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# Stimulation of mitochondrial proton conductance by hydroxyn

**(i) Synopsis**

Mild uncoupling of oxidative phosphorylation, caused by a leak of protons back into the matrix, limits mitochondrial production of reactive oxygen species. This proton leak can be induced by lipid peroxidation products of reactive oxygen species, such as 4-hydroxynonenal (HNE). HNE activates uncoupling proteins (UCP1, UCP2 and UCP3) and the adenine nucleotide translocase, thereby providing a negative feedback loop. The mechanism of activation and the conditions necessary to induce uncoupling by HNE are unclear. We find that activation of proton leak by HNE in rat and mouse skeletal muscle mitochondria is dependent upon incubation with respiratory substrate. In the presence of HNE, mitochondria energised with succinate become progressively more leaky to protons over time compared to mitochondria in the absence of either HNE or succinate. Energised mitochondria must attain a high membrane potential to allow HNE to activate uncoupling; a drop of 10 – 20 mV from the resting value is sufficient to blunt induction of proton leak by HNE. Uncoupling occurs through UCP3 (11%), adenine nucleotide translocase (64%) and other pathways (25%). Our findings show that exogenous HNE only activates uncoupling at high membrane potential. They suggest that both endogenous HNE production and high membrane potential are required before mild uncoupling will be triggered to attenuate mitochondrial ROS production.

Key words: Adenine nucleotide translocase; ANT; HNE; proton leak; UCP3; uncoupling protein;

Abbreviations used: ANT, adenine nucleotide translocase; BSA, bovine serum albumin; HNE, 4-hydroxy-2-nonenal; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; ROS, reactive oxygen species; TPMP, triphenylmethylphosphonium; UCP, uncoupling protein; KO, knockout; WT, wild type; state 4, mitochondrial condition with respiratory substrate but no ADP or exogenous uncoupler present.









resolved in [16]). This discrepancy may highlight the condition-dependence of the interaction of HNE with uncoupling pathways and may be caused by differences in energisation regimes between the present work and [7]. Such differences may also explain differences in activation of UCP1 by HNE observed in different laboratories [10, 11]. The discrepancy may also reflect possible differences in the requirements for high membrane potential of activation of UCP3 and ANT by HNE - further work is needed to resolve this point. The observation that UCP3 catalyses about 11% of the effect of HNE while ANT catalyses about 64%, even though UCP3 is present at about 4 pmol/mg protein [18], while ANT is present at about 2 nmol/mg protein [19], suggests that the HNE-activated proton conductance of UCP3 is about 100-fold higher than that of ANT, providing a rationale for an important role of UCP3 in regulating proton conductance and ROS production even though ANT is also activatable and present in excess.

The present paper explores the conditions under which HNE stimulates the uncoupling of skeletal muscle mitochondria. Firstly, we find that extent of incubation time during energisation is important. Secondly, we find that a high membrane potential must be achieved during this energisation. Physiologically, uncoupling by HNE is thought to be involved in the negative feedback control of mitochondrial ROS production [7] which is strongly attenuated if protonmotive force drops [3, 4]. We propose that the requirement for a high membrane potential for stimulation of proton conductance by HNE ensures that this feedback uncoupling only occurs at very high membrane potential, and prevents the uncoupling of mitochondria by non-mitochondrial ROS and its peroxidation products produced during high workload when mitochondria are functioning at a lower membrane potential.

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**Figure 1 Energisation-dependent stimulation of proton leak in rat skeletal muscle mitochondria by 50  $\mu$ M HNE**

Proton leak kinetics were measured in rat skeletal muscle mitochondria as described in the experimental section. (A) Timings of additions to the incubation chamber. Mitochondria plus inhibitors (M) were added at  $t = -10$  min, 50  $\mu$ M HNE (where present) was added at  $t = -9$  min, 4 mM succinate (S) was added at the times indicated, then leak kinetics (LK) were measured between  $t = 0$  and  $t = 3$  min by titration of membrane potential and oxygen consumption rate with malonate from 0.14 to 2.3 mM. Kinetic response of mitochondrial proton leak rate (measured as oxygen consumption rate) to membrane potential measured after energisation with 4 mM succinate for 0.75 (circles), 2.5 (triangles) or 5.0 min (squares) was measured minus (B) or plus (C) 50  $\mu$ M HNE. (D) Proton leak rate interpolated at 166 mV (solid vertical lines in (B) and (C)); the average highest protonmotive force common to all curves generated in the experiment. Data are means  $\pm$  S.E.M. of duplicate experiments performed on five separate preparations. One-way ANOVA was performed for (D);  $p = 0.0001$ , with Tukey's Multiple Comparison Test used posthoc: \*significantly different:  $p < 0.05$ ; \*\*,  $p < 0.001$ .

**Figure 2 Sensitivity of HNE-stimulated proton leak in rat skeletal muscle mitochondria to membrane potential**

(A-C), effect of malonate. (D-F), effect of KCN. (A,B,D,E), proton leak kinetics. 4 mM succinate was added at  $t = -1.5$  min (see Fig. 1A) in the absence (open symbols) or presence (closed symbols) of 50  $\mu$ M HNE. Proton leak kinetics were measured between  $t = 0$  and  $t = 3$  min after no treatment (A, D) or addition of 430  $\mu$ M malonate (B) or 43  $\mu$ M KCN (E) immediately prior to addition of succinate. (C, F), inhibition of HNE-stimulated proton leak by malonate and KCN. The increase in proton conductance at  $t = 0$  caused by addition of 50  $\mu$ M HNE was interpolated (solid vertical lines, (A,B,D,E)) at the highest common membrane potential, 164 mV in (C) and 169 mV in (F). 'X' indicates the drop in membrane potential measured at  $t = 0$  caused by pre-addition of malonate or KCN. (G), effect of this drop in membrane potential on HNE-stimulated proton conductance. The increases in proton leak rate from (C) and additional experiments (not shown) using 140  $\mu$ M and 290  $\mu$ M malonate (circles), and from (F) and additional experiments (not shown) using 14  $\mu$ M and 29  $\mu$ M KCN (triangles) are plotted against the drop ('X') in membrane potential at  $t = 0$  in the absence of HNE. Data are means  $\pm$  S.E.M. of 5 (A-C) or 6 (D-F) experiments each performed in duplicate. \*\*Significantly different from zero addition by paired Student's  $t$  test:  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Lines in (G) are linear regressions.

**Figure 3 Contribution of UCP3 to HNE-stimulated proton leak after 2.5 min energisation in mouse skeletal muscle mitochondria**

(A, B) proton leak kinetics. (A) mitochondria from wild-type mice. (B), mitochondria from *Ucp3*KO mice. 4 mM succinate was added at  $t = -2.5$  min, minus (open symbols) or plus (closed symbols) 50  $\mu$ M HNE. (C), proton leak due to UCP3 measured by interpolation at 160 mV (solid vertical lines in (A,B)) as (A) minus (B). Data are means  $\pm$  S.E.M. of 12 experiments, each performed in duplicate. \*Significantly different from control by paired Student's  $t$  test:  $p < 0.05$ .

**Figure 4 Contribution of ANT to HNE-stimulated proton leak after 2.5 min energisation in *Ucp3*KO mouse skeletal muscle mitochondria**

(A, B) proton leak kinetics in mitochondria from *Ucp3*KO mice. 4 mM succinate was added at  $t = -5$  min, minus (open symbols) or plus (closed symbols) 50  $\mu$ M HNE in the absence (A) or presence (B) of 7 nmol/mg protein carboxyatractylate. (C), proton leak due to ANT measured by interpolation at 157 mV (solid vertical lines in (A,B)) as (A) minus (B). Data are means  $\pm$  S.E.M. of 6 experiments, each performed in duplicate. \*Significantly different from control by paired Student's  $t$  test:  $p < 0.03$ .

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Figure 1

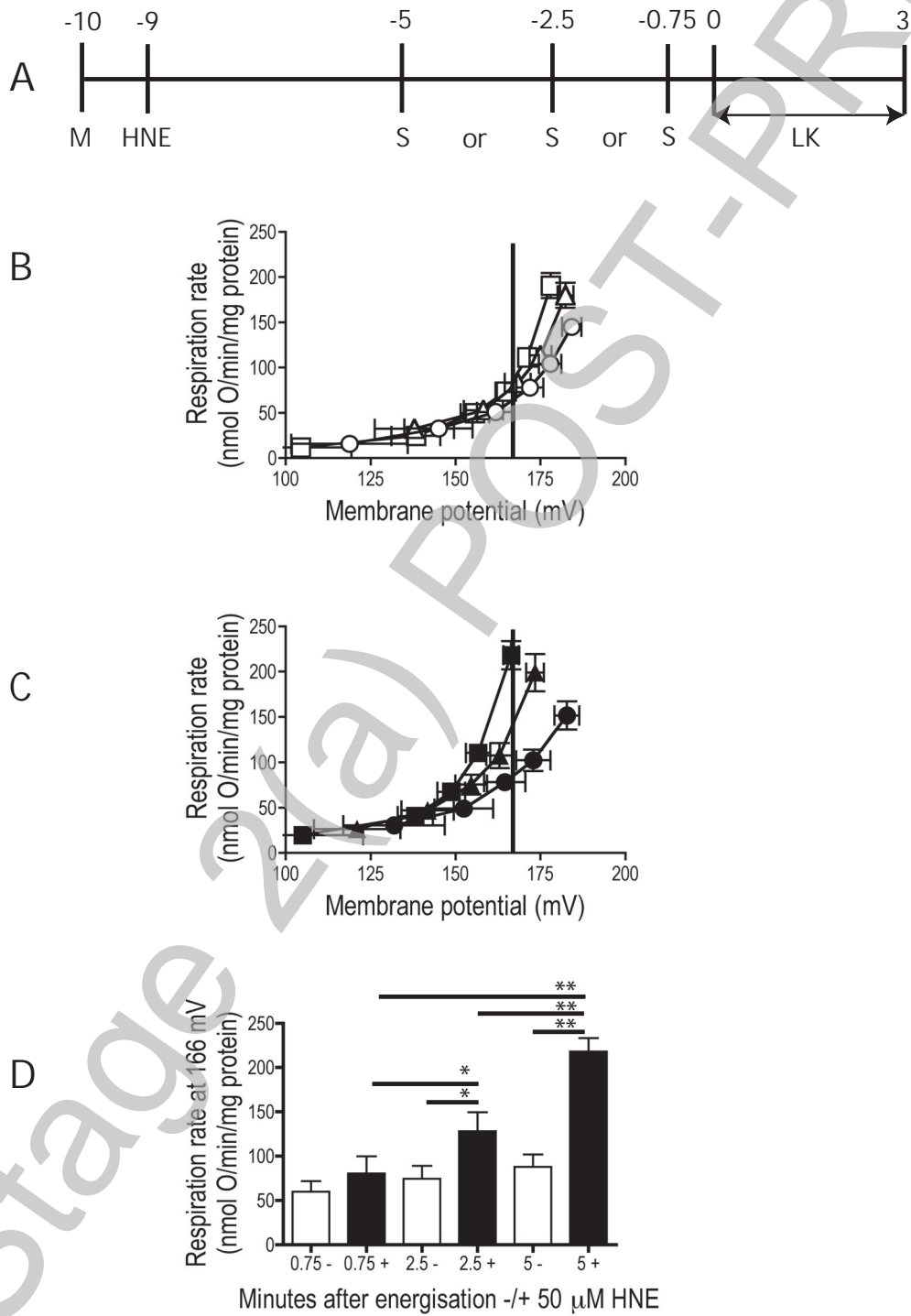
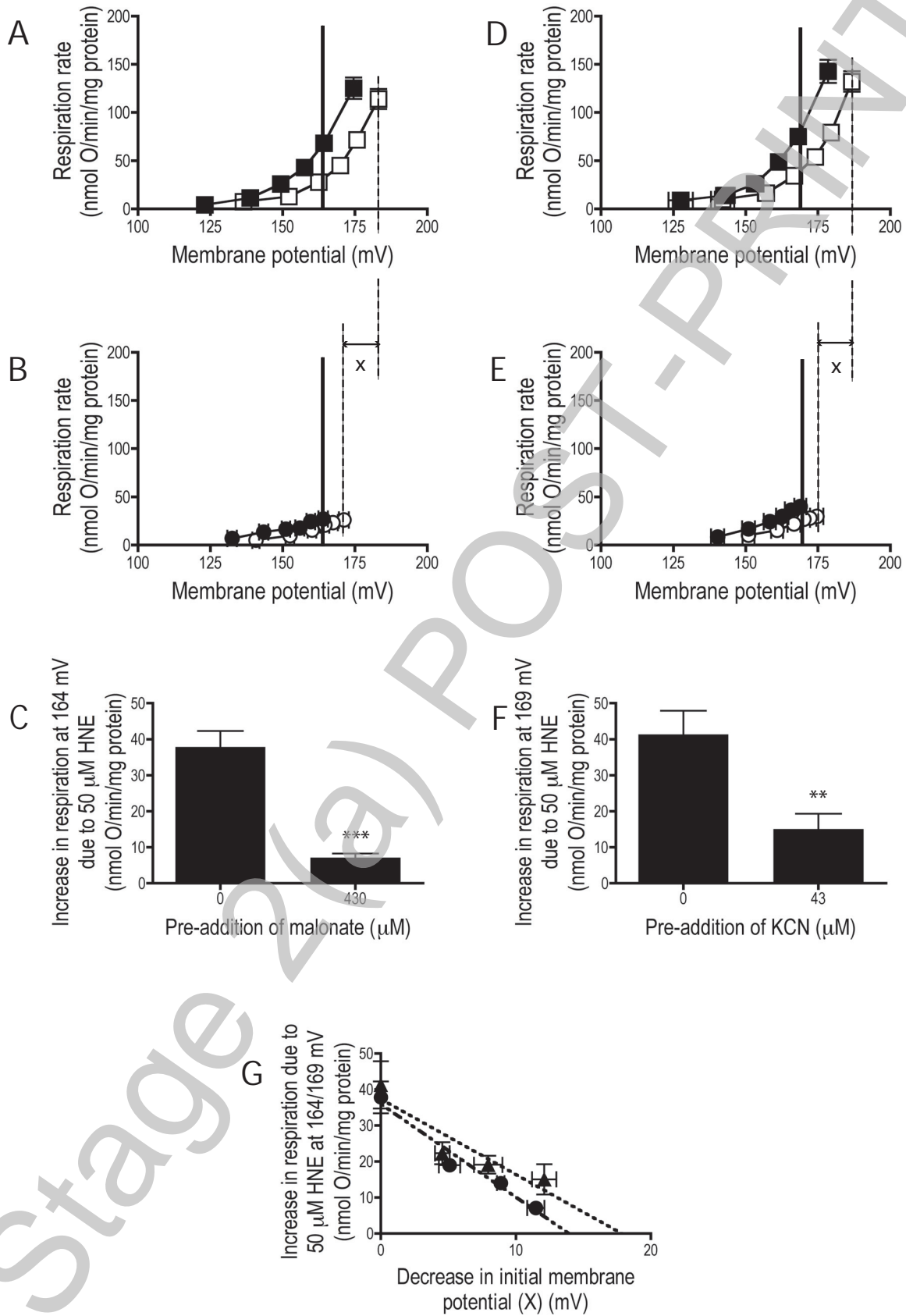
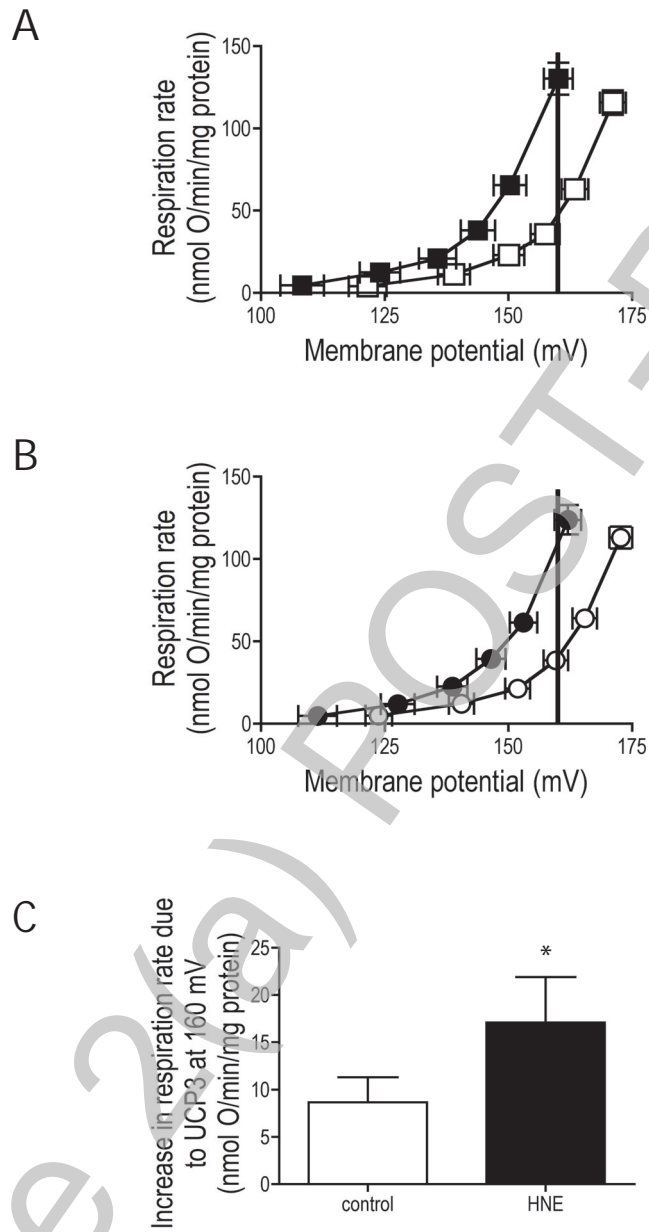


Figure 2



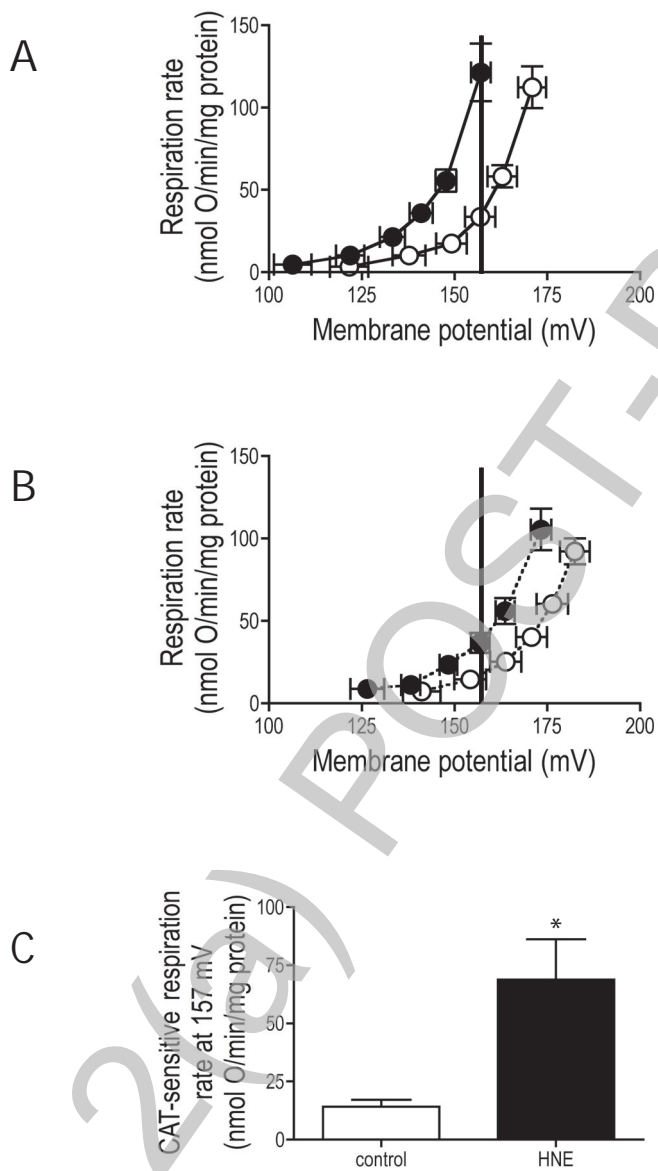
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Figure 3



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Figure 4



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