Published in final edited form as:

Science. 2012 May 18; 336(6083): 918–922. doi:10.1126/science.1215327.

The ancient drug salicylate directly activates AMP-activated protein kinase

Simon A. Hawley¹, Morgan D. Fullerton², Fiona A. Ross¹, Jonathan D. Schertzer², Cyrille Chevtzoff¹, Katherine J. Walker¹, Mark W. Pegg³, Darya Zibrova³, Kevin A. Green¹, Kirsty J. Mustard¹, Bruce E. Kemp⁴, Kei Sakamoto^{3,5}, Gregory R. Steinberg^{2,4}, and D. Grahame Hardie¹

¹Division of Cell Signalling & Immunology, College of Life Sciences, University of Dundee, Dundee, DD1 5EH, Scotland, UK.

²Divisions of Endocrinology and Metabolism, Department of Medicine, and Division of Biochemistry and Biomedical Sciences, Department of Pediatrics, McMaster University, 1200 Main St. W., Hamilton, ON, L8N 3Z5, Canada

³MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Dundee, DD1 5EH, Scotland, UK.

⁴St Vincent's Institute of Medical Research and Department of Medicine, University of Melbourne, 41 Victoria Parade, Fitzroy, Vic 3065 Australia.

Abstract

Salicylate, a plant product, has been in medicinal use since ancient times. More recently it has been replaced by synthetic derivatives such as aspirin and salsalate, both rapidly broken down to salicylate *in vivo*. At concentrations reached in plasma following administration of salsalate, or aspirin at high doses, salicylate activates adenosine monophosphate-activated protein kinase (AMPK), a central regulator of cell growth and metabolism. Salicylate binds at the same site as the synthetic activator, A-769662, to cause allosteric activation and inhibition of dephosphorylation of the activating phosphorylation site, Thr172. In AMPK knockout mice, effects of salicylate to increase fat utilization and lower plasma fatty acids *in vivo* were lost. Our results suggest that AMPK activation could explain some beneficial effects of salsalate and aspirin in humans.

The medicinal effects of willow bark have been known since the time of Hippocrates. The active component is salicylate, a hormone produced by plants in response to pathogen infection (1). For medicinal use it was largely replaced by aspirin (acetyl salicylate), which is rapidly broken down to salicylate *in vivo* (2, 3). Salicylate can also be administered as salsalate, which shows promise for treatment of insulin resistance and type 2 diabetes (4, 5). Aspirin and salicylate inhibit cyclo-oxygenases and hence prostanoid biosynthesis (6), as well as the protein kinase IKK β in the NF- κ B pathway (7). However, some effects of these drugs are still observed in mice deficient in these pathways (8).

Adenosine monophosphate-activated protein kinase (AMPK) is a cellular energy sensor conserved throughout eukaryotes. This heterotrimeric enzyme is composed of catalytic α

Address for correspondence: D. Grahame Hardie, Division of Cell Signalling & Immunology, College of Life Sciences, University of Dundee, Dow Street, Dundee, DD1 5EH, Scotland, UK; Tel: +44 (1382) 384253; FAX: +44 (1382) 385507; d.g.hardie@dundee.ac.uk.

⁵present address: Nestlé Institute of Health Sciences SA, Campus EPFL, 1015 Lausanne, Switzerland

Experimental Procedures Experimental procedures used in this paper are presented in a Supplementary file, as are Figures S1-S7.

subunits and regulatory β and γ subunits (9, 10). Once activated in response to metabolic stress, AMPK phosphorylates targets that switch off adenosine triphosphate (ATP) consuming processes, while switching on catabolic pathways that generate ATP. AMPK is activated >100-fold by phosphorylation at Thr172 in the α subunit by the tumour suppressor protein kinase, LKB1, or the Ca^{2+} -dependent kinase, CaMKK β (9, 10). Binding of AMP or adenosine diphosphate (ADP) to the γ subunit triggers a conformational change that promotes phosphorylation and inhibits dephosphorylation (11-15), causing a switch to the active form. Binding of AMP (but not ADP) to a second site (15) causes further allosteric activation, leading to >1,000-fold activation overall (16). Most drugs or xenobiotics that activate AMPK work by inhibiting mitochondrial ATP synthesis and increasing the concentration of AMP and ADP (17). However, a synthetic activator, A-769662 (18), which also causes allosteric activation and inhibits Thr172 dephosphorylation, binds directly to AMPK at distinct site(s) (19-21).

Salicylate, but not aspirin, activated AMPK when applied to HEK-293 cells, with its effects being significant at 1 mM and above (Fig. 1A; it appears that the esterases that catalyse breakdown of aspirin to salicylate are not expressed in these cells). This was associated with increased phosphorylation of Thr172 on AMPK- α , and the downstream target of AMPK, acetyl-CoA carboxylase (ACC); in the latter case the effects were evident at 1 mM and above (Fig. 1A). Salicylate can uncouple mitochondrial respiration (22), so we suspected that it might activate AMPK by decreasing cellular ATP and increasing AMP and ADP. To test this, we used isogenic cell lines expressing wild type AMPK (WT cells) or a mutated enzyme in which an R531G substitution in γ 2 renders AMPK insensitive to AMP or ADP (RG cells) (15, 17). At concentrations <10 mM, salicylate caused similar increases in AMPK phosphorylation or activation in WT and RG cells, showing that the effect was not dependent on changes in AMP or ADP. However, at 10 mM and above there was a greater activation/phosphorylation in WT than in RG cells, suggesting that AMP- or ADP-dependent effects were also occurring at these higher concentrations (Fig. 1B). Concentrations >1 mM salicylate increased cellular oxygen uptake in both WT and RG cells. This effect was not additive with the effect of a concentration of dinitrophenol causing a maximal increase in oxygen uptake, suggesting that at these concentrations salicylate, like dinitrophenol, could dissipate the proton gradient and thus uncouple the respiratory chain from ATP synthesis (Fig. 1C). However, unlike effects of another AMPK activator (H_2O_2) any increases in cellular ADP:ATP ratio at salicylate concentrations below 30 mM were very small (Fig. 1D). Thus, mitochondria appear to compensate for mild uncoupling by increasing respiration. Salicylate does not activate AMPK through the Ca^{2+} -CaMKK β pathway (12), because the CaMKK inhibitor STO-609 had no effect on responses to salicylate, although it blocked responses to a Ca^{2+} ionophore, A23187 (Fig. S1A/S1B, supplementary data).

Using a physiological concentration of ATP in assays (2 mM), salicylate caused a 1.6-fold allosteric activation with a half-maximal effect ($A_{0.5}$) at 1.0 ± 0.2 mM (Fig. 2A). A large activation by AMP was observed at all salicylate concentrations up to 30 mM (Fig. 2B), and salicylate did not affect the concentration of AMP causing half-maximal activation ($A_{0.5} = 18 \pm 3$ or 13 ± 2 μM , with or without 10 mM salicylate, Fig. 2C). By contrast, increasing concentrations of salicylate progressively antagonized activation by 30 and 100 nM A-769662 (Fig. 2D), and 10 mM salicylate increased $A_{0.5}$ for A-769662 by >4-fold (from 39 ± 4 to 172 ± 24 nM, Fig. 2E). These results suggest that salicylate is a partial agonist acting at the same site as A-769662, causing a small activation on its own but antagonizing the larger activation by A-769662. If it binds at the same site, salicylate should protect against Thr172 dephosphorylation, as A-769662 does (19, 20). Indeed, salicylate protected AMPK (human $\alpha 1\beta 1\gamma 1$ complex) against dephosphorylation and inactivation by protein phosphatase-2C α to the same extent as did AMP and A-769662 (Fig. 2F), although it had no

effect on PP2C α assayed using a peptide substrate (Fig. S2). An S108A substitution in the β 1 subunit abolishes effects of A-769662 on dephosphorylation, and β 2-containing complexes are resistant to the drug (20, 21). As expected, an S108A substitution in the α 1 β 1 γ 1 complex abolished the effects of A-769662 and salicylate, but not that of AMP (Fig. 2G), whereas there was no effect of salicylate on dephosphorylation of an α 1 β 2 γ 1 complex, although there was a small effect of A-769662 (Fig. 2H). These results lend further support to the idea that salicylate binds at the same site(s) as A-769662.

To examine effects of salicylate on AMPK phosphorylation and activation in intact cells, we used HEK-293 cells carrying a Flp recombinase target site to generate isogenic lines expressing FLAG-tagged WT β 1, a β 1-S108A mutant, or WT β 2. Using an antibody that recognizes both isoforms, endogenous β 1 and β 2 could be detected in the parental cells, but (as observed previously when expressing α subunits using this system (17)) these were largely replaced by FLAG-tagged β 1 and β 2 (with reduced electrophoretic mobility) in cells expressing recombinant β 1/ β 2; the expression of α 1, α 2 and γ 1 subunits was unaffected (Fig. 3A). A-769662 and salicylate caused increased activation/phosphorylation of AMPK in cells expressing WT β 1 (Fig. 3B), but their effects were greatly reduced in cells expressing the β 1-S108A mutant (Fig. 3C) or WT β 2 (Fig. 3D). By contrast, the effects of quercetin (which acts by increasing AMP (17)) were unaffected.

To test whether salicylate has metabolic effects *in vivo* through AMPK, we used β 1 knockout mice (23). Fatty acid oxidation in isolated WT hepatocytes was stimulated by salicylate or A-769662, and was associated with increased phosphorylation of AMPK and ACC (the latter regulating fat oxidation (24)). All effects were reduced or eliminated in hepatocytes from β 1-KOs (Figs. 3E/F, S3). There were no changes in ADP:ATP ratios in WT cells in response to 1 to 10 mM salicylate (Fig. S4A)

We injected mice with salicylate or A-769662 at the start of a period of fasting to assess metabolism *in vivo*. The dose used generated plasma salicylate of 2.4 ± 0.4 and 2.0 ± 0.1 mM (mean $\pm$ SEM, $n = 7$ and 3) in WT and β 1-KO mice respectively. Both agents caused phosphorylation of liver AMPK and ACC in WT but not β 1-KO mice (Fig. 4A/B), although there were no changes in hepatic AMP/ATP or ADP/ATP ratios in WT mice (Fig. S4B). In WT mice, salicylate also caused phosphorylation and activation of AMPK in soleus muscle and adipose tissue (Fig. S5). Both agents depressed the respiratory exchange ratio (RER) for 6 hours after injection, consistent with a switch from carbohydrate to fat utilization; these effects were lost in β 1-KOs (Fig. 4C through F). Significant depressions in RER, in WT mice only, were evident by calculating the area under the curve (Figs. S6A and S6B). When we calculated fat and carbohydrate utilization, both salicylate and A-769662 increased fat utilization in WT but not β 1-KO mice (Fig. 4G); A-769662 also decreased carbohydrate utilization in WT mice (Figs. S6C and D). Both salicylate and A-769662 reduced serum non-esterified fatty acids in WT but not β 1-KO mice (Fig. 4H). We also studied glucose homeostasis in mice made insulin-resistant by high-fat feeding, followed by daily salicylate injections for 2 weeks. However, effects of salicylate to improve fasting glucose, fasting insulin, glucose tolerance and insulin resistance (HOMA-IR), were retained in β 1-KO mice (Fig. S7), indicating that they were independent of AMPK.

Our results show that salicylate can directly activate AMPK, primarily by inhibiting Thr-172 dephosphorylation. The plasma salicylate concentrations in humans treated with oral salsalate (4) or high-dose aspirin (30-90 mg/kg) (25, 26) are 1 to 3 mM. At these concentrations, salicylate activated AMPK in WT and RG HEK-293 cells to the same extent, and did not increase cellular ADP:ATP ratios, indicating an AMP-independent mechanism. Thus, the natural product salicylate can activate AMPK via a mechanism closely related to that of A-769662, a synthetic activator derived from a high-throughput

screen (which, unlike salicylates, has poor oral availability (18)). Although the exact site(s) occupied by salicylate and A-769662 on AMPK remain unidentified, our finding that the S108A mutation abolishes activation by both agents suggests that the binding sites overlap.

Effects of salicylate on fat oxidation *in vivo* appear to require activation of AMPK- β 1 complexes. Aspirin also reduces circulating lipids in obese rats and improves insulin sensitivity (7). However, in agreement with previous studies (7, 27), our results using long-term salicylate treatment of fat-fed mice (Fig. S7) indicate that effects on AMPK-independent pathways, such as IKK β or JNK, are also important.

After oral administration, aspirin is rapidly broken down by liver, erythrocyte, and plasma esterases to salicylate (3), whose peak plasma concentrations and half-life are orders of magnitude greater than those of aspirin (2). Our findings raise the possibility that other effects of aspirin, like protective effects against development of cancer (28), may be mediated in part by AMPK. AMPK is activated by the anti-diabetic drug metformin (17, 29), and treatment of diabetics with metformin is also associated with reduced cancer incidence (30). Our results show that one thing that salicylates and metformin hold in common is their ability to activate AMPK. However, one caveat is that the doses of aspirin required to activate AMPK *in vivo* may be higher than those used in most human studies.

One-sentence summary

Salicylate (the natural product from which aspirin is derived) is a direct activator of AMP-activated protein kinase – this novel target for salicylate drugs might explain some of their beneficial affects in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

DGH was supported by a Programme Grant (080982) from the Wellcome Trust, and KS by the Medical Research Council. DGH and KS were also funded by the companies (AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck Serono and Pfizer) supporting the Division of Signal Transduction Therapy, University of Dundee. The studies were also supported by grants and fellowship from the Canadian Institutes of Health Research (MDF, GRS), the Canadian Diabetes Association (JDS, GRS), the National Health and Medical Research Council of Australia (GRS, BEK) and the Australian Research Council and the Victorian Government's Operational Infrastructure Support Program (BEK). JDS is a DeGroote Academic Fellow (McMaster University) and MDF a Banting Postdoctoral Fellow (CIHR). GRS is a Canada Research Chair in Metabolism and Obesity.

References

1. Reymond P, Farmer EE. Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* Oct.1998 1:404. [PubMed: 10066616]
2. Higgs GA, Salmon JA, Henderson B, Vane JR. Pharmacokinetics of aspirin and salicylate in relation to inhibition of arachidonate cyclooxygenase and antiinflammatory activity. *Proc. Natl. Acad. Sci. USA.* Mar.1987 84:1417. [PubMed: 3103135]
3. Williams FM. Clinical significance of esterases in man. *Clin. Pharmacokinet.* Sep-Oct;1985 10:392. [PubMed: 3899454]
4. Fleischman A, Shoelson SE, Bernier R, Goldfine AB. Salsalate improves glycemia and inflammatory parameters in obese young adults. *Diabetes Care.* Feb.2008 31:289. [PubMed: 17959861]
5. Goldfine AB, et al. The effects of salsalate on glycemic control in patients with type 2 diabetes: a randomized trial. *Ann. Intern. Med.* Mar 16.2010 152:346. [PubMed: 20231565]

6. Higgs GA, Moncada S, Vane JR. Eicosanoids in inflammation. *Ann. Clin. Res.* 1984; 16:287. [PubMed: 6100035]
7. Yuan M, et al. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science*. Aug 31.2001 293:1673. [PubMed: 11533494]
8. Cronstein BN, Montesinos MC, Weissmann G. Salicylates and sulfasalazine, but not glucocorticoids, inhibit leukocyte accumulation by an adenosine-dependent mechanism that is independent of inhibition of prostaglandin synthesis and p105 of NFkappaB. *Proc. Natl. Acad. Sci. USA*. May 25.1999 96:6377. [PubMed: 10339595]
9. Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat. Rev. Mol. Cell Biol.* Oct.2007 8:774. [PubMed: 17712357]
10. Steinberg GR, Kemp BE. AMPK in health and disease. *Physiol. Rev.* Jul.2009 89:1025. [PubMed: 19584320]
11. Oakhill JS, et al. AMPK is a direct adenylate charge-regulated protein kinase. *Science*. Jun 17.2011 332:1433. [PubMed: 21680840]
12. Hawley SA, et al. 5'-AMP activates the AMP-activated protein kinase cascade, and Ca²⁺/calmodulin the calmodulin-dependent protein kinase I cascade, via three independent mechanisms. *J. Biol. Chem.* 1995; 270:27186. [PubMed: 7592975]
13. Oakhill JS, et al. beta-Subunit myristoylation is the gatekeeper for initiating metabolic stress sensing by AMP-activated protein kinase (AMPK). *Proc. Natl. Acad. Sci. USA*. Nov 9.2010 107:19237. [PubMed: 20974912]
14. Davies SP, Helps NR, Cohen PTW, Hardie DG. 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2Ca and native bovine protein phosphatase-2A_C. *FEBS Lett.* 1995; 377:421. [PubMed: 8549768]
15. Xiao B, et al. Structure of mammalian AMPK and its regulation by ADP. *Nature*. Apr 14.2011 472:230. [PubMed: 21399626]
16. Suter M, et al. Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. *J. Biol. Chem.* Oct 27.2006 281:32207. [PubMed: 16943194]
17. Hawley SA, et al. Use of cells expressing gamma subunit variants to Identify diverse mechanisms of AMPK activation. *Cell Metab.* Jun 9.2010 11:554. [PubMed: 20519126]
18. Cool B, et al. Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab.* Jun.2006 3:403. [PubMed: 16753576]
19. Goransson O, et al. Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. *J. Biol. Chem.* Nov 9.2007 282:32549. [PubMed: 17855357]
20. Sanders MJ, et al. Defining the mechanism of activation of AMP-activated protein kinase by the small molecule A-769662, a member of the thienopyridone family. *J. Biol. Chem.* Nov 9.2007 282:32539. [PubMed: 17728241]
21. Scott JW, et al. Thienopyridone drugs are selective activators of AMP-activated protein kinase beta1-containing complexes. *Chem. Biol.* Nov 24.2008 15:1220. [PubMed: 19022182]
22. Brody TM. Action of sodium salicylate and related compounds on tissue metabolism in vitro. *J. Pharmacol. Exp. Ther.* May.1956 117:39. [PubMed: 13320299]
23. Dzakmo N, et al. AMPK beta1 deletion reduces appetite, preventing obesity and hepatic insulin resistance. *J. Biol. Chem.* Jan 1.2010 285:115. [PubMed: 19892703]
24. Abu-Elheiga L, Matzuk MM, Abo-Hashema KA, Wakil SJ. Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science*. 2001; 291:2613. [PubMed: 11283375]
25. Day RO, et al. Concentration-response relationships for salicylate-induced ototoxicity in normal volunteers. *Br. J. Clin. Pharmacol.* Dec.1989 28:695. [PubMed: 2611090]
26. Hundal RS, et al. Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes. *J. Clin. Invest.* May.2002 109:1321. [PubMed: 12021247]
27. Gao Z, Zuberi A, Quon MJ, Dong Z, Ye J. Aspirin inhibits serine phosphorylation of insulin receptor substrate 1 in tumor necrosis factor-treated cells through targeting multiple serine kinases. *J. Biol. Chem.* Jul 4.2003 278:24944. [PubMed: 12714600]

28. Rothwell PM, et al. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet*. Jan 1.2010 377:31. [PubMed: 21144578]
29. Zhou G, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J. Clin. Invest.* Oct.2001 108:1167. [PubMed: 11602624]
30. Ogunleye AA, Ogston SA, Morris AD, Evans JM. A cohort study of the risk of cancer associated with type 2 diabetes. *Br. J. Cancer*. Oct 6.2009 101:1199. [PubMed: 19690547]

Figure 1.

Effects of salicylate in HEK-293 cells. (A) Effects of salicylate or aspirin on AMPK activity (mean $\pm$ SD, $n = 4$) and phosphorylation of AMPK (Thr172) and ACC (Ser79) ($n = 2$). (B) Effects of salicylate on AMPK activity (mean $\pm$ SD, $n = 6$) and phosphorylation ($n = 2$) in HEK-293 cells stably expressing wild type (WT) $\gamma 2$ or an R531G substitution (RG). In Figs. 1A and 1B, the activity is plotted on a logarithmic scale as % of control without drug, and effects significantly different from control without drug (2-way ANOVA, with Bonferroni's test comparing each drug concentration to control without drug) are shown (* $p < 0.05$, *** $p < 0.001$). (C) Effect of salicylate on oxygen uptake in WT and RG cells (mean $\pm$ SD, $n = 7$ to 13; significant differences by 2-way ANOVA, using Bonferroni's test to compare with basal values without salicylate or DNP, are shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)). (D) Effects of salicylate or H₂O₂ (1 mM) on ADP:ATP ratios (means of duplicate cell incubations).

Figure 2.

Effect of salicylate on AMPK in cell-free assays. (A-E) Effects of salicylate on activity of purified rat liver AMPK; (A) effect of salicylate; (B) effect of salicylate $\pm$ 200 μ M AMP; (C) effect of AMP $\pm$ 10 mM salicylate; (D) effect of salicylate $\pm$ 30 and 100 nM A-769662; (E) effect of A-769662 $\pm$ 10 mM salicylate. Data points in (A-E) are means of duplicate assays; lines were generated by fitting data to the equation: $Y = \text{basal} + (\text{basal} * (\text{activation} - \text{basal}) * X / (A_{0.5} + X))$; values obtained for $A_{0.5}$ and activation are quoted in the text. (F-H) Effects of AMP, A-769662 and salicylate on dephosphorylation of bacterially expressed human AMPK complexes by PP2C α (all incubations contained PP2C α , but control lacked Mg²⁺); bar graphs show AMPK activity (% of control without Mg²⁺, mean $\pm$ SD, $n = 6$; pictures show Thr172 phosphorylation ($n = 2$). (F) WT $\alpha 1\beta 1\gamma 1$ complex; (G) $\alpha 1\beta 1\gamma 1$ complex with $\beta 1$ S108A substitution; (H) WT $\alpha 1\beta 2\gamma 1$ complex. Significant differences from the control plus Mg²⁺, using 1-way ANOVA with Dunnett's multiple comparison test, are shown: ** $p < 0.01$, *** $p < 0.001$. Also shown are significant differences in the size of the effect of A-769662 or salicylate between the $\alpha 1\beta 1\gamma 1$ and $\alpha 1\beta 2\gamma 1$ complexes (2F and 2H): ††† $p < 0.001$. For the latter comparisons, the difference between the “+Mg²⁺+A-76922” and “+Mg²⁺ only” columns was first expressed as a fraction of the difference between the “+Mg²⁺+AMP” and “+Mg²⁺ only” columns.

Figure 3.

Effects of salicylate and A-769662 in intact cells. (A) Expression of β subunits assessed using pan- β antibody in parental cells or cells stably expressing $\beta 1$ WT, $\beta 1$ -S108A, or $\beta 2$ WT, and of endogenous $\alpha 1$, $\alpha 2$ and $\gamma 1$ in the same cells. (B-D) Activity and phosphorylation of AMPK after treatment with various activators in cells expressing: (B) $\beta 1$ WT; (C) $\beta 1$ -S108A; (D) $\beta 2$ WT. Kinase assays [mean $\pm$ SEM, $n = 6$ except for 100 μ M A-769662 ($n = 4$) and quercetin ($n = 2$); significantly different from control without drug, by 1-way ANOVA with Dunnett's multiple comparison test, *** $p < 0.001$] and Western blots ($n = 2$) were of immunoprecipitates made using anti-FLAG antibody. (E) Palmitate oxidation in hepatocytes isolated from $\beta 1$ -KO mice and WT controls (mean $\pm$ SEM, $n = 6$ to 14, significantly different from control without drug by 2-way ANOVA with Bonferroni's test, *** $p < 0.001$; †††significantly different from WT, $p < 0.001$). (F) Phosphorylation of AMPK and ACC in hepatocytes isolated from $\beta 1$ -KO or WT mice.

Figure 4.

Effects of salicylate and A-769662 treatment in $\beta 1$ -KO and WT mice in vivo. (A) Phosphorylation/expression of ACC, AMPK and GAPDH in liver of mice treated with salicylate or A-769662 (doublet in ACC blots represents ACC1/ACC2). (B) Quantification of phosphorylation of ACC (pACC:total ACC, mean $\pm$ SEM; $n = 6$ or 7 for WT, $n = 3$ for $\beta 1$ -KO; statistical significance by 1-way ANOVA with Bonferroni's test compared with vehicle only are shown (* $p < 0.05$, *** $p < 0.001$). (C)-(F) Respiratory exchange ratio measured in $\beta 1$ -KO and WT mice after injection of vehicle, salicylate (250 mg/kg) or A-769662 (30 mg/kg) at the start of a period of fasting. Results are mean $\pm$ SEM ($n = 6$ to 13). By 2-way ANOVA, effects of salicylate ($p < 0.05$) or A-769662 ($p < 0.001$) were only significant in WT mice; significant differences by Bonferroni's test at individual time points are shown (* $p < 0.05$, ** $p < 0.01$). (G) Fatty acid utilization calculated from data in (C)-(F). Results are mean $\pm$ SEM ($n = 7$ or 8). Significant differences by 2-way ANOVA with Bonferroni's test are shown (* $p < 0.05$, ** $p < 0.01$). (H) Plasma non-esterified fatty acids in mice treated with salicylate or A-769662 for 90 min. Results are mean $\pm$ SEM ($n = 7$ or 8). Significant differences by 2-way ANOVA with Bonferroni's test are shown (* $p < 0.05$, ** $p < 0.01$).