NEFAs Influence the NF- κ B Inflammatory and Insulin Signaling Pathways Through TLR4 in Bovine Hepatocytes In Vitro

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Abstract

Dairy cows are often in a state of negative energy balance (NEB), because of decreased dry matter intake and increased energy requirements, initiating lipid mobilization and leading to high serum β -hydroxybutyrate and nonesterified fatty acid (NEFA) levels, which can induce ketosis and fatty liver in dairy cows. We all have known that inflammation and insulin resistance are also common diseases in perinatal period of dairy cows. So what is the relationship between negative energy balance, insulin resistance and inflammation in cow? In order to study the role of NEFAs in the NF- κ B inflammatory and insulin signaling pathways through TLR4, we cultured bovine primary hepatocytes and added different concentrations of NEFAs to test the mRNA and protein levels of inflammatory and insulin signaling pathways. Our experiments indicated that NEFAs could activate the NF- κ B inflammatory signaling pathway and influence insulin resistance through TLR4. However, an inhibitor of TLR4 alleviated the inhibitory effects of NEFAs on the insulin pathway. In conclusion, all of these results indicate that high-dose NEFAs can activate the TLR4/NF- κ B inflammatory signaling pathway, and reduce the sensitivity of the insulin pathway through the TLR4/PI3K/AKT metabolic axis.

Introduction

Ketosis and fatty liver, common nutritional and metabolic diseases in perinatal cows, are characterized by negative energy balance (NEB) often accompanied by insulin resistance and inflammation [1]. Under NEB conditions, dairy cows mobilize their body fat to produce a large amount of nonesterified fatty acids (NEFAs) into the blood. Incompletely oxidized NEFAs in the liver may become β -hydroxybutyric acid (BHBA), the most important factor in ketosis, as excessive NEFAs oxidation can cause ketosis. When fatty acid metabolism accelerates, excess acetyl coenzyme A produced by β -oxidation is transported to hepatocytes for ketosis. Fatty acids that do not undergo β -oxidation are re-esterified back into triglycerides (TGs) and released into the bloodstream in the form of very low-density lipoproteins. Studies in obese or high-fat diet-fed mice have demonstrated that NEFAs are closely related to inflammation and insulin resistance and act as signaling molecules that can directly regulate lipid metabolism in hepatocytes [2].

Insulin is a regulator of fat production [3]. In early lactation, the skeletal muscle and adipose tissue may exhibit hypoinsulinemia and decreased insulin sensitivity, resulting in increased glucose, which can be used by the breast [4]. Free fatty acids (FFAs) are increased in obese individuals and play an important role in insulin resistance, it is clear that FFAs can induce insulin resistance in the muscle and in type 2 diabetes [5]. However, the mechanism by which FFAs induce NEB and liver insulin resistance in the perinatal period is not clear.

TLRs are a class of pathogen recognition receptors that activate the NF- κ B signaling pathway and promote the transcription of pro-inflammatory factors [6]. Inflammatory factors can influence the transmission of insulin signaling pathway to downstream by different pathway to induce the phosphorylation of insulin receptor substrates at serine residues, which can reduce the physiological

function of insulin and result in insulin resistance [7]. Recent studies indicate that TLR4 plays an important role in insulin resistance. In the tissue, skeletal muscle, pancreas and liver, insulin resistance is induced by the inflammatory response [8, 9]. Furthermore, studies have indicated that NEFAs can activate TLR4 in vitro and in vivo [10]. However, no studies on the relationship between high-dose NEFAs, the TLR4/NF- κ B inflammatory signaling pathway and liver insulin resistance, nor how NEFAs cause insulin resistance in bovine hepatocytes, have been carried out.

In this study, we added NEFAs to calf primary hepatocytes in vitro to detect the effects of NEFAs on inflammatory and insulin signaling pathways and illuminate whether NEFAs mediate cow hepatocyte inflammation and insulin resistance through TLR4.

Results

NEFAs affected the NF- κ B inflammatory pathway through the TLR4 protein

We added TAK-242, and inhibitor of TLR4, and detected its effect on the inflammatory pathway. Hepatocytes were divided into four groups: NEFAs + TAK-242 group, TAK-242 group, NEFAs (2.4 mM) group and DMSO group. The TLR4 and p-P65 protein levels in the NEFAs group were higher than those in the DMSO group ($p < 0.05$); however, after addition of the inhibitor, namely, in the NEFAs + TAK-242 group, the protein level of TLR4 was significantly lower than that in the NEFAs group ($p < 0.05$). However, the level of P65 showed no obviously change ($p > 0.05$) (Fig. 3).

Analysis of the effects of NEFAs on the NF- κ B inflammatory pathways through TLR4 by cell immunofluorescence

To further test whether NEFAs affects inflammatory pathways through TLR4, we used immunofluorescence to detect the effects of NEFAs and TAK-242 on the nuclear transfer of P65. The expression of P65 in the nucleus and cytoplasm was obviously higher in the NEFAs groups than that in the control group. The expression of P65 in the nucleus and cytoplasm was lower NEFAs + TAK-242 group than in the high-concentration group (Fig. 4).

NEFAs affected inflammatory factors through TLR4 at the mRNA level

We analyzed the effects of NEFAs on the inflammatory factors IL-6 and TNF α to see if they occurred through TLR4 at the mRNA level. The primer sequences specific for IL-6 and TNF α are listed in Table 1. The IL-6 mRNA level showed no obvious difference between low-concentration group and control group, but the medium- and high-concentration groups contained IL-6 mRNA levels clearly higher than those in the control group ($p < 0.05$). However, the addition of TAK-242 obviously decreased the mRNA level of IL-6

compared to that in the NEFAs group ($p < 0.05$). There was no difference in IL-6 mRNA level between the DMSO and control groups ($p > 0.05$) (Fig. 5A). The mRNA level of TNF α increased with increasing NEFA concentration ($p < 0.05$) and was highest in the high-concentration group ($p < 0.05$). Furthermore, the mRNA level of TNF α was lower in the TAK-242 + NEFAs group than in the high-concentration group and control group ($p < 0.05$), but there was no difference in TNF α mRNA levels between the TAK-242 and DMSO groups ($p > 0.05$) (Fig. 5B).

Table 1

Primers used for qRT-PCR

Primers used for PCR (5' - 3')	
β-actin	F: GCT AAC AGT CCG CCT AGA AGC A
	R: GTC ATC ACC ATC GGC AAT GAG
IL-6	F: AAC GAG TGG GTA AAG AAC GC
	R: CTG ACC AGA GGA GGG AAT GC
TNF-α	F: CTG CCG GAC TAC CTG GAC TAT
	R: CCT CAC TTC CCT ACA TCC CTA A
G6-Pase	F: AGC AAG TGG TTC CCG TTT C
	R: ACC CAG GCG AGG CAG TA
PEPCK	F: AAG TAC CTT GAG GAG CAA GTG AA
	R: GGT GCG TTG TAT GGA TTG GA

Effect of NEFAs on inflammatory cytokines in the cell supernatant

To verify that NEFAs influence inflammatory pathways though TLR4, we detected the IL-6 and TNF α levels in the cell supernatant by ELISA. The IL-6 and TNF α levels were obviously increased in the high-concentration group ($p < 0.05$) and lower in the TAK-242 + NEFAs group than in the high-concentration group ($p < 0.05$). However, there were no difference between the low- and medium-concentration groups and control group ($p > 0.05$) (Fig. 6).

NEFAs effect the insulin pathway through TLR4 at the protein level

In this experiment, we divided cells into 11 groups: the NEFAs group (2.4 mM), TAK-242 group (1 μ M), NEFAs + TAK-242 group, DMSO group (1 μ M), INS group (100 nm), 0.6 mM NEFAs + INS group, 1.2 mM NEFAs + INS group, 2.4 mM NEFAs + INS group, TAK-242 + INS group, 2.4 mM NEFAs + TAK-242 + INS group, and DMSO + INS group.

In the NEFAs group, the phosphorylation levels of GSK-3 β and IRS1 were higher ($p < 0.05$), and the protein levels of p-AKT, p-IRS2, GLUT4 and PI3K were significantly lower than those in the DMSO group ($p < 0.05$). There were no significant difference in IRS1, IRS2 or AKT levels between the groups ($p > 0.05$). The protein level of GSK-3 β in the NEFAs group was lower than that in the DMSO group ($p < 0.05$), but there was no difference between the other groups ($p > 0.05$). After the addition of the TLR4 inhibitor, the levels of phosphorylated IRS1 and GSK-3 β in the NEFAs + TAK-242 group were lower than those in the NEFAs group; however, the protein levels of p-AKT, p-IRS2, GLUT4 and PI3K were significantly higher than those in the NEFAs group ($p < 0.05$). There was no difference in protein levels between the TAK-242 and DMSO groups ($p > 0.05$) except for those of p-GSK-3 β and GLUT4 (Fig. 7).

We simultaneously added insulin to some cells as a control and found that the expression of p-IRS1 and IRS1 was no different between the groups with and without insulin ($p > 0.05$). The protein level of p-IRS2 increased as the NEFA concentration gradually increased but was lower in the high-concentration group than in the medium-concentration group and similar to the level in the low-concentration group. However, the expression of p-IRS2 in the NEFAs + TAK-242 group was lower than that in the 2.4 mM NEFAs group ($p < 0.05$). There was no difference in p-IRS2 levels between the DMSO and blank control groups, but it was higher in the TAK-242 group than in the blank control group. There was no difference in IRS2 level between the groups ($p > 0.05$). The PI3K protein level showed no obvious difference between the NEFAs groups ($p > 0.05$), but after the addition of TAK-242, it was higher than that in the 2.4 mM NEFAs group ($p < 0.05$). The expression levels of p-AKT in the low- and medium-concentration groups were higher than that in the blank control group, but p-AKT expression in the high-concentration group was significantly lower than that in the medium- and low-concentration groups ($p < 0.05$). After the addition of TAK-242, the p-AKT level was obviously higher in the NEFAs + TAK-242 groups than in the high-concentration group ($p < 0.05$), and p-AKT levels in the TAK-242 and DMSO groups were higher than those in the blank control group ($p < 0.05$), but there was no difference in p-AKT level between the TAK-242 and DMSO groups ($p > 0.05$). The protein level of AKT did not significantly differ between the NEFAs groups ($p > 0.05$) and was higher in the TAK-242 group than in the blank control group ($p < 0.05$). The protein level of p-GSK-3 β increased as the NEFA concentration increased and was highest in the medium-concentration group but was lower in the high-concentration group than in the medium-concentration group ($p < 0.05$) and change in a dose-dependent manner. There was no significant difference in p-GSK-3 β protein level between the other groups ($p > 0.05$). With increasing NEFA concentration, the protein level of p-GSK-3 β increased and peaked in the medium-concentration group but was reduced in the high-concentration group, and this change was dose dependent. After the addition of an inhibitor, there was no significant difference in p-GSK-3 β protein level between the groups ($p > 0.05$). The protein levels of GSK-3 β in the low- and medium-concentration groups were not significant different from that in the blank control group ($p > 0.05$), and the protein level in the high-concentration group was lower than that in the blank control group and NEFAs + TAK-242 group ($p < 0.05$). The protein expression of GLUT4 decreased with increasing NEFA concentration. After the addition of an inhibitor, GLUT4 protein expression was higher in the NEFAs + TAK-242 group than in the high-concentration group ($p < 0.05$), and that in the TAK-242 and DMSO group did not significantly differ from that in the blank control group ($p > 0.05$) (Fig. 8).

NEFAs affect the expression of PEPCK and G6-Pase through TLR4 at the mRNA level

PEPCK and G6-Pase are key enzymes in the gluconeogenesis process. The mRNA levels of PEPCK in the high- and medium-concentration groups were higher than that in the blank control group ($p < 0.05$), but there was no difference in PEPCK mRNA level between the low-concentration group and blank control group ($p > 0.05$). PEPCK mRNA levels in the NEFAs + TAK-242 group were lower than those in the blank control group, and there were no difference in PEPCK mRNA level between the TAK-242, DMSO and blank control groups ($p > 0.05$) (Fig. 9A). The mRNA level of G6-Pase increased with increasing NEFA concentration and was highest in the high-concentration group ($p < 0.05$). The expression level in the NEFAs + TAK-242 group was lower than that in the high-concentration group ($p < 0.05$), and there were no difference in expression between the other groups ($p > 0.05$) (Fig. 9B). After the addition of insulin, the mRNA level of PEPCK increased with increasing NEFA concentration, and PEPCK mRNA levels in the NEFAs + TAK-242 group were lower than those in the high-concentration group ($p < 0.05$), which was in accordance with the levels in the group without insulin (Fig. 10A). The mRNA level of G6-Pase reduced with increasing NEFA concentration; G6-Pase mRNA levels in the NEFAs + TAK-242 group were lower than those in the high-concentration group ($p < 0.05$), and there were no difference in G6-Pase level between TAK-242, DMSO and blank control groups ($p > 0.05$) (Fig. 9B).

Discussion

Ketosis is one of the most common metabolic diseases in perinatal cows [11]. Furthermore, ketosis-related diseases, including fatty liver, abomasum displacement, infectious diseases and other productive diseases, have long plagued agricultural development [12, 13]. Perinatal cows are physically characterized by NEB [14]. The initial stage of NEB in cows is characterized by low glucose and high NEFA levels [15]. Cows under NEB conditions mobilize a large amount of fat to provide more ATP to oxidize NEFAs in the cow liver [16]. However, high NEFA concentrations in the blood of perinatal cows trigger some NEB-related metabolic disorders and diseases, such as liver lipid accumulation, ketosis, abomasum displacement and retained fetal membranes [17]. Clinical data show that fatty liver in cows often manifests as insulin resistance. Cows with type I ketosis often exhibit low insulin levels, and those with type II often exhibit high insulin levels with decreased insulin sensitivity. After recovery, the effects of insulin cannot correct NEB-mediated liver lipid metabolic disorder [18]. Therefore, specific liver regulation of the insulin signaling pathway is crucial to explaining the pathogenesis of cow ketosis and fatty liver. Postpartum cows often exhibit insulin resistance mainly caused by inadequate energy intake. However, the intake of too much energy may lead to obesity and insulin resistance, which are also closely related. TLRs are pattern recognition receptors that can affect microbial pathogens by promoting inflammatory signaling pathways such as the NF- κ B pathway in the innate immune system [19]. The NF- κ B pathway can promote the synthesis of inflammatory cytokines and chemokines [20]. Obesity is one of the most important factors that affects insulin resistance. Insulin resistance caused by obesity is associated with cytokines secreted by fat cells such as adiponectin and resistin and inflammatory factors such as TNF- α .

and IL-6 [21]. Obesity is characterized by long-term inflammatory pathway activation, and inflammatory diseases are associated with insulin resistance in obesity. However, the mechanism of this inflammatory pathway activation is not clear in obese patients. The levels of blood FFAs in obese patients tend to be increased, and a variety of hypotheses regarding insulin resistance caused by FFAs have now been proposed. High NEFA and BHBA levels are clinicopathological characteristics of NEB; however, it is unclear whether insulin resistance in perinatal cows with NEB is associated with high blood NEFA levels.

NEFAs affect the NF- κ B inflammatory signaling pathway through TLR4

Studies in humans and mice have shown a very close connection between inflammation and insulin resistance [22–24]. Rats in which TLR4 was specifically knocked out fed a high-fat diet exhibit fatty liver, but this high-fat diet can obviously increase insulin sensitivity, reducing fat accumulation and inflammation in the blood [25]. The metabolic characteristics of cows are different from those of humans and rats, and the relationship between insulin resistance and inflammation in bovines is not clear. In this study, we assumed that NEFAs can bind TLR4 on the cell membrane of hepatocytes and influence the NF- κ B and insulin signaling pathways.

We found that NEFAs obviously increased the initial calf hepatocyte TLR4 protein expression level, and with increasing concentrations of NEFAs, the P65 phosphorylation level also gradually increased (Fig. 1). Because the activation of NF- κ B is mainly achieved through the phosphorylation of p65, NEFAs can activate the TLR4 and NF- κ B pathways. After activation of the NF- κ B pathway, a large number of inflammatory factors such as IL-6, IL-1 β , and TNF- α are produced. To detect whether NEFAs affect the NF- κ B inflammatory pathway by binding TLR4, we added a TLR4 inhibitor group and the corresponding control group (Fig. 3). Our experimental results showed that the protein expression of TLR4 and p-P65 in the group mixed with a TLR4 inhibitor and NEFAs was significantly lower than that in the NEFAs group, and nuclear expression of the nuclear transcription factor p65 decreased significantly after the addition of inhibitor (Fig. 4). Similarly, the mRNA expression and supernatant concentration of the proinflammatory factors IL-6 and TNF- α , were significantly increased in the NEFAs group but decreased after the addition of TLR4 inhibitors (Fig. 5, 6). This indicates that the inhibitor of TLR4 could inhibit not only TLR4 but also P65, a key factor in the inflammatory pathway of NF- κ B, and can also inhibit the expression and secretion of proinflammatory factors. Overall, these findings indicate that NEFAs can activate the NF- κ B pathway through TLR4, promote the release of proinflammatory factors such as IL-6 and TNF- α , and then promote the inflammatory response.

NEFAs affect insulin pathway through TLR4

In patients with diabetes, metabolically active cells cannot use glucose, so they heavily accumulate in the blood [26]. Glucose is mainly used in targeted tissues through two pathways—the PI3K and AMPK pathways [27]. The PI3K/AKT pathway promotes the expression of GLUT4 in muscle cells and adipocytes and plays an important role in glucose metabolism [28]. AMPK is activated mainly through drug

stimulation, such as stimulation with metformin, or through exercise, effectively promoting glucose utilization and insulin sensitivity [29]. Therefore, the PI3K/AKT and AMPK pathways may be potential targets for regulating insulin resistance to glucose metabolism in type 2 diabetes and obesity. In this study, we studied the PI3K/AKT pathway. IRS1 is a key regulatory factor downstream of the insulin receptor in insulin signaling. IRS1-deficient mice showed normal glucose tolerance and insulin resistance [30]. Nakamura A (2012) et al indicated that although severe hyperinsulinemia occurred in IRS1^{-/-} rats fed a high-fat diet, there was no liver lipid degeneration [31]. The activation of PI3K in the PI3K pathway depends on the binding of two SH2 regions to the tyrosine-phosphorylated IRS protein [32]. Knockout of the PI3K regulatory subunits p85 α and p85 β increased insulin sensitivity [33, 34]. Previous studies have shown that liver-specific knockout of PRSS8 reduced the phosphorylation of AKT in the liver, resulting in insulin resistance [35]. GSK-3 β is a key enzyme that regulates insulin signaling through glycogen synthase (GS) [36]. GSK-3 β can be activated after phosphorylation of AKT, and AKT/GSK-3 β signaling plays an important role in insulin resistance [37].

In this study, we first examined whether NEFAs could affect the insulin pathway in primary calf hepatocytes. High concentrations of NEFAs increased the protein expression of p-IRS1 (Ser307), p-IRS2, IRS2 and GLUT4 but decreased the expression of PI3K, p-AKT and p-GSK (Fig. 2). Therefore, we determined that high concentrations of NEFAs can induce insulin resistance in primary calf hepatocytes. To determine whether NEFAs affect the insulin pathway through TLR4, we detected changes in the expression of key proteins in the insulin pathway after adding TLR4 inhibitors and prepared a non-insulin group as a control. After the addition of insulin, the expression levels of key proteins in the insulin pathway were greater than those in the non-insulin group; for example, the decrease in PI3K and p-AKT was more pronounced than that in the non-insulin group (Fig. 7, 8). In general, the addition of TLR4 inhibitors affected the insulin pathway, indicating that NEFAs can affect the insulin pathway by binding TLR4. PECK and G6-Pase are two key enzymes involved in gluconeogenesis. Previous studies have shown that the activation of AKT in mouse liver can induce insulin-mediated gluconeogenesis, accompanied by downregulation of PECK and G6-Pase expression [38]. To further verify the effect of TLR4 inhibitors on genes downstream of the insulin pathway, we detected the mRNA expression levels of PECK and G6-Pase in hepatocytes. In the absence of insulin, the mRNA expression levels of PECK and G6-Pase were increased in the NEFAs group and decreased in the inhibitor mixed group, but after the addition of insulin, G6-Pase showed the opposite change (Figs. 9, 10). Because PECK and G6-Pase are downstream of the insulin pathway, their sensitivity to insulin is not particularly accurate, so this finding is not enough to explain its effect on the insulin pathway.

Consequently, these results indicate that NEFAs can regulate the insulin pathway through the TLR4/PI3K/AKT metabolic axis.

Declarations

Author contribution

Qinghua Deng contributed to the conception of the study and wrote the manuscript.

Liyin Du: performed the data analyses.

Yuming Zhang: performed the experiment.

Guowen Liu: helped perform the analysis with constructive discussions.

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Conflicts of interest

The authors declare no conflict of interest.

Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Code availability Not applicable.

Ethics approval The study protocol was approved by the Ethics Committee on the Use and Care of Animals at the Inner Mongolia University for Nationalities (Tongliao, China).

Consent to participate Not applicable.

Consent for publication Not applicable.

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Figures

Figure 1

Effects of NEFAs on TLR4 and the inflammatory pathway. Protein expression levels after incubation with NEFAs were determined by western blotting. Hepatocytes were divided into four groups: the control group (0 mM NEFAs), low-concentration group (0.6 mM NEFAs), medium-concentration group (1.2 mM NEFAs) and high-concentration group (2.4 mM NEFAs).

Figure 2

Effects of NEFAs on the expression of related proteins in the insulin signaling pathway. Protein expression levels after incubation with NEFAs were determined by western blotting. Hepatocytes were divided into four groups: the control group (0 mM NEFAs), low-concentration group (0.6 mM NEFAs), medium-concentration group (1.2 mM NEFAs) and high-concentration group (2.4 mM NEFAs).

NEFAs (2.4 mM)	+	-	+	-
TAK-242 (1μM)	+	+	-	-
DMSO (1μM)	-	-	-	+
INS	-	-	-	-

Figure 3

NEFAs affected the NF-κB inflammatory pathway through the TLR4 protein. Protein expression levels were determined by western blotting. Hepatocytes were divided into four groups: the NEFAs+TAK-242 group, TAK-242 group, NEFAs (2.4 mM) group and DMSO group.

Figure 4

Results of immunofluorescence analysis of P65 in bovine hepatocytes (800×). Hepatocytes were divided into four groups: NEFAs+TAK-242 group, TAK-242 group, NEFAs (2.4 mM) group and DMSO group.

Figure 5

Effects of NEFAs and TAK-242 on the mRNA expression levels of IL-6 and TNF α Values are the means $\pm$ SEs (n=6), and different letters indicate a statistically significant difference; p<0.05.

Figure 6

Effects of NEFAs on IL-6 and TNF α in the culture supernatant Values are the means $\pm$ SEs (n=6), and different letters indicate statistically significant differences; p<0.05.

NEFAs (2.4 mM)	+	-	+	-
TAK-242 (1μM)	-	+	+	-
DMSO (1μM)	-	-	-	+
INS	-	-	-	-

Figure 7

Effects of NEFAs and TAK-242 on key proteins in the insulin signaling pathway

Figure 8

Effects of NEFAs, TAK-242 and INS on key proteins in the insulin signaling pathway

Figure 9

Effects of NEFAs and TAK-242 on the mRNA expression levels of PEPCK and G6-Pase. Values are the means $\pm$ SEs (n=6), and different letters indicate statistically significant differences; $p < 0.05$.

Figure 10

Effects of NEFAs, TAK-242 and INS on the mRNA expression levels of PEPCK and G6-Pase. Values are the means $\pm$ SEs (n=6), and different letters indicate statistically significant differences; $p < 0.05$.

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