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Original Research Article

Propionate promotes gluconeogenesis by regulating mechanistic target of rapamycin (mTOR) pathway in calf hepatocytes

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ABSTRACT

Enhancing hepatic gluconeogenesis is one of the main modes of meeting the glucose requirement of dairy cows. This study attempted to determine whether the gluconeogenesis precursor propionate had an effect on the expression of the main genes involved in gluconeogenesis in calf hepatocytes and elucidate the associated mechanisms. Calf hepatocytes were obtained from 5 healthy calves (1 d old; 30 to 40 kg) and exposed to 0-, 1-, 2.5-, or 5-mM sodium propionate (NaP), which is known to promote the expression of genes involved in the gluconeogenesis pathway, including fructose 1,6-bisphosphatase, phosphoenolpyruvate carboxykinase, and glucose-6-phosphatase. With regard to the underlying mechanism, propionate promoted the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha, hepatocyte nuclear factor 4, and forkhead box O1 (transcription factors that regulate the expression of hepatic gluconeogenic genes) by promoting mammalian target of rapamycin complex 1 (mTORC1), but inhibiting mTORC2 activity ($P < 0.01$). We also established a model of palmitic acid (PA)-induced hepatic injury in calf hepatocytes and found that PA could inhibit the gluconeogenic capacity of calf hepatocytes by suppressing the expression of gluconeogenic genes, inhibiting mTORC1, and promoting the activity of mTORC2 ($P < 0.01$). In contrast, NaP provided protection to calf hepatocytes by counteracting the inhibitory effect of PA on the gluconeogenic capacity of calf hepatocytes ($P < 0.05$). Collectively, these findings indicate that NaP enhances the gluconeogenic capacity of calf hepatocytes by regulating the mTOR pathway activity. Thus, in addition to improving the glucose production potential, propionate may have therapeutic potential for the treatment of hepatic injury in dairy cows.

1. Introduction

Many studies have reported that gluconeogenesis is the main pathway for the production of glucose in cows. Several rate-limiting enzymes are involved in regulating the process of hepatic gluconeogenesis, including fructose 1,6-bisphosphatase (FBP1/2), cytosolic and mitochondrial phosphoenolpyruvate carboxykinase (PCK1/2), and glucose 6-phosphatase (G6PC) (Aschenbach et al., 2010; Pilkis et al., 1988; Pilkis and Granner, 1992), which are strictly regulated by the transcription factors peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), hepatocyte nuclear factor 4 (HNF4), c-AMP response binding protein (CREB), and forkhead box O1 (FOXO1) (Aschenbach et al., 2010; He et al., 2009; Koo et al., 2004; Yoon et al., 2001). Further, ruminal production of propionate, the main substrate for glucose synthesis, contributes as much as 60% to 74% to hepatic gluconeogenesis in cows (Annison and Bryden, 1999; He et al., 2009; Lomax and Baird, 1983). There is growing evidence that hepatic glucose production is proportional to propionate availability in cows (Baird et al., 1980; Zhang et al., 2015). Further, it has been reported that feed intake and supplementation with

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monensin, which increases efficiency of milk production (Richards et al., 2022), may affect hepatic gluconeogenic capacity through an increase in rumen propionate production and *PCK1* expression (Agca et al., 2002; Greenfield et al., 2000; Karcher et al., 2007). In addition to monensin, supplementation with propylene glycol in cows leads to its ruminal metabolization into propionate. Alternatively, propylene glycol may be absorbed and directly used in glucose production in the liver. Thus, propylene glycol supplementation can be used to prevent glucose deficiency (Nielsen and Ingvartsen, 2004; Zhang et al., 2016). In fact, studies have reported that propionate promotes gluconeogenesis by promoting the expression of several genes involved in hepatic gluconeogenesis in dairy cows (Caputo Oliveira et al., 2020; Zhang et al., 2015). However, the effect of propionate on certain key genes and transcription factors involved in gluconeogenesis and the mechanism underlying these effects have not been examined yet.

The mammalian target of rapamycin (mTOR) pathway is an important intracellular metabolic pathway. The mTOR protein is a serine/threonine protein kinase of size 289 kDa that belongs to the phosphatidylinositol-3-hydroxyl kinase-related kinase family. In mammals, mTOR can bind to other proteins to form two structurally distinct complexes — mTORC1 and mTORC2 — which have different functions (Wang et al., 2022a). The mTORC1 protein senses intracellular nutrient and energy status to maintain catabolic and anabolic homeostasis (Dunlop and Tee, 2013). In particular, it plays a role in calf liver cell gluconeogenesis through PGC1α (Wang et al., 2022b). In contrast, mTORC2 has been found to negatively regulate gluconeogenesis through FOXO1 and HNF4 (Koo et al., 2004; Puigserver et al., 2003; Yoon et al., 2001). Thus, the effects of propionate on glucogenesis could be mediated via the mTOR pathway.

The main objective of this study was to investigate the direct effect of propionate on hepatic gluconeogenic viability. Through the analysis of multiple signaling pathways, we determined whether sodium propionate (NaP) regulates gluconeogenesis in calf hepatocytes by modulating the activity of mTORC1 and mTORC2. In addition, we examined the role of hepatic injury induced by free fatty acid palmitic acid (PA) on hepatic gluconeogenesis and investigated whether NaP has an effect on PA-induced hepatic injury.

2. Materials and methods

2.1. Animal ethics statement

This study has received the approval of the Ethics Committee on the Use and Care of Animals of Northwest Agriculture and Forestry University (Approval no. NWAFU-2020-1131) and was conducted by keeping in mind the relevant guidelines and regulations.

2.2. Hepatocyte isolation and culture

Primary calf hepatocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 (HyClone, UT, USA) containing 5% fetal bovine serum (FBS) (Gibco, Grand Island, USA) and 1% penicillin-streptomycin (Solarbio, Beijing, China) in a CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA). Primary hepatocytes were obtained from five healthy 1-day-old Holstein calves (weight, 30 to 40 kg) that were purchased from a commercial dairy farm (Baoji, China).

Hepatocytes were isolated using a previously described protocol (Liu et al., 2014). Briefly, the thickest blood vessel was perfused in perfusate A (140 mM NaCl [Sangon, Shanghai, China], 6.7 mM KCl [Sangon], 10 mM HEPES [Sangon], 2.5 mM glucose [Sangon], 0.5 mM EDTA [Sangon]), perfusate B (140 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 2.5 mM glucose, 5 mM $CaCl_2$ [Sangon]), and perfusate C (0.2 g/L type IV collagenase [Solarbio, Beijing, China] dissolved in perfusate B). The digestion was terminated with a medium containing 10% FBS. The hepatocyte suspension was centrifuged at 50 × *g* for 3 min at 4 °C and washed 3 times. Finally, the cells were suspended in RPMI 1640 and seeded on 10 cm dishes (NEST, Shanghai, China).

2.3. PA and NaP preparation

A stock solution of 100 mM PA (P0500; Sigma–Aldrich, MO, USA) was prepared as described in a previous study (Cousin et al., 2001). Briefly, PA was dissolved in 0.1 M NaOH at 70 °C, and this solution was mixed with 10% fatty acid-free bovine serum albumin (BSA) at 55 °C for 30 min to achieve a final concentration of 100 mM. The stock solution of 100 mM PA was diluted in RPMI 1640 basic medium containing 2% BSA to prepare a working solution. The control treatment comprised an equivalent amount of BSA. NaP (A600882, Sangon) was dissolved in RPMI 1640 basic medium to achieve a final concentration of 100 mM.

2.4. Cell culture and treatment

Cells of the human hepatocyte lines LO2 and HepG2 were purchased from American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% FBS at 37 °C in 5% CO_2.

The PA concentration used in this study was based on the reported serum concentrations of fatty acids during the early postpartum period (Rukkwamsuk et al., 1999) and the NaP concentrations used were also based on previously reported data (Donkin and Armentano, 1995; McCoun et al., 2021). Cells were maintained in RPMI 1640 basic medium containing 2% BSA and treated with different concentrations of PA (0, 100, 200, or 400 μM) and NaP (0, 1, 2.5, or 5 mM), alone or in combination, for 12 h.

A 2 × 2 factorial arrangement was applied for the experiments: primary hepatocytes were treated with NaP (2.5 mM), the mTORC1 inhibitor rapamycin (100 nM) (V900930; Sigma Aldrich, MO, USA), the mTORC1 activator MHY1485 (2 μM) (S7811; Selleck, Shanghai, China), and the PGC1α inhibitor SR-18292 (20 μM) (S8528; Selleck) for 12 h to observe the effect of mTORC1 and PGC1α on the mRNA expression of gluconeogenic genes. The hepatocytes were also treated with NaP (2.5 mM) and PA (400 μM), alone or in combination, for 12 h to observed the potential protective effect of propionate against the inhibitory effect of PA on gluconeogenesis.

2.5. Hepatic glucose output

The gluconeogenesis assay of the hepatocytes was performed as previously described (Chow and Jesse, 1992). Briefly, after 48 h of cell isolation (Wang et al., 2023), the RPMI 1640 medium was replaced with glucose-free medium (11966025, Gibco) containing 5 mM pyruvate. Next, the medium was collected after adding NaP and PA, alone or in combination, for 6 h. After centrifugation, the supernatant was collected, and the glucose content was detected with the Glucose Assay Reagent kit (S0201S; Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, a solution containing 15 μL supernatant mixed with 185 μL Glucose Assay Reagent was heated at 95 °C for 8 min, and 190 μL of the mixed solution was transferred to a 96-well plate. The plates were scanned with a plate reader at 630 nm (TECAN, Spark, Switzerland), and finally, the glucose concentration was calculated from the standard curve.

2.6. Protein extraction and Western blotting

Protein content from the cells was extracted as described previously (Liu et al., 2010). The BCA Protein Assay Kit (TIANGEN, Beijing, China) was used to quantify the total protein concentration. The extracted proteins were run on SDS-PAGE gels and transferred to a nitrocellulose filter membrane (0.45 μm, GE, California, USA). The membranes were blocked with 5% non-fat milk powder dissolved in PBS and incubated overnight at 4 °C with primary antibodies against the following target proteins (diluted to 1:1000 to 1:2000): pT389-S6K1 (9234S/L), p-S6 (4858S), S6K1 (9202S), S6 (2217S), pS473-AKT (9271), AKT (9272), p-4EBP1 (2855), pS2448-mTOR (2971), mTOR (2972), p-p38 (9211), p38 (9212), p-ERK1/2 (4370), ERK1/2 (4695) and pT86-SIN1 (14716) (Cell Signaling Technology, Danvers, USA). Antibodies against β-Actin (66009-1-Ig) and PGC1α (66369-1-Ig) antibody were obtained from Proteintech (Chicago, USA). Following the reaction with the primary antibodies, the membranes were washed with PBS containing 0.1% Tween-20 (PBST, Sangon) and incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma Aldrich, MO, USA) diluted to 1:5000 at room temperature. The membranes were washed again with PBST, and protein detection was performed using the Bio-Rad imaging system (Hercules, CA, USA). β-Actin was used as the internal control. All the protein bands detected were evaluated with Image-Pro Plus (Media Cybernetics).

2.7. RNA extraction and real-time quantitative PCR

Total RNA was isolated from primary hepatocytes with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. The RNA purity (OD_{260}/OD_{280}) and concentration of each sample were determined with the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was confirmed by electrophoresis on a denaturing agarose gel. RT-qPCR analysis was performed in technical triplicate with the TB Green RT-qPCR kit (RR820A; Takara, Dalian, China) on a Roche LightCycler 96 RT-qPCR system (Roche, Basel, Switzerland). cDNA from triplicate wells for each condition were used for obtaining data. The comparative Ct method was used for calculating the relative quantity of the target gene mRNA, which was normalized to β-actin mRNA expression and expressed as fold change ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). The RT-qPCR protocol was as follows: 30 s at 95 °C, and 40 cycles of 10 s at 95 °C and 30 s at 60 °C. The sequences of the primers are listed in Tables 1 and 2.

2.8. Statistical analysis

Data for each calf were independently analyzed, and hepatocytes from each calf were used as biological replicates. Further, each experimental condition was independently reiterated three times, and three technical replicas of each biological replicate were made. The results of the experiments in which hepatocytes were treated with PA (400 μM) were analyzed using a *t*-test with the GraphPad Prism software, version 8.5 (GraphPad InStat Software, San Diego, CA). The effects of various NaP doses (0, 1, 2.5, and 5 mM), treatment time (0, 3, and 6 h), and various PA doses (0, 100, 200, and 400 μM) were analyzed using one-way ANOVA followed by Tukey's tests, and the distribution of variables (normal versus skewed) was assessed using the Shapiro–Wilk test. For independent variables, a 2 × 2 factorial arrangement was used for analysis, with NaP, the mTORC1 inhibitor rapamycin, the mTORC1 activator MHY1485, the PGC1α inhibitor SR18292, and PA as the factors; two levels were considered for each factor: "yes" (it was added) and "no" (it was not added). The model was used to determine the effects of NaP, rapamycin, MHY1485, SR18292, and PA, and the interactions between sets of two factors: NaP × rapamycin, NaP × MHY1485, NaP × SR18292, and NaP × PA. The results were expressed as the mean ± standard error (SEM). Statistical significance was set at $P < 0.05$.

Table 1
Primer sequences for RT-qPCR of cattle.

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
ACSS1	CTGGACGCCTACTTCGAGAC	GCAGCTTCTCCCTTGATGTC
PCCA	TGGGCCAACATTCTCCCATGA	TGGTGAGGATACGCACCTTGT
MCEE	GGAGTGTCCGTCGTTTTTGT	CTGGTTTTCCATGTGCTCCT
MMUT	ATGCAACTCGAGCAAGATGT	ACAAGAAGACGAGGTCTGCG
SUCLG2	GCCTTTGAAAAACCAGGCTGC	CGGAATTCTGCGTTGTCATCA
FBP1	TCCTGCCCTCACCGAGTATG	TCATACAGTAGTCTCAGCTTTCCA
FBP2	AAAGAAGTTTCCTGAGGACGGC	CTGCTCGATGATGTAGGCCA
PCK1	GACGGCCTCAACTACTCAGC	AGTGAGAGCCAACCAGCAGT
PCK2	GACGGCCTCAACTACTCAGC	ATACCAGGGGGACTCCTTTGG
PGC1α	TGGAGTGACATCGAGTGTGCTG	ACTGCTAGCAAGTTTGCCTCA
G6PC	ACTCCTCTGGGTAGCTGTGAT	ACATGACATTCAAGCACCGAAAT
HNF4A	GAATCAACGGCGACATTCGG	AAGGCTGGGATGTACTTGGC
CREB	AGTCCAGACAGTTCAGATTTCA	CAATCCTTGGCACTCCTGGT
FOXO1	GCAACGCGTGGGGCAACCTGT	GGGCACGCTCTTCACCATCCACTC
β-Actin	AAGGACCTCTACGCCAACACG	TTTGCGGTGGACGATGGAG

ACSS1 = acyl-CoA synthetase short-chain family member 1; *PCCA* = propionyl-CoA carboxylase alpha chain; *MCEE* = methylmalonyl-CoA epimerase; *MMUT* = methylmalonyl-CoA mutase; *SUCLG2* = succinate-CoA ligase; *FBP1* = fructose 1,6-bisphosphatase 1; *PCK1* = phosphoenolpyruvate carboxykinase; *PGC1α* = peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *G6PC* = glucose-6-phosphatase; *HNF4A* = hepatocyte nuclear factor 4; *CREB* = c-AMP response binding protein; *FOXO1* = forkhead box O1.

Table 2
Primer sequences for RT-qPCR of human.

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
ACSS1	CGAGAGCGTTGCTTTGATCT	GGGCATGTAGATGGCAACAC
PCCA	CGTGGAGTTCCTTGTGGACT	CAGCTTGTTTGTGCCTGAGA
MCEE	GGAGCACATGGAAAACCAGT	TGGAAGCAGTGAAGGACTCA
MMUT	GAGTGGAGCATATCGCCAGG	CACTTCACGAGGAGTCTGGAA
SUCLG2	TCACAGCTGATCCTAAGGTTG	GCGACGTTCTCTTGGATACC
FBP1	CCTGCCGTCACTGAGTACAT	CAGCAGTCTCAGCTTTCCAT
FBP2	AAGAAATTCCCTGAGGATGGCAG	GCCACGGGATTGCATTCATAC
PCK1	CCTGACCGCAGAGAGATCAT	CCGCCAGGTACTTCTTCTCA
PCK2	CCACTGGCATTCGAGATTTT	CCCGCTGAGAAGGAGTTACA
G6PC	ACTCCTCTGGGTAGCTGTGAT	ACATGACATTCAAGCACCGAAAT
β-Actin	AAGGACCTCTACGCCAACACG	TTTGCGGTGGACGATGGAG

ACSS1 = acyl-CoA synthetase short-chain family member 1; *PCCA* = propionyl-CoA carboxylase alpha chain; *MCEE* = methylmalonyl-CoA epimerase; *MMUT* = methylmalonyl-CoA mutase; *SUCLG2* = succinate-CoA ligase; *FBP1* = fructose 1,6-bisphosphatase 1; *PCK1* = phosphoenolpyruvate carboxykinase; *G6PC* = glucose-6-phosphatase.

3. Results

3.1. Increase in gluconeogenesis in calf hepatocytes treated with propionate

The addition of NaP (0, 1, and 2.5 mM) significantly increased glucose production in calf hepatocytes (Fig. 1A) and increased the expression of the gluconeogenesis-associated genes *FBP1*, *FBP2*, *PCK1*, *PCK2*, and *G6PC* (Fig. 1B–F). Furthermore, we found that NaP (0, 1, and 2.5 mM) treatment resulted in a significant increase in the gene expression of the key enzymes involved in the conversion of propionic acid to pyruvate, including *ACCS1*, *SUCLG2*, *MCEE*, *MMUT*, and *PCCA* (Fig. 1G–K). These findings were confirmed in human-derived hepatocytes, as NaP was found to significantly promote the expression of *FBP1*, *FBP2*, *PCK1*, *PCK2*, and *G6PC* in HepG2 cells (Fig. S1A–E) and LO2 cells (Fig. S1F–J) in a dose-dependent

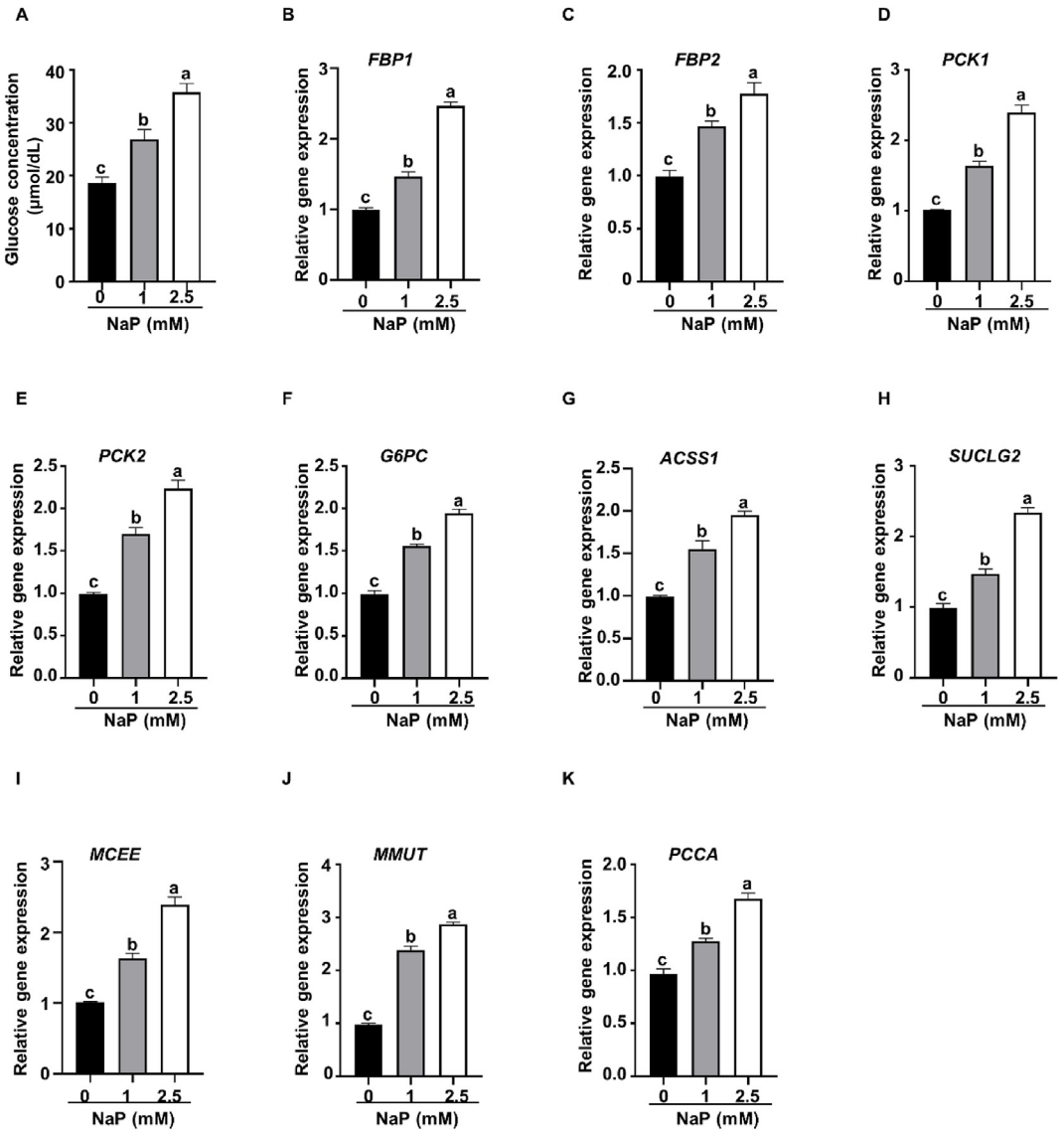

Fig. 1. Increase in gluconeogenesis in calf hepatocytes treated with propionate. Calf hepatocytes were treated with indicated concentration of sodium propionate (NaP) for 6 h, and the glucose concentration was detected using a kit (A). Calf hepatocytes were treated with indicated concentration of NaP for 12 h, and expression levels of fructose 1,6-bisphosphatase 1 (*FBP1*) (B), *FBP2* (C), phosphoenolpyruvate carboxykinase 1 (*PCK1*) (D), *PCK2* (E), glucose-6-phosphatase (*G6PC*) (F), acyl-CoA synthetase short-chain family member 1 (*ACSS1*) (G), succinate-CoA ligase (*SUCLG2*) (H), methylmalonyl-CoA epimerase (*MCEE*) (I), methylmalonyl-CoA mutase (*MMUT*) (J), and propionyl-CoA carboxylase alpha chain (*PCCA*) (K) were detected by real-time quantitative PCR (RT-qPCR). Data were analyzed by one-way ANOVA. Statistical significance was determined as the mean ± SEM. [a, b, c]Bars with a different letter mean a significant difference ($P < 0.05$).

manner. Further, NaP significantly promoted the expression of *ACCS1*, *SUCLG2*, *MCEE*, *MMUT*, and *PCCA* in HepG2 cells, and this regulatory effect was also concentration-dependent (Fig. S1K–O). These results indicate that propionate promotes the expression of the genes involved in hepatic gluconeogenesis and thus, enhances hepatocyte gluconeogenesis.

3.2. Increased expression of key transcription factors of gluconeogenesis in calf hepatocytes treated with propionate

To investigate the molecular mechanism by which NaP promotes hepatic gluconeogenesis, we first studied the influence of NaP on the expression of key transcription factors involved in gluconeogenesis. We found that NaP could significantly promote the mRNA expression of *HNF4A*, *FOXO1*, *CREB*, and *PGC1α* (Fig. 2A–D). Further, examination of the protein levels of PGC1α by immunoblotting assay demonstrated that the protein levels of PGC1α were dramatically enhanced with increase in NaP concentration (Fig. 2E and F). For example, when the concentration of NaP reached 2.5 mM, the protein levels of PGC1α increased to about five-fold the NaP concentration in untreated hepatocytes (Fig. 2F). To confirm this observation, we repeated this experiment in human-derived hepatocytes and found that NaP also significantly promoted the protein levels of PGC1α in HepG2 cells (Fig. 2G and H) and LO2 cells (Fig. 2I and J) in a concentration-dependent manner, with the protein levels of PGC1α increasing to more than three- and five-fold, respectively, at the NaP concentration of 2.5 mM (Fig. 2H and J). These results suggest that propionate possibly enhances the expression of key gluconeogenic genes and increases glucose production in hepatocytes by promoting the expression of key transcription factors that are involved in hepatic gluconeogenic gene expression.

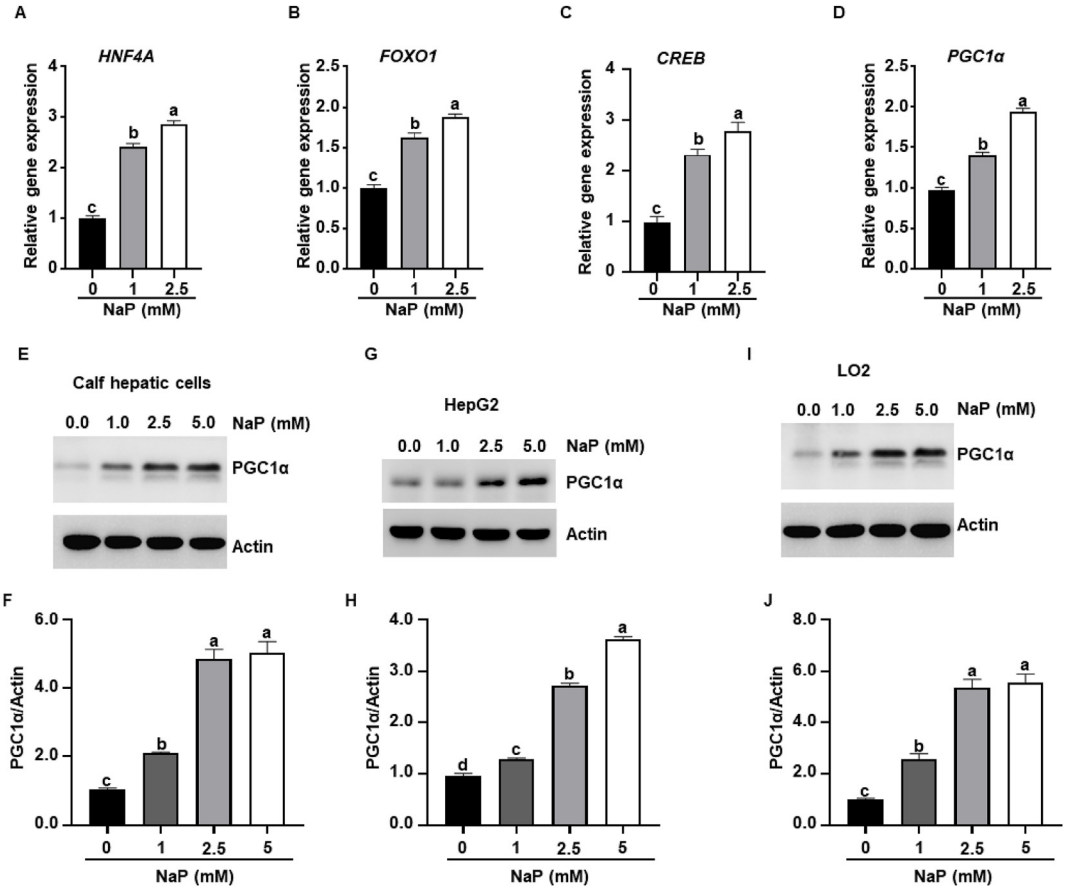

Fig. 2. Increased expression of key transcription factors of gluconeogenesis in calf hepatocytes treated with propionate. Calf hepatocytes were treated with indicated concentration of NaP for 12 h, and expression levels of hepatocyte nuclear factor 4 (*HNF4A*) (A), forkhead box O1 (*FOXO1*) (B), c-AMP response binding protein (*CREB*) (C), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC1α*) (D) were detected by RT-qPCR. Calf hepatocytes (E, F), HepG2 (G, H), and LO2 (I, J) cells were treated with indicated concentration of NaP for 12 h, the indicated proteins were detected by Western blotting (E, G, I), and quantified by ImageJ (F, H, J). Data were analyzed by one-way ANOVA. [a, b, c, d]Bars with a different letter mean a significant difference ($P < 0.05$).

3.3. Regulation of mTOR activity by propionate

To determine the molecular mechanism by which propionate regulates gluconeogenesis, we treated calf hepatocytes with different concentrations of NaP (0, 1, 2.5, and 5.0 mM). Changes were detected in several upstream signaling molecules, including mTORC1, mTORC2, and MAPK. The pT389-S6K, p-S6, and p-4EBP1 levels are considered as important indicators of mTORC1 activity, while mTORC2 activity is indicated by changes in the phosphorylation of serine at position 473 of AKT (pS473-AKT), the phosphorylation of threonine at position 86 of SIN1 (pT86-SIN1), and the phosphorylation of serine at position 2448 of mTOR (pS2448-mTOR). Further, the phosphorylation levels of ERK and p38 are considered as markers of changes in the MAPK pathway. Our results showed that NaP promoted the levels of pT389-S6K, p-S6 and p-4EBP1 in a concentration-dependent manner. Further, when the concentration of NaP reached 2.5 mM, the levels of pT389-S6K, p-S6, and p-4EBP1 all increased by more than 3-fold (Fig. 3A–D). In contrast, NaP addition inhibited mTORC2 activity in a dose-dependent manner, with the levels of pS473-AKT, pT86-SIN1, and pS2448-mTOR decreasing by more than half at a NaP concentration of 2.5 mM (Fig. 3F–I). Interestingly, the addition of NaP did not affect MAPK activity, as the phosphorylation levels of ERK and p38 remained unchanged even at NaP concentrations of 5.0 mM (Fig. 3A, E, F and J).

Apart from the effects of different concentrations of NaP, we explored the effects of different treatment times on the signaling pathways. In line with previous data, our results showed a dramatic increase in pT389-S6K and p-S6 with increase in treatment time, and the increase was more than 3-fold with treatment for 6 h (Fig. S2A–C). In contrast, the levels of pS473-AKT and pT86-SIN1 were significantly decreased, and they were decreased by more than half with the 6-h treatment (Fig. S2A, D and E). These results suggest that NaP positively regulates mTORC1 activity and negatively regulates mTORC2 activity in a dose- and time-dependent manner.

3.4. Role of mTORC1 and PGC1α in propionate-mediated regulation of the expression of gluconeogenesis-related genes in calf hepatocytes

To determine whether propionate regulates gluconeogenic gene expression in calf hepatocytes through mTORC1 and PGC1α, we first treated cells with rapamycin, an inhibitor of mTORC1, or NaP. NaP significantly promoted *FBP1* expression, while rapamycin inhibited *FBP1* expression. More importantly, we found that rapamycin was able to completely block the promoting effect of NaP on *FBP1* gene expression (Fig. 4A). Similar results were obtained for *PCK1* and *G6PC* (Fig. 4B and C). Rapamycin was able to not only negatively regulate the expression of *PCK1* and *G6PC* but also

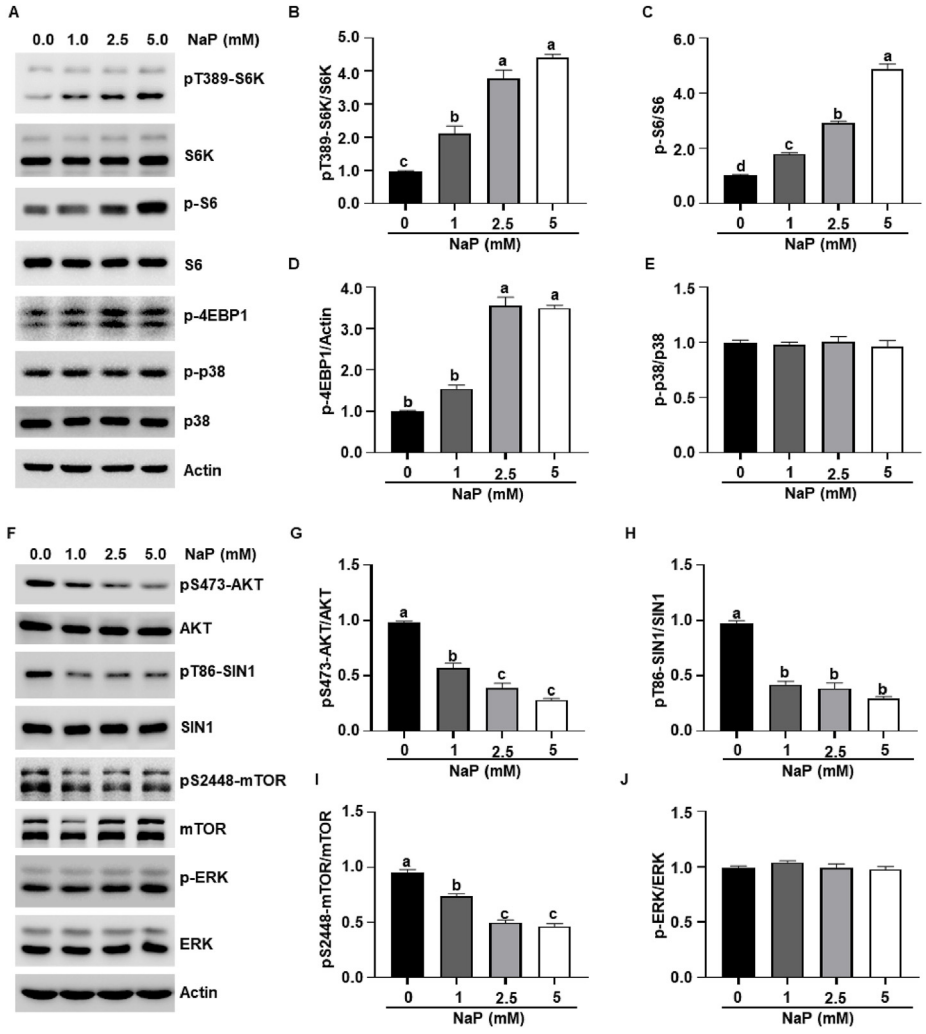

Fig. 3. Regulation of mTOR activity by propionate. Calf hepatocytes were treated with indicated concentration of sodium propionate (NaP) for 12 h. The indicated proteins were detected by Western blotting (A, F), and quantified by ImageJ (B–E and G–J). Data were analyzed by one-way ANOVA. $^{a, b, c, d}$Bars with a different letter mean a significant difference ($P < 0.01$).

significantly counteract the effect of NaP on the expression of these two key gluconeogenic genes (Fig. 4B and C). We also treated the hepatocytes with MHY1485, an agonist of mTORC1, and the results showed that MHY1485 was able to promote the expression of *FBP1*, *PCK1*, and *G6PC*. Further, although NaP was also able to promote the expression of these three key gluconeogenic genes, this effect of NaP was not observed in MHY1485-treated calf hepatocytes (Fig. 4D–F). The findings were corroborated by similar experiments in LO2 cells (Fig. S3A–F) and confirm that propionate controls the expression of calf hepatocyte gluconeogenic genes via mTORC1.

In the next set of experiments, the role of PGC1α in the mechanism via which NaP regulates gluconeogenic gene expression was examined. Therefore, we examined the mRNA expression of *FBP1*, *PCK1*, and *G6PC* in calf hepatocytes, by using SR18292 (an inhibitor of PGC1α) alone or in combination with NaP. The findings indicated that the mRNA expression of *FBP1*, *PCK1*, and *G6PC* was decreased with SR18292 treatment (Fig. 4G–I). Importantly, SR18292 treatment blocked the NaP-induced increase in gluconeogenic gene expression (Fig. 4G–I), and similar results were obtained in LO2 cells (Fig. S3G–I). Thus, our data suggest that propionate regulates gluconeogenic gene expression in calf hepatocytes, at least in part, through the mTORC1 pathway and PGC1α.

3.5. Regulation of mTOR activity and expression of gluconeogenesis-related genes in calf hepatocytes treated with PA

When calf hepatocytes were treated with 0, 100, 200, or 400 μM of PA, the levels of pT389-S6K and p-S6 decreased significantly in a dose-dependent manner (Fig. 5A–C). Further, when the concentration reached 400 μM, the levels of pT389-S6K and p-S6 decreased by more than 70% and 60%, respectively (Fig. 5B and C), while the levels of pS473-AKT and pT86-SIN1 increased more than 3-fold (Fig. 5A, D and E). We also found that PA could regulate the expression of key transcription factors associated with gluconeogenesis, including *FOXO1*, *CREB* and *PGC1α* (Fig. 5F–H). Importantly, the mRNA expression of *FBP1*, *FBP2*, *PCK1*, *PCK2*, and *G6PC* decreased with PA treatment (Fig. 5I–M). PA also induced a decrease in the expression of key enzymes that convert propionic acid to pyruvate, such as *ACSS1*, *SUCLG2*, *MCEE*, *MMUT*, and *PCCA* (Fig. 5N–R). These results suggest that PA may negatively regulate gluconeogenic gene

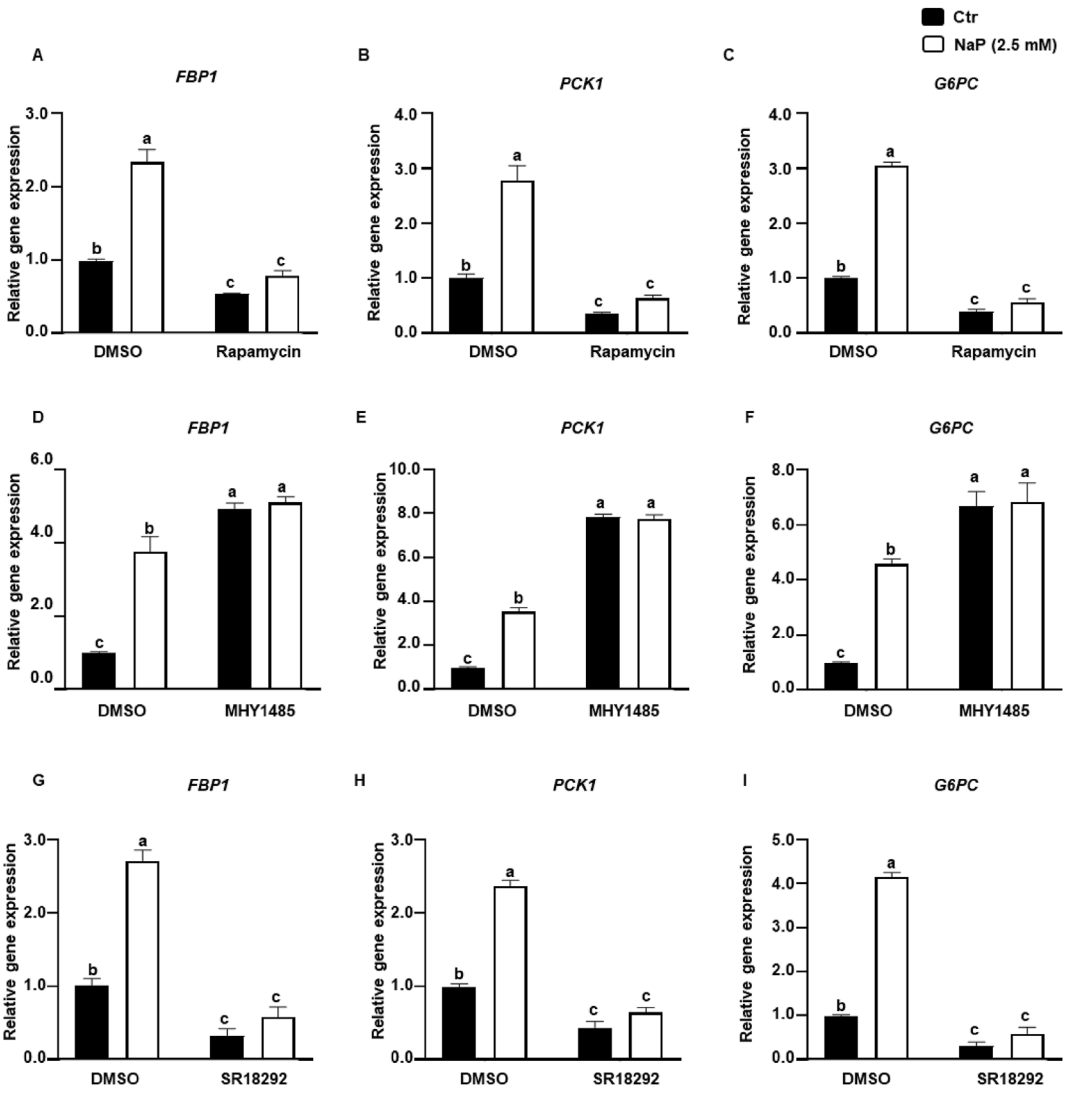

Fig. 4. Role of mammalian target of rapamycin complex 1 (mTORC1) and PGC1α in propionate-mediated regulation of the expression of gluconeogenesis-related genes in calf hepatocytes. (A–C). Calf hepatocytes were treated with NaP and rapamycin (100 nM). The expression levels of *FBP1* (A), *PCK1* (B), and *G6PC* (C) were detected by RT-qPCR. (D–F). Calf hepatocytes were treated with NaP and MHY1485 (2 μM). The expression levels of *FBP1* (D), *PCK1* (E), and *G6PC* (F) were detected by RT-qPCR. (G–I). Calf hepatocytes were treated with NaP and SR18292 (20 μM). The expression levels of *FBP1* (G), *PCK1* (H), and *G6PC* (I) were detected by RT-qPCR. Data were analyzed by two-way ANOVA. [a, b, c]Bars with a different letter mean a significant difference ($P < 0.05$).

expression by promoting mTORC1 activity and inhibiting mTORC2 activity.

3.6. Decrease in the inhibitory effect of PA on gluconeogenesis genes in propionate-treated calf hepatocytes

Next, we investigated whether NaP mitigates the reduction in hepatic gluconeogenic capacity induced by PA. To this end, we assessed mTORC1 and mTORC2 activity in calf hepatocytes treated with PA alone or in combination with NaP. The results showed that although PA was able to significantly inhibit the activity of mTORC1, NaP was able to block the inhibitory effect of PA on mTORC1 activity; further, the mTORC2-promoting effect of PA was also significantly counteracted by NaP (Fig. 6A–C). In addition, we found that NaP was able to significantly block the negative regulatory effects of PA on key gluconeogenic genes, including *FBP1*, *PCK1*, and *G6PC* (Fig. 6D–F). More importantly, when we examined the effects of PA and NaP on calf hepatocyte glucose synthesis, consistent with the above results, PA was able to significantly inhibit calf hepatocyte glucose synthesis and NaP was able to significantly alleviate these negative regulatory effects of PA (Fig. 6G). Thus, the above findings imply that propionate can alleviate the adverse effects of PA on calf hepatocytes and may, therefore, have potential in the treatment of liver injury caused by exposure to high concentrations of PA.

4. Discussion

The present study showed that propionate could enhance gluconeogenesis in calf hepatocytes. These findings are consistent with those of previous studies on dairy cows that demonstrated the effect of propionate on the mRNA expression of gluconeogenic genes, such as *PCK1/2*, pyruvate carboxylase (*PC*), and *G6PC*, and the glucose synthesis ability of the liver (Caputo Oliveira et al., 2020; Mann et al., 2018; Zhang et al., 2020). However, the expression of enzymes that regulate the conversion of propionate to pyruvate, such as *ACSS1*, *MCEE*, *PCCA*, *MMUT*, and *SUCLG2*, and the expression of key gluconeogenic genes, such as *FBP1/2*, were not analyzed in

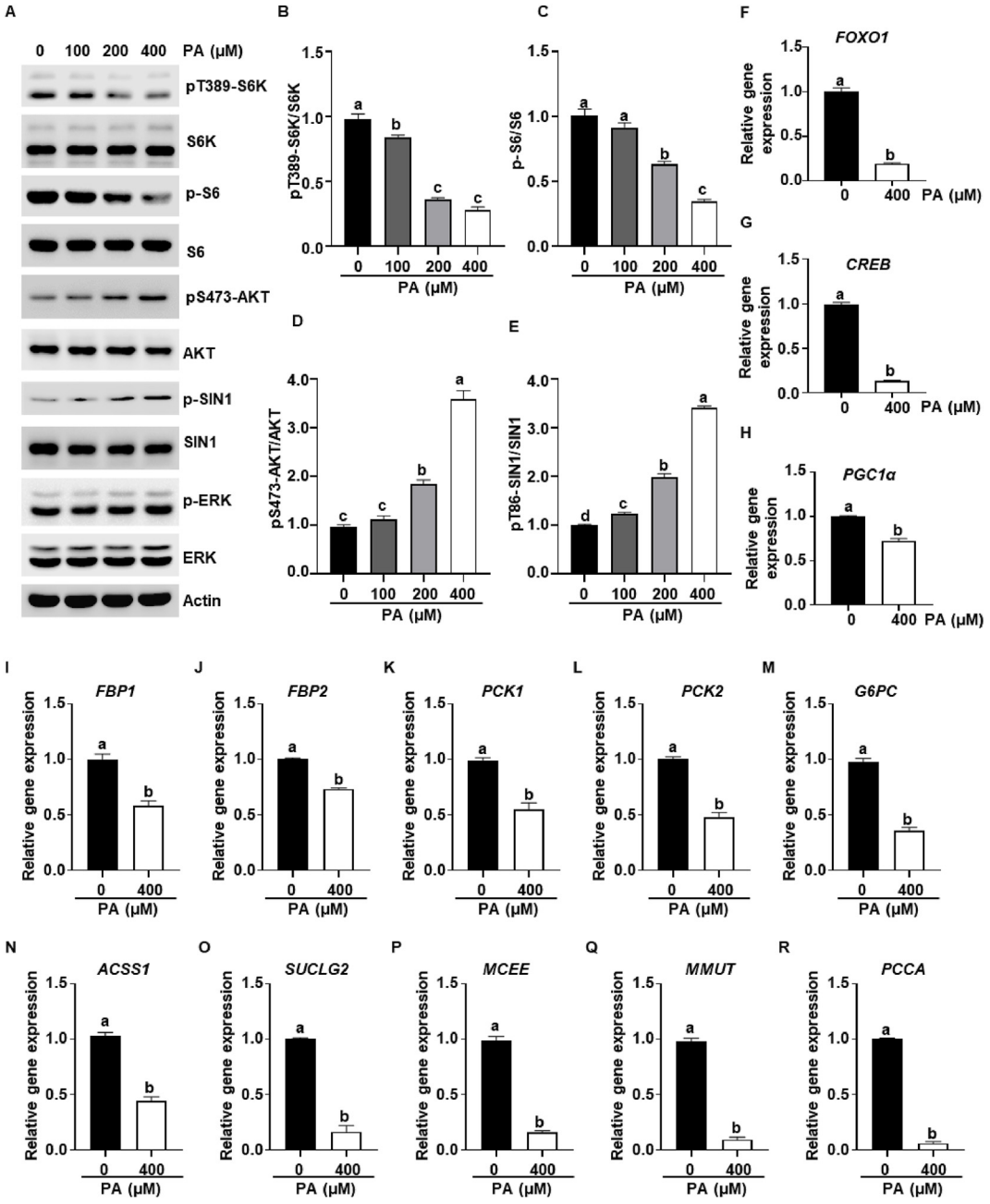

Fig. 5. Regulation of mTOR activity and expression of gluconeogenesis-related genes in calf hepatocytes treated with palmitic acid (PA). Calf hepatocytes were treated with indicated concentration of PA for 12 h, the indicated proteins were detected by Western blotting (A), and quantified by ImageJ (B–E). Calf hepatocytes were treated with PA for 12 h, and expression levels of *FOXO1* (F), *CREB* (G), *PGC1α* (H), *FBP1* (I), *FBP2* (J), *PCK1* (K), *PCK2* (L), *G6PC* (M), *ACCS1* (N), *SUCLG2* (O), *MCEE* (P), *MMUT* (Q), and *PCCA* (R) were detected by RT-qPCR. Data were analyzed by one-way ANOVA (B–E) and *t*-test (F–R). $^{a, b, c, d}$Bars with a different letter mean a significant difference ($P < 0.05$).

these previous studies. Moreover, the mechanisms underlying the regulatory effect of propionate on the expression of gluconeogenic genes were not elucidated in a large number of previous studies on this topic (Aiello and Armentano, 1987; Lin et al., 2022; Zhang et al., 2015, 2016). In the current study, we not only explored the effect of propionate on key transcription factors regulating gluconeogenic genes such as *HNF4A*, *PGC1α*, *FOXO1*, and *CREB* but also examined the upstream signaling pathways regulating these transcription factors.

In order to examine the mechanisms of propionate, in the current study, we focused on the effect of propionate on the mTOR- and MAPK- signaling pathways. The results showed that propionate was able to promote the activity of mTORC1, which is known to negatively regulate cellular autophagy. This result is consistent with that of a previous study (Gao et al., 2021). Cellular autophagy is a very complex process that is regulated by multiple pathways, including the mTOR/AMPK pathway (Kim and Guan, 2011; Yang and Klionsky, 2010), where AMPK is able to influence cellular autophagy through multiple pathways. However, the role of AMPK was not investigated in this study, so further studies are needed to determine whether propionate promotes cellular autophagy through the AMPK pathway. In addition, our study showed that propionate promotes *CREB* mRNA expression. CREB activity is reported to be regulated by the cAMP-PKA signaling axis (Mayr and

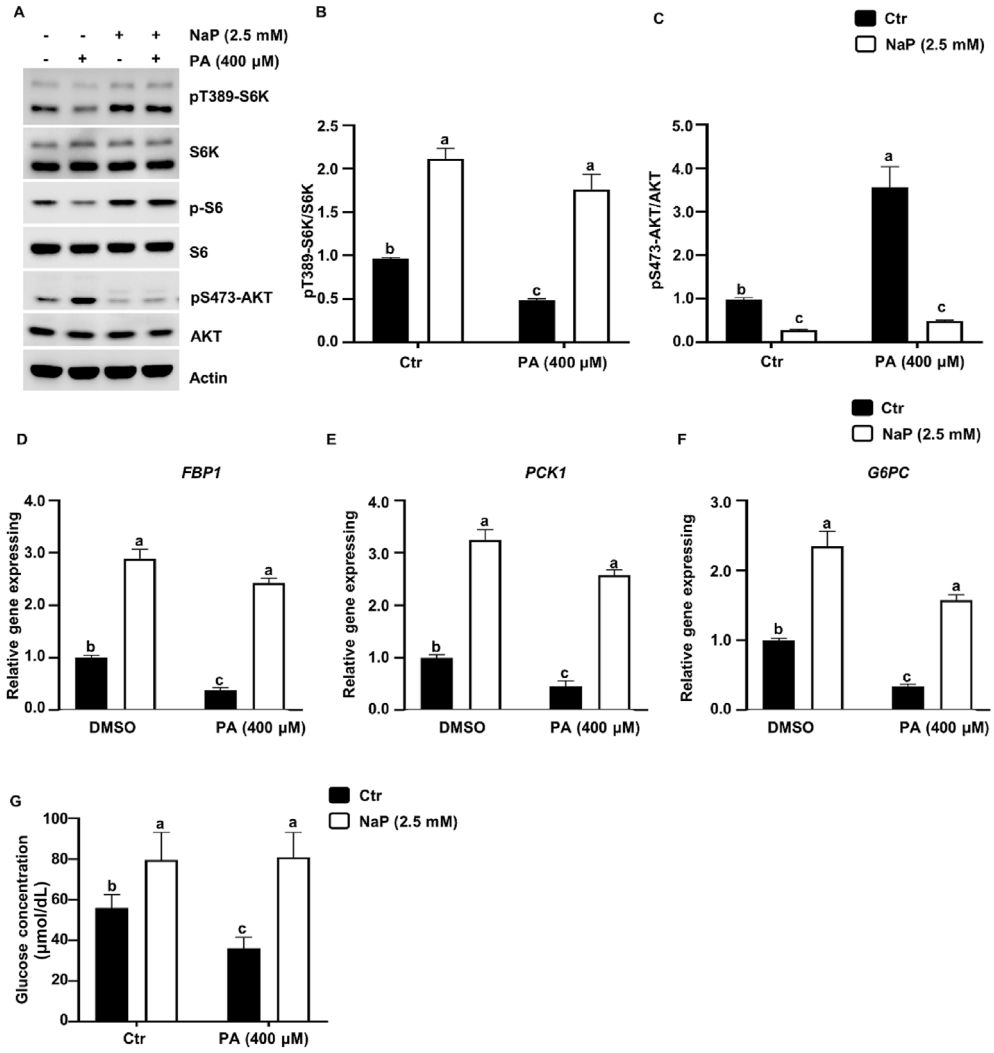

Fig. 6. Decrease in the inhibitory effect of palmitic acid (PA) on gluconeogenesis genes in propionate-treated calf hepatocytes. Calf hepatocytes were treated with sodium propionate (NaP) and PA, the indicated proteins were detected by Western blotting (A), and quantified by ImageJ (B, C). Calf hepatocytes were treated with NaP and PA, the expression levels of *FBP1* (D), *PCK1* (E), and *G6PC* (F) were detected by RT-qPCR. (G) Calf hepatocytes were treated with NaP and PA, and the glucose concentration were detected by kit. Data were analyzed by two-way ANOVA. [a, b, c]Bars with a different letter mean a significant difference ($P < 0.05$).

Montminy, 2001; Shaywitz and Greenberg, 1999; Xu et al., 2007). However, our study did not find any effect of propionate on the cAMP-PKA-signaling axis (data not shown). Therefore, the mechanism underlying this effect of propionate on *CREB* expression needs to be further investigated.

The mTOR pathway is relatively well studied, and the classical theory is that mTORC2 is present upstream of mTORC1 (Battaglioni et al., 2022; Sarbassov et al., 2005). The mTORC2 protein is able to inhibit the activity of mTORC1 by activating AKT, which in turn phosphorylates multiple sites of the protein TSC2 (an important negative regulator of mTORC1) (Garami et al., 2003; Inoki et al., 2002, 2003; Manning et al., 2002; Menon et al., 2014). However, in our study, we observed that propionate was able to inhibit the activity of mTORC2 while promoting the activity of mTORC1. We reasoned that this result might imply that propionate triggers a pathway to activate the mTORC1 pathway that is not dependent on mTORC2 activity. For example, the AMPK, Wnt, and NF-κB signaling pathways are able to inhibit TSC2 activity independent of the mTORC2 pathway (Inoki et al., 2006; Lee et al., 2007; Wang et al., 2022a). Therefore, future studies need to determine whether propionate plays a role in the Wnt and NF-κB signaling pathways and whether it activates the mTORC1 pathway through the Wnt and NF-κB signaling pathways.

Liver injury could negatively impact hepatic gluconeogenesis (Bobe et al., 2004). Increasing ruminal propionate production by supplementation with feed additives (e.g., propylene glycol) is a common prophylactic treatment used to treat circulating glucose deficiency (Nielsen and Ingvartsen, 2004), but the mechanism of hepatic gluconeogenesis by propionate is not well understood. Accordingly, the aim of this study was to explore the potential of therapeutic strategies to alleviate the reduced capacity for gluconeogenesis caused by liver injury-induced by high concentrations of free fatty acids, such as PA. By treating calf hepatocytes isolated from healthy calves with different concentrations of PA, we found that PA inhibited gluconeogenic viability, mainly in the form of downregulation of gluconeogenic genes; promoted mTORC1 activity; and inhibited mTORC2 activity. In addition, we found that propionate treatment significantly countered the inhibitory effect of PA on gluconeogenesis by blocking the inhibitory effect of PA on the expression of gluconeogenic genes. Previous studies have shown that PA challenge can inhibit autophagy in calf liver cells (Feng et al., 2022; Gao et al., 2021), so it would be interesting to

explore whether propionate also affects the autophagy of calf liver cells under conditions of liver damage.

There are some shortcomings in this work that need to be mentioned. For one, we found that propionate regulates the mTORC2 pathway, as well as the expression of *HNF4A*, *FOXO1*, or *CREB*, which are transcription factors associated with gluconeogenesis. However, these findings need to be validated using RNAi technology to specifically knock down the mTORC2 pathway, *HNF4A*, *FOXO1*, or *CREB*, in order to conclusively determine that they are involved in the gluconeogenesis regulation mechanisms of propionate. These experiments were, unfortunately, beyond the scope of the present study. Moreover, although the present findings indicate that propionate can regulate the mTOR pathway, the associated mechanism still needs to be elucidated. In particular, the role of the propionate receptor GPR43 needs to be examined.

5. Conclusions

Taken together, our results suggest that the role of propionate, via modulation of the activity of the mTOR pathway, may be significant in ameliorating liver injury in dairy cows.

Author contributions

Guoyan Wang: Conceptualization, Methodology, Writing—original draft, Investigation. **Senlin Qin:** Conceptualization, Methodology, Writing—original draft, Investigation. **Yining Zheng:** Methodology, Investigation. **Huijun Geng:** Investigation. **Lei Chen:** Project administration and Funding acquisition. **Junhu Yao:** Writing—review and editing, Resources, Supervision, Project administration and Funding acquisition. **Lu Deng:** Conceptualization, Methodology, Writing—original draft, Investigation, Writing—review and editing, Resources, Supervision, Project administration and Funding acquisition.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.07.001.

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