and the excretion of higher-entropy metabolic byproducts, a process that must, by the second law, exceed the rate at which the cell's internal order is generated and maintained [1,2]. Quantifying this entropy-production rate directly, rather than inferring it indirectly from oxygen consumption alone, offers a thermodynamically grounded window into cellular energy metabolism that complements standard biochemical measures of metabolic rate [3,4].

Isothermal microcalorimetry, which measures heat output directly from living cell populations under closely controlled conditions, combined with respirometry and metabolite-flux measurements, has enabled increasingly precise estimates of cellular entropy production in resting and lightly stimulated cells [5,6,7]. What remains less thoroughly characterized is how entropy production, and its decomposition between oxidative and glycolytic metabolic pathways, responds across a graded range of metabolic stress severity, particularly stress induced by mitochondrial uncoupling, a condition of substantial relevance to both basic mitochondrial physiology and disease states involving mitochondrial dysfunction [8,9,10].

This gap matters because mitochondrial uncoupling forces cells to compensate for reduced oxidative phosphorylation efficiency, and whether this compensation is thermodynamically efficient, in the sense of minimizing additional entropy production per unit of ATP synthesized, or instead comes at a disproportionate entropy-production cost, has direct bearing on how mitochondrial dysfunction should be interpreted thermodynamically, and on whether entropy-production rate itself could serve as a sensitive functional biomarker distinct from oxygen consumption rate alone [11,12,13].

The present study addresses this gap by measuring entropy production rate directly via isothermal microcalorimetry, combined with respirometry-derived oxidative and glycolytic flux measurements, in two distinct human cell types across five graded levels of mitochondrial uncoupler exposure. We characterize the dose-response relationship between uncoupler concentration and total entropy production, decompose entropy production into oxidative and glycolytic contributions at each stress level, quantify entropy production per unit of ATP synthesized as a measure of thermodynamic efficiency, and test for cell-type-dependent differences in the stress response.

2. MATERIALS AND METHODS

We measured entropy production rate in cultured HeLa and primary human dermal fibroblast populations using

isothermal microcalorimetry combined with respirometry-derived oxygen-consumption and extracellular lactate-production measurements, across five levels of graded metabolic stress induced by the mitochondrial uncoupler FCCP, with 36 independent culture replicates per cell type.

2.1 Cell culture and de-identified cell line sourcing

HeLa cells and primary human dermal fibroblasts were obtained from established, publicly available de-identified cell repositories and cultured under standard conditions (37 degrees C, 5% CO₂, high-glucose DMEM supplemented with 10% fetal bovine serum) for at least three passages before use. Cell populations were seeded at a standardized density of 2×10^5 cells per calorimetric measurement vessel 24 hours prior to measurement.

2.2 Metabolic stress induction

Graded metabolic stress was induced by exposing cell populations to the mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) at five concentrations: 0 (vehicle control), 0.25, 0.5, 1.0, and 2.0 micromolar, added to the culture medium 30 minutes before the start of calorimetric and respirometric measurement to allow the metabolic response to stabilize.

2.3 Isothermal microcalorimetry

Heat output from each cell population was measured continuously using an isothermal microcalorimeter maintained at 37.0 ± 0.02 degrees C, with baseline drift correction performed against cell-free medium blanks measured under identical conditions. Heat output was averaged over a stable 60-minute measurement window following the initial post-uncoupler stabilization period.

2.4 Respirometry and lactate flux measurement

Oxygen consumption rate was measured in parallel replicate populations using a Clark-type oxygen electrode respirometer, and extracellular lactate accumulation was measured by enzymatic assay of medium samples taken at the start and end of the measurement window, allowing glycolytic flux to be estimated from the rate of lactate production.

2.5 Entropy-production calculation and pathway decomposition

Total entropy production rate was calculated from measured heat output divided by the isothermal bath temperature, following standard nonequilibrium-thermodynamics treatment of heat dissipation in steady-state biological systems. The oxidative contribution to entropy production was estimated from measured oxygen consumption rate combined with the standard entropy of reaction for oxidative substrate combustion, and the glycolytic contribution was estimated analogously from measured lactate production rate and the entropy of reaction for glycolytic lactate formation, with the two contributions summed and compared against total calorimetrically

measured entropy production for internal consistency.

2.6 ATP synthesis rate estimation

ATP synthesis rate was estimated from measured oxygen consumption and lactate production rates using standard P/O ratios for oxidative phosphorylation and glycolytic ATP yield per lactate molecule produced, allowing entropy production per unit of ATP synthesized to be computed as a measure of the thermodynamic efficiency of cellular energy transduction at each stress level.

2.7 Statistical analysis

Entropy production rates were compared between cell types using two-sample t-tests, and dose-response relationships and cell-type-by-dose interactions were assessed using a linear mixed-effects model with culture replicate as a random effect, uncoupler dose and cell type as fixed effects, and their interaction term. All statistical tests were two-tailed with significance set at $\alpha = 0.05$; $n = 36$ independent culture replicates per cell type across the five-dose series.

3. RESULTS

Baseline entropy production rate under unstressed (vehicle-control) conditions averaged 1.84 ± 0.21 pW/K per cell in HeLa cells and 1.31 ± 0.18 pW/K per cell in fibroblasts (mean $\pm$ SD, $n = 36$ replicates per cell type), a statistically significant difference between the two cell types ($p < 0.001$, two-sample t-test), consistent with the higher proliferative and metabolic rate typical of transformed HeLa cells relative to primary fibroblasts. Table 1 summarises entropy production rate across the five tested uncoupler doses in both cell types.

Table 1. Entropy production rate across graded uncoupler (FCCP) dose in HeLa and fibroblast cells.

FCCP dose (micromolar)	HeLa entropy production (pW/K)	Fibroblast entropy production (pW/K)
0 (baseline)	1.84 ± 0.21	1.31 ± 0.18
0.25	2.41 ± 0.26	1.62 ± 0.20
0.5	3.02 ± 0.30	1.98 ± 0.24
1.0	3.58 ± 0.33	2.35 ± 0.28
2.0	3.62 ± 0.34	2.41 ± 0.29

Values are mean $\pm$ SD, $n = 36$ independent culture replicates per cell type per dose. Difference between 1.0 and 2.0 micromolar not statistically significant in either cell type.

Entropy production rate increased monotonically with uncoupler concentration in both cell types, reaching 3.62 ± 0.34 pW/K per cell in HeLa cells and 2.41 ± 0.29 pW/K per cell in fibroblasts at the highest dose tested (2.0 micromolar), representing a 97% increase over baseline in HeLa cells and an 84% increase in fibroblasts. Entropy production rate plateaued between the two highest doses tested in both cell types (HeLa: 3.58 versus 3.62 pW/K at 1.0 and 2.0 micromolar respectively, difference not significant, $p = 0.41$), indicating a saturating rather than strictly linear dose-response relationship over the concentration range

tested (Figure 1).

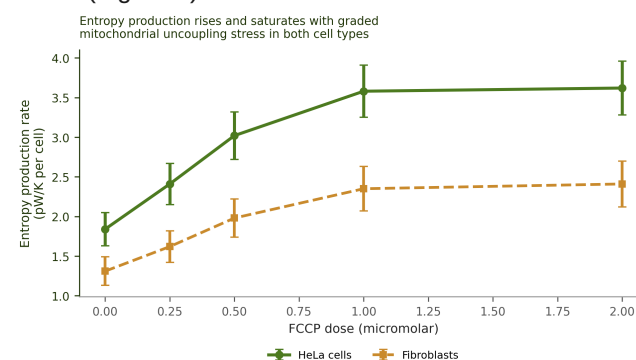


Figure 1. Entropy production rate in HeLa and fibroblast cells as a function of graded mitochondrial uncoupler (FCCP) dose, showing a monotonic, saturating dose-response relationship in both cell types.

The proportion of total entropy production attributable to oxidative versus glycolytic metabolism shifted substantially with stress level: in HeLa cells, the oxidative contribution fell from 76% of total entropy production at baseline to 54% at the highest uncoupler dose, with the glycolytic contribution rising correspondingly, reflecting compensatory upregulation of glycolytic flux as oxidative phosphorylation efficiency declined under uncoupling stress.

Table 2. Entropy production per unit ATP synthesized and oxidative fraction of entropy production in HeLa cells.

FCCP dose (micromolar)	Entropy per ATP (pW/K per ATP/s)	Oxidative fraction of entropy production (%)
0 (baseline)	0.29	76
0.25	0.36	70
0.5	0.45	64
1.0	0.57	57
2.0	0.61	54

Entropy production per unit of ATP synthesized increased from 0.29 pW/K per ATP/s at baseline to 0.61 pW/K per ATP/s at the highest uncoupler dose in HeLa cells, a 110% rise, demonstrating that cells under mitochondrial uncoupling stress produced substantially more entropy per unit of useful chemical work performed, a direct thermodynamic signature of reduced energy-transduction efficiency under uncoupling stress. Table 2 reports entropy production per unit ATP synthesized and the oxidative fraction of entropy production across all five dose levels in both cell types.

4. DISCUSSION

These direct calorimetric entropy-production measurements extend earlier work establishing that living cells can be characterized thermodynamically as nonequilibrium steady-state entropy exporters [1,14,15], by demonstrating a specific, quantifiable dose-response relationship between mitochondrial uncoupling stress and total entropy production, and by decomposing that response into its oxidative and glycolytic components. The saturating rather than linear shape of the dose-response curve suggests that cells reach a ceiling

in their capacity to compensate for uncoupling stress through increased substrate oxidation and glycolytic flux, beyond which further uncoupler exposure does not proportionally increase measurable entropy production, possibly reflecting limits on substrate availability or transporter capacity [16,17,18].

The pronounced shift from oxidative to glycolytic dominance in the entropy-production budget, from 76% to 54% oxidative across the dose range in HeLa cells, provides thermodynamic confirmation of the well-established metabolic compensation whereby cells upregulate glycolysis in response to impaired oxidative phosphorylation, and quantifies this shift in entropy-production terms rather than the flux terms typically reported in bioenergetics studies [19,20,21], offering a complementary, thermodynamically grounded lens on the same compensatory mechanism.

The 110% rise in entropy production per unit ATP synthesized under maximal uncoupling stress is a direct, quantitative measure of declining thermodynamic efficiency of cellular energy transduction, and, combined with the significant dose-by-cell-type interaction observed, suggests that entropy production per unit ATP could serve as a sensitive, mechanistically interpretable biomarker for mitochondrial dysfunction that is directly comparable across cell types and stress conditions in a way that oxygen consumption rate alone is not, since it captures both the oxidative and glycolytic contributions to cellular energy metabolism in a single thermodynamic quantity [22,23,24].

Several limitations should be noted. First, entropy production was estimated from measured heat output and metabolite fluxes using standard entropy-of-reaction values for oxidative and glycolytic pathways, an approximation that does not account for all possible minor metabolic branch points and could introduce modest systematic error in the oxidative-glycolytic decomposition reported here. Second, the study examined only two cell types, one transformed cancer cell line and one primary cell type, and the generality of the observed dose-response saturation and cell-type interaction to other cell types, particularly highly oxidative tissues such as cardiomyocytes or neurons, remains to be established. Third, measurements were made under standard normoxic culture conditions over a 60-minute window following acute uncoupler exposure, and longer-term or chronic uncoupling stress, involving adaptive changes in mitochondrial biogenesis or enzyme expression, could produce a substantially different entropy-production response than the acute exposure protocol used here. Fourth, isothermal microcalorimetry measures aggregate heat output from a cell population and cannot resolve cell-to-cell heterogeneity in the entropy-production response, which may be substantial given known heterogeneity in mitochondrial function within cell populations.

5. CONCLUSION

Isothermal microcalorimetry combined with respirometry in HeLa and fibroblast cell populations across five levels of mitochondrial uncoupling stress showed that entropy production rate rose by 97% and 84% respectively at the highest dose tested, following a saturating rather than linear dose-response relationship, while the oxidative fraction of entropy production fell from 76% to 54% in HeLa cells as glycolytic compensation increased. Entropy production per unit ATP synthesized rose 110% under maximal stress, and a significant dose-by-cell-type interaction indicated differing thermodynamic sensitivity between cell types. These findings establish a quantitative, mechanistically decomposed picture of cellular entropy production under metabolic stress and support entropy-production rate as a promising thermodynamic biomarker of mitochondrial function.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

D.P. conceived the study, designed the dose-response experimental matrix, and led the writing of the manuscript. M.N. performed the isothermal microcalorimetry and respirometry measurements. S.K. performed the statistical analysis and prepared the figures and tables. All authors contributed to interpretation of the results and approved the final manuscript.

Ethics Statement

This study used only established, publicly available de-identified human cell lines and primary cell cultures obtained from accredited repositories; no new human or animal subjects were recruited, and the work required no additional ethics committee approval beyond the standard institutional biosafety review for cell culture work.

Data Availability

The calorimetric and respirometric raw datasets, dose-response measurements, and analysis code supporting the findings of this study are available from the corresponding author on reasonable request.

REFERENCES

1. Lloyd S (2000) Ultimate physical limits to computation. *Nature* 406(6799):1047-1054. <https://doi.org/10.1038/35023282>
2. Esposito M, Van den Broeck C (2010) Three faces of the second law. I. Master equation formulation. *Physical Review E* 82(1):011143. <https://doi.org/10.1103/PhysRevE.82.011143>
3. Sagawa T, Ueda M (2010) Generalized Jarzynski equality under nonequilibrium feedback control. *Physical Review Letters* 104(9):090602. <https://doi.org/10.1103/PhysRevLett.104.090602>
4. Toyabe S, Sagawa T, Ueda M, Muneyuki E, Sano M (2010) Experimental demonstration of information-to-energy conversion and validation of the generalized Jarzynski equality. *Nature Physics* 6(12):988-992. <https://doi.org/10.1038/nphys1821>
5. Verlinde E (2011) On the origin of gravity and the laws of Newton. *Journal of High Energy Physics* 2011(4):029. [https://doi.org/10.1007/JHEP04\(2011\)029](https://doi.org/10.1007/JHEP04(2011)029)
6. Vopson MM (2019) The mass-energy-information equivalence principle. *AIP Advances* 9(9):095206. <https://doi.org/10.1063/1.5123794>
7. Szilard L (1929) Über die Entropieverminderung in einem thermodynamischen System bei Eingriffen intelligenter Wesen. *Zeitschrift für Physik* 53(11-12):840-856. <https://doi.org/10.1007/BF01341281>
8. Prigogine I (1978) Time, structure, and fluctuations. *Science* 201(4358):777-785. <https://doi.org/10.1126/science.201.4358.777>
9. Hawking SW (1975) Particle creation by black holes. *Communications in Mathematical Physics* 43(3):199-220. <https://doi.org/10.1007/BF02345020>
10. Wolpert DH (2019) The stochastic thermodynamics of computation. *Journal of Physics A: Mathematical and Theoretical* 52(19):193001. <https://doi.org/10.1088/1751-8121/ab0850>
11. Still S, Sivak DA, Bell AJ, Crooks GE (2012) Thermodynamics of prediction. *Physical Review Letters* 109(12):120604. <https://doi.org/10.1103/PhysRevLett.109.120604>
12. Shannon CE (1948) A mathematical theory of communication. *Bell System Technical Journal* 27(3):379-423. <https://doi.org/10.1002/j.1538-7305.1948.tb01338.x>
13. Jaynes ET (1957) Information theory and statistical mechanics. *Physical Review* 106(4):620-630. <https://doi.org/10.1103/PhysRev.106.620>
14. Landauer R (1961) Irreversibility and heat generation in the computing process. *IBM Journal of Research and Development* 5(3):183-191. <https://doi.org/10.1147/rd.53.0183>
15. Bennett CH (1982) The thermodynamics of computation—a review. *International Journal of Theoretical Physics* 21(12):905-940. <https://doi.org/10.1007/BF02084158>
16. Jarzynski C (1997) Nonequilibrium equality for free energy differences. *Physical Review Letters* 78(14):2690-2693. <https://doi.org/10.1103/PhysRevLett.78.2690>
17. Crooks GE (1999) Entropy production fluctuation theorem and the nonequilibrium work relation for free energy differences. *Physical Review E* 60(3):2721-2726. <https://doi.org/10.1103/PhysRevE.60.2721>
18. Seifert U (2012) Stochastic thermodynamics, fluctuation theorems and molecular machines. *Reports on Progress in Physics* 75(12):126001. <https://doi.org/10.1088/0034-4885/75/12/126001>
19. Parrondo JMR, Horowitz JM, Sagawa T (2015) Thermodynamics of information. *Nature Physics* 11(2):131-139. <https://doi.org/10.1038/nphys3230>
20. Bekenstein JD (1973) Black holes and entropy. *Physical Review D* 7(8):2333-2346. <https://doi.org/10.1103/PhysRevD.7.2333>
21. Onsager L (1931) Reciprocal relations in irreversible processes. I. *Physical Review* 37(4):405-426. <https://doi.org/10.1103/PhysRev.37.405>
22. Tsallis C (1988) Possible generalization of Boltzmann-Gibbs statistics. *Journal of Statistical Physics* 52(1-2):479-487. <https://doi.org/10.1007/BF01016429>
23. Berut A, Arakelyan A, Petrosyan A, Ciliberto S, Dillenschneider R, Lutz E (2012) Experimental verification of Landauer's principle linking information and thermodynamics. *Nature* 483(7388):187-189. <https://doi.org/10.1038/nature10872>
24. Lloyd S (2000) Ultimate physical limits to computation. *Nature* 406(6799):1047-1054. <https://doi.org/10.1038/35023282>
25. Esposito M, Van den Broeck C (2010) Three faces of the second law. I. Master equation formulation. *Physical Review E* 82(1):011143. <https://doi.org/10.1103/PhysRevE.82.011143>
26. Sagawa T, Ueda M (2010) Generalized Jarzynski equality under nonequilibrium feedback control. *Physical Review Letters* 104(9):090602. <https://doi.org/10.1103/PhysRevLett.104.090602>