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Coffee Bioactive *N*-Methylpyridinium: Unveiling Its Antilipogenic Effects by Targeting De Novo Lipogenesis in Human Hepatocytes

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Scope: Type 2 diabetes and nonalcoholic fatty liver diseases (NAFLDs) are promoted by insulin resistance (IR), which alters lipid homeostasis in the liver. This study aims to investigate the effect of *N*-methylpyridinium (NMP), a bioactive alkaloid of coffee brew, on lipid metabolism in hepatocytes.
Methods and results: The effect of NMP in modulating lipid metabolism is evaluated at physiological concentrations in a diabetes cell model represented by HepG2 cells cultured in a high-glucose medium. Hyperglycemia triggers lipid droplet accumulation in cells and enhances the lipogenic gene expression, which is transactivated by sterol regulatory element binding protein-1 (SREBP-1). Lipid droplet accumulation alters the redox status and endoplasmic reticulum (ER) stress, leading to the activation of the unfolded protein response and antioxidative pathways by X-Box Binding Protein 1(XBP-1)/eukaryotic Initiation Factor 2 alpha (eIF2α) Protein Kinase RNA-Like ER Kinase and nuclear factor erythroid 2-related factor 2 (NRF2), respectively. NMP induces the phosphorylation of AMP-dependent protein kinase (AMPK) and acetyl-CoA carboxylase α (ACACA), and improves the redox status and ER homeostasis, essential steps to reduce lipogenesis and lipid droplet accumulation.
Conclusion: These results suggest that NMP may be beneficial for the management of T2D and NAFLD by ameliorating the cell oxidative and ER homeostasis and lipid metabolism.

1. Introduction

Diabetes is one of the most severe and challenging health problems. According to the 10th edition of the Diabetes Atlas published by the International Diabetes Federation (IDF), in 2021 about 537 million people worldwide had diabetes.[1] The overwhelming majority of people with diabetes have type 2 diabetes (T2D), in which hyperglycemia derives from the inability of the body's cells to fully respond to insulin, a condition termed insulin resistance (IR).[1]
In the healthy liver, insulin signaling causes suppression of gluconeogenesis and stimulation of de novo lipogenesis (DNL). Although IR promotes excessive gluconeogenesis, it paradoxically promotes DNL, leading to excessive deposition of fat in the liver, a condition known as nonalcoholic fatty liver disease (NAFLD).[2–5] Several mechanisms have been proposed to explain the paradoxical activation of lipogenesis in the liver under IR, although none seem exhaustive.[6]

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The endoplasmic reticulum (ER) is a subcellular organelle involved in protein folding, assembly, and secretion. Disruption of ER homeostasis leads to the accumulation of misfolded or unfolded proteins in the ER lumen, a condition referred to as ER stress.[7] During ER stress, three intracellular signaling pathways, Protein Kinase RNA-Like ER Kinase (PERK)-eIF2α, Inositol-Requiring Protein 1 (IRE1α)-XBP1, and Activating Transcription Factor 6 (ATF6)Heme-oxygenase 1, drive the unfolded protein response (UPR) to restore ER homeostasis.[8] ER stress represents a causative mechanism of lipogenesis activation and lipid accumulation in hepatocytes.[7,9–12] We found that ER stress occurs in HepG2 cells in hyperglycemia condition and triggers the expression and the trans-activation activity of the sterol regulatory element binding protein-1 (SREBP-1), a major transcriptional regulator of lipogenic genes, thus promoting lipogenesis and lipid accumulation.[2] Any strategy helping to decrease lipid accumulation in the liver may provide a protective effect against NAFLD and T2D. Natural compounds from vegetables and fruits have been considered an adjuvant therapy for their antidiabetic, antiobesity, antioxidant, antiinflammatory, and lipid-lowering actions. These compounds may have the potential to relieve clinical complications in subjects with T2D and NAFLD.[13,14]

Coffee brew is one of the most popular beverages in the world, with its popularity and consumption linked to its pleasant aroma and stimulating effects on the central nervous system. Many coffee phytochemicals are bioactive compounds with beneficial effects on some types of cancer, cardiovascular diseases, blood pressure, inflammation, and blood cholesterol levels.[15–19] Moreover, epidemiological studies concur in an association between habitual coffee consumption and a lower risk of T2D and NAFLD.[20,21] However, few studies have been conducted to explain the molecular mechanisms of the antidiabetic properties of coffee brews. *N*-methylpyridinium (NMP) represents an interesting bioactive molecule of the coffee brew. It derives from the decomposition of the alkaloid trigonelline during the roasting process of green coffee beans.[22]

The present work aimed to analyze thoroughly the effect of NMP, at physiologically achievable concentrations, in modulating the lipid synthesis pathway in a widely used experimental diabetes model obtained by incubating HepG2 cells with 30 mM glucose (high glucose [HG]).[23,24] Here, we reported that hyperglycemia triggered the activation of the XBP-1 and SREBP-1, two transcriptional factors that orchestrate the expression of genes implied in DNL. Accordingly, the transactivation of lipogenic genes, including citrate carrier (CiC), ATP-cytrate lyase (ACLY), cytosolic acetyl-CoA carboxylase α (ACACA), stearoyl-CoA desaturase 1 (SCD1), and diacylglycerol *o*-acyltransferase 2 (DGAT2), was observed as well. NMP treatment efficaciously counteracted the activation of lipogenic genes triggered by HG. We also provide evidence that the antilipogenic mechanism of NMP was explained primarily by the strong phosphorylation of ACACA, which can be explained by the NMP-mediated intervention of AMP-dependent protein kinase (AMPK). Overall, the findings presented here reveal an antilipogenic effect of NMP on the DNL pathway through molecular mechanisms acting on ER stress and the AMPK/ACACA axis.

2. Results

2.1. Effect of NMP Treatment on Lipid Accumulation in High Glucose-Treated HepG2 Cells

As shown in **Figure 1**A, when compared to LG-cells, the addition of HG to the cells enhanced cell viability. Treatment of HG-cells with NMP up to 0.25 μM did not change cell viability, whereas NMP concentrations in the range 0.5–5 μM reduced cell viability compared to HG-cells, even though it was not significantly different from LG-cells. A significant reduction of cell viability was observed in HG-cells treated with NMP at 10 μM. Compared to cells cultured in LG medium, 24-h incubation with HG caused a significant accumulation of triglycerides (TGs) (Figure 1B, C). In cells treated with NMP at 0.1 or 0.25 μM, lipid accumulation was significantly reduced compared to HG-cells (Figure 1B, C).

2.2. Effect of NMP Treatment on Oxidative Stress in Hyperglycemic HepG2 Cells

Compared to the LG-cells, the fluorescence measured in HG-cells increased, indicating an imbalance in ROS production (**Figure 2**A, B). Incubation of HG-cells with 0.25 μM NMP was effective in attenuating oxidative stress triggered by HG, as suggested by the rescue of fluorescence, whose level was similar to that of control LG-cells (Figure 2A, B).

In HG-cells, oxidative stress was caused by an imbalance between the production of ROS and their removal by enzymatic and nonenzymatic antioxidant systems. These systems, among others, include several antioxidant enzymes whose expression is controlled by the nuclear factor erythroid 2-related factor 2 (NRF2) transcription factor.[25] Therefore, nuclear levels of NRF2 were evaluated by Western blotting in HG-NMP-treated cells and compared with the HG- and LG-cells. Results showed that the NRF2 level in the nuclei was barely detected in LG-cells, whereas it was remarkably high in HG-cells (Figure 2C). The augmented transactivation activity of NRF2 in cells upon exposure to HG was supported by luciferase reporter assay, carried out with the 2xARE-pGL3Prom construct, containing two copies of the NRF2 binding site in tandem upstream of the SV40 promoter (Figure 2D). Accordingly, the abundance of mRNA of NRF2 target genes, Heme-oxygenase 1 (*Ho-1*), NAD(P)H Quinone Dehydrogenase 1 (*Nqo1*), Superoxide dismutase 1 (*Sod1*), Superoxide dismutase 2 (*Sod2*), and Catalase (*Cat*), was incremented by about 1.3–1.8 times more in HG-cells than in LG-cells. The nuclear level of NRF2, NRF2 transactivation activity, and the mRNA abundance of NRF2 target genes did not change in HG-cells incubated with NMP compared to the HG-cells incubated without NMP (Figure 2C, E). Compared to LG-cells, SOD1 and SOD2 enzymatic activity was reduced in HG-cells. The activity of all enzymes augmented in HG-NMP-treated cells (Figure 2F).

2.3. NMP Treatment Attenuated Endoplasmic Reticulum Stress Triggered by the Hyperglycemic Condition

Previous findings have reported that the ER stress and the UPR signaling pathway play a crucial role both in lipid droplet accumulation[10–12] and in the establishment of oxidative

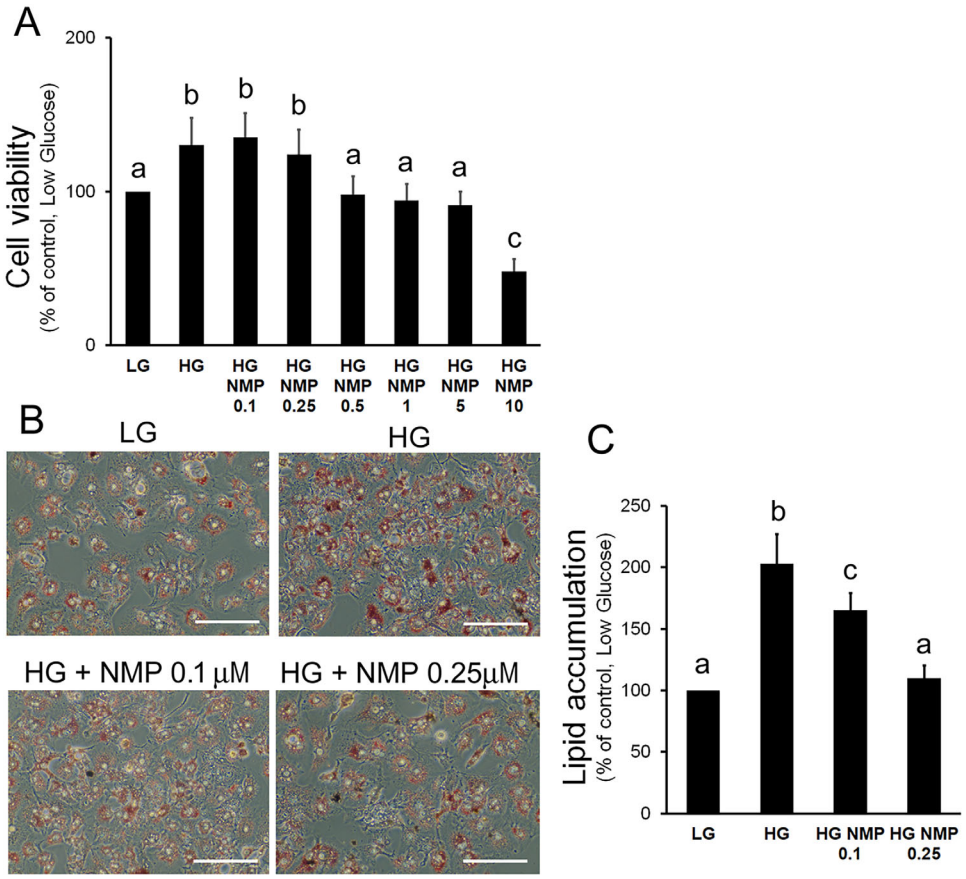

Figure 1. Effect of NMP on lipid accumulation in HepG2 cells cultured in HG medium. A) HepG2 cells were incubated in LG, HG, and HG in the presence of concentrations of NMPranging from 0 to 10 µM for 24 h. Cell viability was assessed by MTT assay, and results were expressed as a percentage of the control cells incubated in LG medium. B) The micrographs depict the lipid droplet accumulation, visualized by Oil Red O staining, in cells incubated in LG, HG, or HG with NMP at the concentration indicated in the figure. The bars in micrographs correspond to 100 µm. Images are representative of three independent experiments. C) After Oil Red O staining, the incorporated stain was solubilized and quantified by spectrophotometry at 510 nm. Values were reported in histograms as percentages with respect to the control, represented by HepG2 incubated in LG medium. Data are presented as the mean ± SD ($n = 3$) and experiments were repeated three times independently. The data underwent analysis using ANOVA followed by the Bonferroni/Dunn post hoc test ($p < 0.05$). ANOVA, analysis of variance; LG, low glucose; HG, high glucose; NMP, *N*-methylpyridinium; SD, standard deviation.

stress.[26] ER stress and UPR trigger the expression of lipogenic transcription factor SREBP-1 through an efficient mRNA translation mediated by a Cap-independent mechanism.[27] Therefore, we evaluated the effect of HG and HG together with NMP on the modulation of ER stress markers, the transcription factors XBP-1, ATF6*α*, and CHOP/GADD153, and the chaperonine GRP78/BIP (**Figure** 3A). We found that the levels of all ER-stress markers were higher in HG-cells when compared with LG-cells, whereas the addition of 0.25 µM NMP resolved ER-stress induction. XBP1 is known to upregulate ER stress-related genes, such as *Dnajb9, Pdia3,* and *Hrd1*. When compared to LG-cells, an increase of the ER stress-related genes was observed in HG-cells but not in HG-cells treated with NMP (Figure 3B).

EIF2*α* is the *α* subunit of the initiation factor eIF2, and its phosphorylated form represents a marker of ER stress. The level of P-eIF2*α* and the ratio P-eIF2*α*/eIF2*α* were remarkably increased in HG-cells (Figure 3C). Treatment with 0.25 µM NMP reduced eIF2*α* phosphorylation to the level of LG-cells (Figure 3C).

2.4. NMP Treatment Negatively Regulated the Expression of the Lipogenic SREBP-1 Transcription Factor

The molecular basis of the lipid-lowering property of NMP was deepened by investigating the expression of the SREBP-1, the master transcription factor involved in lipogenic gene regulation. HG condition caused a 1.7 times increase in SREBP-1 gene expression compared to LG-cells (**Figure** 4A). When compared to HG-cells, a reduction of SREBP-1 mRNA abundance was observed in HG-cells treated with NMP. Similarly, the protein level of SREBP-1 was higher in cells incubated in HG medium than in control cells. NMP treatment was effective in reducing SREBP-1 expression compared to cells in the HG medium (Figure 4A). The translation of SREBP-1 mRNA was monitored using a luciferase assay performed with the pGL3S1a construct, containing the 5′UTR of human SREBP-1 mRNA upstream of the firefly luciferase (FL) gene reporter.[27] FL activity was about 2.8-fold higher in HG-cells compared to the control cells. By contrast, FL activity was significantly reduced in HG-cells in the presence

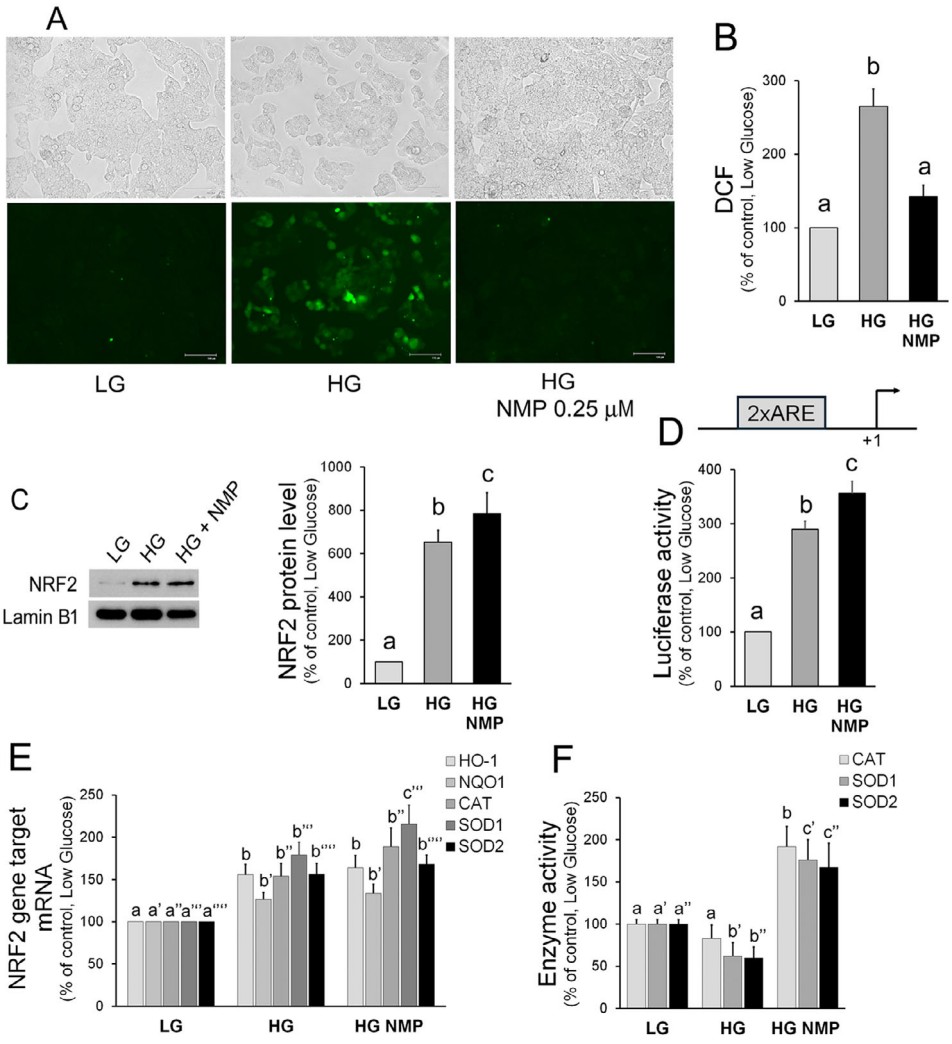

Figure 2. Effects of HG and NMP treatment on oxidative stress and the expression and trans-activation activity of NRF2. HepG2 cells were incubated in LG, HG, and HG in the presence of 0.25 µM NMP for 24 h. A) ROS production was evaluated using the cell-permeant H_2DCFDA. Micrographs depict ROS production in cells incubated in LG, HG, or HG in the presence of NMP. The bars in micrographs correspond to 100 µm. Images are representative of three independent experiments. B) ROS production was quantified by fluorimetry. C) The nuclear content of NRF2 from treated cells was quantified by Western blotting and expressed as percentage relative to control cells (LG). D) Cells were transiently transfected with the NRF2 reporter construct, depicted in the upper panel. After 24 h, HepG2 cells were incubated in LG, HG, or HG and 0.25 µM NMP for 24 h, and firefly luciferase activity was measured and normalized to β-galactosidase activity and protein concentration. Normalized luciferase activity was expressed as percentage relative to the control cells (LG). E) Total RNA was extracted and the abundance of mRNA of NRF2-target genes (*Ho-1*, *Nqo1*, *Sod1*, and *Cat*) was quantified by RT-qPCR. F) The enzymatic activity of CAT, SOD1, and SOD2 were measured in LG-, HG-, and HG/NMP-cells. Data are presented as the mean ± SD ($n = 3$), and experiments were repeated three times independently. The data underwent analysis using ANOVA followed by the Bonferroni/Dunn post hoc test ($p < 0.05$). ANOVA, analysis of variance; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HG, high glucose; NMP, *N*-methylpyridinium; NRF2, nuclear factor erythroid 2-related factor 2; SD, standard deviation.

of NMP (Figure 4B). The pCiC1484-Luc construct, containing a previously characterized SREBP-1-binding site (E-box),[28] was used to follow the transactivation activity of SREBP-1. Results indicated that the SREBP-1 transactivation activity was increased in HG compared to control cells grown in the LG medium. Compared to HG-cells, the transactivation activity of SREBP-1 was reduced by NMP treatment to the level observed in LG-cells (Figure 4C). The analysis of the expression of SREBP-1 target genes, i.e., mitochondrial *Cic*, ATP-citrate lyase (*Acly*), *Acaca*, *Scd1*, and *Dgat2*, was also investigated. When compared to the control LG-cells, an increase in lipogenic gene expression was observed in HG-cells but not in HG-cells treated with NMP (Figure 4D).

2.5. NMP Treatment Promotes Phosphorylation of AMP-Dependent Kinase and Acetyl-CoA Carboxylase α

A crucial point in controlling DNL by hormones and nutrients is represented by the enzyme ACACA, which catalyzes the first committed step in fatty acid synthesis. Indeed, AMP-dependent kinase (AMPK) catalyzes the phosphorylation of

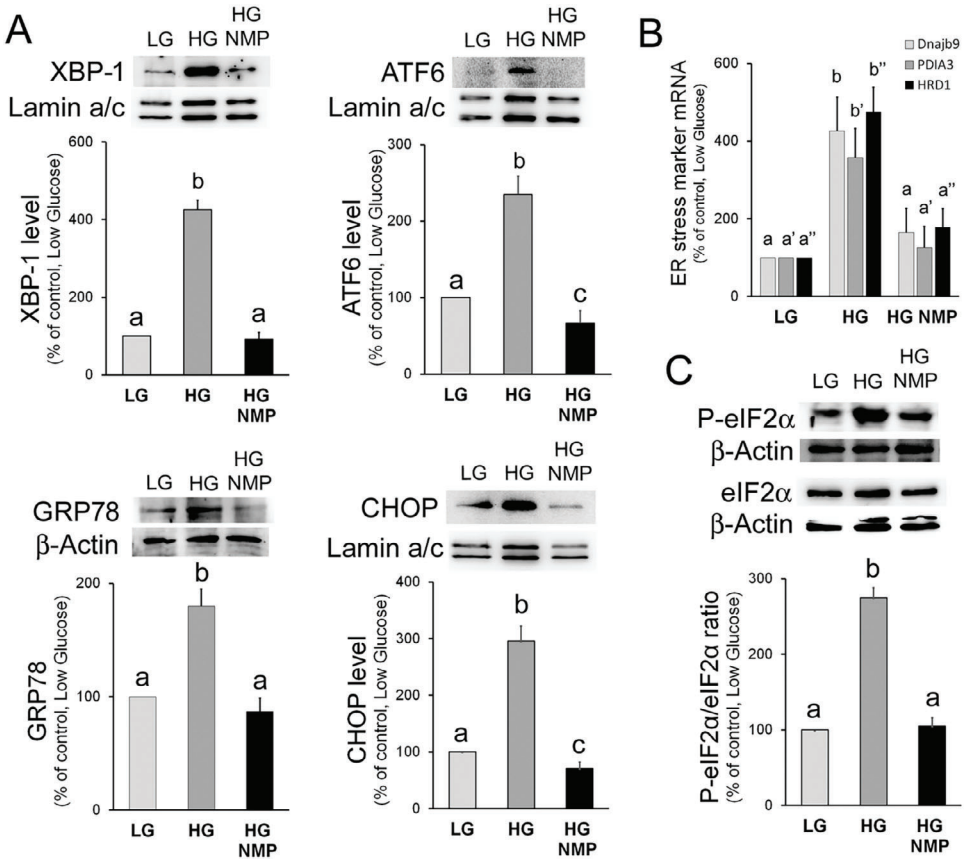

Figure 3. Effects of NMP treatment on endoplasmic reticulum stress induced by hyperglycemia. HepG2 cells were incubated in LG, HG, or HG in the presence of 0.25 μM NMP for 24 h. A) Cytosolic and nuclear proteins were extracted from the cells and separated by SDS/PAGE. After incubation with antibodies against XBP-1, ATF6, GRP78, and CHOP, the content of the respective protein was quantified by densitometric analysis and expressed as percentage with respect to control cells (LG). B) Total RNA was extracted and the abundance of mRNA of ER stress-target genes (*Dnajb9*, *Pdia3*, and *Hrd1*) was quantified by RT-qPCR. C) The levels of total eIF2α and P-eIF2α were determined by Western blotting. The P-eIF2α/eIF2α ratio was reported as percentage with respect to control cells (LG). The data are presented as the mean ± SD ($n = 3$) and experiments were repeated three times independently. The data underwent analysis using ANOVA followed by the Bonferroni/Dunn post hoc test ($p < 0.05$). ANOVA, analysis of variance; ER, endoplasmic reticulum; HG, high glucose; NMP, *N*-methylpyridinium; SD, standard deviation.

ACACA at the Ser79 residue, causing the inhibition of its enzymatic activity.[29] Here, we investigated the effect of NMP treatment on the level of P-ACACA. Western blots showed that the ratio P-ACACA/ACACA was reduced in HG-cells while it was strongly augmented in HG-cells treated with NMP (**Figure 5**). We evaluated the putative role of AMPK in the modulation of the level of p-ACACA in LG and untreated HG and NMP-treated HG-cells. When compared to LG, the ratio P-AMPK/AMPK was reduced in HG-cells and augmented in HG-cells treated with NMP (Figure 5).

3. Discussion

T2D represents a severe public health concern with a considerable impact on human life and health expenditures. Epidemiological and experimental evidence suggests that an unbalanced Western diet, rich in saturated fatty acids and poor in vegetables, fruits, and fish, is one of the leading causes of the onset of the disease.[30] Moreover, NAFLD occurs very commonly in patients with T2D, with a prevalence of 55%–68%.[31,32] The etiopathology of these coexisting metabolic diseases is complex and constitutes a classical "chicken or egg" causality dilemma. Indeed, studies have provided opposite results, supporting one of the two pathological conditions as the cause of the second one.[31,33] IR in the liver undoubtedly plays a crucial role in fostering specific tracts of T2D and NAFLD.[4] Although insulin promotes anabolic metabolism in the liver by increasing glucose consumption and lipid synthesis, individuals affected by IR fail to inhibit hepatic glucose production and have increased hepatic lipid synthesis, leading to hyperglycemia, hyperlipidemia, and NAFLD.[4] Paradoxically, both hyperglycemia and hyperlipidemia are among the causes triggering IR.[34] The vicious circle of IR thus represents a crucial point that needs to be interrupted in the treatment of T2D and NAFLD. Nutraceuticals may be appropriate as a supplementary treatment for T2D and NAFLD due to their biological properties.[2,35]

Both caffeinated and decaffeinated coffee brew consumption has been associated with a reduced risk of T2D.[36] Apart from the above-mentioned epidemiological evidence, studies on coffee compounds, including NMP at physiological concentrations

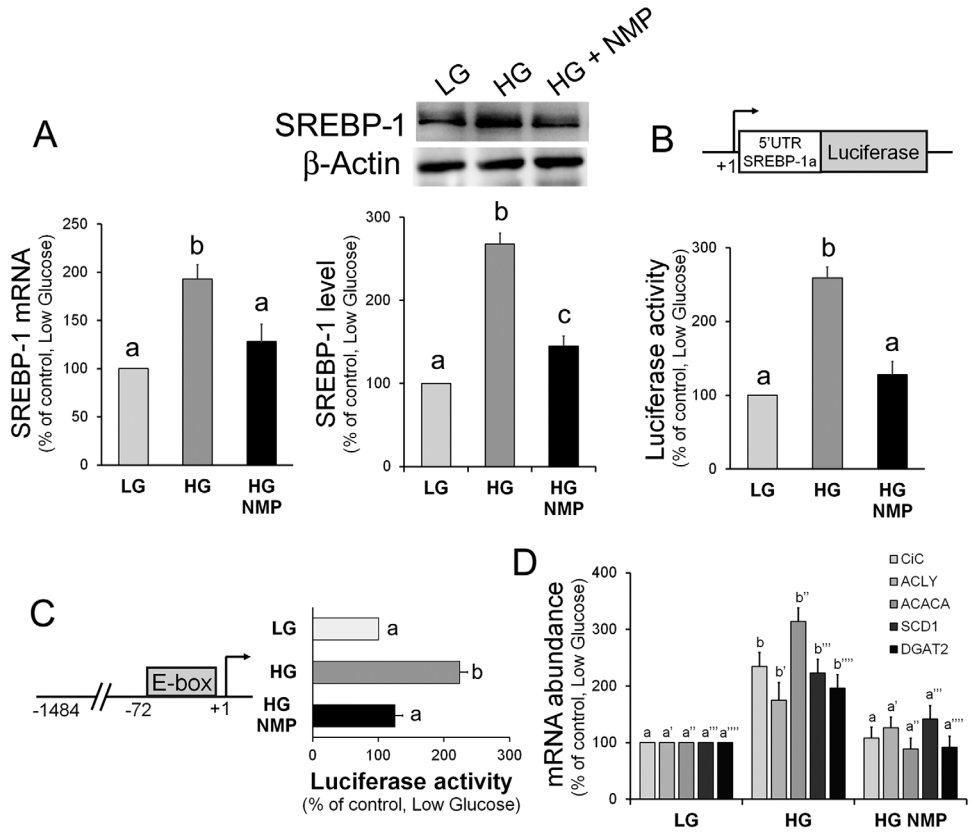

Figure 4. Effects of HG and NMP treatment on the expression and trans-activation activity of SREBP-1. A) Left panel, the abundance of SREBP-1 mRNA was quantified by RT-qPCR. Right panel, the content of SREBP-1 was quantified in LG, HG, and HG/NMP and reported in histograms as a percentage of control cells (LG). B) The depiction shows the pGL3S1a construct with the 5′UTR SREBP-1a upstream of the firefly luciferase gene. Cells were transiently cotransfected with the pGL3S1a together the *β*-galactosidase reference plasmid. After 24 h, HepG2 cells were incubated in LG or HG, in the presence or in the absence of 0.25 μM NMP for 24 h, and luciferase activity was normalized to *β*-galactosidase activity and protein concentration. The translation efficiency drove by 5′UTR SREBP-1 in HG and in HG-NMP cells was expressed as a percentage of the control cells (LG). C) Cells were transiently cotransfected with the CiC promoter-luciferase constructs together with *β*-galactosidase reference plasmid. CiC promoter activation was expressed as a percentage of the control cells (LG). D) The abundance of mRNA for SREBP-1 target genes, *Cic*, *Acly*, *Acaca*, *Scd1*, and *Dgat2*, was quantified by RT-qPCR. The data are presented as the mean ± SD ($n = 3$), and experiments were repeated three times independently. The data underwent analysis using ANOVA followed by the Bonferroni/Dunn post hoc test ($p < 0.05$). *Acaca*, acetyl-CoA carboxylase *α*; *Acly*, ATP-citrate lyase; ANOVA, analysis of variance; CiC, citrate carrier; *Dgat2*, diacylglycerol O-acyltransferase 2; LG, low glucose; HG, high glucose; NMP, *N*-methylpyridinium; *Scd1*, stearoyl-CoA desaturase 1; SD, standard deviation; SREBP-1, sterol regulatory element binding protein-1.

and molecular mechanisms involved in glucose homeostasis, are poorly deepened. It has been reported that NMP improves insulin signaling while reducing inflammation in adipocytes, allowing its qualification as an antiinflammatory molecule.[15]

The objectives here pursued concerned the evaluation of NMP effects on lipid metabolism dysregulation, oxidative stress, and ER stress, three pathophysiological tracts of T2D and NAFLD that are profoundly interconnected. For these objectives, we used a hyperglycemic hepatic cell model represented by HepG2 cells incubated under HG concentration. This experimental model has been evaluated since hyperglycemia is often found in obese patients with NAFLD and plays a role in liver toxicity by activating oxidative and ER stress, IR, steatosis, and cellular demise.[37] Even though this model cannot replace the in vivo hyperglycemic hepatocytes, it offers the possibility to study specific molecular mechanisms of glucotoxicity under controlled experimental conditions.[23,24] Here, we found that culturing HepG2 cells in an HG medium caused the accumulation of lipid droplets in the cytoplasm, which corroborates the assessment of liver steatosis in T2D patients.[5] Since glucose represents a primary carbon source for DNL and TGs synthesis, its high availability forces the activation of the lipogenic pathway and intracellular accumulation of lipid droplets.

The biological action of NMP against lipid synthesis and accumulation in hyperglycemic cells was analyzed by testing different concentrations in the 0.1–10 μM range. Cell viability assay indicated that a cytotoxic effect is evident only in cells incubated with the 10 μM NMP. However, treatment with NMP at low concentrations (0.1–0.25 μM) was sufficient to inhibit the lipid accumulation in HG-cells, reducing it to the level of the untreated cells (Figure 1). Notably, these NMP concentrations range in those measured in subjects' blood after consumption of one or three espresso coffees, something fully aligned with common coffee consumption patterns.[38,39]

Our results showed that the HG condition caused an alteration of the redox status in hepatocytes with high production of ROS

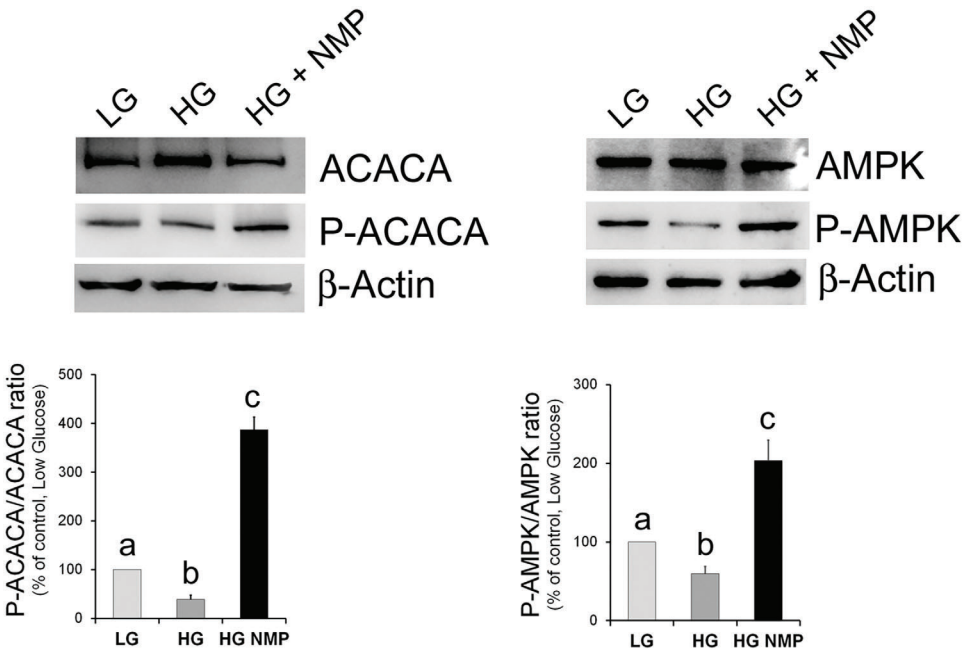

Figure 5. Effects of HG and NMP treatment on the AMPK and ACACA phosphorylation. HepG2 cells were incubated in low glucose (LG), in high glucose (HG), or in HG in the presence of 0.25 μM NMP for 24 h. Total proteins were extracted from the cells and separated by SDS/PAGE. The ratio of P-ACACA/ACACA (left panel) and P-AMPK/AMPK was expressed as a percentage of control cells (LG). The data are presented as the mean ± SD ($n = 3$) and experiments were repeated three times independently. The data underwent analysis using ANOVA followed by the Bonferroni/Dunn post hoc test ($p < 0.05$). ACACA, acetyl-CoA carboxylase α; AMPK, AMP-dependent protein kinase; ANOVA, analysis of variance; HG, high glucose; NMP, *N*-methylpyridinium; SD, standard deviation.

(Figure 2), mainly correlated to mitochondrial dysfunction.[40] Notably, the oxidative status is recognized as a major cause of diabetes clinical complications,[40] including the accumulation of lipid droplets in the liver.[41] The redox homeostasis in cells is regulated by the NRF2, a redox-sensitive transcription factor belonging to the cap'n'collar (CNC) basic-leucine zipper (bZIP) family. In normal conditions, inactive NRF2 is in the cytoplasm bound to the cytoskeletal-anchoring protein Kelch-like ECH-associated protein 1 (KEAP1). It has been reported that high levels of ROS lead to the release of NRF2 from KEAP1.[42] In the nuclei, NRF2 binds to the antioxidant response element (ARE) and activates the expression of genes coding for antioxidant enzymes like NAD(P)H:quinone oxidoreductase 1 and proteins for glutathione synthesis to restore redox homeostasis.[42]

As a consequence of increased ROS, we found that nuclear NRF2 content was augmented in HG-cells compared to control LG-cells (Figure 2). Apart from the shuttling into the nuclei, the NRF2 transactivation in HG-cells was demonstrated by NRF2/ARE reporter gene assay results and mRNA quantification of NRF2-target genes. Although these results indicated the activation of NRF2 target genes *Sod1, Sod2,* and *Cat* in HG-cells, their enzymatic activity was inhibited under the same conditions, justifying the persistence of the altered redox status in HG-cells. Indeed, it has been reported that the enzymatic activities of SOD and catalase were inhibited in the liver in db/db mice, a T2D model, by *O*-GlcNAcylation posttranslational modification.[43,44]

When compared to the control, elevated nuclear level of NRF2 triggered by the oxidative status was not only observed in HG-cells but also in HG/NMP-treated cells, where the levels of ROS returned to the levels of control LG-cells (Figure 2). This result can be explained by the fact that NMP is a modulator of NRF2.[45] The NMP activation of NRF2 and its target genes, including *Sod1, Sod2, and Cat*, was paralleled by the rescue and further increase of SOD activity, contributing to the decrease in ROS compared to HG-cells.

Other molecular mechanisms different from that mediated by NRF2 contribute to the recovery by NMP of the HG-induced oxidative stress, and lipid accumulation ER is a subcellular organelle involved in several functions, including the synthesis of lipids and the correct folding of proteins residing within and transiting along the secretory pathway.[46] The UPR pathway, activated during ER stress, is composed of three signaling branches, PERK-eIF2α, IRE1-XBP1, and ATF6, being XBP-1 and ATF6 two transcription factors that control the expression of genes involved in ER proteostasis. eIF2α is the α subunit of the translation initiation factor IF2, and its phosphorylation results in a severe decline in de novo protein synthesis, an important strategy against stressful insults. Previous reports have highlighted ER stress among the factors involved in the etiology of NAFLD.[47] Notably, the phosphorylation of eIF2α and the activation of XBP1-ATF6 transcription factors have also been correlated with increased DNL,[48] even though other findings indicate XBP-1 as an antilipogenic transcription factor.[49] In the present study, we showed a remarkable increment of P-eIF2α, and of nuclear XBP-1 and ATF6 in HG-cells (Figure 3). Moreover, compared to LG-cells, in HG-cells an increment of the expression of XBP-1 target genes, *Dnajb9*, *Pdia3*, and *Hrd1*, was observed. According to previous findings, activation of ER-stress fosters the upregulation of SREBP-1 and lipogenic gene expression (Figure 4).[9,10,27] Conversely, when compared to

HG-cells ER stress was reduced in HG-NMP cells, as suggested by the lower levels of ER-stress markers, i.e., the transcription factors XBP1, ATF6, and CHOP, the chaperonine GRP78, and the phosphorylated translation factor P-eIF2α. Following the recovery of ER stress, the levels of SREBP-1 and lipogenic gene targets in HG-NMP returned to the levels of control LG-cells. Since ER stress induces the production of ROS in the ER and mitochondria,[50] the resolution of ER stress may constitute a key molecular mechanism of antioxidant action for NMP. Among the genes activated in HG-cells is ACACA, which plays a crucial role in the control of lipid homeostasis. ACACA catalyzes the synthesis of the precursor of fatty acid synthesis, malonyl-CoA, an allosteric molecule that prevents mitochondrial fatty acid oxidation by inhibiting the carnitine-palmitoyltransferase 1 enzyme. The increase of lipid droplets observed in HG-cells can be a consequence of both the activation of lipogenesis and the reduction of β-oxidation of fatty acids. Compared to the HG-cells, treatment with NMP reduced the expression of ACACA (Figure 5), explaining the reduction of lipid accumulation.

AMPK is a metabolic sensor composed of a catalytic (α) subunit and two regulatory (β, γ) subunits, and its activation requires phosphorylation of the α subunit. AMPK catalyzes the phosphorylation at the Ser79 residue of ACACA, determining the inhibition of ACACA enzymatic activity.[51,52] We found that incubation with NMP was effective in increasing the P-ACACA level compared to HG-treated cells, thus nullifying the ACACA stimulation by HG (Figure 5). A similar trend of P-ACACA levels in HG- and HG-NMP-treated cells was followed by the active AMPK (P-AMPK), suggesting a mechanism of NMP action mediated by the AMPK-ACACA axis. The importance of the AMPK-ACACA axis as a mechanism of action of NMP is supported by findings reported for other natural bioactive compounds, including the polyphenols quercetin and some catechins.[2,53] The mechanism of activation of AMPK by NMP is currently unknown. We hypothesize that NMP could act upstream of the AMPK signaling pathway by directly stimulating the deacetylase SIRT1.[51] Alternatively, NMP could inhibit AMPK-specific phosphatases, through a mechanism similar to that described for quercetin.[2] For these or other hypotheses about the mechanism of action of NMP, further in-depth studies are required. Overall, our findings indicate that the inhibition of ACACA through Ser79 phosphorylation mediated by AMPK probably constitutes the basic molecular mechanism of the action of NMP in reducing the harmful effects related to HG concentrations in hepatocytes. Following the treatment of HG-cells with physiological concentrations of NMP, the reduced expression of ACACA, mainly in the phosphorylated form, suggests a potent inhibition of the malonyl-CoA production and, therefore, a reduction of the biosynthesis of fatty acids and TGs. The lack of lipid accumulation by NMP action would, in turn, determine the relief of both ER stress and oxidative stress in cells induced by HG. Since NMP acts on the AMPK/ACACA axis, the effect of NMP in both normoglycemic and hyperglycemic hepatocytes could affect glucose and lipid catabolism as well as mitochondrial respiration and energy production, important aspects that need further investigation. Moreover, in vivo studies are needed to confirm these observations in more complex biological systems and to investigate the potential benefits of coffee components on T2D and NAFLD prevention.

4. Experimental Section

Cell Culture, Treatment With a High Concentration of Glucose and NMP, and Determination of Cell Viability, Oxidative Stress, and Lipid Droplet Accumulation

Human hepatocellular carcinoma cells (HepG2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg L^{-1} glucose (Sigma–Aldrich, Milano, Italy), supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), penicillin G (100 units mL^{-1}), and streptomycin (100 µg mL^{-1}), and kept at 37 °C in a humidified atmosphere containing 5% CO_2. For each experimental condition, three 100 mm dishes were seeded with 10^6 cells each. After 24 h, cells were incubated in DMEM with 1000 mg L^{-1} glucose for 24 h (referred to as LG-cells) (Sigma–Aldrich, Milano, Italy). On the third day, hyperglycemic conditions were induced by incubating the cells with glucose 30 mM (referred to as HG-cells). For the treatment with NMP (Merck, Darmstadt, Germany), cells were first pretreated at the indicated concentrations in LG medium for 1 h, then the LG medium was changed to HG medium supplemented with NMP at the same physiologically achievable concentration[38] for a further 24 h. 0.5 mmol L^{-1} NMP stock solution was prepared in sterile water. Cell viability was assessed using the MTT assay as previously described.[54] Oxidative stress was evaluated by using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen, Monza, Italy), following the indications by the manufacturer. Intracellular lipid droplet staining and TG content in lipid droplets were determined using the Oil red O stain.[55]

Isolation of RNA From Cultured Cells, Real-Time qPCR, and Western Blot Analysis

Total RNA and protein extraction, quantification of mRNA levels, and Western blot analysis were performed as previously described.[2] The primers used for real-time PCR analysis are reported in Supporting Information Table S1. The blots were incubated with specific primary antibodies against Acetyl-CoA Carboxylase alpha (ACACA), phosphorylated-Ser79 acetyl-CoA carboxylase alpha (P-ACACA), NRF2, XBP-1, SREBP-1, ATF6α, β-actin, Lamin B1, lamin A/C, CHOP/GADD153, GRP78, eIF2α (sc-137104, sc-271965, sc-365949, sc-8015, sc-13551, sc-166659, sc-47778, sc-374015, sc-7292, sc-166682, sc-13539, sc-133132, Santa Cruz Biotechnologies, Dallas, TX, USA), and P-eIF2α (3597S, Cell Signaling Technology, Danvers, MA, USA). Densitometric analysis was carried out on the Western blots using the NIH Image 1.62 software (National Institutes of Health, Bethesda, Rockville, MD, USA), normalizing to β-actin or lamin used as a control.

Cell Transfection and Luciferase Assay

For transient transfection, 5×10^5 cells were seeded into 12-well plates 24 h before transfection. HepG2 cells were transiently transfected in LG medium with luciferase reporter construct using Metafectene® Pro (Biontex Laboratories, München, Germany) following the manufacturer's recommendations. After the 24-h transfection period, the cells were subjected to HG or NMP treatment as indicated above. After the treatment, cells were lysed, and FL activities were measured using the Luciferase Reporter Assay System (Promega, Milano, Italy). For the transfection normalization, the pcDNA3.1/His/lacZ plasmid, coding for a β-galactosidase, was used.

SOD1 and SOD2 Activities Determination

Cytosol and mitochondria were isolated as previously described.[56] Briefly, cells were collected and resuspended in hypotonic buffer (10 mmol L^{-1} NaCl, 2.5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} Tris base, pH 7.5) and homogenized on ice with a glass homogenizer. The homogenates were then centrifuged at 1300 × g for 5 min at 4 °C. The supernatant was centrifuged at

17 000 × *g* for 15 min at 4 °C, obtaining the mitochondria pellet and the cytosolic fractions. The enzymatic activities of catalase, cytosolic SOD1, and mitochondrial SOD2 were measured using the Catalse Assay Kit and the SOD Activity Assay Kit (Sigma–Aldrich, Milano, Italy).

Statistical Analysis

All data are presented as means ± SD for the number of experiments indicated in each case. Statistical analysis was performed using one-way analysis of variance (ANOVA) and a Bonferroni/Dunn post hoc test. Values sharing a different letter differ significantly. Differences were considered statistically significant at $p < 0.05$.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

L.G. and E.S. contributed equally to this work. F.D., S.Q., L.S., and M.M. contributed to the conceptualization; F.D., M.M., L.S., and S.Q. contributed to the methodology; B.D.C.S., E.S., F.S., and L.G. contributed to the investigation and data analysis; F.D. contributed to the writing the original draft; D.D.R., F.D., L.S., M.M., P.M, E.S., and B.D.C.S. contributed to the writing review, F.D., L.S., MM., D.D.R. contributed to the resources.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

coffee, de novo lipogenesis, endoplasmic reticulum stress, fatty liver, oxidative stress, plant bioactive compounds, pyridine alkaloid, SREBP-1, type 2 diabetes

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