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RETRACTION

Retraction: Ethanol sensitizes mitochondria to the permeability transition by inhibiting deacetylation of cyclophilin-D mediated by sirtuin-3

Nataly Shulga and John G. Pastorino

Retraction of: *J. Cell Sci.* **123**, 4117-4127

This article has been retracted at the request of the corresponding author, John G. Pastorino.

This notice updates and replaces a recent Expression of Concern, published on 15 February 2016.

Journal of Cell Science was alerted to potential blot duplication and reuse in the following five papers published in Journal of Cell Science by John G. Pastorino:

Sirtuin-3 deacetylation of cyclophilin D induces dissociation of hexokinase II from the mitochondria
Nataly Shulga, Robin Wilson-Smith and John G. Pastorino
J. Cell. Sci. (2010) **123**, 894-902

Ethanol sensitizes mitochondria to the permeability transition by inhibiting deacetylation of cyclophilin-D mediated by sirtuin-3
Nataly Shulga and John G. Pastorino
J. Cell. Sci. (2010) **123**, 4117-4127

GRIM-19-mediated translocation of STAT3 to mitochondria is necessary for TNF-induced necroptosis
Nataly Shulga and John G. Pastorino
J. Cell. Sci. (2012) **125**, 2995-3003

Sirtuin-3 modulates Bak- and Bax-dependent apoptosis
Manish Verma, Nataly Shulga and John G. Pastorino
J. Cell. Sci. (2013) **126**, 274-288

Mitoneet mediates TNFα-induced necroptosis promoted by exposure to fructose and ethanol
Nataly Shulga and John G. Pastorino
J. Cell. Sci. (2014) **127**, 896-907

These concerns were relayed to Dr Pastorino, the corresponding author, who responded with an explanation and original data. Following review of these data, we felt unable to resolve this matter at a distance, so contacted the authors' institution (Rowan University) and requested that they investigate further.

Following their assessment, Rowan University required that Dr Pastorino retract all of the above named papers published in Journal of Cell Science. Dr Pastorino also entered a Voluntary Exclusion Agreement with The Office of Research Integrity (ORI); the agreement can be found here: http://ori.hhs.gov/content/case-summary-pastorino-john-g.

ORI found that Dr Pastorino intentionally falsified and/or fabricated data and, specifically, that he "duplicated images, or trimmed and/or manipulated blot images from unrelated sources to obscure their origin, and relabelled them to represent different experimental results in:"

- Figures 2A,C; 3B; 5A; 7B; 8A in *J. Cell. Sci.* (2010a), **123**, 894-902.
- Figures 2B; 5A; 6A,B in *J. Cell. Sci.* (2010b), **123**, 4117-4127.
- Figures 1A; 2A,B; 4C; 5A,B; 6A; 7A–C in *J. Cell. Sci.* (2012) **125**, 2995-3003.
- Figures 4F; 5H; 6A in *J. Cell. Sci.* (2013) **126**, 274-288.
- Figures 1B; 2B,C; 3A,B; 4D in *J. Cell. Sci.*(2014) **127**, 896-907.

Ethanol sensitizes mitochondria to the permeability transition by inhibiting deacetylation of cyclophilin-D mediated by sirtuin-3

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Summary

Ethanol increases the vulnerability of mitochondria to induction of the mitochondrial permeability transition (MPT). Cyclophilin-D activity enhances the potential for the permeability transition pore (PTP) to open. In the present study, we demonstrate that ethanol and its metabolism sensitize the PTP to opening, in part by increasing the acetylation and activity of cyclophilin-D. This effect of ethanol is mediated by inhibiting the activity of sirtuin-3, an NAD^+ dependent deacetylase that is localized to the mitochondrial matrix. The ethanol-enhanced acetylation of cyclophilin-D also increases the interaction of cyclophilin-D with the adenine nucleotide translocator-1 (ANT-1) and is dependent on ethanol metabolism. Moreover, activation of AMPK, a known positive modulator of sirtuin activity, prevented the ethanol-induced suppression of sirtuin-3 activity and the attendant increase of cyclophilin-D acetylation, activity and association with ANT-1. Additionally, AMPK reactivation of sirtuin-3 prevented the sensitization to the MPT and the enhancement of cell killing by TNF in cells exposed to ethanol.

Key words: Sirtuin-3, Ethanol, Mitochondria, Cyclophilin-D

Introduction

Sirtuins are NAD^+ dependent histone and/or protein deacetylases that have been implicated in a number of cellular processes including control of gene expression, longevity and metabolic regulation (Saunders and Verdin, 2007; Schwer and Verdin, 2008). Sirtuin-1 activity is enhanced by increases in NAD^+ levels that occur during caloric restriction. It is believed that ethanol metabolism brings about a decrease in the NAD^+/NADH ratio due to the activity of alcohol dehydrogenase, potentially resulting in the inhibition of sirtuin activities. Indeed, is has been demonstrated that ethanol exposure inhibits the activity of sirtuin-1, leading to an increase in the acetylation and consequent stimulation of sterol regulatory element binding protein (SREBP-1c) (You et al., 2008a; You et al., 2008b). The phytoalexin resveratrol, which activates sirtuin-1, alleviates the onset of alcoholic fatty liver in mice fed an ethanol-containing diet (Ajmo et al., 2008; Hou et al., 2008). Additionally, activation of AMPK (AMPK-AMP-dependent protein kinase) by 5-aminoimidazole-4-carboxamide (AICAR), countered the increase in SREBP-1c activity stimulated by ethanol exposure (You et al., 2004). This action of AMPK might be mediated through stimulation of sirtuin-1, because AMPK activation partially enhances sirtuin-1 activity by increasing cellular NAD^+ levels (Ajmo et al., 2008; Yang, H. et al., 2007a).

There are seven know sirtuins. Like cyclophilin-D, sirtuin-3 is localized to the mitochondrial matrix and is known to deacetylate proteins involved in metabolic pathways, such as the acetyl CoA synthetase 2 pathway (Ahn et al., 2008; Cooper and Spelbrink, 2008; Hallows et al., 2008; Shi et al., 2005). The present study demonstrates that ethanol exposure decreases the activity of sirtuin-3. In turn, the decline of sirtuin-3 activity is accompanied by an increase in the acetylation and activity of cyclophilin-D, thereby lowering the threshold for opening of the permeability transition pore (PTP). Moreover, the effects of ethanol on cyclophilin-D are prevented by activation of AMPK, which reactivates sirtuin-3 in ethanol-exposed cells and blunts the stimulation of cyclophilin-D activity provoked by ethanol exposure. Additionally, AMPK activation prevents the ethanol-induced sensitization to onset of the PTP and potentiation of tumor necrosis factor (TNF)-induced cytotoxicity through a sirtuin-3 dependent pathway.

Results

Ethanol increases the activity of cyclophilin-D and sensitizes mitochondria to onset of the permeability transition

H4IIEC3 cells were exposed to 25 mM of ethanol for 24 and 48 hours. Mitochondria were then isolated and cyclophilin-D peptidyl-prolyl cis-trans isomerase activity was determined. As shown in Fig. 1A (left graph), ethanol exposure provoked a 47% increase of cyclophilin-D activity at 24 hours of exposure and a 71% increase in activity at 48 hours. The stimulation of cyclophilin-D activity by ethanol was dependent on ethanol metabolism. Inhibition of ethanol metabolism by 4-methylpyrazole (4-MP), an inhibitor of alcohol dehydrogenase, prevented the ethanol-induced increase of cyclophilin-D activity detected at both 24 and 48 hours (Fig. 1A, left graph).

Mitochondria were isolated and opening of the permeability transition pore measured by a decrease in absorbance. As shown in Fig. 1B, mitochondria isolated from control cells were able to sustain three doses of 50 μM Ca^{2+} before onset of the permeability transition occurred. By contrast, mitochondria isolated from cells exposed to ethanol for 48 hours were sensitized to the permeability transition, with mitochondrial swelling triggered by only one dose of 50 μM Ca^{2+}. Importantly, the sensitization to the mitochondrial permeability transition (MPT) by ethanol exposure was prevented

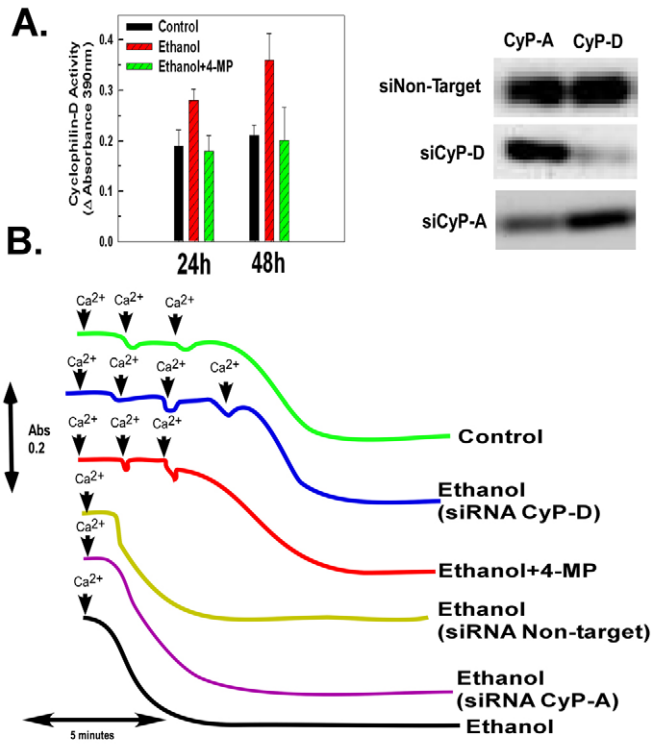

Fig. 1. Ethanol exposure stimulates the peptidyl-prolyl cis-trans isomerase activity of cyclophilin-D and sensitizes the mitochondria to the MPT. (**A**) H4IIEC3 cells were either left untreated or exposed to 25 mM of ethanol in the absence or presence of 5 mM 4-MP. Following 24 or 48 hours of incubation, the cells were harvested and mitochondria isolated. Alternatively, cells were transfected with siRNA targeting CyP-A or CyP-D. The western blot on the left shows mitochondrial extracts that were assayed for cyclophilin-D activity and cyclophilin-D or A expression. The graph on the right shows the quantification of these experiments. Values are the means from triplicate samples, and the error bars indicate standard deviations. $P<0.05$ for control versus ethanol and ethanol versus ethanol+4-MP by one way ANOVA and Scheffe's post-hoc test. (**B**) H4IIEC3 cells were either left untreated or transfected with 50 nM of a non-targeting siRNA or an siRNA targeting sirtuin-3. After 24 hours, cells were either left untreated or exposed to 25 mM ethanol in the absence or presence of 4-MP. After 48 hours the cells were harvested and the mitochondria isolated. Mitochondrial respiration was initiated by the addition of 1 mM malate and 1 mM glutamate. To trigger mitochondrial swelling, 50 μM Ca^{2+} was added at the points indicated. The change in absorbance was measured spectrophotometrically.

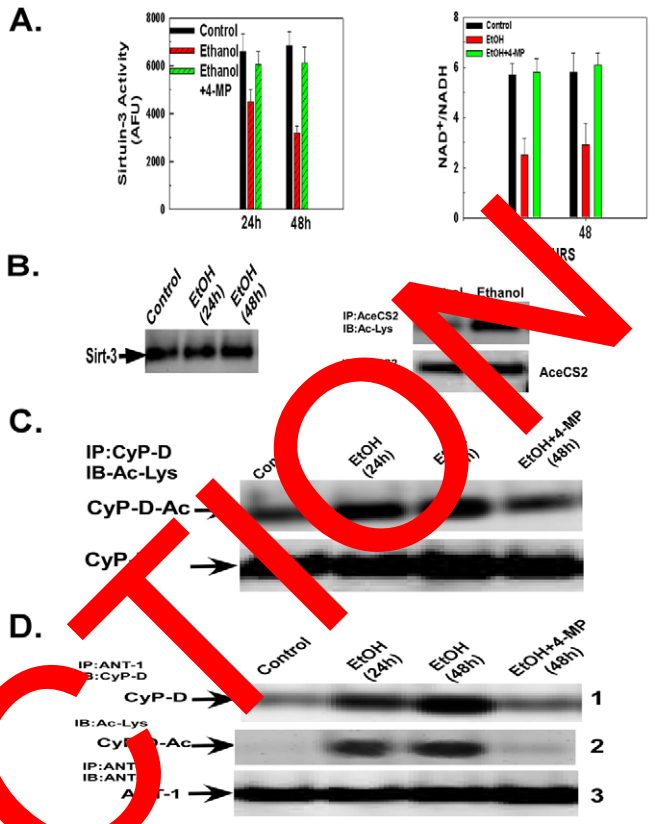

Fig. 2. Ethanol exposure inhibits sirtuin-3 activity and promotes cyclophilin-D acetylation and binding to the adenine nucleotide translocator-1. (**A**) H4IIEC3 cells were left untreated or exposed to 25 mM of ethanol for 24 or 48 hours in the absence or presence of 4-MP. The cells were harvested and the NAD^+:NADH and sirtuin-3 activity was determined in whole-cell and mitochondrial extracts, respectively. The values are the means from triplicate samples, and the error bars indicate standard deviations. $P<0.05$ for control versus ethanol and ethanol versus ethanol+4-MP by one-way ANOVA and Scheffe's post-hoc test. (**B**) H4IIEC3 cells were left untreated or exposed to 25 mM of ethanol for 24 or 48 hours in the absence or presence of 4-MP. The cells were harvested and the mitochondria isolated. Sirtuin-3 expression was determined by western blotting. Acetyl-CoA sythetase 2 (AceCS2) was immunoprecipitated from mitochondrial extracts. The western blots of the immunoprecipitates were probed with antibody against acetylated lysine, stripped and then re-probed with antibody against AceCS2. (**C**) H4IIEC3 cells were left untreated or exposed to 25 mM of ethanol for 24 or 48 hours in the absence or presence of 4-MP. The cells were harvested and the mitochondria isolated. Cyclophilin-D was immunoprecipitated from mitochondrial extracts. The western blots of the immunoprecipitates were probed with antibody against acetylated lysine, stripped and then re-probed with antibody against cyclophilin-D. (**D**) H4IIEC3 cells were either left untreated or exposed to 25 mM of ethanol for 24 or 48 hours. The cells were harvested and mitochondria isolated. ANT-1 was immunoprecipitated from mitochondrial extracts. The western blots of the immunoprecipitates were probed with antibodies against cyclophilin-D or ANT-1. To access cyclophilin-D acetylation, the blots were stripped and then re-probed with antibody against acetylated lysine.

by inhibition of ethanol metabolism with 4-MP (Fig. 1B). The ethanol-induced sensitization to the MPT was also dependent on cyclophilin-D. Cyclophilin-D expression was suppressed in H4IIEC3 cells by RNA interference (RNAi), small interfering RNA (siRNA) targeting cyclophilin-A was used as a control (Fig. 1A, left). The mitochondria of H4IIEC3 cells in which cyclophilin-D expression was suppressed were resistant to the sensitizing effects of ethanol on the MPT (Fig. 1B). These results are in keeping with the ability of cyclophilin-D to enhance the opening of the PTP, but are consistent with development of the MPT occurring even in the absence of cyclophilin-D expression under more stringent conditions (Basso et al., 2005). Importantly, transfection with non-target control siRNA or siRNA targeting cyclophilin-A did not prevent the sensitizing effects of ethanol on opening of the PTP.

Ethanol decreases sirtuin-3 activity, and increases the acetylation and binding of cyclophilin-D to ANT-1

Sirtuin activity is controlled in part by the NAD^+/NADH ratio. Ethanol exposure has been demonstrated to inhibit sirtuin-1 activity in the cytosol (Ajmo et al., 2008; Lieber et al., 2008). As shown in Fig. 2A (left panel), in comparison with control cells, ethanol

exposure caused a 31% decrease in sirtuin-3 activity in isolated mitochondria at 24 hours of exposure and a 53% decrease after 48 hours. Importantly, 4-MP prevented the ethanol-induced inhibition of sirtuin-3 activity, indicating that the effect of ethanol depends on its metabolism. Importantly, in parallel with the suppression of sirtuin-3 activity, ethanol exposure provoked a decrease in the NAD^+:NADH ratio at 24 and 48 hours of exposure, which was prevented by inhibition of ethanol metabolism with 4-MP (Fig. 2A, right graph). Importantly (as shown in Fig. 2B, right panel), the effect of ethanol in decreasing sirtuin-3 activity was not caused by a reduction in sirtuin-3 protein expression. Additionally, ethanol exposure provoked a marked increase in the acetylation level of acetyl-CoA synthetase 2 (AceCS2), a known substrate of sirtuin-3 (Fig. 2B, left).

We next wanted to determine whether the ethanol-induced inhibition of sirtuin-3 activity was accompanied by an elevation of cyclophilin-D acetylation. Cyclophilin-D is basally acetylated in control cells (Fig. 2C, lane 1). However, subsequently the acetylation of cyclophilin-D is elevated in cells exposed to ethanol for 24 and 48 hours (Fig. 2C, lanes 2 and 3). Importantly, similar to the ethanol-induced increase of cyclophilin-D activity, the ethanol-induced acetylation of cyclophilin-D was suppressed by 4-MP (Fig. 2C, lane 4).

Cyclophilin-D has been shown to bind to adenine nucleotide translocator-1 (ANT-1) (Bauer et al., 1999; Crompton et al., 1998; Woodfield et al., 1998). As shown in Fig. 2D, panel 1, ethanol exposure at 24 and 48 hours promoted a progressive increase in the level of cyclophilin-D that was co-immunoprecipitated with ANT-1 (lanes 2 and 3). Moreover, the ethanol-induced increase of cyclophilin-D binding to ANT-1 was prevented by 4-MP (Fig. 2D, panel 1, lane 4). Importantly, cyclophilin-D bound to ANT-1 is acetylated. The blot was stripped and re-probed using antibody against acetylated lysine. As shown in Fig. 2D, panel 2, the cyclophilin-D that is bound to ANT-1 in ethanol-exposed cells is mostly acetylated (lanes 2 and 3), which is prevented by the inhibition of ethanol metabolism with 4-MP (lane 4). These data suggest that the ethanol-induced inhibition of sirtuin-3 results in an enhancement of cyclophilin-D acetylation, resulting in an increase in cyclophilin-D associated with ANT-1.

Suppression of sirtuin-3 expression recapitulates the effect of ethanol on cyclophilin-D acetylation, activity and binding to ANT

We used RNAi to determine whether suppression of sirtuin-3 expression and therefore activity would recapitulate the effects of ethanol on cyclophilin-D. As shown in Fig. 3A, left, panel 1, sirtuin-3 expression was suppressed by siRNA targeting sirtuin-3, which as a non-targeting control siRNA or siRNA against sirtuin-1 had no effect. As shown in Fig. 3A, left, panel 2, lane 1, the non-target siRNA did not increase cyclophilin-D acetylation. Similarly, cyclophilin-D acetylation was not affected by suppression of sirtuin-1 (Fig. 3A, left, panel 2, lane 2). By contrast, suppression of sirtuin-3 expression significantly elevated cyclophilin-D acetylation (Fig. 3A, left, panel 2, lane 3). The non-target siRNA and the siRNA against sirtuin-1 or sirtuin-3 had no effect on cyclophilin-D expression (Fig. 3A, left, panel 3). Moreover, as with ethanol exposure, suppression of sirtuin-3 levels also induced an elevation in cyclophilin-D activity caused by non-target siRNA or siRNA targeting sirtuin-1 (Fig. 3A, right graph).

The stimulation of cyclophilin-D acetylation induced by suppression of sirtuin-3 expression was accompanied by a concomitant increase in the level of cyclophilin-D co-immunoprecipitated with ANT-1. As shown in Fig. 3B, panel 1, non-target siRNA and siRNA against sirtuin-1 did not produce an

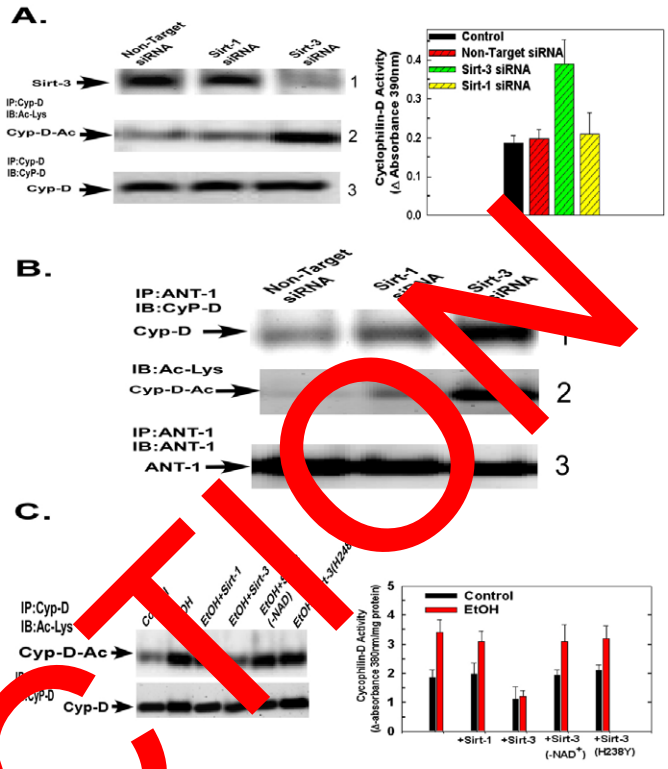

Fig. 3. Suppression of sirtuin-3 expression recapitulates the effects of ethanol exposure on cyclophilin-D acetylation, activity and binding to the ANT-1 complex. H4IIEC3 cells were transfected with 50 nM siRNA targeting sirtuin-1, 3 or a non-targeting control. Following 48 hours of incubation, the cells were harvested and mitochondria isolated. Mitochondrial extracts were separated by SDS-PAGE and then electroblotted onto PVDF membranes. Western blots were probed with antibody against sirtuin-3 (panel 1). Alternatively, cyclophilin-D was immunoprecipitated from mitochondrial extracts. The immunoprecipitates were separated by SDS-PAGE and electroblotted onto PVDF membranes. The western blots were probed with antibody against acetylated lysine, then stripped and re-probed with an antibody against cyclophilin-D (panels 2 and 3). Cyclophilin-D activity was determined fluorescently in mitochondrial extracts. The values are the means of three samples, and error bars indicate standard deviations. $P<0.05$ for non-target siRNA versus sirtuin-3 siRNA. (**B**) H4IIEC3 cells were transfected with 50 nM of siRNA targeting sirtuin-1, 3 or a non-targeting control. Following 48 hours of incubation, cells were harvested and mitochondria isolated. ANT-1 was immunoprecipitated from mitochondrial extracts. The immunoprecipitates were separated by SDS-PAGE and electroblotted onto PVDF membranes. Western blots were then probed with antibodies against cyclophilin-D or ANT-1. To access cyclophilin-D acetylation, the cyclophilin-D blots were stripped and then re-probed with antibody against acetylated lysine. (**C**) (Left) Western blot of H4IIEC3 cells that were either left untreated or exposed to 25 mM of ethanol for 48 hours. Cyclophilin-D was immunoprecipitated from mitochondrial extracts and incubated with recombinant sirtuins. The immunoprecipitates were then run out on SDS-PAGE gels and electroblotted onto PVDF membranes. Blots were then probed with antibody against acetylated lysine, stripped and re-probed with antibody against cyclophilin-D. (Right) Quantification of cyclophilin-D activity. Cyclophilin-D immunoprecipitates that had been incubated with recombinant sirtuins. Cyclophilin-D activity was determined fluorescently as described in Materials and Methods. Values are the means from triplicate samples, and error bars indicate standard deviations. $P<0.05$ for control versus ethanol and ethanol versus ethanol+sirtuin-3 by one-way ANOVA and Scheffe's post-hoc test.

increase in the association of cyclophilin-D with ANT-1. However, suppression of sirtuin-3 resulted in an increase in the amount of cyclophilin-D co-immunoprecipitated with ANT-1 (Fig. 3B, panel 1, lane 3). The western blot was stripped and re-probed with antibody against acetylated lysine. Importantly, cyclophilin-D that is bound to ANT-1 is acetylated (Fig. 3B, panel 2, lane 3).

We next wanted to determine directly whether cyclophilin-D is deacetylated by sirtuin-3. Cyclophilin-D was immunoprecipitated from ethanol treated cells and then incubated with sirtuin-1 or sirtuin-3 in vitro. As shown in Fig. 3C, left panel, lane 3, sirtuin-1 did not cause significant deacetylation of cyclophilin-D. By contrast, incubation with sirtuin-3 caused a marked reduction in the level of acetylated cyclophilin-D to a level similar to that seen in control cells (lane 4). Importantly, sirtuin-3 in the absence of its required cofactor, NAD^+, or enzymatically inactive sirtuin-3(H238Y) – which carried a His238 to Tyr point mutation – were unable to deacetylate cyclophilin-D (Fig. 3C, left, lanes 5 and 6). The deacetylation of cyclophilin-D by sirtuin-3 was paralleled by a decrease of cyclophilin-D activity. Incubation of cyclophilin-D immunoprecipitated from ethanol-exposed cells with sirtuin-3 resulted in a dramatic reduction of cyclophilin-D activity, whereas incubation with sirtuin-1 had little effect (Fig. 3C, right graph). Importantly, incubation with sirtuin-3(H238Y) or sirtuin-3 in the absence of NAD^+ did not decrease cyclophilin-D activity (Fig. 3C, right graph).

Increased cyclophilin-D acetylation and decreased sirtuin-3 activity in mitochondria isolated from ethanol-fed rats and mouse hepatocytes exposed to ethanol

Rats were placed on the Lieber-DeCarli liquid diet in which ethanol constitutes 36% of calories (Pastorino and Hoek, 2008; Pastorino et al., 1999). The control animals were given a similar liquid diet with maltodextrin isocalorically replacing ethanol. As shown in Fig. 4A, left panel, mitochondria isolated from the liver of ethanol-fed rats displayed a marked increase in acetylation of cyclophilin-D compared with control-fed animals. However, as with the H4IIIEC3 cells, sirtuin-3 expression was not elevated (Fig. 4A, right panel). The increased acetylation of cyclophilin-D seen in the mitochondria of ethanol-fed animals was paralleled by an increase of cyclophilin-D activity and decline of sirtuin-3 activity (Fig. 4B).

As shown in Fig. 4C, left panel, mouse hepatocytes that had been exposed to 25 mM of ethanol for 48 hours displayed increased levels of cyclophilin-D acetylation. Similarly, transfection of mouse hepatocytes with siRNA targeting sirtuin-3 resulted in an increase of cyclophilin-D acetylation. The increase of cyclophilin-D acetylation in ethanol-exposed hepatocytes was accompanied by an inhibition of sirtuin-3 activity and stimulation of cyclophilin-D cis/trans isomerase activity that was prevented by 4-MP (Fig. 4C, right panel). Importantly, the suppression of sirtuin-3 activity in mouse hepatocytes exposed to ethanol was accompanied by an increase in sensitivity to induction of the permeability transition. As shown in Fig. 4D, left panel, mitochondria isolated from mouse hepatocytes exposed to ethanol displayed an increased sensitivity to PTP induction that was prevented by suppression of cyclophilin-D. Suppression of sirtuin-3 expression also increased sensitivity to PTP formation and, in ethanol-exposed hepatocytes, did not result in an additive or synergistic effect. The enhanced sensitivity to PTP induction was accompanied by a potentiation of TNF-induced cytotoxicity. As shown in Fig. 4D, right panel, mouse hepatocytes exposed to ethanol exhibited an increase in TNF-induced cytotoxicity that was prevented by suppressing cyclophilin-D. Importantly, like ethanol exposure, suppression of sirtuin-3 expression also promoted TNF-induced cytotoxicity in mouse hepatocytes that was not additive with the effect of ethanol.

AICAR can stimulate AMPK and sirtuin-3 activities in ethanol-exposed cells

AMPK has been shown to activate sirtuin-1 by modulating the NAD^+:NADH ratio (Canto et al., 2009). We wanted to determine whether activation of AMPK can reverse the inhibitory effects of ethanol on sirtuin-3 activity. AMPK activation was monitored by the phosphorylation levels of Thr172. H4IIIEC3 cells were exposed to 25 mM of ethanol for 48 hours followed by treatment with AICAR for an additional 8 hours. As shown in Fig. 5A, left panel, AICAR stimulated AMPK phosphorylation in control or ethanol-exposed cells. The level of phosphorylation of AMPK by AICAR treatment was blunted in ethanol-exposed cells, consistent with previous observations (Liangpunsakul et al., 2009; Liangpunsakul et al., 2008). AICAR reversed the decline in the NAD^+:NADH ratio in cells exposed to ethanol for 48 hours (Fig. 5A, right graph). Additionally, as shown in Fig. 5B, the stimulation of AMPK phosphorylation by AICAR was accompanied by an enhancement of AMPK activity measured over an 8-hour time course. Cells exposed to ethanol for 48 hours displayed a 50% decrease of basal AMPK activity compared with unexposed control cells (0 hour). Treatment of control cells with AICAR resulted in maximal activation of AMPK at 8 hours, when the cells exhibited a 54% increase of AMPK activity over the basal level. Ethanol exposure blunted AICAR stimulation of AMPK. Cells exposed to ethanol for 48 hours and subsequently treated with AICAR exhibited maximal stimulation of AMPK activity at 8 hours when AMPK activity was 120% above the basal level of activity seen in ethanol-exposed cells, but only 23% above the basal level of activity seen in control cells.

The AICAR-induced activation of AMPK was accompanied by a reversal in the decline of sirtuin-3 activity seen in ethanol exposed cells. As shown in Fig. 5C, cells exposed to ethanol for 48 hours exhibited a 52% reduction in basal sirtuin-3 activity compared with control cells (0 hours). Importantly, cells exposed to ethanol for 48 hours and subsequently treated with AICAR displayed sirtuin-3 re-activation, with maximal sirtuin-3 stimulation occurring at 8 hours, when sirtuin-3 activity was 128% above the basal activity seen in cells exposed to ethanol and 18% above the basal level of activity seen in control cells.

AMPK activation in ethanol-exposed cells prevents the increase of cyclophilin-D acetylation, activation and binding to ANT-1

The AICAR-induced stimulation of AMPK activity in ethanol-exposed cells prevented the elevation of cyclophilin-D acetylation. As shown in Fig. 6A, lane 2, exposure of cells to ethanol for 48 hours resulted in a marked acetylation of cyclophilin-D. Treatment of control cells with AICAR for 8 hours slightly reduced the levels of acetylated cyclophilin-D (Fig. 6A, lane 3). By contrast, in cells exposed to ethanol for 48 hours, subsequent treatment with AICAR for 8 hours markedly reversed the ethanol-induced increase of cyclophilin-D acetylation (Fig. 6A, lane 4). The ability of AICAR to prevent the increased acetylation of cyclophilin-D by ethanol exposure was dependent on sirtuin-3. Transfection with siRNA to suppress sirtuin-3 expression prevented AICAR from reversing the stimulation of cyclophilin-D acetylation in ethanol-exposed cells (Fig. 6A, lane 5).

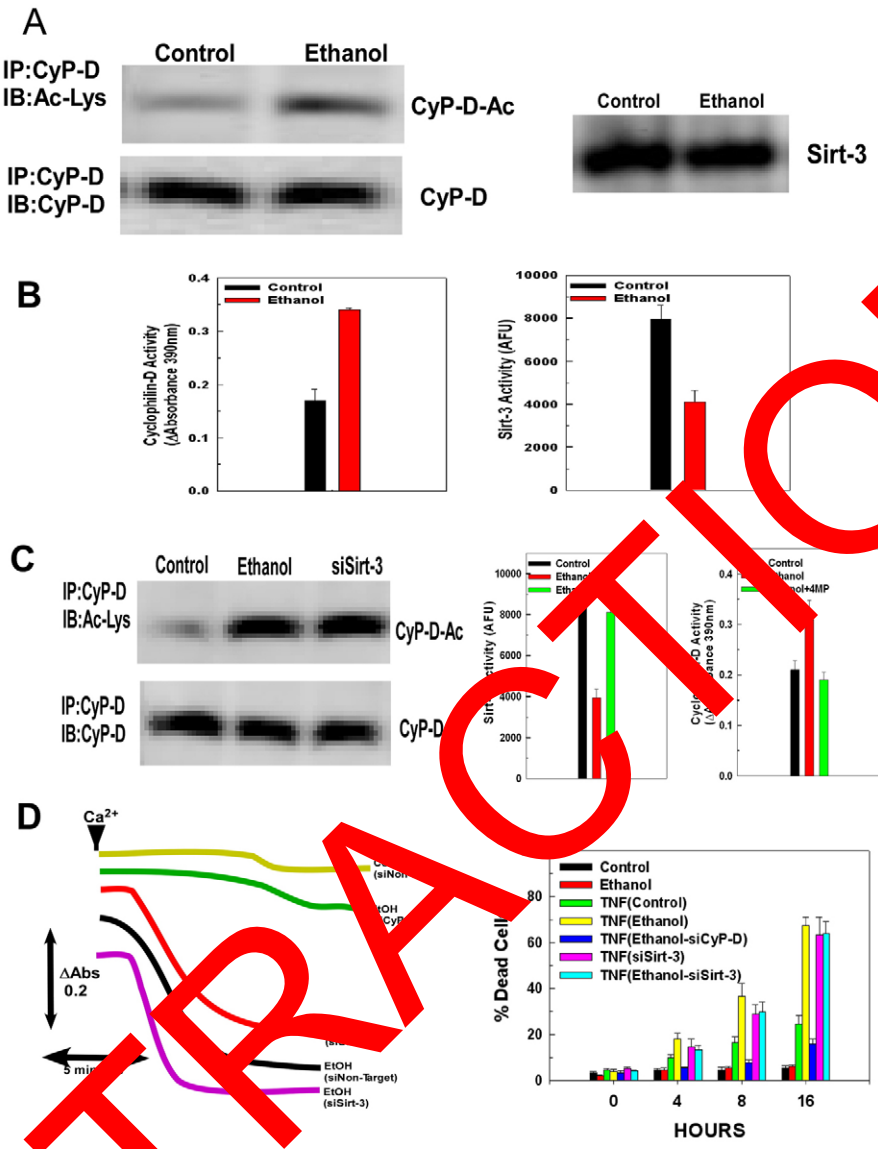

Fig. 4. Increased cyclophilin-D acetylation and decreased sirtuin-3 activity in mitochondria isolated from ethanol-fed rats and mouse hepatocytes exposed to ethanol. (**A**) Western blots of mitochondria that had been isolated from the liver of control or ethanol-fed rats. Cyclophilin-D was immunoprecipitated from mitochondrial extracts. The immunoprecipitates were separated by SDS-PAGE and electroblotted onto PVDF membranes. Blots were probed with antibody against acetylated lysine, then stripped and re-probed with an antibody against cyclophilin-D. Alternatively, mitochondrial extracts were used to determine the expression of sirtuin-3 by using an antibody against sirtuin-3 antibody. (**B**) Quantification of cyclophilin-D and sirtuin-3 activity. Mitochondria that had been isolated from the livers of control or ethanol-fed rats. Cyclophilin-D or sirtuin-3 activity was determined fluorescently in mitochondrial extracts. The values are the means from triplicate samples, and the error bars indicate standard deviations. $P<0.05$ for control versus ethanol. (**C**) (Left) Western blots (left) of mouse hepatocytes that had been left untreated or were transfected with siRNA targeting sirtuin-3 or exposed to ethanol. Following 48 hours, the hepatocytes were harvested and mitochondria isolated. Cyclophilin-D was immunoprecipitated from mitochondrial extracts. The immunoprecipitates were then separated by SDS-PAGE and electroblotted. Blots were then probed with antibody against acetylated lysine, stripped and re-probed with antibody against cyclophilin-D. (Right) Quantification of sirtuin-3 and cyclophilin-D activity. Mouse hepatocytes were untreated or exposed to ethanol for 48 hours in the absence or presence of 4-MP. Sirtuin-3 or cyclophilin-D activity was determined fluorescently in mitochondrial extracts as described in Materials and Methods. The values are the means from triplicate samples, and the error bars indicate standard deviations. $P<0.05$ for control versus ethanol and ethanol versus ethanol+4-MP by one-way ANOVA and Scheffe's post-hoc test. (**D**) (Left) Mouse hepatocytes were transfected with 50 nM of siRNA targeting sirtuin-3, cyclophilin-D or a non-targeting control; 24 hours after transfection, cells were left untreated or exposed to 25 mM of ethanol for 48 hours. The cells were then harvested and mitochondria isolated. Where shown (arrows), 50 μM Ca^{2+} was added. The change in absorbance was measured spectrophotometrically at 540 nm. (Right) At 24 hours after transfection, mouse hepatocytes were left untreated or exposed to 25 mM of ethanol for 48 hours. Cells were then treated with 10 ng/ml of TNF. At the times indicated, the viability of the cells was assessed. Values are the means of three samples, and the error bars indicate standard deviations. $P<0.05$ for TNF(control) versus TNF(ethanol), TNF(ethanol) versus TNF(ethanol-siCyP-D) and TNF(control) versus TNF(siSirt-3) by one-way ANOVA and Scheffe's post-hoc test.

AICAR also prevented the ethanol-induced increase in the association of cyclophilin-D with ANT-1. As shown in Fig. 6B, panel 1, lane 2, ethanol exposure for 48 hours induced an increase in the level of cyclophilin-D that co-immunoprecipitates with ANT-1. AICAR alone slightly alter interaction of cyclophilin-D with ANT-1 in control cells (Fig. 6B, panel 1, lane 3). However, in cells

exposed to ethanol for 48 hours and subsequently treated with AICAR for 8 hours, the enhanced interaction of cyclophilin-D with ANT-1 was reversed (Fig. 6B, panel 1, lane 4). The effect of AICAR was dependent on sirtuin-3, with suppression of sirtuin-3 expression preventing the ability of AICAR to reverse the increase in the interaction of cyclophilin-D with ANT-1 in ethanol-exposed cells (Fig. 6B, panel 1, lane 5). The western blot was stripped and re-probed antibody against acetylated lysine as indicated in Fig. 6B, panel 2. Importantly, the cyclophilin-D associated with ANT-1 was largely acetylated.

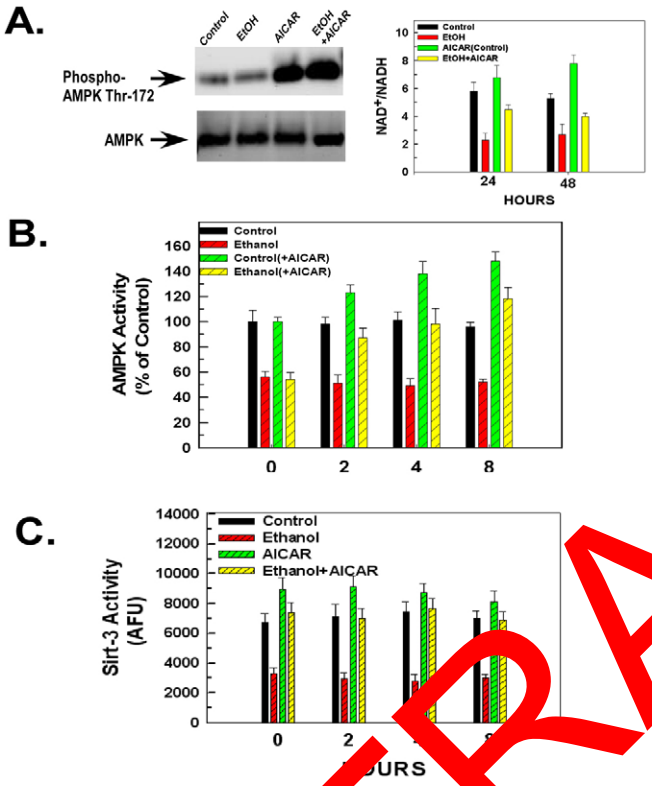

Fig. 5. Activation of AMPK by AI[illegible]verses the inhi[illegible]ry effect of ethanol exposure on sirtuin-3 a[illegible]vity. (A[illegible]eft) Western bl[illegible]s of H4IIEC3 cells that had been either left [illegible]treated or expo[illegible]d to 25 mM of ethanol for 48 hours. Where indicated, ce[illegible]had been treated wi[illegible]5 mM of AICAR for another 8 hours. The ce[illegible]were harvested and cell e[illegible]cts were separated by SDS-PAGE and ele[illegible]blotted ont[illegible]VDF membranes. Blots were probed with antibodies agai[illegible]AMPK[illegible]specific for AMPK phosphorylated on Thr172. (Right) Quantifi[illegible]of the NAD[illegible]NADH ratio. Cell extracts were used to det[illegible]e NAD[illegible]ADH ra[illegible]luorescently as described in Materia[illegible]nd Met[illegible]ds. Values [illegible]th[illegible]eans of three samples, error bars indi[illegible]e standard d[illegible]iations. $P<0.[illegible]$ for control versus ethanol and ethanol versus ethanol+[illegible]-way ANOVA and Scheffe's post-hoc test. (**B**) Qua[illegible]i[illegible]on of AMPK activity. H4IIEC3 cells were either left untreated or exposed[illegible]25 mM of ethanol for 48 hours. Where indicated cells were subsequently t[illegible]ed with 0.5 mM of AICAR. At the time points indicated the cells were harves[illegible]and AMPK activity was determined. The values are the means of three samples, error bars indicate standard deviations. $P<0.05$ for control versus ethanol, control versus control+AICAR and ethanol versus ethanol+AICAR by one-way ANOVA and Scheffe's post-hoc test. (**C**) Quantification of sirtuin-3 activity. H4IIEC3 cells were either left untreated or exposed to 25 mM of ethanol for 48 hours. Where indicated, cells were treated with 0.5 mM of AICAR. At the time points indicated, the cells were harvested and mitochondria isolated. Sirtuin-3 activity was measured in mitochondrial extracts. Values are the means of three samples, error bars indicate standard deviations. $P<0.05$ for control versus ethanol and ethanol versus ethanol+AICAR by one-way ANOVA and Scheffe's post-hoc test.

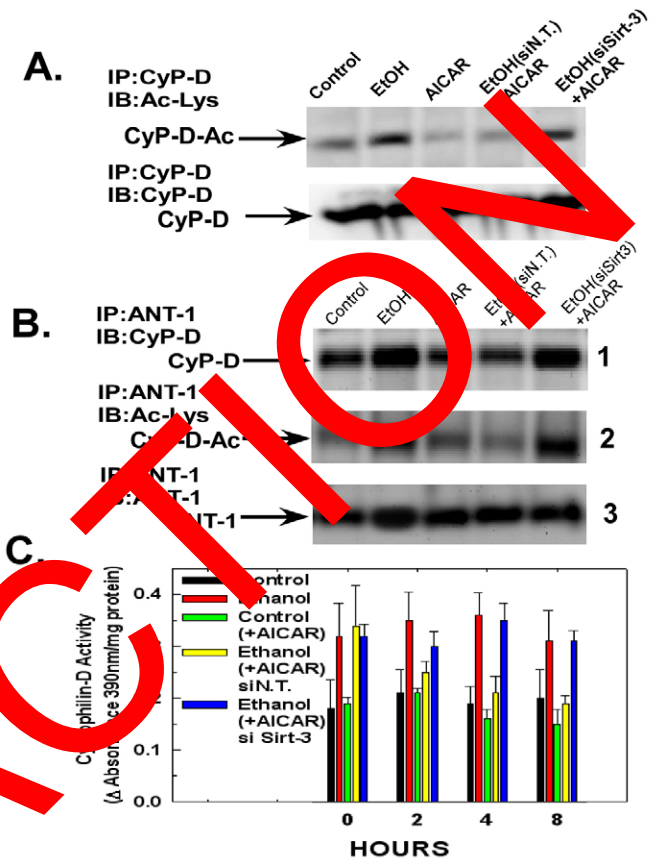

Fig. 6. Sirtuin-3 is necessary for AICAR to reverse the ethanol-induced activation, acetylation and binding of cyclophilin-D to ANT-1. (**A**) Western blots of H4IIEC3 cells that had been transfected with non-targeting siRNA or siRNA against sirtuin-3. After 24 hours, the cells were either left untreated or exposed to 25 mM of ethanol for 48 hours. Following ethanol exposure, were indicated, the cells were treated with 0.5 mM of AICAR for 8 hours. The mitochondria were then isolated and mitochondrial extracts were immunoprecipitated with cyclophilin-D antibody. The immunoprecipitates were separated by SDS-PAGE and electroblotted onto PVDF membranes. Blots were probed with antibody against acetylated lysine, then stripped and re-probed with an antibody against cyclophilin-D. (**B**) Western blots of H4IIEC3 cells that had been transfected with non-targeting siRNA or siRNA against sirtuin-3. After 24 hours, the cells were either left untreated or exposed to 25 mM of ethanol for 48 hours. Where indicated, cells were subsequently treated with 0.5 mM of AICAR for 8 hours. Mitochondrial extracts were immunoprecipitated with antibody against ANT-1. The immunoprecipitates were separated by SDS-PAGE and electroblotted onto PVDF membranes. Blots were then probed with antibodies against cyclophilin-D or ANT-1. To access cyclophilin-D acetylation, the cyclophilin-D blots were stripped and then re-probed with antibody against acetylated lysine. (**C**) Quantification of cyclophilin-D activity. H4IIEC3 cells were transfected with non-target control siRNA or siRNA against sirtuin-3. After 24 hours, the cells were either left untreated or exposed to 25 mM of ethanol for 48 hours. Subsequently, where indicated, the cells were treated with 0.5 mM of AICAR. At the times indicated, cells were harvested and mitochondria isolated. Cyclophilin-D activity was measured in mitochondrial extracts as described in Materials and Methods. The values are the means of three samples, error bars indicate standard deviations. $P<0.05$ for control versus ethanol, ethanol versus ethanol+AICAR siN.T. and ethanol+AICAR siN.T. versus ethanol+AICAR siSirt-3 by one-way ANOVA and Scheffe's post-hoc test.

AICAR also inhibited the ethanol-induced stimulation of cyclophilin-D activity. As shown in Fig. 6C, ethanol exposure for 48 hours induced an 50% elevation of basal cyclophilin-D activity compared with untreated cells (0 hours). The stimulation of cyclophilin-D activity by ethanol was reversed by treatment with AICAR. Cells exposed to ethanol for 48 hours and subsequently treated with AICAR exhibited a drop in cyclophilin-D activity over an 8-hour time course. At 4 hours and 8 hours, AICAR treatment decreased cyclophilin-D activity by 38% and 44%, respectively, in ethanol-exposed cells. The ability of AMPK activation by AICAR to reverse the ethanol-induced stimulation of cyclophilin-D is dependent on sirtuin-3 expression. Suppression of sirtuin-3 prevented AICAR from reversing the ethanol-induced enhancement of cyclophilin-D activity. As shown in Fig. 6C, when sirtuin-3 expression was suppressed by siRNA, AICAR treatment was unable to reverse the elevation of cyclophilin-D activity in cells that were exposed to ethanol for 48 hours.

Sirtuin-3 is necessary for AMPK activation to prevent ethanol-induced sensitization to the MPT- and TNF-induced cell killing

As shown in Fig. 7A, mitochondria isolated from ethanol-exposed cells required only one dose of 50 µM Ca^{2+} to provoke opening of the PTP. By contrast, mitochondria isolated from cells exposed to ethanol for 48 hours and subsequently treated with AICAR for 8 hours exhibited sensitivity to MPT induction identical to that of control cells, requiring three doses of 50 µM Ca^{2+} to trigger the MPT. Repression of sirtuin-3 expression with siRNA prevented AICAR from reversing the sensitizing effects of ethanol on MPT induction, with only one dose of 50 µM Ca^{2+} triggering induction of the PTP. Importantly, suppression of sirtuin-3 and concurrent ethanol exposure did not result in an additive or synergistic effect for PTP induction.

It has been demonstrated that the increased sensitivity of mitochondria to MPT caused by ethanol is partly responsible for the enhanced cytotoxicity elicited by TNF in ethanol-exposed cells (Pastorino and Hoek, 2000). As shown in Fig. 7B, control cells exhibited a 24% incidence of cell death at 16 hours after TNF exposure. By contrast, cells exposed to ethanol for 48 hours and subsequently treated with TNF exhibited a marked potentiation of TNF-induced cytotoxicity, with a 34% loss of viability at 8 hours and a 67% loss in viability at 16 hours. Treatment with AICAR was able to reverse the sensitizing effects of ethanol on TNF-induced cytotoxicity. Cells that had been exposed to ethanol for 48 hours and were subsequently treated with AICAR for 8 hours exhibited marked protection against TNF-induced cytotoxicity, with only 35% of the cells showing viability after 16 hours of TNF treatment. Suppression of sirtuin-3 expression prevented AICAR from exerting a protective effect against TNF-induced cytotoxicity in ethanol-exposed cells, with 63% of the cells dead after 16 hours of treatment (Fig. 7B). Importantly, as would be expected, suppression of sirtuin-3 expression was sufficient to potentiate TNF-induced cytotoxicity. When sirtuin-3 levels were suppressed with siRNA in cells not exposed to ethanol, treatment with TNF-induced cytotoxicity in 65% of the cells after 16 hours (Fig. 7B). Importantly, concurrent suppression of sirtuin-3 and exposure to ethanol did not provoke an additive or synergistic effect on TNF-induced cytotoxicity, indicating that they are acting through the same mechanism of inhibiting sirtuin-3 activity. These data thus indicate that sirtuin-3 is a crucial mediator of cellular sensitivity to TNF.

Acetylation of lysine-145 controls sensitivity to PTP induction and TNF cytotoxicity in ethanol-exposed cells

We have demonstrated that cyclophilin-D is acetylated on Lys145 and controls its cis-trans isomerase activity (Shulga et al., 2010). Two point mutants were generated, CyP-D(K145Q) and CyP-D(K145R), which mimic constitutive acetylation and deacetylation, respectively. Stable cell lines expressing either cyclophilin-D were generated. As shown in Fig. 8A, mitochondria isolated from cells expressing CyP-D(K145R) were resistant to the sensitizing effects of ethanol exposure to induction of the PTP. By contrast, cells expressing CyP-D(K145Q) exhibited enhanced sensitivity to PTP induction, even when not exposed to ethanol. Additionally, as shown in Fig. 8B, expression of CyP-D(K145R) prevented ethanol exposure or sirtuin-3 suppression from sensitizing the cells to

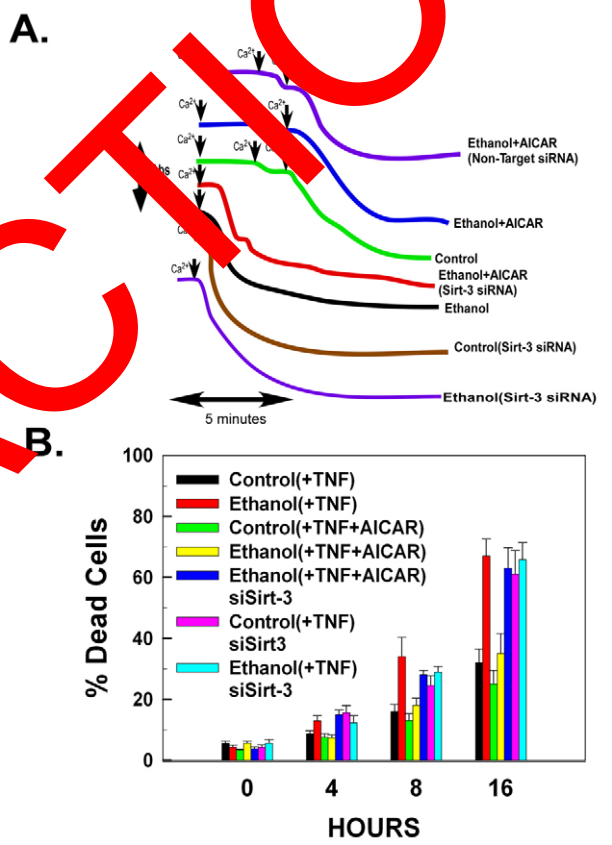

Fig. 7. Sirtuin-3 is required for AMPK activation to reverse the ethanol-induced sensitization to onset of the MPT and TNF-induced cytotoxicity. (**A**) H4IIEC3 cells were transfected with non-target control siRNA or siRNA against sirtuin-3. After 24 hours, the cells were left untreated or exposed to 25 mM of ethanol for 48 hours. Where indicated, the cells were subsequently treated with 0.5 mM of AICAR for 8 hours. Mitochondria were isolated. Where shown, 50 µM of Ca^{2+} was added. The change in absorbance was measured at 540 nm. (**B**) H4IIEC3 cells were transfected with non-target control siRNA or siRNA against sirtuin-3. After 24 hours, the cells were left untreated or exposed to 25 mM of ethanol for 48 hours. Where indicated, the cells were subsequently treated with 0.5 mM of AICAR for 8 hours. Cells were then treated with 10 ng/ml of TNF. At the times indicated, the viability of the cells was assessed. The values are the means of three samples, and error bars indicate standard deviations. $P<0.05$ for control(+TNF) versus ethanol(+TNF), ethanol(+TNF) versus ethanol(+TNF+AICAR), ethanol(+TNF+AICAR) versus ethanol(+TNF+AICAR)siSirt-3 and control(+TNF) versus control(+TNF)siSirt-3 by one-way ANOVA and Scheffe's post-hoc test.

TNF-induced cytotoxicity. By contrast, expression of CyP-D(K145Q) by itself was sufficient to enhance TNF-induced cytotoxicity. Importantly, knockdown of CyP-D expression prevented suppression of sirtuin-3 levels from enhancing TNF-induced cytotoxicity (Fig. 8B). These data thus indicate that the control exerted by sirtuin-3 on CyP-D acetylation influences the ability of CyP-D to induce the permeability transition. Moreover, the promotion of TNF-induced cytotoxicity generated by the decrease of sirtuin-3 activity is dependent on CyP-D, which is crucial for induction of the MPT and TNF-induced cell death.

Maintenance of the NAD^+:NADH ratio prevents the ethanol-induced decline of sirtuin-3 activity

Ethanol exposure induced a decline in the NAD^+:NADH ratio that could account for the inhibition of sirtuin-3 activity. Therefore, cells were supplemented with acetoacetate (AcA), whose metabolism increases the NAD^+:NADH ratio. As shown in Fig. 9A, left graph, addition of AcA markedly blunted the decline in the NAD^+:NADH ratio in ethanol-exposed cells at 24 and 48 hours. Importantly, AcA also prevented the increase of cyclophilin-D acetylation induced by ethanol exposure (Fig. 9A, right, lane 4). Similarly, AcA prevented both the decrease of sirtuin-3 and the increase of cyclophilin-D activities induced by ethanol exposure (Fig. 9B). The inhibition of cyclophilin-D activation by AcA also prevented the ethanol-induced sensitization to PTP induction and TNF-induced cytotoxicity (Fig. 9C, left and right graphs, respectively).

Discussion

Exposure of cells to ethanol and its metabolism ha[s] bee[n] demonstrated to cause a myriad of alterations in cellular phys[iology] and mitochondrial function (Cunningham et al., 1990; Hoek [et al.], 2002; Lieber, 2004; Diehl, 1999; Rashid et [al.], 1[9]). Such [an] alteration leads to steatosis, an initial ma[nifestation] of excessive ethanol consumption. These changes in [cellular] me[tabolism] have been implicated in enhancing the ev[entual] [de]velopment of more serious consequences of excessive [e]thanol inta[ke] such as the onset of alcoholic steatohepatitis and [cir]rhosis. An impo[rtant] consequence from this cascade of malfun[ctio]ns is [an] increased sen[sit]ivity to cell death exhibited by cells exposed to eth[an]ol.

Sirtuins have em[erg]ed as importa[nt] components in the modulation of the [ef]fects of ethanol on cel[l] metabolism. It has been demonstrat[ed] in hepat[oc]ytes that sirtuin activity is decreased by exposure to eth[an]ol, p[os]sibly beca[us]e of the decline in the ratio of NAD^+:NADH ind[uc]ed by me[ta]bolized ethanol (You et al., 2008b; [Yo]u et [al.], 2008). The in[vo]lvement of sirtuins in the effects of eth[an]ol are [re]inforced [by] the finding that resveratrol, which act[iva]tes sirtu[in]s, alleviates the development of alcoholic fatty liver [di]sease (Ajmo [et] al., 2008; You and Crabb, 2004). Interesti[ng]ly, knockdown of sirtuin-3 has recently been demonstrate[d] to inhibit mitochondrial fatty acid oxidation (Hirschey et al., 2010).

The effects of ethanol exposure on sirtuin activities are highlighted by the hyperacetylation of a number of cellular proteins including acetyl-CoA synthetase 2 (AceCS2) (Kannarkat et al., 2006; Picklo, 2008; Shepard and Tuma, 2009). AceCS2 is a mitochondrial matrix protein that is deacetylated by sirtuin-3 and, as demonstrated here, whose acetylation is increased by exposure to ethanol (Fig. 2B) (Hallows et al., 2006).

Like the sirtuin-3 substrate AceCS2, cyclophilin-D is localized to the mitochondrial matrix. Cyclophilin-D is a peptidyl-prolyl cis-trans isomerase that has been shown to promote opening of the MPT pore. We and others have demonstrated that the mitochondria of cells exposed to ethanol are more susceptible to triggering of the MPT (Pastorino et al., 1999; Higuchi et al., 2001; Wu and Cederbaum, 2001). This could be owing to a number of alterations to mitochondrial function induced by ethanol exposure, such as an increase in the formation of reactive oxygen species and a decline of mitochondrial glutathione (Cahill et al., 1997; [Ca]hill et al., 1998; Nagy et al., 1994; Tsukamoto et al., 2001). Howeve[r], [in]hibition of cyclophilin-D activity with cyclosporin A has been show[n] to prevent the sensitization to MPT induction exhibit[ed] by [mi]tochondria [iso]lated from cells exposed to ethanol, suggesting [tha]t cyclophilin D is a significant component to MPT ind[uc]tion (Pasto[rino] et al., 1999).

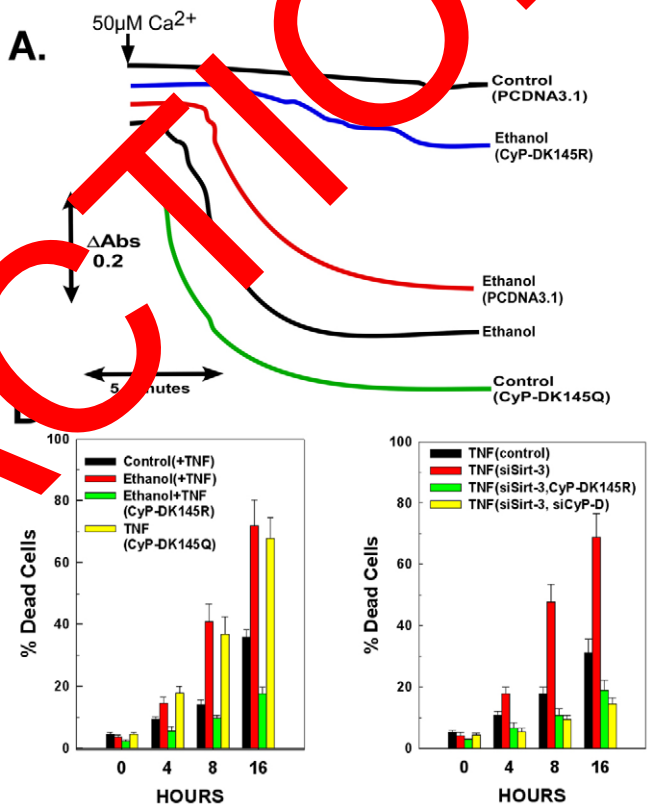

Fig. 8. Acetylation of the Lys145 residue in cyclophilin-D controls sensitivity to PTP induction and TNF cytotoxicity in ethanol-exposed cells. (**A**) H4IIEC3 cells expressing CyP-D(K145R) or CyP-D(K145Q) were generated. Cells were then either left untreated or exposed to 25 mM of ethanol for 48 hours. Mitochondria were then isolated. Mitochondrial respiration was initiated by the addition of 1 mM malate and 1 mM glutamate. To trigger mitochondrial swelling, 50 μM Ca^{2+} was added as indicated. The change in absorbance was measured at 540 nm. (**B**) (Left) H4IIEC3 cells stably expressing CyP-D(K145R) or CyP-D(K145Q) were exposed to 25 mM of ethanol for 48 hours. Cells were then treated with 10 ng/ml TNF. Alternatively, cells expressing CyP-D(K145R) were transfected with siRNA against sirtuin-3. Following 48 hours, the cells were treated with TNF. (Right) Cells were transfected with siRNA targeting sirt-3 and Cyp-D. Following 48 hours, cells were treated with 10 ng/ml TNF. At the times indicated, the viability of the cells was assessed. The values are the means of three samples, and error bars indicate standard deviations. $P<0.05$ for control+TNF versus ethanol+TNF, ethanol+TNF versus ethanol+TNF(CyP-DK145R), control+TNF versus TNF(CyP-DK145Q) and TNF(siSirt-3) versus TNF(siSirt-3,CyP-DK145R), TNF(siSirt-3) versus TNF(siSirt-3, siCyP-D) by one-way ANOVA and Scheffe's post-hoc test.

Mitochondria isolated from cyclophilin-D knockout mice display a greatly reduced sensitivity to MPT induction (Baines et al., 2005; Basso et al., 2005; Schweizer et al., 1993). The mechanism by which cyclophilin-D promotes induction of the MPT is currently unclear. However the cis-trans isomerase activity of cyclophilin-D is thought to mediate alterations in the conformation of mitochondrial inner membrane proteins to promote formation and opening of the PTP. This is supported by the ability of cyclosporin-A to suppress onset of the MPT. Cyclosporin-A binds to cyclophilin-D and inhibits its cis-trans isomerase activity (Broekemeier et al., 1989; Crompton et al., 1998; Halestrap et al., 1997; Nicolli et al., 1996). However, the proteins that mediate formation of the PTP are currently unknown. ANT-1 is the most abundant inner mitochondrial membrane protein. Some evidence suggests that ANT-1 or ANT-3 is a component of the PTP (Bauer et al., 1999; Pereira et al., 2007; Yang, Z. et al., 2007). In the present study, ethanol exposure promotes an enhancement in the binding of cyclophilin-D to ANT-1. However, studies by Kokoszka and colleagues, who have used mice in which ANT is not expressed, indicate that the PTP still forms (Kokoszka et al., 2004). However, the same group has demonstrated that even though ANT-1 might not be a component of the PTP per se, it can control susceptibility to MPT induction (Lee et al., 2009). So, even though the composition of the PTP is unclear, the current data demonstrate that an increase in the acetylation and activity of cyclophilin-D can enhance the interaction of cyclophilin-D with a protein of the mitochondrial inner membrane that modulates sensitivity to PTP opening.

Activation of AMPK by AICAR was able to reverse the inhibitory effects of ethanol on sirtuin-3 activity. The AMPK induced re-activation of sirtuin-3 in ethanol-exposed cells was accompanied by a consequent decline of cyclophilin-D acetylation, activity and binding to the ANT-1. AMPK has been shown to stimulate sirtuin-1 activity by increasing [illegible] levels. Indeed, in the present study, the decline of NAD^+ levels in ethanol-exposed cells was partially reversed by treatment with AICAR (Fig. 5A). Additionally, AcA prevented the ethanol-induced decline in the NAD^+:NADH ratio. AcA also reversed the ethanol-induced inhibition of siturin-3 activity, activation of cyclophilin-D and increased sensitivity to PTP induction and TNF-induced cytotoxicity (Fig. 9).

In the present study, we have shown that an increase in cyclophilin-D activity enhanced cell death by TNF by sensitizing the mitochondria to induction of the MPT. This is consistent with studies demonstrating that cyclophilin-D overexpression potentiates

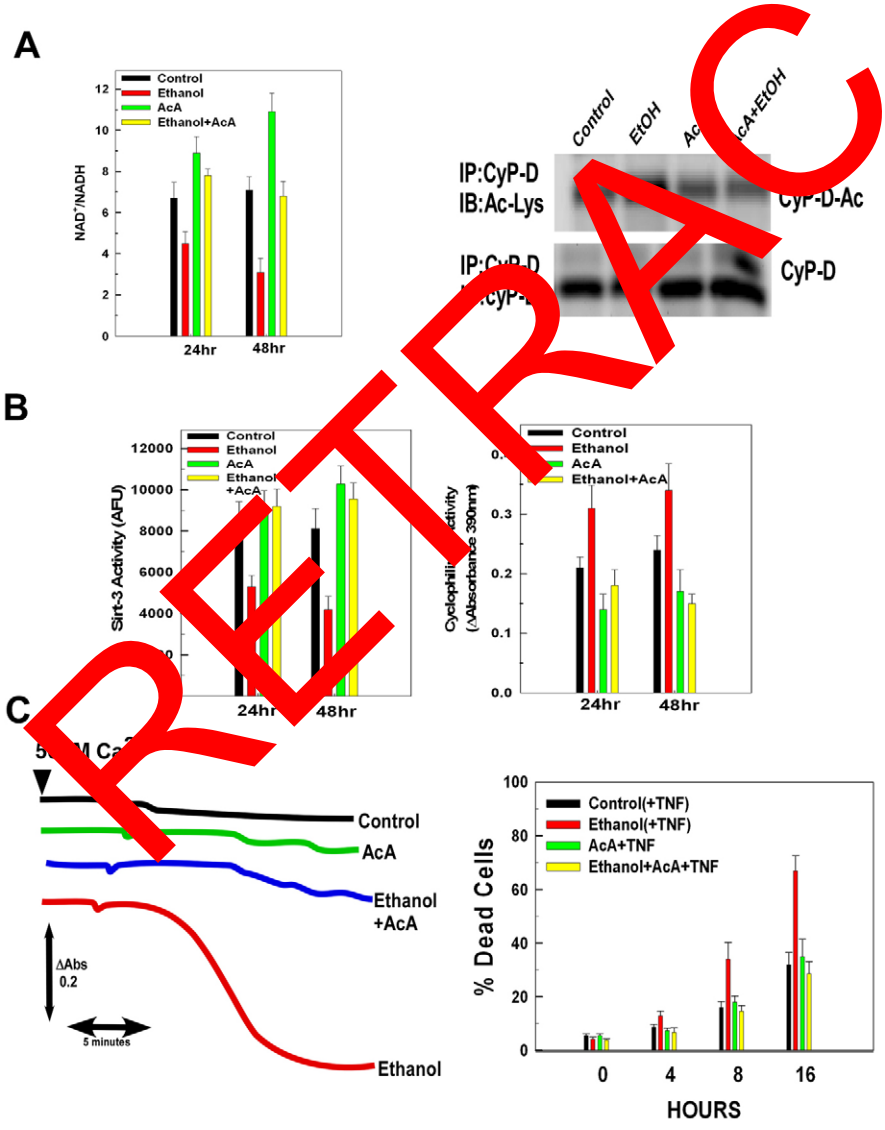

Fig. 9. Maintenance of the NAD^+:NADH ratio prevents the ethanol-induced decline of sirtuin-3 activity. (**A**) (Left) H4IIEC3 cells were untreated or exposed to 25 mM of ethanol in the absence or presence of 10 mM of acetoacetate (AcA) for 48 hours. Cell extracts were prepared to determine the NAD^+:NADH ratio. (Right) Western blots of H4IIEC3 cells treated as above. Cyclophilin-D was immunoprecipitated from mitochondrial extracts. The immunoprecipitates were then separated by SDS-PAGE and electroblotted onto PVDF membranes. Blots were then probed with antibody against cyclophilin-D, stripped and reprobed with antibody against acetylated lysine. The values are the means of three samples, and the error bars indicate standard deviations. $P<0.05$ for control versus ethanol, ethanol versus ACA and ethanol versus ethanol+AcA by one-way ANOVA and Scheffe's post-hoc test. (**B**) H4IIEC3 cells were untreated or exposed to 25 mM of ethanol in the absence or presence of 10 mM of AcA. After 24 or 48 hours incubation, cyclophilin-D or sirtuin-3 activity was determined in mitochondrial extracts as described in Materials and Methods. The values are the means of three samples, error bars indicate standard deviations. $P<0.05$ for control versus ethanol, ethanol versus AcA and ethanol versus ethanol+AcA by one-way ANOVA and Scheffe's post-hoc test. (**C**) (Left) H4IIEC3 cells were untreated or exposed to 25 mM of ethanol in the absence or presence of 10 mM AcA. Following a 48-hour incubation, mitochondria were isolated. Where shown (arrows), a 50 μM Ca^{2+} was added. (Right) H4IIEC3 cells were treated with 10 ng/ml TNF. At the times indicated, the viability of the cells was assessed. The values are the means of three samples and the error bars indicate standard deviations. $P<0.05$ for control(+TNF) versus ethanol(+TNF) and ethanol(+TNF) versus ethanol+TNF+AcA by one-way ANOVA and Scheffe's post-hoc test.

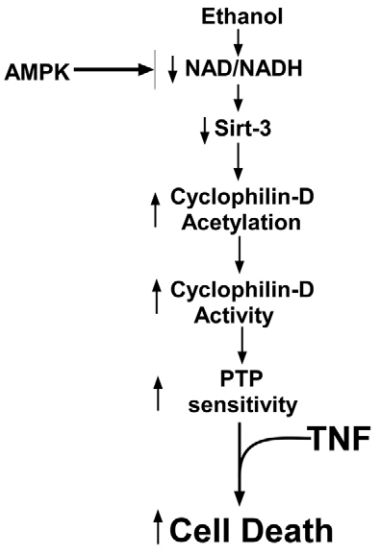

Fig. 10. Ethanol-induced decline of sirtuin-3 activity sensitizes mitochondria to MPT induction by TNF. Ethanol metabolism induces a decline of sirtuin-3 activity, causing an increase in cyclophilin-D acetylation and activity, which results in a sensitization to induction of the permeability transition by TNF.

necrotic cell death and that TNF-induced cytotoxicity is mediated by the permeability transition (Bradham et al., 1998; Crompton et al., 1998; Li et al., 2004; Pastorino and Hoek, 2000; Pastorino et al., 1996; Woodfield et al., 1998). However, in some instances cyclophilin-D has been demonstrated to prevent apoptotic cell death through modulation of Bcl-2 and by promoting the binding of hexokinase II to the mitochondria (Li et al., 2004; Schubert and Grimm, 2004). Indeed, we have demonstrated that a decrease of sirtuin-3 activity enhances the binding of hexokinase II to the mitochondria by increasing cyclophilin-D activity, potentially making the cells resistant to apoptosis (Shulga et al., 2010).

In summary, the present study identifies sirtuin-3 as a target through which ethanol exposure enhances the sensitivity of mitochondria to induction of the MPT (Fig. 10). Ethanol exposure decreases the cellular NAD^+:NADH ratio, thereby contributing to an inhibition of sirtuin-3 activity. The inhibition of sirtuin-3 causes an increase in the acetylation activity and binding of cyclophilin-D to ANT-1. The increased activity of cyclophilin-D promotes onset of the MPT that has been shown to mediate TNF-induced cytotoxicity (Bradham et al., 1998; Pastorino and Hoek, 2000; Pastorino et al., 1996). Importantly, AMPK activation by AICAR reversed the effects of ethanol exposure on sirtuin-3 activity and, consequently, cyclophilin-D. AMPK activation also reversed the enhanced sensitivity of mitochondria isolated from ethanol-exposed cells to induction of the MPT and prevented the increased sensitivity to TNF-induced cytotoxicity exhibited by ethanol-exposed cells.

Materials and Methods

Cell culture and treatments

H4IIEC3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin under an atmosphere of 95% air, 5% CO_2 at 37°C. Cells were subcultured 1:5 once a week. The cells were treated with 25 mm ethanol for 24 or 48 hours. Where indicated, cells were first exposed to 25 mm ethanol, with or without 5 mM 4-MP. The culture medium was replaced every 24 hours with fresh medium containing 25 mM of ethanol in the absence or presence of 4-MP. To prevent evaporation of ethanol, a plastic vessel was placed in the incubator, containing a mixture of water and ethanol. The level of ethanol in the culture medium was monitored spectrophotometrically by an alcohol dehydrogenase assay. On the day of the experiment, the cells were washed and placed in DMEM in the presence of 25 mm ethanol, with or without 5 mM 4-MP. Where indicated, AICAR or TNF was dissolved in phosphate-buffered saline (PBS) and added to the wells in a 0.2% volume to give a final concentration of 0.5 mM or 10 ng/ml, respectively (22 units/ml).

Isolation of mitochondrial fraction and MPT measurement

Following treatments, the cells were harvested by trypsinization and centrifuged at 600 ***g*** for 10 minutes at 4°C. The cell pellets were washed once in PBS and then resuspended in 3 volumes of isolation buffer [20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na^+-EDTA, 1 mM dithiothreitol (DTT), and 10 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 10 μM antipain] in 250 mM sucrose. After chilling on ice for 3 minutes, cells were disrupted by 40 strokes of a glass homogenizer. The homogenate was centrifuged twice at 600 ***g*** at 4°C to remove intact cells and nuclei. The mitochondrion-enriched fraction (heavy membrane fraction) was then pelleted by centrifugation at 12,000 ***g*** for 30 minutes. Mitochondrial integrity was determined by the respiratory control ratio as oxygen consumption in states three and four respiration, by using a Clark oxygen electrode with 1 mM glutamate and 1 mM malate as respiratory substrates. Mitochondria were incubated in a KCl-based medium (120 mM KCl, 25 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 20 mM HEPES pH 7.4, 1 mM glutamate and 1 mM malate were added as respiratory substrates. Mitochondrial swelling was monitored at 540 nm on a Helios spectrophotometer.

Measurement of sirtuin-3 and cyclophilin-D activity

Sirtuin-3 activity was measured in mitochondrial extracts by using the Cyclex sirtuin-3 assay kit (MBL). A sirtuin-3 peptide substrate that is acetylated and fluorescently labeled was incubated with the mitochondrial extract. Deacetylation of the substrate by sirtuin-3 activity sensitizes it to lysyl endopeptidase, which cleaves the peptide releasing a quencher of the fluorophore. Fluorescence intensity was measured on a fluorescence plate reader with excitation at 340 nm and emission at 440 mm.

Cyclophilin-D isomerase activity was determined colorimetrically by using a peptide substrate in which the rate limiting conversion of cis to trans of a proline residue in the peptide makes it susceptible to cleavage by chymotrypsin, resulting in the release of the chromogenic group p-nitroanilide. The absorbance change at 390 nm was monitored over a 10 minute period with data collected every 0.2 seconds. Additionally, cyclophilin-D was immunoprecipitated from mitochondrial extracts that had been isolated from cells incubated in glucose-based medium. The immunoprecipitated cyclophilin-D was incubated with recombinant sirtuins in sirtuin reaction buffer (50 mM Tris-HCl pH 8.8, 4 mM $MgCl_2$, 0.5 mM DTT). The resultant proteins were then separated by SDS-PAGE and electro-blotted onto PVDF membranes. The western blots were developed using antibody against acetylated lysine (Cell Signaling).

Immunoprecipitation of ANT and cyclophilin-D

Cyclophilin-D was immunoprecipitated from mitochondrial extracts. The immunoprecipitates were then separated by SDS-PAGE and electro-blotted onto PVDF membranes. The western blots were developed using antibody against acetylated lysine, then stripped and reprobed with antibody against anti-cyclophilin-D (Cell Signaling).

ANT-1 was immuno-captured from mitochondrial extracts by using monoclonal antibody against ANT-1 crosslinked to agarose beads. (MitoSciences). The immunocomplexes were eluted with SDS buffer, and separated by 12% SDS-PAGE and electro-blotted onto PVDF membranes. The western blots were then probed with antibody against cyclophilin-D and stained with horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000); detection was carried out by enhanced chemiluminescence. The western blots were then stripped and re-probed using antibodies against acetylated lysine or ANT-1.

Transfection of siRNAs

siRNAs targeting sirtuins 3, 1, 4, 5, cyclophilin-D or a non-targeting control were delivered by a lipid-based method supplied from a commercial vendor (Gene Therapy Systems) at a final siRNA concentration of 50 nM. After formation of the siRNA-liposome complexes, the mixture was added to H4IIEC3 cells or mouse hepatocytes for 24 hours. Afterwards, the medium was aspirated and complete medium was added back.

Measurements of cell viability and the NAD^+:NADH ratio

Cell viability was determined by Trypan Blue exclusion and the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium (MTS). NADH levels were detected fluorescently utilizing a non-fluorescent detection reagent that is oxidized in the presence of NADH to produce the fluorescent analog and NAD^+. NAD^+ levels were detected by converting NAD^+ to NADH in an enzyme-coupled reaction. Levels of NAD^+ and NADH were 2 nmole per 10^6 cells and 0.35 nmole per 10^6 cells, respectively, in control cells not exposed to ethanol.

Measurement of AMPK activity

Assays were performed at 30°C and with 5 μg of cell lysates in reaction buffer, 40 mM HEPES pH 7.0, 80 mM NaCl, 5 mM Mg^{2+} acetate, 1 mM DTT, 8% glycerol, 0.8 mM EDTA, 200 μM AMP and ATP and 2 μCi [γ-^{32}P] ATP with or without SAMS peptide. Following 30 minutes of incubation, reactions were spotted onto phosphocellulose filter paper that was then washed with phosphoric acid. The radioactivity on the filter paper was measured by scintillation counting.

Statistical analysis

Results are expressed as means ± s.d. of at least three independent experiments. Statistical difference between test groups was analyzed by one-way ANOVA followed by Scheffe's post-hoc test. Statistical significance was defined at $P<0.05$.

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