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Da Silva Morais et al.

The development of hepatic fibrosis occurs normally in AMPK deficient mice.

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AMPK and murin HSC 1

Da Silva Morais et al.

Abstract

Inhibition or blockade of hepatic stellate cells (HSCs), the main matrix producing cells involved in wound healing response, represents an attractive strategy for the treatment of liver fibrosis. *In vitro* studies have shown that activation of the AMP-activated protein kinase (AMPK), a key player in the regulation of cellular energy homeostasis, inhibits proliferation of myofibroblasts derived from HSC. If AMPK is a true regulator of fibrogenesis then defective AMPK activity would enhance fibrogenesis and hepatic fibrosis. To test this, *in vitro* studies were performed on mouse primary HSCs treated or not with AMPK activator AICAR or isolated from mice lacking the AMPK $\alpha 1$ catalytic subunit (AMPK $\alpha 1^{-/-}$) or their littermates (AMPK $\alpha 1^{+/+}$). Liver fibrosis was induced *in vivo* in AMPK $\alpha 1^{-/-}$ and $+/+$ mice by repeated injections of CCl₄. During culture activation of HSC, AMPK protein and activity significantly increased and regulatory AMPK $\gamma 3$ mRNA was specifically up-regulated. Stimulation of AMPK activity by AICAR inhibited HSC proliferation, as expected, as well as collagen- $\alpha 1$ expression. Importantly, AMPK $\alpha 1$ deletion inhibited proliferation of HSC, but not fibrogenesis *in vivo*. Moreover, AMPK $\alpha 1$ deletion was not associated with enhanced carbon tetrachloride-induced fibrosis *in vivo*. Our data demonstrate that HSC trans-differentiation is associated with increased AMPK activity that could relate to the stabilization of AMPK complex by the $\gamma 3$ subunits. Activation of AMPK in HSC inhibits *in vitro* fibrogenesis. By contrast low AMPK activity does not prevent HSC activation *in vitro* nor *in vivo* fibrosis.

Key words: Hepatic stellate cells, trans-differentiation, collagen, mouse, AICAR

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Da Silva Morais et al.

Introduction

Studies in rodents have demonstrated that adiponectin has a suppressive effect on liver fibrogenesis: Mice lacking adiponectin develop more severe carbon tetrachloride (CCl₄)-induced hepatic fibrosis than the wild-type mice while injection of adenovirus encoding for adiponectin prevents CCl₄- fibrosis in wild-type mice. In cultured hepatic stellate cells (HSC), adiponectin inhibits proliferation, migration, and expression of fibrogenic genes, and might induce apoptosis of activated cells [1;2]. In human with non-alcoholic steatohepatitis (NASH), decreased adiponectin levels have also been associated with the degree of hepatic steatosis, necroinflammation and fibrosis [3].

Adiponectin mediates most of its metabolic effects through receptor-dependent activation of AMP-activated protein kinase (AMPK) and peroxisome proliferator activated receptor alpha (PPAR α) [4]. AMPK, a serine/threonine heterotrimeric protein kinase composed by one catalytic (α) and two regulatory (β and γ) subunits, is a fuel-sensing enzyme important for the regulation of cellular energy homeostasis [5]. Upon activation, AMPK stimulates ATP-generating catabolic pathways, such as glycolysis and lipid oxidation, and turns off energy-consuming processes, such as glycogen, lipid and protein synthesis, to restore energy balance [6;7]. In addition, AMPK also regulates both cell growth and proliferation and cell cycling in several cell types [8-10].

Recent studies from Brenner's and Marra's groups have highlighted that the activation of AMPK by high molecular weight adiponectin, aminoimidazole carboxamide ribonucleotide (AICAR), metformin or adenovirus-mediated expression of a constitutively active form of AMPK inhibited proliferation of human immortalized HSC cell line and myofibroblasts derived from primary human or rat HSCs in response to platelet-derived growth factor (PDGF) [11;12]. By contrast, AMPK activation had little impact on the production of extracellular matrix components such as collagen I- α 1 [11]. Those studies thus suggest that activation of AMPK could represent a mechanism to inhibit fibrosis progression, although *in vivo* data to support this are currently lacking.

The aims of this study are to evaluate AMPK activity during trans-differentiation of primary mouse hepatic stellate cells *in vitro*, to determine the impact of AMPK activation or deficiency in this phenomenon and finally to evaluate hepatic fibrosis *in vivo* in mice lacking AMPK α 1.

Da Silva Morais et al.

Materials and methods

Animals studies

Female AMPK α 1 null-mice (AMPK α 1^{-/-}) or their littermates (AMPK α 1^{+/+}) [13] were used for in vitro and in vivo studies. They have a SV19/C57Bl6 original mixed background and have been backcrossed into C57Bl6. Balb/C (Wt) mice were used for in vitro studies as reference strain for the study of primary HSC. All animals were kept in a light cycle, temperature and humidity-controlled environment and had *ad libitum* access to water and diet. Animals were handled according to the guidelines for humane care for laboratory animals in accordance with EU Regulations and the study protocol was approved by the local ethics committee.

For in vitro studies, primary HSCs were isolated as previously described [14]. Primary HSC from BalbC mice were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere with 5% CO₂ and 95% air for 24 hours [14]. Classically, compared with BalbC mice, isolation of HSC from C57Bl6 mice yields less cells and their initial adherence on plastic is poorer. To compensate for poor initial adherence, AMPK^{+/+} and AMPK^{-/-} primary HSC were incubated in DMEM supplemented with 20% FBS. After 24 hours, the medium was removed and replaced by DMEM supplemented with 5% FBS. The purity of cultures was evaluated by examining the characteristic stellate shape of the cells with phase-contrast microscopy and the presence of lipid droplets by auto-fluorescence using an excitation wavelength of 320 nm. After the first replacement of medium (day 1), purity was above 90%. Medium was renewed every 2 days. To activate AMPK, HSCs were exposed to AICAR 250 μ M (BIOMOL International, USA) for 48 hours between day 3 and day 5. At this dose, AICAR consistently activates AMPK and does not cause cell toxicity (not shown).

For in vivo studies, 10 weeks-old AMPK α 1^{-/-} and ^{+/+} mice were injected CCl₄ (400 μ L/kg body weight, intraperitoneally) or vehicle three times a week for 1 week or 4 weeks (n=5 per group). At the time of sacrifice (48 h after the last injection of CCl₄), blood was collected by cardiac puncture and the liver and abdominal subcutaneous fat rapidly excised. Part of the liver was fixed in 4% formalin and embedded in paraffin; the remaining liver and the subcutaneous adipose tissue were snap frozen in liquid nitrogen and kept at -80°C.

Histology and immunohistochemistry.

Liver sections were stained with hematoxylin/eosin or sirius red (to visualize collagen deposition) using standard techniques.

Detection of α -smooth muscle actin (α -SMA) was performed on liver sections and on HSC. The latter were seeded on plastic Thermanox™ Coverslips (Nunc, USA) and after determined time and culture conditions, and fixed by immersion in formaldehyde 4% at 4°C for 5 min. After inhibition of endogenous peroxidases with 0.1% H₂O₂ in methanol, and blocking in 10% Normal Goat Serum for 20 min, slides and coverslips were incubated with mouse monoclonal anti α -SMA antibody (1:300) (Clone 1A4, Dako, Belgium) overnight at 4°C. A peroxidase-conjugated secondary antibody was then applied for 1 hour at room temperature and peroxidase activity revealed with 3-amino-9-ethylcarbazole (DakoCytomation). Slides were then counter stained in haematoxylin-eosin and examined under the microscope [14].

Biochemical assays.

Hydroxyproline content was determined on liver samples hydrolyzed in 5mL 6M HCl at 110 degrees for 18-24 hours as previously reported [15] using hydroxyproline 40 μ g/ml (Sigma Aldrich, Belgium) as a standard.

Da Silva Morais et al.

HSC proliferation

Murin primary HSC were cultured in 96-well plates at a density of 2000 cells per well. At day 3, HSCs were exposed or not to AICAR 250 μ M for 48 hours. At day 4 (i.e. 24 hours after initiation of this treatment), Bromo-deoxyuridin (BrdU) was added to all the culture wells. HSC proliferation was evaluated at day 5 by BrdU incorporation using the Cell Proliferation ELISA (Roche Diagnostics, Belgium).

Total RNA extraction, Reverse Transcription and Quantitative PCR

Total RNA was extracted from cultured HSCs or liver wedges using the TRIPure Isolation Reagent (Roche Diagnostics, Belgium). cDNA was obtained by reverse transcription using random primers and M-MLV reverse transcriptase (Gibco BRL, Belgium). mRNA expression levels were quantitated by realtime PCR (RT-qPCR) as previously described [14]. Primer pairs for transcripts of interest were designed using the Primer Express design software (Applied Biosystems) and listed in Table 1. RPL19 RNA was chosen as an invariant standard. Results were expressed as fold expression relative to expression in the control group (value set at 1) using the delta Ct method [16].

Preparation of cell lysates, Western Blot analyses and AMPK activities

Lysates for western blot analyses and AMPK activity were obtained from cells or liver wedges as described [17]. Proteins were separated by SDS-PAGE and transferred onto PVDF membrane (PolyScreen, NEN Life Science Products, USA). The membranes were then exposed to primary antibodies: AMPK α 1 or AMPK α 2 (generous gift from G. Hardie, Dundee, Scotland), phospho-(Thr172)-AMPK α , p70S6K, phospho-(Thr389)-p70S6K (Cell Signaling, USA) and Heat Shock Protein 90 (HSP-90) (BD Transduction Laboratories, USA), then to the appropriate secondary antibody and revealed by chemiluminescence. The quantification of immune-reactive proteins was obtained by densitometry using the Gel DocTM XR System 170-8170 device and software (Bio-Rad, USA). Intensity of HSP-90 band was used as a loading control.

Total AMPK activity was assayed after precipitation with 10% (w/v) poly(ethylene glycol) 6000 (Merck, Belgium) as described [18]. To measure AMPK α 1 and α 2 activities, 100 μ g protein were immunoprecipitated with protein-G-sepharose and isoform specific antibodies to the α 1 or α 2 catalytic subunits of AMPK (kindly provided by G. Hardie, Dundee, Scotland) for 1h at 4°C and activities measured as described [19].

Statistical analysis

Results are expressed as mean $\pm$ standard deviation (SD). Statistical differences between groups were tested using one way analysis of variance (ANOVA). Statistical significance was assumed for p values <0.05.

Da Silva Morais et al.

Results

AMPK expression and activity in cultured HSCs

Three days after plating on plastic, murine primary HSC expressed AMPK α 1 but little of the AMPK α 2 catalytic subunit and mainly in their non-phosphorylated (inactive) form (Figure 1A). After 7 days in culture, there was a significant increase in AMPK α 1 and α 2 protein expression, with AMPK α 1 being higher than α 2 (Figure 1A). During culture activation of the cells, AMPK phosphorylation and the activity of both catalytic subunits also increased (Figure 1 A and B).

Gene expression for the catalytic (α) and regulatory (β and γ) subunit of the AMPK heterotrimeric complex [5] has been evaluated during time in culture. As depicted in Figure 1, AMPK γ 3 mRNA increased significantly with time in culture, while AMPK α 1/2, β 1/2, γ 1/2 mRNA did not vary significantly (Figure 1C), implying that increased AMPK protein and activity does not rely on increased transcription.

Impact of AMPK activation on HSC trans-differentiation

To evaluate the impact of AMPK activation on HSC trans-differentiation, primary HSCs were incubated with AICAR (250 μ M) or vehicle for 48 hours between day 3 and day 5. AICAR treatment significantly increased total AMPK activity (Figure 2A) and Thr172-phosphorylation of AMPK (Figure 2B).

AMPK participates to the inhibition of protein synthesis and cell proliferation [20;21]. Activated AMPK phosphorylates and inactivates mammalian Target of Rapamycin (mTOR), thereby reducing phosphorylation of p70S6K and consequently protein synthesis [20]. Activation of AMPK in HSC by AICAR abrogated p70S6Kinase (p70S6K) phosphorylation (Figure 2B) and inhibited HSC proliferation as assessed by cell counting and BrdU incorporation (Figures 2 E and F). It is of note that both AMPK and p70S6K protein levels are increased in activated HSC compared to quiescent HSC and reduced upon AICAR treatment (Figure 2B). Also, the physiological up-regulations of collagen- α I mRNA and α -smooth muscle actin (α -SMA) expression that occur during culture activation of HSCs are profoundly repressed upon exposure to AICAR (Figures 2C and D).

Taken together, those data demonstrate that activation of primary HSC is associated with enhanced AMPK activity and that additional pharmacological stimulation of AMPK during this process represses protein expression (including those of the AMPK signalling pathway) and inhibits fibrogenesis and HSC proliferation.

Effect of AMPK α 1 deletion on HSC activation

HSCs isolated from AMPK α 1^{-/-} mice that only express the AMPK α 2 catalytic subunit were used to test the hypothesis that decreased AMPK activity will enhance in vitro fibrogenesis.

As observed in Figure 3A and B, collagen- α 1 and α -SMA mRNA expressions increased similarly during culture-activation of AMPK α 1^{-/-} and ^{+/+} HSCs. By contrast, cell proliferation was profoundly impaired in primary AMPK α 1^{-/-} HSC as assessed by the lack of cell expansion and lack of increase in BrdU incorporation between day 3 and day 5, compared to AMPK α 1^{+/+} cells (Figures 3C & D). After passage, the proliferation of myofibroblastic AMPK α 1^{+/+} and AMPK α 1^{-/-} cells was not different.

Thus upon plastic activation of HSC lacking AMPK α 1, cell proliferation is delayed but the induction of profibrotic genes is not altered.

Lack of AMPK α 1 catalytic subunit did not alter hepatic fibrosis in vivo

To evaluate the in vivo consequences of low AMPK activity on hepatic fibrogenesis, we compared CCl₄-induced liver fibrosis in AMPK α 1^{-/-} and ^{+/+} mice. Thr172 phosphorylation of

AMPK and murin HSC 6

Da Silva Morais et al.

AMPK was similar in CTL and CCl₄-treated AMPK α 1^{+/+} livers (Figure 4A); CCl₄ tended to increase total AMPK activity and to decrease AMPK α 2 activity, but those changes did not reach statistical significance (Figure 4B and C). CCl₄ administered for 4 weeks induced hepatic fibrosis as shown by the formation of collagen bridges between central veins, visualized by Sirius Red staining, and the increased number of α -SMA positive cells, corresponding to activated HSCs (Figures 5A and C). In AMPK α 1^{-/-} mice, hepatic AMPK activity is 75% lower than in AMPK α 1^{+/+} due to the abolition of AMPK α 1 expression and activity (Figure 4A-B). The deletion of the AMPK α 1 catalytic subunit did not significantly alter the development of liver fibrosis in these mice as shown by similar collagen matrix deposition and α -SMA positive cells whether early the fibrogenic process (after 1 week challenge with CCl₄- Supplementary Figure 1) or in a later stage of fibrosis development (after a 4week challenge with CCl₄ - Figures 5B and D). In keeping with the histological findings, hydroxyproline content was as high in AMPK α 1^{-/-} and AMPK α 1^{+/+} livers after 4 weeks CCl₄ (Figure 5E).

Fibrosis in the CCl₄ model is classically accompanied by significant increased in collagen- α 1, α -SMA, transforming growth factor beta (TGF β)-1 [22], associated with HSCs activation, as well as monocyte chemoattractant protein-1 (MCP-1) [23] and intracellular adhesion molecule-1 (ICAM-1) [24] expressions, reflecting inflammatory cell recruitment during the wound healing process. As expected, this was the case in CCl₄-treated AMPK α 1^{+/+} mice (Figure 6, Supplementary Figure 1). The expression of these genes was not altered in mice lacking AMPK α 1 (Figure 6, Supplementary Figure 1), confirming that life-long low AMPK activity does not perturb the inflammatory components and the kinetics of fibrogenesis during the wound healing response.

Da Silva Morais et al.

Discussion

HSC activation is a key initial event for hepatic fibrogenesis. This process, characterized by cell proliferation and important morphological and phenotypical alterations, is energetically demanding [9]. At a cellular level, AMPK gauges energy status, and accordingly operates a coordinated switch between ATP-consuming and energy producing pathways [5;7]. It also controls cell cycle progression and protein synthesis in close adequacy with the availability of energy resources. Our results show that culture-activation of primary mouse HSC, mimicking the activation process occurring *in vivo* during fibrogenesis, is accompanied by an increased AMPK activity, mainly sustained by AMPK α 1. Increased AMPK protein and kinase activities are not dependent on enhanced gene expression. Rather, up-regulation of γ 3 subunit may induce stabilisation of the AMPK complex. As AMPK γ 3 is an isoform preponderantly seen in muscle cells [25], its up-regulation may be part of the molecular changes associated with myofibroblastic transformation of HSCs.

AMPK operates an inhibitory control on protein synthesis and cell growth partly through the inhibition of the mTOR pathway [26;27] in conditions of energy shortage. AMPK can inactivate mTOR serine/threonine kinase by 2 means: direct inhibitory phosphorylation of mTOR/Raptor and phosphorylation and activation of TSC2. Inhibition of mTOR results in a decrease phosphorylation of the p70 Ribosomal S6 Kinase (p70S6K) [20;28]. During culture activation of HSCs, increased AMPK activity was however associated with increased phosphorylation of p70S6K and cell proliferation. By contrast, decreased phosphorylation of p70S6K and inhibition of cell proliferation occurred when AMPK was further activated by AICAR. The explanation for this remains elusive and remains to be addressed experimentally. Inhibition of mTOR/p70S6K may only occur above a certain threshold of AMPK activity, reached upon AICAR stimulation but not during spontaneous activation of HSC. Depending on energy availability and on the stimulus for AMPK activation, preferential or selective downstream pathways may be activated and cellular response would be different whether AMPK is activated in response to AICAR, to a change in AMP/ATP ratio, or by an up-stream kinase CaMKK, LKB-1 or cytokine-dependent activation of TAK-1 [29]. AMPK activators suppress proliferation in vascular smooth muscle cells [8;30] and cancer cells [31] and protect against the development of tumours also when mTOR is activated via reduced expression of PTEN and LKB1 [32]. This suggested that AMPK regains the control over the mTOR pathway when it is activated at "supra-physiological" levels. It has recently been shown that AMPK activation, by adiponectin, AICAR, metformin or constitutively active AMPK, inhibits and negatively modulates the activated phenotype and proliferation of both human and rat HSC-derived myofibroblasts [11;12]. Similarly, activation of AMPK induced by treatment with AICAR also inhibits cell proliferation, α -SMA expression and collagen- α 1 production during plastic activation of primary HSC. This is associated with the inhibition of p70S6K protein synthesis pathway. AMPK activation, such as obtained by adiponectin or by a constitutively active AMPK α 2 isoform, has also been shown to inhibit TGF- β -SMAD3 signalling [33]. Inhibition of this potent fibrogenic signal for HSC [22;34] represents thus a potentially additive mechanisms of repression of protein synthesis and cell proliferation upon AMPK stimulation. Altogether, these results support the concept that drugs activating AMPK, which have a proven beneficial action on liver glucose and lipid metabolism, also have *in vitro* anti-fibrotic properties.

To test whether inhibition of or low AMPK activity would enhance fibrogenesis, we used AMPK α 1^{-/-} mice, in which AMPK α 1 is absent and residual AMPK activity only supported by the AMPK α 2 subunit. *In vitro*, we showed that HSC lacking AMPK α 1 retain their trans-differentiation and fibrogenic capacities although early proliferation is delayed. Moreover, we showed that the fibrotic response at two time points during repeated injections of CCl₄ is not altered in AMPK α 1^{-/-} mice *in vivo*. Deposition of scar tissue and increased number of α -SMA

AMPK and murin HSC 8

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Da Silva Morais et al.

positive myofibroblastic cell occur to a similar extent in AMPK α 1^{+/+} and AMPK α 1^{-/-} mice and up-regulation of pro-fibrotic cytokines and chemotatic factors is comparable whether early (1week) or later (4weeks) in the fibrogenic process.

Thus, while AMPK stimulation inhibits HSC proliferation and fibrogenesis, the lack of AMPK α 1 and low AMPK activity do not alter the fibrogenic and wound-healing response to chronic liver injury. AMPK α 1 mice may have adapted to chronic loss of AMPK, residual AMPK activity, supported by AMPK α 2, may be sufficient to compensate for the loss of the main catalytic subunit, or cytokines, chemokines and other pro-fibrotic factors in the wound-healing environment may overcome AMPK deficiency to ensure normal fibrotic response. These possibilities need to be addressed experimentally. Alternatively, AMPK is not required for fibrogenesis, but its activation over a threshold level operates a break on the fibrogenic process. This remains however to be demonstrated in vivo to validate AMPK as a potential target for antifibrotic therapy.

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Da Silva Morais et al.

Acknowledgments

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Accepted Manuscript

AMPK and murin HSC 10

Da Silva Morais et al.

Legends to the Figures

Figure 1: Increased AMPK activity and protein expression during HSC trans-differentiation

(A) Representative western blot for total AMPK, phospho(Thr172)-AMPK (p-AMPK), AMPK α 1 and α 2 catalytic subunits and HSP-90 in quiescent (3 days in culture) and activated (7 days in culture) primary HSC isolated from Balb/C mice. Bar graphs representing total AMPK/HSP-90 and p-AMPK/total AMPK ratio, determined by densitometric analysis (mean $\pm$ SD; n=5 per group). (B) Hepatic AMPK α 1 and AMPK α 2 activities. (C) Bar graph representing the mRNA expression of AMPK subunits quantitated by RT-qPCR. Data are normalised to the expression of RPL19 mRNA regarded as an invariant control and expressed as fold induction (mean $\pm$ SD on n=12 culture dishes from 3 different cell preparations) compared to freshly isolated HSC.. ** p<0.05 and *** p<0.001 compared to day 3 HSC.

Figure 2: Activation of AMPK by AICAR inhibits fibrogenesis and proliferation of primary HSC.

Bar graphs represent (A) total AMPK activity, (C) collagen- α 1 and (D) α -SMA mRNA expressions, quantitated by RT-qPCR, and (E) cell count, (F) BrdU incorporation assessing cell proliferation and (B) representative western blot for total AMPK, phosphor(Thr172)-AMPK (p-AMPK), p70S6K, phosphor(Thr389)-p70S6K (p-p70S6K) (with densitometric analysis in bar graphs), measured in primary HSC isolated from BalbCmice exposed or not to AICAR 250 μ M between day 3 and day 5. Results are means $\pm$ SD on n=10 culture dishes from 3 different cell preparations. ** p<0.01 and *** p<0.001 compared to CTL, ## p<0.01 and ### p<0.001 compared to AICAR. (B) Data normalized to HSP-90 for western blot are expressed as mean $\pm$ SD (n=5 per group). ** p<0.01 and *** p<0.001 compared to HSC day 3 and ## p<0.01 and ### p<0.001 compared to HSC day 5 in CTL conditions.

Figure 3: Impact of AMPK α 1 deletion on HSC trans-differentiation

Cultures of primary HSC isolated from AMPK α 1^{+/+} and AMPK α 1^{-/-} mice analysed after 3 days, 5 days or after passage 1 or 2. mRNA levels of Collagen- α 1 (A) and α -SMA (B) normalised to the expression of RPL19 mRNA regarded as an invariant control and expressed as fold induction (mean $\pm$ SD on n=10 culture dishes from 3 different cell preparations) compared to freshly isolated HSC; cell proliferation assessed by BrdU incorporation (C), representative photomicrographs of α -SMA immunostained HSCs fixed at day 3 and day 5 after isolation (D) *** p<0.001 compared to AMPK α 1^{+/+} HSC cultured for 3 days, ### p<0.001 in AMPK α 1^{+/+} versus AMPK α 1^{-/-} HSC, \$\$\$ p<0.001 compared to AMPK α 1^{-/-} cultured for 3 days.

Figure 4: Impact of repeated CCl₄ on total AMPK and AMPK α 2 activities

AMPK α 1^{+/+} and ^{-/-} mice were treated with corn oil (CTL) or CCl₄ (400 μ l/Kg body weight) 3 X/wk for 4 weeks). (A) Representative western blot for total AMPK, phospho(Thr172)-AMPK (p-AMPK), AMPK α 1 and α 2 catalytic subunits and HSP-90 and bar graphs representing (B) total AMPK and (C) AMPK α 2 activities. Results are expressed as mean $\pm$ SD on n=5 per group. *** p<0.001 versus CTL AMPK α 1^{+/+} livers

Figure 5: AMPK α 1 deficiency does not alter the severity of CCl₄- induced hepatic fibrosis

AMPK and murin HSC 11

Da Silva Morais et al.

AMPK α 1^{+/+} and ^{-/-} mice were treated with corn oil (CTL) or CCl₄ (400 μ l/Kg body weight; 3 X/wk for 4 weeks). Representative photomicrographs of (A and B) Sirius red (original magnification X10) and (C and D) α -SMA immuno-stained liver sections (original magnification X20). Bar graph representing quantification of hepatic hydroxyproline (E). Transcript expression of collagen- α 1, α -SMA, TGF β -1, ICAM-1 and MCP-1 mRNA (F) in AMPK α 1^{+/+} and ^{-/-} mice quantitated by RT-qPCR normalised to the expression of RPL19 mRNA regarded as an invariant control and expressed as fold induction compared to controls (vehicle-treated animals) of the same genotype. Results, expressed as mean $\pm$ SD on n=5 per group. ** p<0.05, *** p<0.001 in CCl₄ vs CTL.

Supplementary Figure 1:

AMPK α 1^{-/-} develop similar fibrotic response than wild type mice in response to CCl₄ injections for 1 week.

AMPK α 1^{+/+} and ^{-/-} mice were treated with corn oil or CCl₄ (400 μ l/Kg body weight; 3 X/wk for 1 week). Representative photomicrographs of Sirius red and α -SMA immuno-stained liver sections (A). Bar graph representing expression of collagen- α 1, α -SMA, mRNA in AMPK α 1^{+/+} and ^{-/-} mice quantitated by RT-qPCR normalised to the expression of RPL19 mRNA regarded as an invariant control and expressed as fold induction compared to controls (vehicle-treated animals) of the same genotype. Results, expressed as mean $\pm$ SD on n=5 per group. ** p<0.05, *** p<0.001 in CCl₄ vs CTL.

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Da Silva Morais et al.

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AMPK and murin HSC 13

Da Silva Morais et al.

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Da Silva Morais et al.

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Da Silva Morais et al.

Table 1

Sequences of Primers

Gene	Primer Sequences	GeneBank accession #
α -SMA	Forward: 5'- TCCTGACGCTGAAGTATCCGATA-3' Reverse: 5'- GGTGCCAGATCTTTTCCATGTC-3'	NM_007392
AMPK α 1	Forward: 5'- TCGGCACCTTCGGGAAA-3' Reverse: 5'- GTTGAGTATCTTCACAGCCACTTTATGT-3'	NM_001013367
AMPK α 2	Forward: 5'- CGCCTCTCATCGCAGACA-3' Reverse: 5'- CTTGGGCTTCGTTGTGTTGA-3'	NM_178143
AMPK β 1	Forward: 5'- AGGACACGGGCATCTCTTGT-3' Reverse: 5'- GTGGTTCAGCATGACGTGGTT-3'	NM_031869
AMPK β 2	Forward: 5'- TGTTATGCTGAACCATCTCTATGCA-3' Reverse: 5'- GCGTGGTGACATACTTCTTCTTGT-3'	NM_182997
AMPK γ 1	Forward: 5'- TGCCATGGTCCGTACTACCA-3' Reverse: 5'- CGGAGACTCGGTGCTGTACA-3'	NM_016781
AMPK γ 2	Forward: 5'- AACGTACAATAACTTGGACATCACAGT-3' Reverse: 5'- GCACTTCACCACACCCCTCAA-3'	NM_145401
AMPK γ 3	Forward: 5'- CACGGGAACAGGTGCATAGG-3' Reverse: 5'- GGAGACCACGCCAGAAAGA-3'	NM_153744
Col- α I	Forward: 5'- TTCACCTACAGCACGCTTGT-3' Reverse: 5'- TCATCGAATACAAAACCAAGA-3'	NM_007742
ICAM-1	Forward: 5'- CCGCAGGTCCAATTACACT-3' Reverse: 5'- CAGAGCGGCAGAGCAAAAG-3'	NM_010493
MCP-1	Forward: 5'- CCACTCACCTGCTGCTACTCAT-3' Reverse: 5'- CTGCTGGTGATCCTCTTGT-3'	NM_011333
RPL19	Forward: 5'- GAAGGTCAAAGGGAATGTGTTCA-3' Reverse: 5'- ACAAGCTGAAGGCAGACAAGG-3'	NM_009078
TGF- β 1	Forward: 5'- CCTGCAAGACCATCGACATG-3' Reverse: 5'- GAGCCTTAGTTTGGACAGGATCTG-3'	NM_011577

α -SMA: alpha smooth muscle actin; AMPK: AMPK-activated protein kinase; Col- α I: Collagen alpha 1; ICAM-1: Inter-Cellular Adhesion Molecule 1; MCP-1: Monocyte chemotactic protein-1; RPL19: ribosomal protein L19; TGF- β 1: Transforming Growth Factor beta-1

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5