Hexokinase II Binding to Mitochondria Is Necessary for Kupffer Cell Activation and Is Potentiated by Ethanol Exposure*

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Background: Kupffer cells mediate alcohol-induced liver disease.

Results: Ethanol exposure potentiates activation of Kupffer cells by promoting the translocation of hexokinase to mitochondria.

Conclusion: Mitochondrial hexokinase is required for activation of Kupffer cells and is potentiated by ethanol.

Significance: Detachment of hexokinase from the mitochondria prevents Kupffer cell activation.

Ethanol exposure promotes the development of steatohepatitis, which can progress to end stage liver disease. Kupffer cells have been documented to play a key role in the genesis and progression of alcoholic liver disease with ethanol exposure enhancing Kupffer cell activation. In the present study, we identified the binding of hexokinase II to the mitochondria as a requirement for stimulation of Kupffer cells. Furthermore, the presence of ethanol enhances the ability of hexokinase II to bind to mitochondria. The results confirm the importance of hexokinase II in the activation of Kupffer cells and provide evidence for its potential role in ethanol-induced liver disease.

This article has been retracted by the publisher. An investigation by the Office of Research Integrity determined that falsified and/or fabricated Western blots were included in Figs. 1B, 3A, 4D, 5E, and 6C (https://ori.hhs.gov/content/case-summary-pastorino-john-g).

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2 The abbreviations used are: TLR, Toll-like receptor; ROS, reactive oxygen species; ASC, apoptosis-associated specklike protein containing a CARD (caspase activation and recruitment domain); NLRP, NOD-like receptor family pyrin domain-containing; NOD, nucleotide-binding oligomerization domain; PPP, pentose phosphate pathway; AMPK, AMP-activated protein kinase; AFC, 7-amino-4-trifluoromethylcoumarin; sirt-3, sirtuin-3; N-HXX II, 1-β-o-ribofuranoside.
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Aerobic glycolysis is critically dependent on the ability of hexokinase I or II to bind to mitochondria through the voltage-dependent anion channel, an outer mitochondrial membrane protein (8, 11, 12). In this position, ATP is selectively channeled to hexokinase II from the mitochondrial matrix where it is synthesized by oxidative phosphorylation, thus greatly augmenting glycolytic flux (13, 14). We and others have demonstrated that detachment of hexokinase II from the mitochondria effectively terminates aerobic glycolysis, leaving cancer cells incapable of rapid division (14–17). Cyclophilin-D is localized to the mitochondrial matrix and through its interaction with the adenine nucleotide carrier on the inner mitochondrial membrane mediates the binding of hexokinase II to voltage-dependent anion channel on the outer mitochondrial membrane (18, 19). We have demonstrated that the activity of cyclophilin-D is modulated by the mitochondrial deacetylase sirtuin-3. Decreased activity of sirtuin-3 brings about an increased acetylation of cyclophilin-D, resulting in stimulation of its peptidyl-prolyl cis-trans isomerase activity, causing increased binding of hexokinase II to the mitochondria.

In the present study, we demonstrated that in liver macrophages (Kupffer cells) the binding of hexokinase II to mitochondria is essential for inflammasome activation. Upon activation of Kupffer cells, there was a rapid translocation of hexokinase II to the mitochondria that was mediated by a LPS- and ethanol-induced decrease of sirtuin-3 activity, resulting in a stimulation of cyclophilin-D. Moreover, exposure to ethanol greatly enhanced Kupffer cell activation. Ethanol greatly enhanced LPS- and ethanol-induced decrease of sirtuin-3 activity. Additionally, activation of hexokinase II by ethanol-induced inhibition of hexokinase I or II to bind to mitochondria through the voltage-dependent anion channel on the outer mitochondrial membrane (18, 19). We have demonstrated that the activity of cyclophilin-D is modulated by the mitochondrial deacetylase sirtuin-3. Decreased activity of sirtuin-3 brings about an increased acetylation of cyclophilin-D, resulting in stimulation of its peptidyl-prolyl cis-trans isomerase activity, causing increased binding of hexokinase II to the mitochondria.

Experimental Procedures

Ethanol Feeding Protocol—Male Sprague-Dawley rats (140–160 g) were obtained from Charles River Laboratories (Raleigh, NC). Lieber-DeCarli ethanol diet was purchased from Dyets Inc. (Bethlehem, PA). Rats were assigned to pair- or ethanol-fed groups. Ethanol-fed rats were allowed free access to a liquid diet containing 17% of calories as ethanol for 2 days, and then the ethanol content of the diet was increased to 35% of the calories for the duration of the 4-week feeding protocol. Controls were pair-fed with a liquid diet in which maltose dextrose were substituted isocalorically for ethanol.

Isolation and Culture of Kupffer Cells—The protocol was adapted from Thakur et al. (20). Livers were perfused with 0.05% collagenase, and the resulting suspension of liver cells was treated with 0.02% Pronase for 15 min at 12 °C. The resulting cell suspension from two rats per treatment group was pooled and then centrifuged three times at 100 × g for 2 min. The pooled supernatant was then purified by centrifugal elutriation. The Kupffer cells were suspended in CMRL medium. After 1 h, non-adherent cells were removed by aspiration, and fresh medium was added.

Measurement of IL-1β and TNFα—Cell culture medium was removed at the times indicated and stored at −20 °C for TNF-α or IL-1β assay using ELISA (R&D Systems, Minneapolis, MN). High binding capacity polystyrene 96-well plates were coated with purified biotin-conjugated anti-murine IL-1β or TNF-α antibody (1 μg/ml) overnight. Avidin-HRP was then added at 1:5,000 for 30 min at room temperature followed by 100 μl/well 3,3’,5,5’-tetramethylbenzidine substrate. A values were read at 450 nm with a 570-nm subtracted correction using a BioTek® plate reader.

Measurement of Caspase-1 Activity—The activity of caspase-1 was measured in cell lysates using the fluorometric substrate Ac-YVAD-AFC. Kupffer cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 0.3% Nonidet P-40, 0.1 mM Na3VO4, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Lysates were then centrifuged at 14,000 × g for 10 min. The supernatants were collected, mixed with 50 μl of reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10% sucrose, 10 mM DTT, and 100 μM Ac-YVAD-AFC), and then incubated at 37 °C for 1 h. Samples were read at 405 nm in a 96-well microtiter plate.

Measurement of Reactive Oxygen Species—Kupffer cells were cultured for 16–18 h and then stimulated with LPS at the times indicated at 37 °C in a 5% CO2 atmosphere. Medium was then replaced with PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 100 μM PMSF). Lysates were then centrifuged at 12,000 × g for 10 min at 4 °C. The resulting supernatant was then centrifuged at 15,000 × g for 15 min at 4 °C. The resulting supernatant was added to the PBL-specific ligand that binds to a specific plasma membrane protein (Qiagen, Qproteome plasma membrane isolation kit). The resulting plasma membrane-enriched vesicles were precipitated using magnetic beads that bound to the PBL ligand. The plasma membrane vesicles were eluted under native conditions in buffer (50 mM Tris, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, and 1 mM EDTA with protease inhibitor mixture). Samples were separated by SDS-PAGE and probed by Western blotting with antibody specific for p67phox. Western blots were probed with antibody to Na,K-ATPase to ensure equal loading of plasma membrane proteins between samples.

Mitochondrial and Cytosolic Isolation—Kupffer cells from two individual wells (~1.0 × 106 cells total) were harvested and centrifuged at 600 × g for 10 min at 4 °C. The cell pellets were resuspended in 3 volumes of isolation buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM aprotinin) in 250 mM sucrose and disrupted by 40 strokes of a glass homogenizer. The homogenate was centrifuged twice at 1,500 × g for 15 min at 4 °C to remove unbroken cells and nuclei. The mitochondrially enriched fraction (heavy membrane fraction) was then pelleted by centrifugation at 12,000 × g for 30 min. The supernatant was removed and filtered through 0.2-μm and then 0.1-μm Ultrafree MC filters (Millipore Corp.) to give cytosolic protein.
Western Blotting—Proteins were immunoblotted onto PVDF membranes using the XCell II Blot module (Invitrogen). Hexokinase II, voltage-dependent anion channel 1, p67phox, and β-actin were detected using mouse monoclonal antibodies (from Cell Signaling Technology and Santa Cruz Biotechnology) at 1:1,000 dilution.

Measurement of Lactate—The lactate concentration was measured enzymatically (BioVision) with a colorimetric assay read at A570 nm, with the concentration of lactate determined by a standard curve.

Measurement of Sirtuin-3 and Cyclophilin-D Activity—Sirtuin-3 activity was measured in mitochondrial extracts using the CycLex sirtuin-3 assay kit (MBL International Corp.). A sirtuin-3 peptide substrate that is acetylated and fluorescently labeled was mixed with the mitochondrial extract. Fluorescence intensity was measured on a fluorescence plate reader with excitation at 340 nm and emission at 440 nm. Cyclophilin-D was immunoprecipitated from mitochondrial extracts that had been isolated from Kupffer cells. Cyclophilin-D peptidyl-prolyl cis-trans isomerase activity was determined colorimetrically by using a peptide in which the rate of cis to trans conversion of a proline residue in the peptide makes it susceptible to cleavage by chymotrypsin, resulting in the release of the chromogenic dye p-nitroanilide.

Measurement of the Cellular NADPH/NADP⁺ Ratio—The NADPH/NADP⁺ ratio was determined using a commercially available NADPH/NADP⁺ assay kit (BioAssay Systems). Kupffer cells were homogenized in either acidic extraction buffer for extraction of NADP⁺ or alkaline extraction buffer for extraction of NADPH. Calculation of total pyridine nucleotide content was using an NADP⁺ standard curve.

RNA Interference—siRNAs targeting sirtuin-3 (sirt-3) and cyclophilin-D and a non-targeting control were delivered into cells using TransIT-TKO at a final concentration of 100 nM. Sixteen hours after plating the siRNA-liposome complexes were added and incubated for 72 h, which the cells were washed twice with PBS and fresh complete medium was added.

Statistical Analysis—Results are expressed as means ± S.D. of at least three independent experiments. Statistical significance was defined at p < 0.05.

RESULTS

Ethanol Potentiates LPS-induced Inflammasome Activation—Kupffer cells were isolated from ethanol-fed rats or rats fed a control diet. To assess inflammasome activation, the Kupffer cells were stimulated with 100 ng/ml LPS. TLRL-4 is known to bind to and be stimulated by LPS. As shown in Fig. 1A, Kupffer cells isolated from control- or ethanol-fed rats did not display a difference in the expression of TLRL-4, and this was not affected by transfection with a non-targeting siRNA, but the expression of TLRL-4 was suppressed by transfection with siRNA targeting TLRL-4. By contrast, the expression of TLRL-2 was not affected. The inflammasome sensor, NLRP-3, is activated by LPS-induced stimulation of TLRL-4 (21). As shown in Fig. 1B, Kupffer cells isolated from ethanol-fed rats did not display an increase in NLRP-3 expression compared with controls. As with TLRL-4, siRNA targeting NLRP-3 brought about its suppression in both control and ethanol-exposed Kupffer cells but had no effect on NLRC-4 expression.

LPS stimulation of Kupffer cells isolated from control-fed rats produced a steady increase in the extracellular concentration of IL-1β detected in the culture medium (Fig. 1C). In contrast to the equivalent expression of TLRL-4 and NLRP-3 in Kupffer cells isolated from control- and ethanol-fed rats, Kupffer cells isolated from rats fed an ethanol-containing diet displayed a marked potentiation of LPS-induced IL-1β secretion compared with controls, reaching a 7-fold elevation of IL-1β levels over an 8-h time course. Caspase-1 activation is required for IL-1β production. Kupffer cells isolated from ethanol-fed rats displayed a 2-fold greater activation of caspase-1 upon LPS stimulation compared with Kupffer cells isolated from control animals (Fig. 1D). Similarly, Kupffer cells isolated from ethanol-fed rats displayed a 3-fold greater increase in TNFα secretion into the culture medium than cells isolated from control-fed rats (1,200 ng/10⁶ cells versus 3,800 ng/10⁶ cells; Fig. 1E). Importantly, suppression of TLRL-4 and NLRP-3 expression mitigated inflammasome activation in control or ethanol-exposed Kupffer cells (Fig. 1F). Kupffer cells isolated from control-fed rats exhibited a 2-fold greater production of IL-1β than cells isolated from control-fed rats treated with diphenyleneiodonium, an NADPH oxidase inhibitor, prevented LPS-induced production of IL-1β in ethanol-exposed Kupffer cells isolated from both control- and ethanol-fed rats.

Ethanol and LPS Modulate Sirtuin-3 and Cyclophilin-D Activity and Stimulate Binding of Hexokinase II to the Mitochondria—Mitochondria were isolated, and the activity of sirtuin-3 was determined. As shown in Fig. 2A, Kupffer cells isolated from control-fed rats exhibited a 40% reduction in sirtuin-3 activity upon stimulation with LPS over an 8-h time course. Significantly, Fig. 2A demonstrates that ethanol exposure suppressed basal sirtuin-3 activity in Kupffer cells. Kupffer cells isolated from ethanol-fed mice displayed a 50% reduction in basal sirtuin-3 activity compared with controls. Moreover, LPS was additive with ethanol in suppressing sirtuin-3 activity with LPS stimulation of ethanol-exposed Kupffer cells resulting in an 80% reduction of sirtuin-3 activity over an 8-h time course. We have demonstrated that a decline of mitochondrial sirtuin-3 activity leads to an increase in the acetylation and activity of cyclophilin-D (19). Importantly, as shown in Fig. 2B, LPS induced a stimulation of cyclophilin-D activity in Kupffer cells isolated from control-fed rats. In parallel to the suppression of sirtuin-3 activity, Kupffer cells isolated from ethanol-fed rats exhibited a 78% increase in basal cyclophilin-D activity, which upon stimulation with LPS induced a further doubling of cyclophilin-D activity. Importantly, as we have found previously, the decline and stimulation of sirt-3 and cyclophilin-D activities brought about by exposure to ethanol and/or LPS were not due to a reduction or increase in their expression, respectively (not shown) (22).

Activation of inflammatory cells is associated with an increase of glycolysis (5). As shown in Fig. 2C, LPS stimulation of Kupffer cells isolated from control-fed rats brought
about a doubling of the lactate concentration in the extracellular medium. Kupffer cells isolated from ethanol-fed rats did not display an increase in the basal extracellular lactate concentration but exhibited greatly amplified lactate production when stimulated with LPS. The accelerated lactate production was accompanied by a translocation of hexokinase II from the cytosol to the mitochondria. Fig. 2D demonstrates that LPS induced a translocation of hexokinase II from the cytosol to the mitochondria in Kupffer cells isolated from control-fed rats (lane 1 versus lane 2). Kupffer cells isolated from ethanol-fed rats displayed no increase in the basal level of mitochondrial hexokinase II but rather exhibited a greater stimulation in the translocation of hexokinase II from the cytosol to the mitochondria (Fig. 2D, lane 3 versus lane 4). Importantly, ethanol exposure potentiated caspase-1 activation and IL-1β secretion in Kupffer cells to the same degree as seen in Kupffer cells transfected with non-targeting siRNA, indicating that the transfection procedure had minimal effect on the responsiveness of control or ethanol-exposed Kupffer cells to LPS (Fig. 2, E and F, versus Fig. 1, D and C, respectively). Similar results were found for TNF-α production (result not shown).
Detachment of Hexokinase II from Mitochondria Abrogates Ethanol- and LPS-induced Inflammasome Activation—Kupffer cells isolated from control- or ethanol-fed rats were transfected with either a non-targeting siRNA or siRNA targeting cyclophilin-D. Alternatively, we have demonstrated that hexokinase II can be detached from the mitochondria by a cell-permeable peptide consisting of the N-terminal region of hexokinase II (N-HXK II) required for its interaction with voltage-dependent anion channel 1 (14, 24, 25). As shown in Fig. 3A (lanes 2 and 3 and lanes 5 and 6), suppression of cyclophilin-D expression or treatment with 20 μM N-HXK II prevented the LPS-induced stimulation of hexokinase II translocation to the mitochondria.
in Kupffer cells isolated from control- or ethanol-fed rats. Importantly, detachment of hexokinase II from the mitochondria by either suppression of cyclophilin-D expression or treatment with N-HXK II prevented the amplified lactate production induced by LPS in control and ethanol-exposed Kupffer cells (Fig. 3B). Additionally, Fig. 3, C and D, demonstrate that suppression of cyclophilin-D expression or treatment with N-HXK II prevented the LPS and ethanol induction of caspase-1 activity (Fig. 3C) and IL-1β and TNF-α (Fig. 3D) production, as well as mitochondrial reactive oxygen species (Fig. 3E). These results provide evidence for a role of hexokinase II in Kupffer cell activation and inflammation through its interaction with the mitochondria and cyclophilin-D.
caspase-1 activity and consequent cleavage and secretion of IL-1β. Similarly, as shown in Fig. 3E, production of TNFα was prevented by suppression of cyclophilin-D expression or detachment of hexokinase II from the mitochondria. Moreover, as shown in Fig. 3F, suppression of cyclophilin-D expression or detachment of hexokinase II from the mitochondria abrogated LPS-induced ROS production, suggesting that the induction of hexokinase II binding to the mitochondria is necessary for NADPH oxidase activation.

**Detachment of Hexokinase II from Mitochondria Prevents Ethanol- and LPS-induced Activation of NADPH Oxidase—Instead of going through the entire glycolytic pathway, some of the glucose 6-phosphate produced from hexokinase II can be shunted to the PPP, also known as the hexose monophosphate shunt. The PPP is up-regulated upon macrophage activation (26–29). As shown in Fig. 4A, LPS stimulation of Kupffer cells isolated from control-fed rats resulted in a 90% increase in the NADPH/NADP⁺ ratio. Importantly, inhibition of glucose-6-phosphate dehydrogenase, the first enzyme of the PPP, with dehydroepiandrosterone prevented the LPS-induced increase of the NADPH/NADP⁺ ratio, indicating that the elevation of NADPH levels is derived from PPP activity (Fig. 4A). Kupffer cells isolated from ethanol-fed rats displayed a marked potentiation of LPS-induced ROS production, both in control and LPS-treated cells (Fig. 4B, lane 2 versus lane 4), but ethanol exposure alone did not result in an appreciable activation of NADPH oxidase (Fig. 4B, lane 3), but ethanol exposure caused a potentiation of LPS-induced NADPH oxidase activation compared with that of controls (Fig. 4B, lane 2 versus lane 4). Importantly, the elevation of NADPH levels and activation of NADPH oxidase and their potentiation
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by exposure to ethanol were dependent on the binding of hexokinase II to the mitochondria. As demonstrated in Fig. 4, C and D, detachment of hexokinase II from the mitochondria brought about by suppression of cyclophilin-D expression or treatment with N-HXK II prevented the LPS-induced increase in the NADPH/NADP⁺ ratio and p67phox translocation to the plasma membrane in Kupffer cells isolated from control- or ethanol-fed rats (Fig. 4D, lanes 2 and 3 and lanes 5 and 6).

Suppression of Sirt-3 Expression Recapitulates the Effects of Ethanol in Potentiating LPS-induced Inflammasome Activation—

We wanted to determine whether suppression of sirtuin-3 expression could reproduce the potentiation of LPS-induced inflammasome activation brought about by ethanol. To accomplish this, we utilized concentrations of siRNA targeting sirtuin-3 that decreased sirtuin-3 levels and activity ~50%. As shown in Fig. 5A, transfection with a non-targeting siRNA did not induce a decline of sirtuin-3 activity in and of itself and did not potentiate the LPS-induced decline of sirtuin-3 activity. Conversely, transfection with a suboptimal concentration of siRNA targeting sirtuin-3 in combination with a non-targeting control siRNA caused a decline of sirtuin-3 activity that approximated that seen in ethanol-exposed cells (Fig. 5A). Treatment with LPS produced a further decline of sirtuin-3 activity that reached the levels seen in ethanol-exposed cells treated with LPS (Fig. 5A). Importantly, as shown in Fig. 5B, transfection with siRNA targeting sirtuin-3 and a non-targeting control did not induce inflammasome activation in and of itself, as measured by IL-1β production. However, LPS treatment of Kupffer cells that were co-transfected with siRNA targeting sirtuin-3 and a non-targeting control siRNA produced a further decline of sirtuin-3 activity in the transfected cells (Fig. 5B).

Suppression of sirtuin-3 expression recapitulates the effects of ethanol in potentiating LPS-induced inflammasome activation. Kupffer cells co-transfected with siRNA targeting sirtuin-3 and a non-targeting control did not induce inflammasome activation in and of itself and did not potentiate the LPS-induced decline of sirtuin-3 activity. Conversely, transfection with a suboptimal concentration of siRNA targeting sirtuin-3 in combination with a non-targeting control siRNA caused a decline of sirtuin-3 activity that approximated that seen in ethanol-exposed cells (Fig. 5A). Treatment with LPS produced a further decline of sirtuin-3 activity that reached the levels seen in ethanol-exposed cells treated with LPS (Fig. 5A). Importantly, as shown in Fig. 5B, transfection with siRNA targeting sirtuin-3 and a non-targeting control did not induce inflammasome activation in and of itself, as measured by IL-1β production. However, LPS treatment of Kupffer cells that were co-transfected with siRNA targeting sirtuin-3 and a non-targeting control siRNA produced a further decline of sirtuin-3 activity in the transfected cells (Fig. 5B).

DISCUSSION

The present study demonstrated that in Kupffer cells LPS-induced inflammasome activation is dependent on a rapid increase of aerobic glycolysis that is potentiated by exposure to ethanol. Critically, the conversion to aerobic glycolysis was reliant on an increase in the levels of hexokinase II bound to the mitochondria with enforced detachment of hexokinase II from the mitochondria abrogating the LPS-induced stimulation of lactate production, ROS formation, and inflammasome activation. Moreover, the LPS-induced promotion of hexokinase II binding to the mitochondria was contingent on a stimulation of cyclophilin-D activity, which in turn was mediated by a decline of sirtuin-3 activity, both of which were potentiated by ethanol exposure. Moreover, stimulation of AMPK resulted in a reversal of the LPS- and ethanol-induced suppression of sirtuin-3 activity, thus precluding the LPS- and ethanol-induced binding of hexokinase II to the mitochondria.
Distinct from conventional glycolysis, aerobic glycolysis takes place in the presence of adequate oxygenation and mitochondrial function, suggesting that it has functional importance distinct from ATP generation (5, 6, 30–33). Indeed, aerobic glycolysis is critical for the generation of metabolic intermediates that are required in cells that are

![Graph A](image1)

**A.** Kupffer cells were plated in 6-well plates at 500,000 cells/well. Following a 16-h incubation, the cells were transfected with 50 nM non-targeting siRNA or 25 nM siRNA targeting sirtuin-3 in tandem with 25 nM non-targeting siRNA. After a 24-h incubation, the cells were treated with 100 ng/ml LPS. Alternatively, Kupffer cells were isolated from ethanol-fed mice. Following a 16-h incubation, the cells were transfected with 50 nM non-targeting siRNA. After a 24-h incubation, the cells were left untreated or treated with 100 ng/ml LPS. At the time points indicated, the cells were harvested, and mitochondria were isolated. Mitochondrial lysates were prepared, and sirtuin-3 activity was determined as described in the Experimental Procedures. Values are the mean of three independent experiments with the error bars indicating S.D.

![Graph B](image2)

**B.** Kupffer cells were plated in 24-well plates at 50,000 cells/well. Following a 16-h incubation, the cells were transfected with 50 nM non-targeting siRNA or 25 nM siRNA targeting sirtuin-3 in tandem with 25 nM non-targeting siRNA. After 24 h, the cells were treated with 100 ng/ml LPS. Alternatively, the cells were pretreated with 20 μM N-HXK II for 30 min prior to LPS exposure. At the time points indicated, aliquots of medium were taken, and the level of IL-1β was determined by ELISA as described in the Experimental Procedures. Values are the mean of three independent experiments with the error bars indicating S.D.

![Graph C](image3)

**C.** Kupffer cells were plated in 24-well plates at 50,000 cells/well. Following a 16-h incubation, the cells were transfected with 50 nM non-targeting siRNA or 25 nM non-targeting siRNA targeting sirtuin-3 in tandem with 25 nM non-targeting siRNA. After 24 h, the cells were treated with 100 ng/ml LPS. Alternatively, the cells were pretreated with 20 μM N-HXK II for 30 min prior to LPS exposure. At the time points indicated, the cells were harvested, and caspase-1 activity was determined fluorscently in whole cell lysates. Values are the mean of three independent experiments with the error bars indicating S.D.

![Graph D](image4)

**D.** Kupffer cells were plated in 24-well plates at 50,000 cells/well. Following a 16-h incubation, the cells were transfected with 50 nM non-targeting siRNA or 25 nM siRNA targeting sirtuin-3 in tandem with 25 nM non-targeting siRNA. After 24 h, the cells were treated with 100 ng/ml LPS. Alternatively, the cells were pretreated with 20 μM N-HXK II for 30 min prior to LPS exposure. At the time points indicated, the cells were harvested, and caspase-1 activity was determined fluorscently in whole cell lysates. Values are the mean of three independent experiments with the error bars indicating S.D.

![Graph E](image5)

**E.** Kupffer cells were plated in 6-well plates at 500,000 cells/well. Following a 16-h incubation, the cells were transfected with 50 nM non-targeting siRNA (siN.T.) or siRNA targeting cyclophilin-D (siCyP-D). After a further 24-h incubation, the cells were treated with 100 ng/ml LPS. Alternatively, the cells were pretreated with 20 μM N-HXK II for 30 min prior to LPS treatment. At 2 h, the cells were harvested. Mitochondrial (Mito.) and cytosolic (Cyt.) fractions were prepared and utilized for Western blotting. Densitometry values are indicated below their respective bands and are the mean of three independent experiments ± S.D. AFU, arbitrary fluorescence units; VDAC-1, voltage-dependent anion channel 1.

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FIGURE 5. Suppression of sirtuin-3 expression replicates the effects of ethanol on Kupffer cell activation. A, Kupffer cells were plated in 6-well plates at 500,000 cells/well. Following a 16-h incubation, the cells were transfected with 50 nM non-targeting siRNA or 25 nM siRNA targeting sirtuin-3 in tandem with 25 nM non-targeting siRNA. After a 24-h incubation, the cells were treated with 100 ng/ml LPS. Alternatively, Kupffer cells were isolated from ethanol-fed mice. Following a 16-h incubation, the cells were transfected with 50 nM non-targeting siRNA. After a 24-h incubation, the cells were left untreated or treated with 100 ng/ml LPS. At the time points indicated, the cells were harvested, and mitochondria were isolated. Mitochondrial lysates were prepared, and sirtuin-3 activity was determined as described under “Experimental Procedures.” Values are the mean of three independent experiments with the error bars indicating S.D. B, Kupffer cells were plated in 24-well plates at 50,000 cells/well. Following a 16-h incubation, the cells were transfected with 50 nM non-targeting siRNA. After a 24-h incubation, the cells were left untreated or treated with 100 ng/ml LPS. The time points indicated, the cells were harvested, and mitochondria were isolated. Mitochondrial lysates were prepared, and sirtuin-3 activity was determined as described under “Experimental Procedures.” Values are the mean of three independent experiments with the error bars indicating S.D.
rapidly synthesizing proteins, lipids, DNA, and RNA (8, 9, 34, 35). Such conditions are prevalent in activated immune cells and in cancer cells. By being positioned at the interface of mitochondrial oxidative phosphorylation and glycolysis, mitochondrially bound hexokinase II is capable of funneling the ATP produced by the efficiency of oxidative phosphorylation to the production of glucose 6-phosphate. However, instead of being exclusively utilized by the glycolytic path-
way, some of the glucose 6-phosphate generated by hexokinese II can be shunted to the pentose phosphate pathway. The PPP, also known as the hexose monophosphate shunt, has been known to be activated in stimulated immune cells (36–38). NADPH is a primary metabolic intermediate generated in the PPP and is critical to meet the demands of reductive biosynthesis and for the stimulation of NADPH oxidase activity, which is partially responsible for the respiratory burst characteristic of activated macrophages. Indeed, we demonstrated that mitochondrially bound hexokinase II is necessary for LPS and ethanol to elevate the NADPH/NADP⁺ ratio with detachment of hexokinase II from the mitochondria preventing NADPH oxidase activation. However, a significant proportion of the glucose 6-phosphate produced proceeds through glycolysis and results in the end product of lactate. Our data demonstrate that LPS and ethanol induced a marked increase in the extracellular concentration of lactate, which was dependent on mitochondrially bound hexokinase II. Importantly, lactate-induced acidosis has been demonstrated to boost TLR-4 signaling (39, 40).

Remarkably, AMPK activation has been shown to have anti-inflammatory effects and prevents the metabolic switch to aerobic glycolysis in LPS-stimulated dendritic cells (7). Our data suggest that this effect of AMPK is mediated by preventing the elevated binding of hexokinase II to the mitochondria. We demonstrated that AMPK activation reverses the inhibitory effect of ethanol exposure on sirtuin-3 activity, thereby preventing the ethanol-induced activation of NADPH oxidase and the increased expression of pro-apoptotic proteins (20, 41–43). Additionally, the ability of AMPK to increase the immediate alcoholic liver disease has been shown to be dependent on inflammasome activation (4). In contrast to Kupffer cells, ethanol exposure sensitizes hepatocytes to injury that is mediated in part through opening of the permeability transition pore (44, 45). The ethanol-induced potentiation of the permeability transition pore is mediated partly by the ethanol-induced suppression of sirtuin-3 activity, leading to a concomitant stimulation of cyclophilin-D (46–48). However, in contrast to Kupffer cells, hepatocytes do not express hexokinase II, resulting in an increase in the sensitivity of ethanol-exposed hepatocytes to TNF-induced cytotoxicity (22, 23). By contrast, when hexokinase II is present, the stimulation of cyclophilin-D activity increases the level of mitochondrially bound hexokinase II, resulting in a decrease in the sensitivity of mitochondria to injury. Therefore, in Kupffer cells, the robust binding of hexokinase II to mitochondria brought about by LPS and ethanol results not only in a stimulation of aerobic glycolysis but may also protect the Kupffer cells from mitochondrial damage and apoptosis brought about by the increased levels of ROS produced by NADPH oxidase and the increased expression of pro-apoptotic proteins.

In summary, our present study positions mitochondrial hexokinase II as a critical factor in Kupffer cell activation (6). LPS and ethanol induced sirtuin-3 activity declined, while cyclophilin-D activity and a resultant loss in hexokinase II to mitochondria. Suppression of cyclophilin-D expression or enforced detachment of hexokinase II from the mitochondria prevented the LPS-induced translocation of hexokinase II to the mitochondria, and inflammasome activation. Ethanol exposure antagonized the decline of sirtuin-3 activity brought about by LPS, thereby amplifying the LPS-induced translocation of hexokinase II to the mitochondria and inflammasome activation. Moreover, activation of AMPK prevented the LPS- and ethanol-induced decline of sirtuin-3 activity, thereby preclud-

**FIGURE 6.** Activation of AMPK reverses the inhibition of sirt-3 activity mediated by LPS and ethanol and the potentiating effect on Kupffer cell stimulation. A, Kupffer cells isolated from control- or ethanol-fed rats were plated in 6-well plates at 500,000 cells/well and incubated for 16 h. Following incubation, the cells were transfected with 50 nm non-targeting siRNA. After 24 h, the cells were then treated with 100 ng/ml LPS. Alternatively, the cells were pretreated with 0.5 mM AICAR for 30 min prior to exposure to LPS. At the time points indicated, the cells were harvested, and mitochondria were isolated. Mitochondrial lysates were prepared, and sirtuin-3 activity was determined as described under “Experimental Procedures.” Values are the means of three independent experiments with the error bars indicating S.D. B, Kupffer cells isolated from control- or ethanol-fed rats were plated in 6-well plates at 500,000 cells/well and incubated for 16 h. The cells were then transfected with 50 nm non-targeting siRNA or siRNA targeting sirtuin-3 and incubated for a further 24 h. The cells were then treated with 100 ng/ml LPS. Alternatively, the cells were pretreated with 0.5 mM AICAR for 30 min prior to exposure to LPS. At the time points indicated, the cells were harvested, and mitochondria were isolated. Mitochondrial lysates were prepared, and sirtuin-3 activity was determined as described under “Experimental Procedures.” Values are the means of three independent experiments with the error bars indicating S.D. C, Kupffer cells isolated from control- or ethanol-fed rats were plated in 6-well plates at 50,000 cells/well and incubated for 16 h. The cells were then transfected with 50 nm non-targeting siRNA or siRNA targeting sirtuin-3 and incubated for a further 24 h. The cells were then treated with 100 ng/ml LPS. Alternatively, the cells were pretreated with 0.5 mM AICAR for 30 min prior to exposure to LPS. At the time points indicated, the cells were harvested, and caspase-1 activity was determined fluorescently in whole cell lysates. Values are the means of three independent experiments with the error bars indicating S.D. D, Kupffer cells isolated from control- or ethanol-fed rats were plated in 6-well plates at 50,000 cells/well and incubated for 16 h. The cells were then transfected with 50 nm non-targeting siRNA or siRNA targeting sirtuin-3 and incubated for a further 24 h. The cells were then treated with 100 ng/ml LPS. Alternatively, the cells were pretreated with 0.5 mM AICAR for 30 min prior to exposure to LPS. At the time points indicated, the cells were harvested, and caspase-1 activity was determined fluorescently in whole cell lysates by ELISA as described under “Experimental Procedures.” Values are the means of three independent experiments with the error bars indicating S.D. AFU, arbitrary fluorescence units; VDAC-1, voltage-dependent anion channel 1.
Hexokinase and Macrophage Activation

![Diagram of Hexokinase and Mitochondria](image_url)

The pathway by which Kupffer cell activation mediates the increased binding of hexokinase II to the mitochondria (Mito-HXK II) and its consequences for reductive biosynthesis, ROS formation, and acidosis are shown. CyP-D, cyclophilin-D.

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