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ARTICLE

Nicotinamide riboside, an NAD^+ precursor, attenuates inflammation and oxidative stress by activating sirtuin 1 in alcohol-stimulated macrophages

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Abstract

Macrophages play an essential role in alcohol-induced inflammation and oxidative stress. We investigated the effects of nicotinamide riboside (NR), a natural nicotinamide adenine dinucleotide (NAD^+) precursor, on alcohol-induced inflammation and oxidative stress in macrophages. NR significantly decreased ethanol-induced inflammatory gene expression, with a concomitant decrease in nuclear translocation of nuclear factor κB p65 in RAW 264.7 macrophages and mouse bone marrow-derived macrophages (BMDMs). In macrophages incubated with ethanol or acetaldehyde, NR abolished the accumulation of cellular reactive oxygen species. Ethanol decreased sirtuin 1 (SIRT1) expression and activity, and cellular NAD^+ level while inducing pro-inflammatory gene expression. However, NR markedly attenuated the changes. SIRT1 inhibition augmented ethanol-induced inflammatory gene expression, but its activation elicited opposing effects. Also, ethanol did not alter glycolysis but increased glycolytic capacity, glycolytic reserve, and non-glycolytic acidification, with concomitant increases in hypoxia-induced factor 1α expression and activity, phosphorylation of pyruvate dehydrogenase, and extracellular lactate levels. Interestingly, ethanol increased mitochondrial respiration and ATP production but decreased maximal respiration and spare respiration capacity. The latter was linked to decreases in mitochondrial copy numbers. NR abolished the ethanol-induced metabolic changes in the glycolytic and oxidative phosphorylation pathways in RAW 264.7 macrophages. In conclusion, NR exerts anti-inflammatory and antioxidant properties by abrogating the inhibitory effects of ethanol on the SIRT1 pathway by increasing *Sirt1* expression and its activator, NAD^+. Also, SIRT1 activation and normalization of ethanol-induced changes in NAD^+/NADH ratios by NR are likely crucial to counteract the changes in energy phenotypes of macrophages exposed to ethanol.

Introduction

Chronic alcohol consumption triggers inflammation and oxidative stress, leading to alcohol-associated diseases, such as alcoholic liver disease [1–3]. Alcohol-induced diseases were responsible for ~6% of all global deaths in 2016, according to a report by the World Health Organization [4]. Macrophages are crucial players for the pathogenesis of alcoholic diseases as they produce pro-inflammatory cytokines and reactive oxygen species (ROS) when exposed to alcohol [2, 3].

Nicotinamide adenine dinucleotide (NAD) is an essential coenzyme for multiple NAD-consuming enzymes involved in mitochondrial oxidative phosphorylation and energy metabolism [5]. During ethanol oxidation to acetate, NAD^+ is consumed, consequently decreasing the ratios of NAD^+/nicotinamide adenine dinucleotide (NADH) [6, 7]. NAD also acts as a cofactor of sirtuin 1 (SIRT1), a class III histone deacetylase, whose activity is controlled by cellular NAD^+/NADH ratio [8, 9]. SIRT1 is known to regulate inflammation and oxidative stress in alcohol-stimulated hepatocytes and macrophages [10–12]. As decreased cellular NAD^+ level may contribute to alcohol-related dysfunctions in cells and tissues [13], the supply of NAD^+ may be necessary to counteract alcohol-induced inflammation and oxidative stress.

Nicotinamide riboside (NR), an NAD^+ precursor, makes up ~40% of pro-vitamin B3 in cow milk [14, 15]. NR becomes bioavailable NAD^+ after the reactions by

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nicotinamide riboside kinase (NRK) and nicotinamide mononucleotide adenylyltransferases (NMNAT) or nicotinamide (NAM) salvage pathway [16]. Mounting evidence shows that NR increases NAD^+ level in metabolically active tissues, e.g., skeletal muscle, liver, and brown adipose tissue, consequently enhancing mitochondrial biogenesis and oxidative metabolism to protect against metabolic diseases [17, 18]. Also, NR prevents and reverses nonalcoholic fatty liver disease via a SIRT1-dependent mitochondrial unfolded protein response, inducing β-oxidation and mitochondrial complex content and activity [19]. Previously, we showed that NR attenuated the development of liver fibrosis in a diet-induced obesity mouse model, which was attributed, at least in part, to its inhibition of hepatic stellate cell activation [20]. Also, NR elevated hepatic NAD^+ levels, preventing alcohol-induced liver damage by activating SIRT1 in mice [10].

Macrophages play an essential role in alcohol-induced cell damages [21, 22]. However, little is known about the effects of NR on alcohol-induced inflammation and oxidative stress in macrophages. In the present study, we explored whether NR can inhibit inflammation and oxidative stress in macrophages exposed to ethanol with a primary focus on the role of SIRT1 therein. Also, we determined the effect of NR on the regulation of energy metabolism, i.e., glycolysis and mitochondrial respiration, to extend our understanding of alcohol-induced changes in the metabotypes of macrophages.

Materials and methods

Cell culture and treatment

Murine RAW 264.7 macrophages (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 media, as we previously described [23]. Bone marrow-derived macrophages (BMDMs) were prepared from the tibia and femur of C57BL/6 J mice and cultured, as previously described [23]. All cells were maintained in 5% CO_2 at 37 °C. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Connecticut.

NR was kindly provided by ChromaDex Corporate (Irvine, CA, USA). Sirtinol, a SIRT1 inhibitor, was purchased from Cayman Chemical (Ann Arbor, MI, USA); and resveratrol, a natural SIRT1 activator, was from Sigma-Aldrich (St. Louis, MO, USA). Cells were treated with 80 mM ethanol (Sigma-Aldrich) for 72 h in the absence or presence of 1 mM of NR with a daily media change. Sirtinol and resveratrol stock solution (10 mM) were prepared in dimethyl sulfoxide and stored at −80 °C until use. Sirtinol and resveratrol were used for experiments at the final concentrations of 15 μM and 10 μM, respectively.

Total RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Reverse transcription for cDNA synthesis and qRT-PCR analysis were performed as previously described [20, 24] using Bio-Rad CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA, USA). Primers were designed according to GenBank database using the Beacon Designer 7 software (PREMIER Biosoft International, Palo Alto, CA, USA), and primer sequences will be available upon request.

Western blot analysis

Total cell lysate preparation and Western blot analysis were conducted as described [20, 24]. Cytosolic and nuclear proteins of cells were isolated using a Nuclear Extraction Kit (Cayman Chemical) according to the company's instruction. Antibodies for p65, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and TATA-binding protein (TBP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for pyruvate dehydrogenase (PDH) and p-PDH were obtained from Abcam (Cambridge, MA, USA). SIRT1 and glucose transporter 1 (GLUT1) antibodies were from Cell Signaling Technology (Danvers, MA, USA). GAPDH, TBP, and REVERT Total Protein Stain (LI-COR, Lincoln, NE, USA) were used as a loading control for data normalization in cytosolic, nuclear, and total protein, respectively. The blots were imaged and quantified using a Chemidoc XRS + system (Bio-Rad) using Image Lab software (Bio-Rad) or an LI-COR Odyssey CLx (LI-COR) with LI-COR Image Studio software.

Cellular ROS measurement

Cellular ROS levels were quantified using 2',7'-dichlorofluorescein (DCFH; Sigma-Aldrich, St. Louis, MO, USA) as we previously described [25].

Enzyme-linked immunosorbent assay (ELISA) for tumor necrosis factor α (TNFα)

After RAW 264.7 macrophages were treated with 80 mM ethanol in the absence or presence of 1 mM of NR for 72 h, a conditioned medium was collected to measure secreted TNFα using a TNF alpha mouse uncoated ELISA kit (eBioscience, San Diego, CA, USA) following the manufacturer's protocol.

SIRT1 activity measurement

Non-cell-based SIRT1 activity was determined using a SIRT1 Direct Fluorescent Screening Assay Kit (Cayman

Chemical) following the manufacturer's protocol. For the measurement of cell-based SIRT1 activity, RAW 264.7 macrophages were treated with 80 mM ethanol in the absence or presence of 1 mM of NR for 72 h with daily media change. Then nuclear protein was extracted for SIRT1 deacetylase activity using a fluorometric activity assay kit (Abcam, Cambridge, MA, USA). The fluorescence signal was measured at 340 nm excitation and 460 nm emission using a BioTek Synergy Mx (BioTek, Winooski, VT, USA) microplate reader.

Cellular NAD^+ measurement

Cellular NAD^+ levels were measured as we previously described [20] and expressed as pmol per 1 $\times 10^6$ cells.

Mitochondrial DNA copy number

Total DNA was isolated from RAW 264.7 macrophages treated with 80 mM ethanol in the absence or presence of 1 mM of NR for 72 h using NucleoSpin Tissue (MACHEREY-NAGEL Inc., Germany). Mitochondrial DNA copy number was determined as we previously described using 16S ribosomal RNA (*16S*) and cytochrome b (*CyB*) as mitochondrial genes and hexokinase 2 (*Hk2*) as a nuclear gene [26].

Measurement of extracellular lactate levels

Conditioned medium was collected after RAW 264.7 macrophages were treated with 80 mM ethanol in the absence or presence of 1 mM of NR for 72 h. Extracellular lactate levels were determined using an L-lactate assay kit (Cayman Chemical) following the manufacturer's protocol.

Energy phenotypes of cells

After RAW 264.7 macrophages were treated with 80 mM ethanol in the absence or presence of 1 mM of NR for 72 h, cells were subjected to an XFe24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA) for Mito Stress test and Glycolysis test (Seahorse Biosciences) as previously described [27]. Once the assay was finished, total DNA was isolated from each well using NucleoSpin Tissue (MACHEREY-NAGEL Inc.) to normalize the data.

Hypoxia-inducible factor 1α (HIF-1α) activity assay

After RAW 264.7 macrophages were treated with 80 mM ethanol in the absence or presence of 1 mM of NR for 72 h, nuclear proteins of cells were collected to measure HIF1α activity using an HIF-1 alpha Transcription Factor Assay Kit (Abcam) following the manufacturer's protocol.

Statistical analyses

One-way analysis of variance (ANOVA) and Newman-Keuls post hoc test or unpaired t-test was used to detect significant differences between groups using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA). Data were considered statistically significant at $P < 0.05$. Values are expressed as mean ± SEM.

Results

NR repressed the induction of pro-inflammatory gene expression by ethanol via the inhibition of the NFκB pathway in macrophages

The ethanol exposure for 72 h significantly increased mRNA levels of interleukin (*Il*)*1b*, *Il6*, and *Tnf*, which were significantly decreased by NR in RAW 264.7 macrophages (Fig. 1A). Consistently, NR significantly decreased TNFα secretion from ethanol-treated macrophages (Fig. 1B). Cytosolic and nuclear NFκB p65 levels were markedly increased by ethanol; however, NR completely abolished the increase in RAW 264.7 macrophages (Fig. 1C). Similar inhibitory effects of NR on inflammatory gene expression were observed in BMDMs (Fig. 1D).

NR repressed ethanol-induced ROS accumulation in macrophages

Alcohol increases ROS accumulation and oxidative stress, as oxidative free radicals and electrons are generated during the oxidation of alcohol [2, 6]. Ethanol and its metabolite, acetaldehyde, significantly increased cellular ROS levels, but NR completely abolished the increase to a basal level in RAW 264.7 macrophages (Fig. 2A). Also, mRNA and protein levels of cytochrome b-245, beta polypeptide (*Cybb*), a ROS-producing NADPH oxidase 2 (NOX2), were increased by ethanol, which was abrogated by NR (Fig. 2B). Similar inhibitory effects of NR on cellular ROS levels and *Cybb* mRNA expression were observed in BMDMs (Fig. 2C). mRNA levels of nuclear factor E2-related factor 2 (*Nfe212*) and other antioxidant genes, including catalase (*Cat*), superoxide dismutase 1 (*Sod1*), glutathione peroxidase 1 (*Gpx1*), and *Gpx4*, were significantly increased by ethanol in RAW 264.7 macrophages (Fig. 2D). NR significantly attenuated the increase. Interestingly, NR decreased *Sod2* mRNA level in RAW 264.7 macrophages stimulated with ethanol, although ethanol did not induce the gene expression.

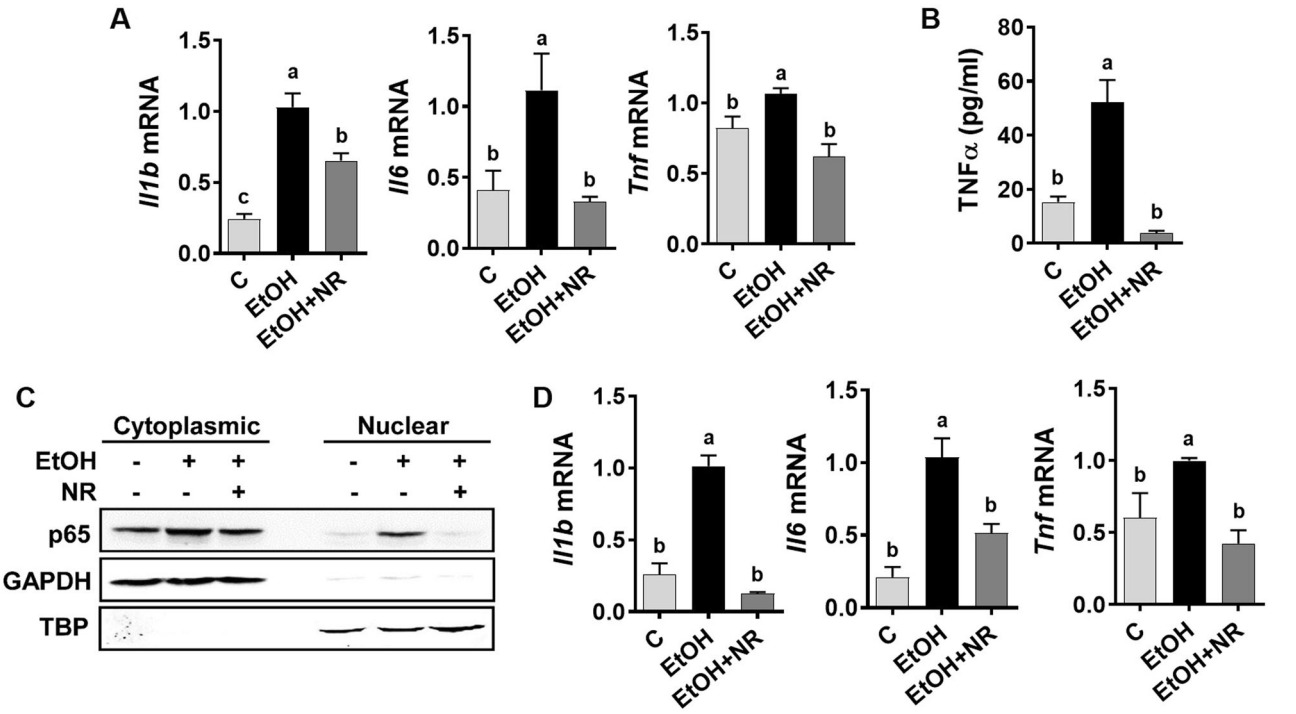

Fig. 1 NR inhibits ethanol-induced inflammation by inhibiting NFκB nuclear translocation in macrophages. RAW 264.7 macrophages were treated with 1 mM NR and 80 mM EtOH for 72 h for gene analysis (**A**), TNFα secretion (**B**) nuclear translocation of NFκB p65 (**C**). GAPDH and TBP were used for the purity of cytoplasmic and nuclear fractions, respectively. A representative blot image is shown. **D** BMDMs were treated with 1 mM NR and 80 mM EtOH for 72 h for gene analysis. C, control. Mean ± SEM. Bars without sharing a common letter are significantly different ($P < 0.05$).

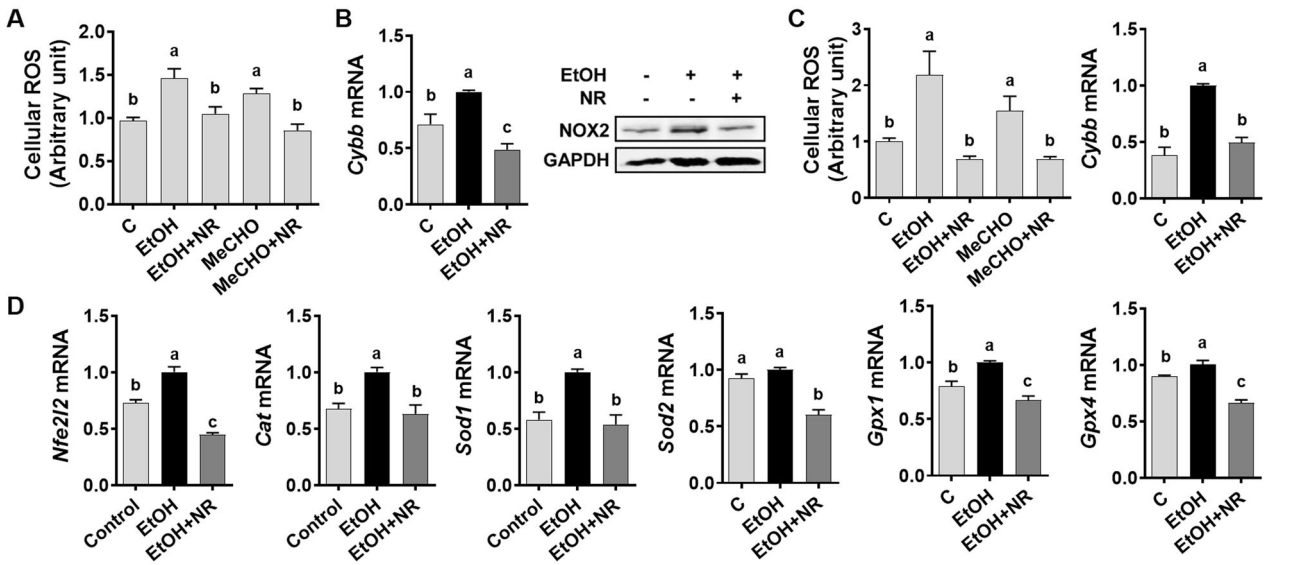

Fig. 2 Increases in cellular ROS accumulation and antioxidant gene expression by ethanol were inhibited by NR in macrophages. RAW 264.7 macrophages were treated with 80 mM EtOH, 200 μM MeCHO, and 1 mM NR for 72 h for ROS accumulation (**A**), *Cybb* mRNA and protein expression (**B**), and antioxidant gene expression (**D**). **C** BMDMs were treated with 80 mM EtOH, 200 μM MeCHO, and 1 mM NR for 72 h for cellular ROS accumulation and *Cybb* expression. C, control. A representative blot image is shown. Mean ± SEM. Bars without sharing a common letter are significantly different ($P < 0.05$).

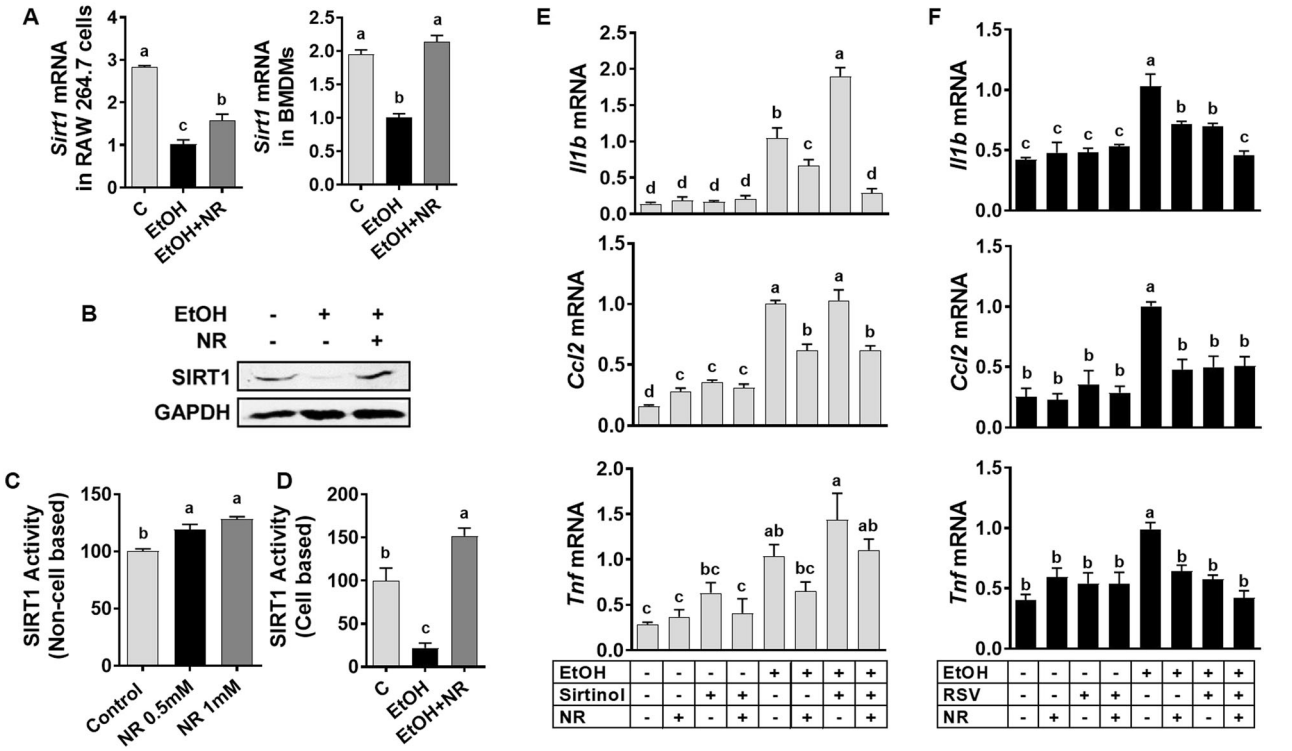

Fig. 3 NR inhibits ethanol-induced decreases in SIRT1 expression and activity in macrophages. **A** RAW 264.7 macrophages and BMDMs were treated with 1 mM NR and 80 mM EtOH for 72 h for *Sirt1* mRNA measurement. **B** RAW 264.7 macrophages were treated as described above for Western blot analysis. A representative blot image is shown. **C** Non-cell-based SIRT1 activity. **D** RAW 264.7 macrophages were treated with NR and EtOH as described above for cell-based SIRT1 activity. RAW 264.7 macrophages were treated with 80 mM EtOH, 1 mM NR in the presence or absence of 15 μM sirtinol (**E**) or 10 μM resveratrol (**F**) for 72 h for gene analysis. C, control. Mean ± SEM. Bars without sharing a common letter are significantly different ($P < 0.05$).

Ethanol decreased SIRT1 expression and activity, which was abolished by NR in macrophages

SIRT1 is known to inhibit the production of pro-inflammatory cytokines and ROS accumulation in macrophages [28, 29]. We demonstrated ethanol metabolites, such as acetaldehyde and acetate, decreased *Sirt1* expression in macrophages [12]. Therefore, we next determined whether NR can abrogate the repressive effect of ethanol on *Sirt1* expression in macrophages. Ethanol decreased *Sirt1* mRNA, and NR attenuated the decrease in RAW 264.7 macrophages and BMDMs (Fig. 3A). Decreased SIRT1 protein by ethanol was restored by NR in RAW 264.7 macrophages (Fig. 3B). In a non-cell-based assay, NR significantly increased SIRT1 activity, indicating a potential direct activation of SIRT1 by NR (Fig. 3C). In a cell-based SIRT1 activity assay, SIRT1 activity decreased profoundly by ethanol (Fig. 3D). However, NR abolished the decrease and further elevated SIRT1 activity in RAW 264.7 macrophages.

The contribution of SIRT1 to the anti-inflammatory effect of NR in macrophages exposed to ethanol was evaluated using sirtinol, a SIRT1 inhibitor [30, 31], and resveratrol, a natural SIRT1 activator [31, 32]. It should be noted that resveratrol also activates AMP-activated protein kinase, which also leads to SIRT1 activation [33, 34]. In unstimulated RAW 264.7 macrophages, sirtinol and NR modestly affected the expression of C-C motif chemokine ligand 2 (*Ccl2*), *Il1b*, and *Tnf* (Fig. 3E). Ethanol significantly increased the pro-inflammatory gene expression, which was further induced by sirtinol in RAW 264.7 macrophages, but NR markedly attenuated the effect of ethanol and sirtinol (Fig. 3E). Resveratrol and NR did not alter the expression of pro-inflammatory genes in unstimulated RAW 264.7 macrophages (Fig. 3F). However, increased *Il1b*, *Ccl2*, and *Tnf* mRNA levels by ethanol were significantly abrogated by NR or resveratrol alone. In the combination of NR and resveratrol, there was no further reduction in ethanol-stimulated *Ccl2* and *Tnf* mRNA levels. Only *Il1b* mRNA level was decreased by NR and resveratrol in combination to a greater extent in ethanol-stimulated macrophages.

Ethanol decreased cellular NAD+ level and the expression of genes involved in the NAD+ salvage pathway, which were abolished by NR in macrophages

As SIRT1 requires NAD^+ as a cofactor for their activation [9, 10], we measured cellular NAD^+ levels in macrophages.

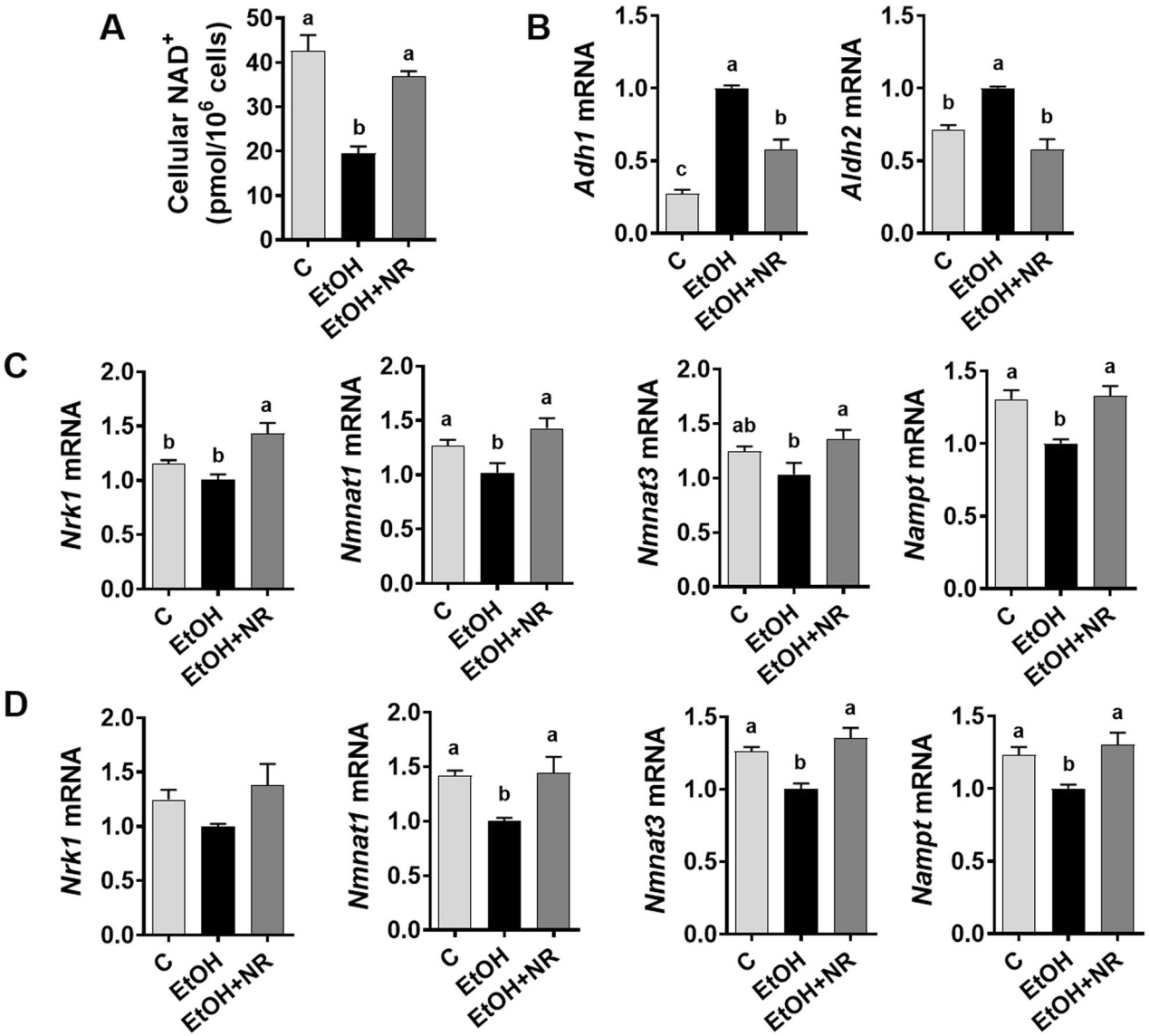

Fig. 4 NR abrogates ethanol-induced decrease in cellular NAD$^+$ levels and genes involved in the NAD$^+$ salvage pathway in macrophages. RAW 264.7 macrophages were treated with 1 mM NR and 80 mM EtOH for 72 h for cellular NAD$^+$ measurement (**A**) and gene analysis (**B**, **C**). **D** BMDMs were treated the same as RAW 264.7 macrophages for gene analysis. C, control. Mean ± SEM. Bars without sharing a common letter are significantly different ($P < 0.05$).

Ethanol significantly reduced cellular NAD$^+$ level by ~50%, but the decrease was restored by NR in RAW 264.7 macrophages (Fig. 4A). Alcohol dehydrogenase 1 (*Adh1*) and acetaldehyde dehydrogenase 2 (*Aldh2*) catalyze the oxidation of ethanol and acetaldehyde by consuming NAD$^+$, respectively [7, 35], leading to an increase in the conversion of NAD$^+$ to NADH. Both *Adh1* and *Aldh2* mRNA abundance was significantly increased by ethanol, but NR completely attenuated the increase in RAW 264.7 macrophages (Fig. 4B).

Next, we measured the expression of enzymes related to the production of NAD$^+$ in the macrophages. NR increased the expression of nicotinamide riboside kinase 1 (*Nrk1*) in RAW 264.7 macrophages (Fig. 4C), which plays an essential role in regulating hepatic NAD$^+$ biosynthesis [36]. NR is converted to nicotinamide mononucleotide (NMN) by NRK1, and NMN becomes NAD$^+$ by nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) in the nucleus and NMNAT3 in mitochondria [16]. Nicotinamide phosphoribosyltransferase (NAMPT) is another important enzyme for NAD$^+$ production, which catalyzes the conversion of nicotinamide (NAM) to NMN, which are metabolites of NR in the NAD$^+$ salvage pathway. While *Nrk1* mRNA was not altered by ethanol, *Nmnat1*, *Nmnat3* and *Nampt* mRNA levels were decreased by ethanol, which was abolished by NR in RAW 264.7 macrophages (Fig. 4C) and BMDMs (Fig. 4D).

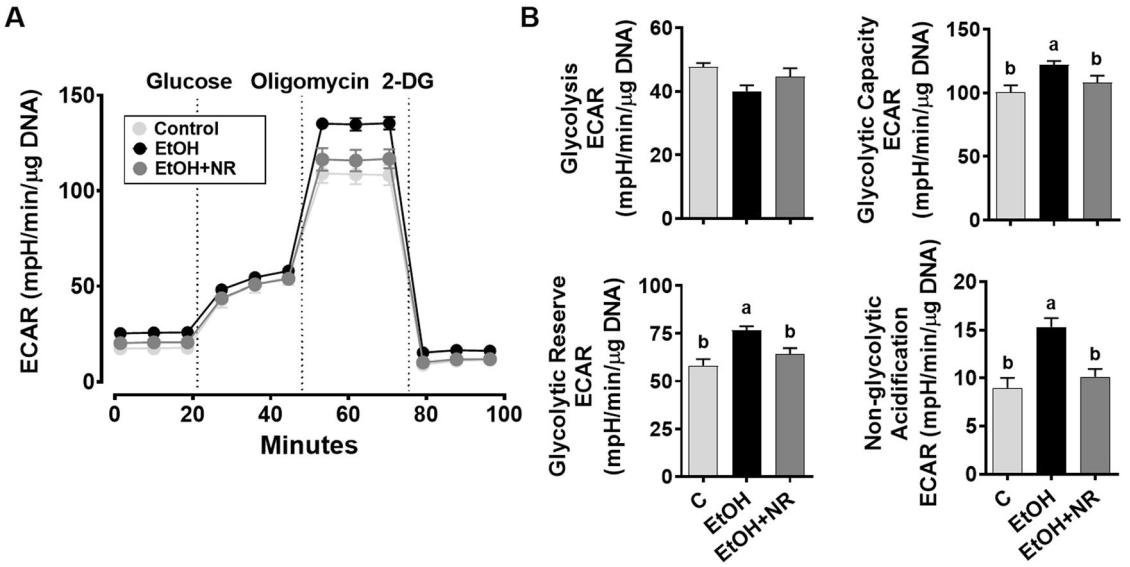

Fig. 5 Increased glycolytic capacity and reserve by ethanol was abrogated by NR in RAW 264.7 macrophages. Cells were treated with 1 mM NR and 80 mM EtOH for 72 h for glycolysis stress tests (**A**, **B**). C, control. Mean ± SEM. Bars without sharing a common letter are significantly different ($P < 0.05$).

NR attenuated the ethanol-induced glycolytic capacity of RAW 264.7 macrophages

Evidence suggests that metabolic pathways to generate energy are closely linked to immune systems, and macrophages can switch metabolic phenotypes depending on extracellular stimuli [37, 38]. Therefore, we investigated the potential role of NR in the modulation of glycolysis in macrophages exposed to ethanol. Glycolysis rates were not significantly altered by ethanol in RAW 264.7 macrophages (Fig. 5A, B). However, ethanol significantly increased glycolytic capacity, glycolytic reserve, and non-glycolytic acidification, all of which were abolished by NR.

Next, we determined the effect of ethanol and NR on the expression of genes that regulate glucose entry to cells and pyruvate to mitochondrial tricarboxylic acid (TCA) cycle. Hypoxia-induced factor 1α (HIF-1α) regulates the expression of glucose transporter 1 (GLUT1), which is the primary cell membrane glucose transporter in macrophages [39]. Hexokinase 1 (HK1) is also vital for glucose entry into macrophages by phosphorylating glucose to glucose-6-phosphate. *Hif1a*, *Glut1*, and *Hk1* mRNA levels were significantly induced by ethanol, but NR completely abrogated the induction in RAW 264.7 macrophages (Fig. 6A). While ethanol increased GLUT1 protein by ~4-fold, NR inhibited the increase close to the basal level (Fig. 6B). Consistently, HIF-1α activity was significantly increased by ethanol but decreased by NR (Fig. 6C).

Pyruvate, the end-product of glycolysis, enters the mitochondria and is converted to acetyl-CoA by pyruvate dehydrogenase (PDH) complex [40]. Phosphorylation of PDH by PDH kinase (PDK) inhibits the enzyme activity [41]. Alternatively, pyruvate is reduced to lactate in the cytosol by lactate dehydrogenase (LDH) by regenerating NAD^+. Therefore, these enzymes determine the metabolic fate of pyruvate. The mRNA levels of *Pdk1* and *Ldha* were significantly increased by ethanol, which was abolished by NR (Fig. 6D). PDH phosphorylation, which decreases the entry of pyruvate to the citric acid cycle, was increased by ethanol but significantly decreased by NR (Fig. 6E). Ethanol reduces the cytosolic NAD^+/NADH ratio by consuming NAD^+ to metabolize ethanol, favoring pyruvate conversion to lactate in the liver [10]. Extracellular lactate levels were increased by ethanol but significantly decreased by NR (Fig. 6F). As pyruvate can be generated from acetaldehyde catalyzed by pyruvate decarboxylase under NAD^+ deficiency condition [42], it is not surprising to observe glycolysis was not altered while lactate was increased with ethanol treatment.

NR regulated ethanol-induced mitochondrial respiration in RAW 264.7 macrophages

We further investigated the effect of ethanol and NR on mitochondrial respiration in RAW 264.7 macrophages. Ethanol increased basal respiration, ATP production, and proton leak while decreasing maximal respiration and spare respiratory capacity, all of which were abolished by NR (Fig. 7A, B). Next, we evaluated the effect of ethanol and NR on the expression of genes important for mitochondrial

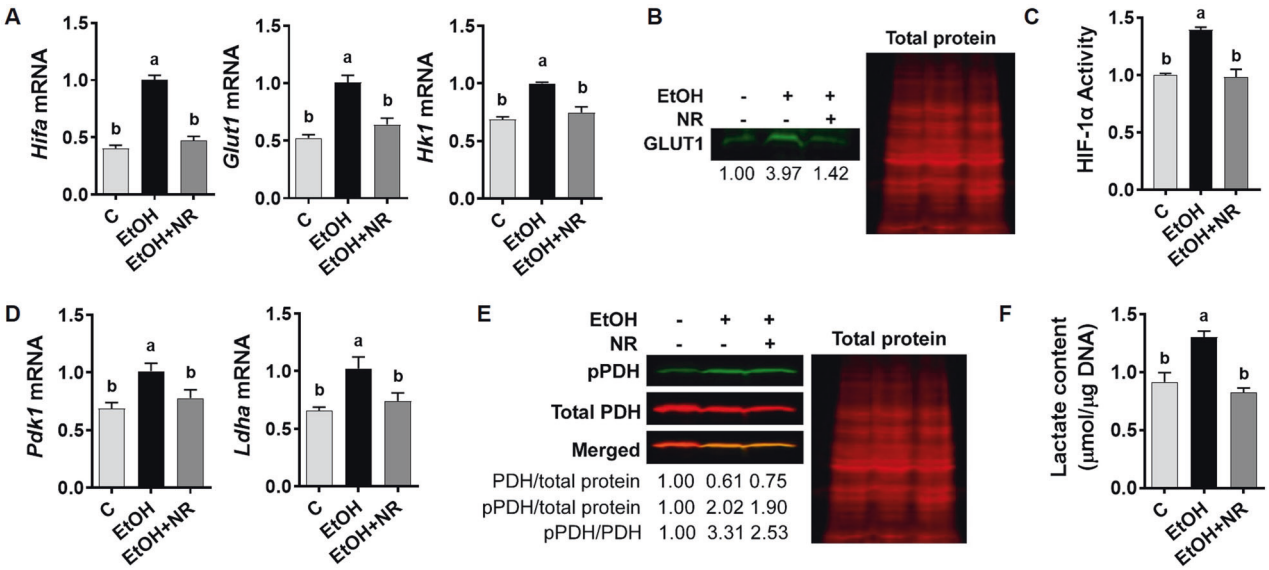

Fig. 6 NR attenuates changes in the factors that control energy substrate flow into glycolysis and TCA cycle in RAW 264.7 macrophages. Cells were treated with 1 mM NR and 80 mM EtOH for 72 h for gene analysis. **A**, **D** Western blot for protein measurement (**B**, **E**), HIF-1α activity assay (**C**), and extracellular lactate contents (**F**). C, control. For Western blot analysis, a representative blot image is shown and densitometry analysis was conducted using total protein for normalization, which are under blot images. Mean ± SEM. Bars without sharing a common letter are significantly different ($P < 0.05$).

biogenesis. mRNA levels of peroxisome proliferator-activated receptor γ coactivator 1α (*Ppargc1a*) and *Ppargc1b*, crucial activators of mitochondrial biogenesis [43, 44], were significantly decreased by ethanol, but NR attenuated the repression (Fig. 7C). Consistently, the ratios of mitochondrial to genomic DNA, indicative of mitochondrial copy numbers, were significantly decreased by ethanol but NR abolished the decrease (Fig. 7D).

Discussion

Increased ROS generation by alcohol disrupts redox homeostasis, which can activate the inflammatory responses in macrophages [2, 3]. We evaluated the effect of NR on inflammation and oxidative stress in ethanol-stimulated macrophages to explore its therapeutic potential for alcohol-associated inflammatory diseases. In the present study, we found that NR activates SIRT1 by inducing its expression with a concomitant increase in cellular NAD^+ levels, consequently inhibiting inflammation and oxidative stress in macrophages exposed to ethanol. Importantly, NR abolished ethanol-triggered changes in glucose metabolism and mitochondrial respiration by regulating SIRT1 activity, which may contribute to the anti-inflammatory and antioxidant properties. As inflammatory macrophages exhibit a metabolic shift to aerobic glycolysis [45, 46], NR may counteract the changes in energy phenotypes of macrophages when they are activated by ethanol.

Ethanol is oxidized into acetaldehyde by ADH, which is further converted to acetate by ALDH [7, 35]. NAD^+ is consumed during the reactions by serving as an intermediate electron carrier and reduced to NADH [2, 47]. As NAD^+ is a cofactor of SIRT1 [9, 10], oxidation of ethanol can diminish SIRT1 activity by depleting cellular NAD^+ levels. Indeed, we found that ethanol decreased cellular NAD^+ levels, along with a significant increase in *Adh1* and *Aldh2* mRNA levels in macrophages. NR treatment abolished the changes and maintained NAD^+ levels in macrophages exposed to ethanol. Notably, NR appeared to increase NAD^+ levels not only by providing an NAD^+ precursor but increasing the expression of genes involved in the NAD^+ salvage pathway for its conversion to NAD^+. NRKs phosphorylate NR to NMN, which is an intermediate of NAD^+ synthesis [48]. In the NAD^+ salvage pathway, nicotinamide (NAM) is recycled by NAMPT to NMN, which is converted to NAD^+ by NMNATs [16, 49]. In the present study, ethanol decreased the expression of enzymes in the NAD^+ salvage pathways, while NR attenuated the decrease. Studies have shown that *Nrk1* overexpression elevates NAD^+ levels in the mouse liver [36], and NAMPT promotes NAD^+ production under oxidative stress to maintain mitochondrial NAD^+ levels [50]. Besides, treatment of NMN, which is an intermediate of NAD^+ synthesis by NMNATs, attenuates oxidative stress in aged cerebromicrovascular endothelial cells [51]. NMN supplementation also promotes neurovascular rejuvenation through SIRT1 activation, mitochondrial protection, and anti-inflammatory effects in aged mice [52] and in the aorta

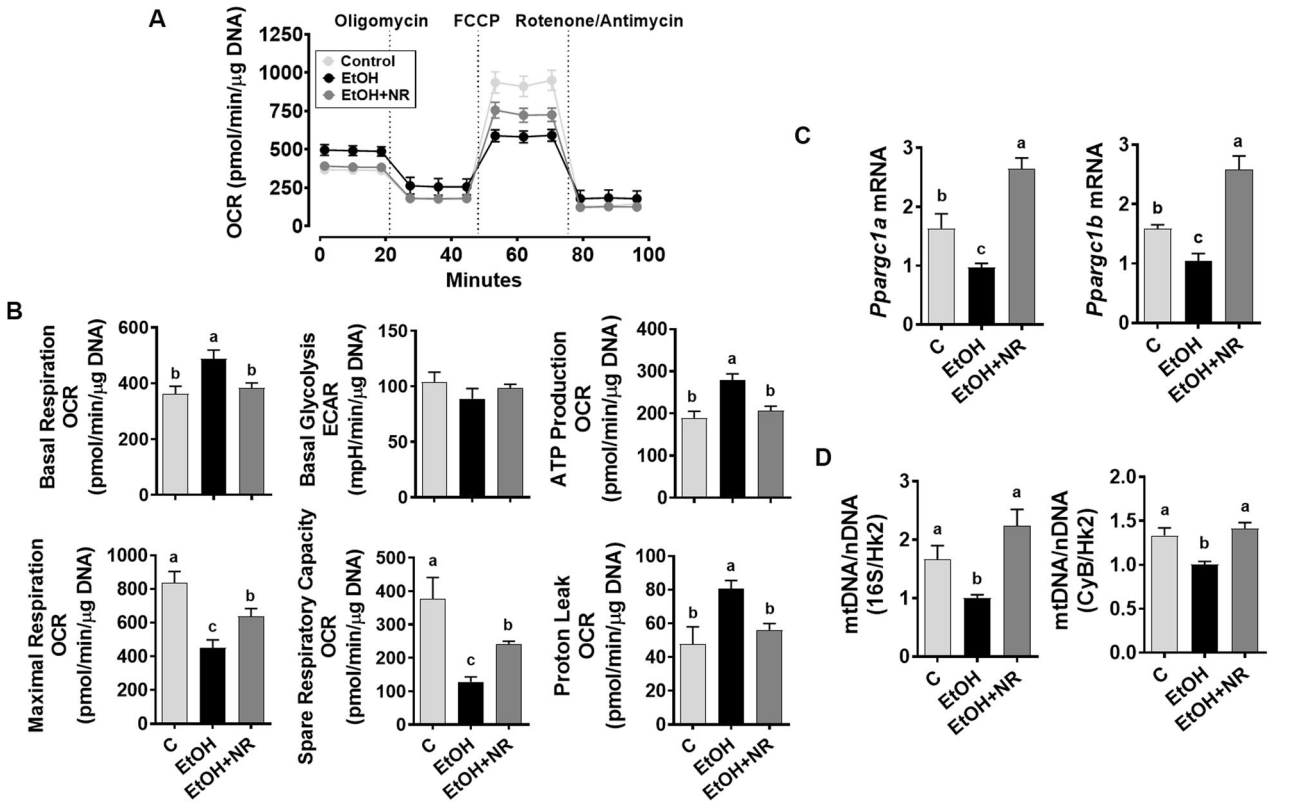

Fig. 7 NR inhibits ethanol-induced alterations in mitochondrial respiration and copy numbers in RAW 264.7 macrophages. A, **B** Cells were treated with 1 mM NR and 80 mM EtOH for 72 h for Mito Stress tests (**A**, **B**), gene analysis (**C**), and (**D**) mitochondrial copy number measurements. Mitochondrial copy numbers were determined using *16S* and *CyB* as a mitochondrial gene and *Hk2* as a nuclear gene. C, control. Mean ± SEM. Bars without sharing a common letter are significantly different ($P < 0.05$).

of aged mice [53]. Therefore, NR counteracts decreased NAD^+ levels by ethanol through the induction of *Nampt* and *Nmnat* expression to facilitate its conversion to NAD^+ in macrophages.

Increased cellular NAD^+ levels by NR and resultant SIRT1 activation are likely responsible for the anti-inflammatory effect of NR in ethanol-treated macrophages. We recently reported that *Sirt1* knockdown elevated ethanol-induced inflammatory gene expression in macrophages [54]. Consistent with our previous study, we found that ethanol significantly decreased the expression of *Sirt1*, which was attenuated by NR. Furthermore, the inhibition of SIRT1 activity increased pro-inflammatory genes, which were reduced with SIRT1 activation. Studies have linked a reduction of NAD^+/SIRT1 to NFκB activation. Poly (ADP-ribose) polymerase-1 (PARP-1), an NAD^+-consuming enzyme, directly interacts with NFκB subunits to form a stable nuclear complex, potentiating NFκB activity [55, 56]. In addition, inhibition of PARP-1 has been reported to improve age-related endothelial dysfunction and neurovascular uncoupling by increasing NAD^+ availability [57]. Ma et al. [58] also reported that resveratrol repressed LPS-induced NFκB activation in alveolar macrophages. Consistently, we found that NR abrogated ethanol-induced nuclear translocation of NFκB p65. Therefore, NR activates the SIRT1 pathway in two ways, i.e., induction of gene expression and provision of NAD^+, to exert its anti-inflammatory effects in ethanol-activated macrophages.

Activated macrophages reprogram energy generation pathways to increase aerobic glycolysis, also known as the "Warburg effect", for effective innate immune responses [37]. Thus, we explored whether ethanol triggers the shift in energy metabolism, favoring aerobic glycolysis in macrophages. HIF-1α plays a crucial role in the metabolic switch in macrophages toward aerobic glycolysis [59]. ROS stabilize HIF-1α [60], and TNFα induces HIF-1α expression through NFκB activation [61, 62], suggesting HIF-1α activation in the milieu of inflammation and oxidative stress. Interestingly, HIF-1α mediates metabolic changes and inflammation triggered by ethanol [63]. In the present study, ethanol increased the expression and activity of HIF-1α and its target gene expression, including *Glut1* and *Hk1*; therefore, one would expect increased glucose entry into macrophages, increasing glycolysis. However, we found no changes in glycolysis by ethanol, although ethanol increased glycolytic capacity, glycolytic reserve, and non-glycolytic acidification in macrophages. Our results are consistent with the report by Hoyt et al. [64] that basal

glycolysis was not significantly altered despite increased lactate in the culture medium in J774 macrophages treated with ethanol for 2 weeks. Ethanol is oxidized to acetaldehyde and then to acetate, which can turn into acetyl-CoA [65]. Some acetaldehydes can become pyruvates by pyruvate decarboxylase. Pyruvate regulates glycolytic rates by inhibiting glycolytic enzymes [66], which may explain no differences in glycolysis with ethanol treatment. Also, acetyl-CoA can activate PDK to phosphorylate PDH for inactivation [67], shifting the conversion of pyruvate to lactate but not to acetyl-CoA. In line with this notion, we found that ethanol increased pPDH/PDH ratios with concomitant increases in *Pdk1* and *Ldha* expression and lactate concentrations in the cell medium. Interestingly, alcoholics frequently have increased circulating lactate concentrations because ethanol metabolism by ADH and ALDH consumes NAD^+, promoting the conversion of pyruvate to lactate [68]. Also, lactate has been shown to induce the HIF-1α signaling, which upregulates glycolysis while suppressing the TCA cycle by inducing PDK1 expression [69–71]. Therefore, ethanol metabolites and NAD^+ depletion due to ethanol metabolism likely have a substantial impact on cellular energy metabolism possibly via the modulation of HIF-1α in macrophages. Significant increases in HIF-1α expression and activity observed in ethanol-treated macrophages support this possibility. It should be noted that NR markedly decreased the expression of *Hif1α*, and its target genes, e.g., *Glut1, Ldha,* and *Pdk1*, along with a reduction in extracellular lactate levels in ethanol-stimulated macrophages. As SIRT1 deacetylates HIF-1α for its inactivation [72], NR may inhibit the activity of HIF-1α, not only by decreasing its expression, but also by de-repressing *Sirt1* expression and increasing NAD^+ in ethanol-treated macrophages to counteract ethanol-induced metabolic switch in macrophages.

Respiratory capacity is another crucial component of a cell's metabolic capacity along with glycolysis. We found that ethanol increased basal respiration, ATP production, and proton leak with no change in basal glycolysis, while it significantly decreased maximal respiration and spare respiratory capacity in macrophages. Also, ethanol significantly decreased the expression of *Ppargc1a* and *Ppargc1b*, which is in line with a concomitant decrease in mitochondrial copy numbers. It is surprising to see the increased basal respiration and ATP production despite decreased mitochondrial copy numbers in ethanol-treated macrophages. The increased mitochondrial respiration by ethanol may be due, at least partly, to the overproduction of NADH during ethanol metabolism because NADH is used for ATP generation via mitochondrial oxidative phosphorylation [37, 73]. Considering that mitochondrial free NADH affects the capacity to regenerate free NADH for energetic supply [74], future studies are warranted to identify the redox state of NADH in mitochondria in ethanol-treated macrophages. It is also presumable that an increased amount of acetate from ethanol provides more carbon substrates for TCA cycles, consequently elevating basal respiration and ATP production in macrophages. Interestingly, we observed a significant increase in non-glycolytic acidification in ethanol-treated macrophages. As protons are generated in the cells from glycolysis (i.e., lactate release) and respiration (carbonic acid formation from CO_2) [75], increased non-glycolytic acidification by ethanol further supports the possible promotion of basal respiration and ATP production by ethanol, even when mitochondrial biogenesis is likely to reduce in ethanol-treated macrophages. NR, by increasing NAD^+ production as its precursor, is likely to negate an ethanol-induced reduction in NAD^+/NADH ratios, attenuating the effects of ethanol on respiratory parameters, including basal respiration, ATP production and proton leak. SIRT1 deacetylates PGC-1α in a NAD-dependent manner to control mitochondrial biogenesis in hepatocytes [76], and PGC-1β is known to inhibit pro-inflammatory cytokine production and primes macrophages for alternative activation [77]. Therefore, the effects of NR on preventing metabolic reprogramming may be related to its positive role in SIRT1 activation as well as the induction of *Ppargc1a* and *Ppargc1b* in ethanol-treated macrophages.

Taken together, the present study demonstrates that NR exerts anti-inflammatory and antioxidant effects in ethanol-stimulated macrophages by inhibiting nuclear translocation of NFκB and reducing cellular ROS accumulation. Notably, the anti-inflammatory and antioxidant effects of NR in macrophages exposed to ethanol are likely related to its activation of SIRT1 by de-repressing SIRT1 expression and providing its activator, NAD^+. Furthermore, SIRT1 activation and normalization of ethanol-induced changes in NAD^+/NADH ratios are presumed to counteract the changes in macrophages' energy phenotypes exposed to ethanol. In vivo studies are warranted to confirm our in vitro findings. Nonetheless, the present study suggests the potential of NR as a therapeutic agent for alcohol-related inflammatory conditions.

Data availability

All data generated or analyzed during this study are included in this published article.

Author contributions HK conducted experiments, analyzed data, and wrote the manuscript. YKP performed experiments and contributed to manuscript preparation. JYL designed experiments, interpreted data, and wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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