The mitochondrial complex one inhibitor R419 potently stimulates AMPK in dorsal root ganglion neurons and reduces incision-evoked pain *in vivo*

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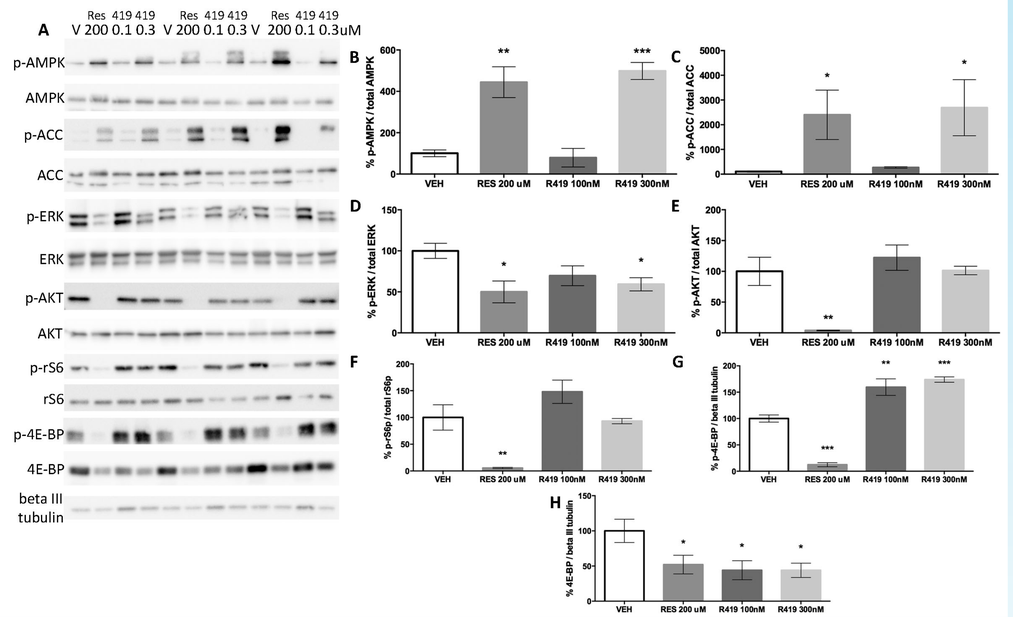
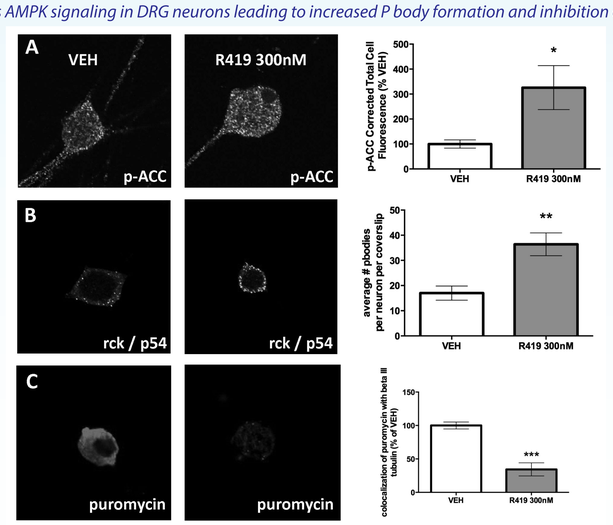
Adenosine monophosphate activated protein kinase (AMPK) is a ubiquitous protein kinase that negatively regulates anabolic pathways such as mechanistic target of rapamycin (mTOR) and mitogen activated protein kinase (MAPK). AMPK activity can be augmented by increasing AMP levels in cells, by positive allosteric modulation or by protection from dephosphorylation. Metformin is a prototypical AMPK activator that interferes with mitochondrial complex one to raise AMP levels in cells and activate AMPK. R419 is a potent complex one inhibitor that has been proposed to activate AMPK in somatic cells leading to beneficial metabolic effects. We tested whether R419 can activate AMPK in dorsal root ganglion (DRG) neurons and if the compound modifies pain hypersensitivity in vivo. Other lines of evidence suggest that AMPK activation can alleviate pain and pain hypersensitivity but potent complex one inhibitors like R419 have not been tested in this context. We find that R419 stimulates AMPK activity and blocks MAPK activity in DRG neurons at concentrations as low as 300nM but has little effect on mTOR signaling. With similar potency, R419 inhibits nascent protein synthesis and induces p body formation in DRG neurons, two signaling events that are induced by other AMPK activators. Moreover, R419 reduces the excitability of DRG neurons exposed to nerve growth factor (NGF) and stimulated with slowly depolarizing ramp currents. Finally we tested if R419 influences NGF- or incision-evoked mechanical hypersensitivity and hyperalgesic priming in mice. R419 co-treatment with NGF blocked mechanical hypersensitivity and hyperalgesic priming in a dose-dependent fashion. R419 treatment by local injection at the time of plantar incision and 24 hrs after also attenuated incision-evoked mechanical hypersensitivity and completely blocked hyperalgesic priming. Pharmacokinetic data from hindpaw samples indicate that R419 has a tissue half-life of approximately 1 or 2 hrs. We conclude that R419 potently activates AMPK in DRG neurons resulting in decreased MAPK activity and cellular excitability and that R419 reduces pain hypersensitivity and the transition to a chronic pain-like state in vivo.

Figure 1: R419 potently activates AMPK in mouse DRG neurons leading to inhibition of ERK signaling



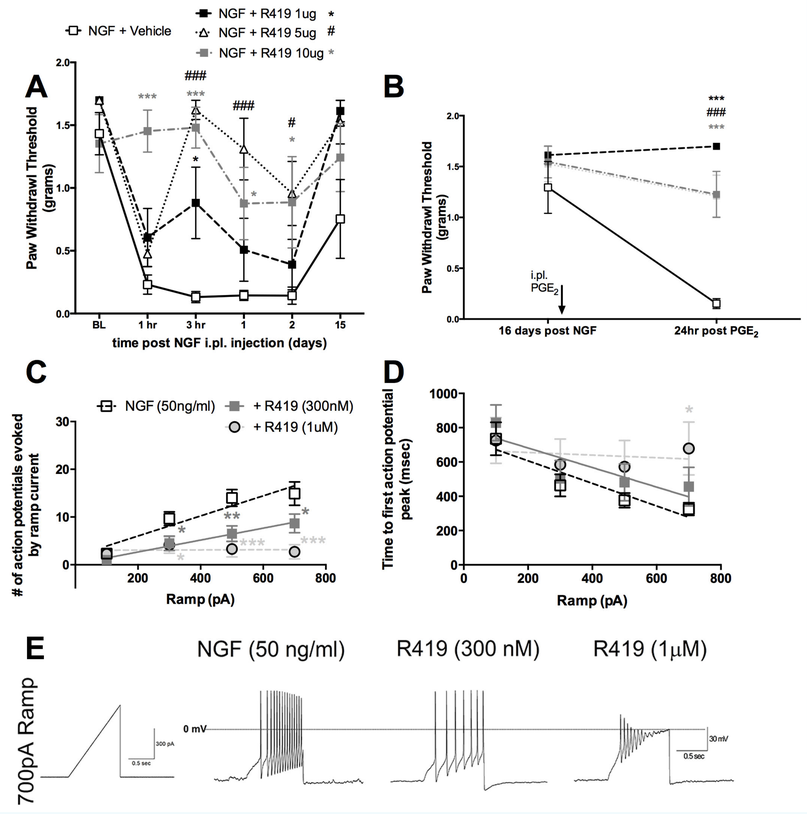
A) Western blots are shown for triplicate samples of DRG neurons in culture for 7 days from Male Swiss Webster mice exposed to vehicle (V), resveratrol (RES, 200 μM), R419 (419, 0.1 and 0.3 μM) for 1 hr. Twenty μg protein was loaded per lane and transferred to membranes that were blotted for phospho and total AMPK (B), ACC (C), ERK (D), AKT (E), rS6 (F) and 4E-BP (G and H) as well as βIII tubulin as a loading control. Data are plotted as the % change for the phosphorylated form of the protein divided by the total protein quantification standardized to the vehicle (VEH) treatment. 4E-BP and p-4E-BP were compared to the βIII tubulin loading control. Ordinary one-way ANOVA was used to assess differences between the vehicle and treatment groups with Fisher's LSD post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N = 3 per group with the exception of panel D where the N = 6.

Figure 2: R419 increases AMPK signaling in DRG neurons leading to increased P body formation and inhibition of nascent protein synthesis



A) DRG neurons in culture taken from male Swiss Webster mice were treated with R419 for 1 hr and then assayed for p-ACC and assessed by confocal microscopy for changes in ACC phosphorylation in neurons labeled with βIII tubulin. N = 9 (VEH) or 10 (R419) coverslips. B) DRG neurons in culture were treated with R419 for 1 hr and then assayed for P body formation with the P body marker rck / p54 and assessed by confocal microscopy for changes in puncta indicative of P body formation. N = 11 (VEH) or 10 (R419) coverslips. C) SUnSET assay was used to examine nascent protein synthesis in DRG neurons treated with R419 for 1 hr. Puromycin incorporation into proteins was measured with a puromycin antibody and assessed in βIII tubulin-positive DRG neurons. N = 6 (VEH) or 8 (R419) coverslips. Differences between groups were assessed by two tailed students t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3: R419 inhibits NGF-evoked mechanical hypersensitivity and NGF-mediated DRG neuron excitability



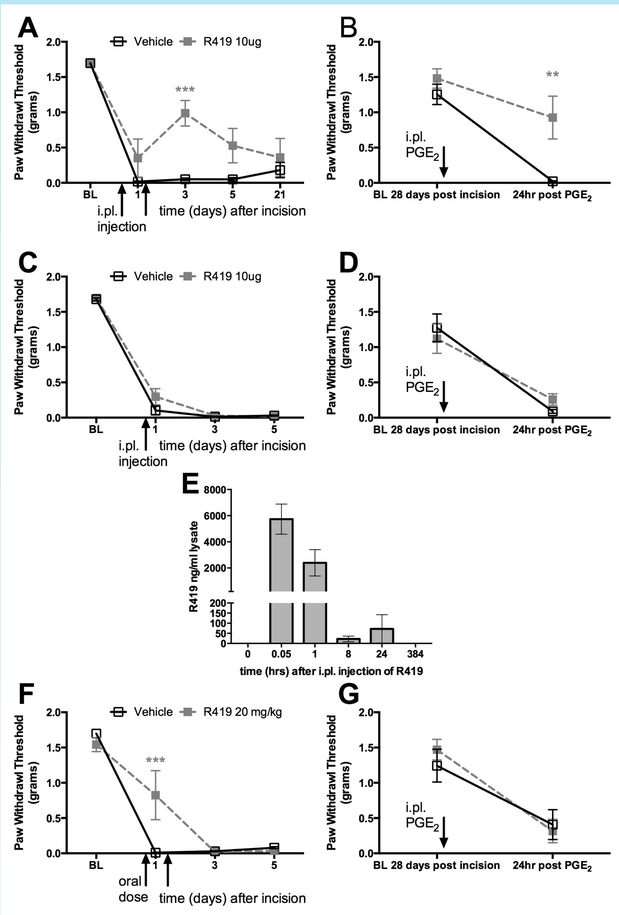
A) Male Swiss Webster mice were treated with NGF (50 ng) +/- R419 at the indicated doses and assessed for mechanical sensitivity by von Frey hair stimulation at the indicated time points. N = 6 per group. B) Mice were subsequently assessed for hyperalgesic priming with injection of PGE2 16 days after NGF +/- R419 treatment. N = 6 mice per group. C) Small diameter DRG neurons were exposed to NGF overnight and then exposed to vehicle or the indicated concentrations of R419 for 1 hr. Ramp currents were used to evoke spiking in recorded neurons. The number of action potentials evoked by the ramp current is shown in (C) and the time to first action potential peak evoked by the ramp current is shown in (D). An example trace for the 3 conditions in C and D for the 700 pA ramp is shown in (E). NGF alone group, N = 12; NGF + R419 300 nM, N = 6; R419 1 μM, N = 7. Behavioral data was analyzed by two-way ANOVA with Bonferroni post hoc test. Electrophysiology data was analyzed by two-way ANOVA with Fisher's LSD post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Conclusions

1) R419 specifically inhibits complex one to activate AMPK, resulting in ERK inhibition and reduced nascent protein synthesis in DRG neurons.
2) R419 inhibits NGF-induced pain in vivo and reduces nociceptor excitability in vitro.
3) R419 has a short tissue half-life in vivo but still reduces incision-induced pain hypersensitivity and hyperalgesic priming.

We conclude that R419 is a promising AMPK activator for the alleviation of post surgical pain via a local mechanism of action.

Figure 4: R419 inhibits incision-evoked mechanical hypersensitivity but has a short tissue half-life



A) Male Swiss Webster mice underwent plantar incision surgery and received intraplantar (i.pl.) injections of R419 (10 μg) or vehicle at the time of incision and 24 hr after incision. Mechanical thresholds were assessed at the indicated time points and hyperalgesic priming (B) was measured by PGE2 response at 28 days after incision, when animals had completely recovered from the initial hypersensitivity. N = 6 per group. C and D) Similar experiments were conducted but with a single i.pl. injection at the time of incision only. N = 6 per group. E) R419 levels in hindpaw skin samples were measured by HPLC at the indicated time points suggesting a t1/2 of approximately 1 hr for R419. N = 4 per group. F and G) Efficacy of R419 given by oral gavage (20 mg/kg) was tested in the plantar incision and hyperalgesic priming model with dosing at 1 hr prior to incision and again 24 hr after surgery. N = 6 per group. ** $p < 0.01$, *** $p < 0.001$.

Lack of off-target activity of R419

Biochemical assay results are presented as the percent inhibition of specific binding to the targets or activity of the enzymes in the presence of 10 μM R419. All assays were performed at Eurofins Panlabs Taiwan, Inc.

Target or ligand	Species	% inhibition at 10μM
CYP450, 1A2	human	0
CYP450, 2C19	human	11
CYP450, 2C9	human	6
CYP450, 2D6	human	1
CYP450, 3A4	human	12
Adenosine A1	human	-5
Adenosine A2A	human	-5
Adenosine A3	human	-2
Adrenergic α1A	rat	29
Adrenergic α1B	rat	41
Adrenergic α1D	human	10
Adrenergic α2A	human	12
Adrenergic β1	human	9
Adrenergic β1	human	9
Androgen (Testosterone), Androgen receptor	rat	-4
Bradykinin B1	human	9
Bradykinin B2	human	2
Calcium Channel L-Type, Benzothiazepine	rat	18
Calcium Channel L-Type, Dihydropyridine	rat	31
Calcium Channel N-Type	rat	1
Cannabinoid CB1	human	-9
Dopamine D1	human	-2
Dopamine D2s	human	1
Dopamine D3	human	6
Dopamine 4.2	human	-8
Endothelin ET_A	human	-1
Endothelin ET_B	human	6
Epidermal Growth Factor (EGF)	human	1
GABA_A, Flunitrazepam, Central	rat	-1
GABA_A, Muscimol, Central	rat	12
GABA_B1A	human	1
Glucocorticoid	human	-8
Glutamate, Kainate	rat	2
Glutamate, NMDA, Agonism	rat	28
Glutamate, NMDA, Glycine	rat	-13
Glutamate, NMDA,Phencyclidine	rat	4
Histamine H1	human	15
Histamine H2	human	12
Histamine H3	human	33
Imidazoline I2, Central	rat	5
Interleukin IL-1	mouse	-8
Leukotriene, Cysteinyl CysLT1	human	-3
Melatonin MT1	human	-3
Muscarinic M1	human	7
Muscarinic M2	human	5
Muscarinic M3	human	-7
Neuropeptide Y Y1	human	-1
Neuropeptide Y Y2	human	11
Nicotinic Acetylcholine	human	8
Nicotinic Acetylcholine α1,Bungarotoxin	human	-1
Opiate δ (OP1, DOP)	human	-1
Opiate κ (OP2, KOP)	human	24
Opiate μ (OP3, MOP)	human	7
Phorbol Ester	mouse	0
Platelet Activating Factor (PAF)	human	26
Potassium Channel [K_ATP]	hamster	4
Potassium Channel hERG	human	35
Prostanoid EP4	human	-7
Purinergic P2χ	rabbit	15
Purinergic P2χ	rat	4
Rolipram	rat	11
Serotonin (5-Hydroxytryptamine) 5-HT1A	human	-3
Serotonin (5-Hydroxytryptamine) 5-HT2B	human	9
Serotonin (5-Hydroxytryptamine) 5-HT3	human	3
Sigma σ1	human	42
Sodium Channel, Site-2	rat	5
Tachykinin NK1	human	13
Thyroid Hormone	rat	4
Transporter, Dopamine (DAT)	human	59
Transporter, GABA	rat	6
Transporter, Norepinephrine (NET)	human	7
Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	human	18

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