Regulating glycolysis, the TLR4 signal pathway and expression of RBM3 in mouse liver in response to acute cold exposure

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ABSTRACT
At low temperatures, the liver increases glucose utilization and expresses RNA-binding motif 3 (RBM3) to cope with cold exposure. In this study, the expression of heat shock protein 70 (HSP70), Toll-like receptor 4 (TLR4), bone marrow differentiation factor 88 (MYD88), and phosphorylated nuclear factor-κB (NF-κB) was consistent with fluctuations in insulin in fasted cold-exposed mice. We also found up-regulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) in acute cold exposure with a decrease in core body temperature. RBM3 transcription and translation were activated 2 h after cold exposure. The anti-apoptotic factor Bcl-2/Bax ratio also increased, while expression of apoptosis factors: cleaved caspase-3, cleaved poly(ADP-ribose)polymerase 1 (PARP-1) and cytochrome-c (Cyt-c) was unchanged. Liver glycogen was depleted after 2 h of cold exposure, and blood glucose decreased after 4 h. Glycogen synthase kinase 3β (GSK3β) phosphorylation continued to increase to promote hepatic glycogen synthesis. We found a high level of protein kinase B (AKT) phosphorylation after 6 h of cold exposure. In addition, we demonstrated that after cold exposure for 2 h, in the liver, continued phosphorylation of fructose-2,6-diphosphate (PFKFB2) and decreased accumulation of glycogen intermediates fructose-1,6-diphosphate (FDP) and pyruvic acid (PA). In summary, the liver responds to cold exposure through a number of different pathways, including activation of HSP70/TLR4 signaling pathways, up-regulation of RBM3 expression, and increased glycolysis and glycogen synthesis. We propose a possible signaling pathway in which regulation of RBM3 expression by the liver affects the AKT metabolic signaling pathway.

Lay Summary
- In response to changes in ambient temperature, mice regulate global metabolism and gene expression through hormones. This study focused on the effects of environmental hypothermia on molecular pathways of glucose metabolism in the liver, which is the important metabolic organ in mice. This provides a basis for further study of mice against cold exposure damage.

Introduction
Cold stress is a major danger for warm-blooded animals, specifically at mild hypothermic temperatures, at which global protein synthesis is known to be inhibited (Dresios et al., 2005). It is thought that cold stress may enhance the instability of atherosclerotic plaques by activating endoplasmic reticulum (ER) stress and enhancing cell apoptosis (Dai et al., 2014). Additionally, changes in ambient temperature can elicit differential changes in metabolic and cardiovascular actions by the hormone, leptin (do Carmo, da Silva, Romero, & Hall, 2017). Cold exposure in mice has been found to trigger a metabolic cascade that orchestrates lipoprotein processing in brown adipose tissue (BAT), hepatic conversion of cholesterol to bile acids via the alternative synthesis pathway, and also causes distinct changes in gut microbiota (Worthmann et al., 2017). The proliferation of both bone-marrow-derived mesenchymal stem cells and prostate tissue changed during hypothermia, and gene expression in skeletal muscle was also affected (Kaija, Pakanen, Kortelainen, & Porvari, 2015; Liu et al., 2017; Zak et al., 2017). In response to cooling, the levels of serum triglycerides and small high-density lipoproteins are increased in the short term (Bartelt et al., 2017) and BAT is activated with the browning of white adipose tissue (WAT) (Wang, Liu, Wang, & Sun, 2015). During cold exposure, the body increases its metabolic rate (Makinen, 2010), and carbohydrates are an important metabolic substrate whose utilization accounts for up to 80% of the total heat produced (Blondin, Péronnet, & Haman, 2010). It has also been reported that the heat produced by shivering, along with adipose tissue and skeletal muscle, enhances glucose uptake during cold exposure, using the 2-deoxyglucose method (Shibata, Perusse, Vallerand, & Bukowiecki, 2008).
The liver is a central metabolic organ in vertebrates, playing key roles in many physiological processes, including detoxification, synthesis of plasma proteins, glucose homeostasis, as well as utilization and cycling of various nutrients (Ghafary et al., 2013). In addition, excess glucose is used to synthesize fatty acids in the liver. The liver also can release glucose to the systemic circulation, either from previously stored glycogen (glycogenolysis) or by generating glucose from precursors such as alanine, lactate, or glycero (gluconeogenesis). This unique ability of the liver to store and release glucose is crucial to endure periods of fasting (Adeva-Andany et al., 2016). Due to profound changes in blood glucose level, the normal physiological processes of the liver are inevitably affected during acute cold exposure.

Previous research has shown that the expression of RNA binding proteins is increased to resist an adverse environment by coordinating necessary intracellular translation and post-translational modification reactions. RNA binding protein 3 (RBM3), a member of the cold-inducible RNA binding protein family (Danno et al., 1997; Derry, Kerns, & Francke, 1995; Dresios et al., 2005; Jackson et al., 2015), has been clearly shown to be induced during hypoxia, cold stress, and ER stress (Jackson et al., 2015; Kita et al., 2002; Ryan, Morey, Ramsdell, & Van Dolah, 2005; Wellmann et al., 2004, 2010; Zhu, Zelmer, Kapfhammer, & Wellmann, 2016). The biological functions of RBM3 include the promotion of global protein synthesis, maintenance of the stability of mRNAs bearing AU-rich elements, induction of stemness via the glycogen synthase kinase (GSK)-3β/Wnt/β-catenin signaling pathway, promotion of skeletal muscle mass, modulation of the cell cycle in G2/M transition, and adjusting the biogenesis of many microRNAs during the Dicer step (Cok, Acton, Sexton, & Morrison, 2004; Ferry, Vanderklish, & Dupont-Versteegden, 2011; Matsuda et al., 2011; Pilotte, Dupont-Versteegden, & Vanderklish, 2011; Smart et al., 2007; Sureban et al., 2008; Venugopal et al., 2016; Wong et al., 2016). RBM3 has also been reported to participate in signaling pathways such as MAPK and serine/threonine kinase (also known as protein kinase B or PKB), all pointing to a general role of RBM3 in cell protection (Neutelings, Lambert, Nusgens, & Colige, 2013; Yang et al., 2017).

In mammals, RBM3 has been shown to be induced in the liver accompanied by metabolic regulation to ensure resistance to cold temperatures. However, the molecular mechanisms which regulate the signaling pathways needed for the expression of RBM3, and whether cold exposure affects liver energy metabolism, remain unclear. In this study, we focused on detecting hypothesized changes in RBM3 expression, glycolytic-related signaling pathways, and intermediates in the liver in response to acute cold exposure.

**Methods**

**Animals**

Pathogen-free male C57BL/6 mice weighing approximately (range) 18–20g, were obtained from the Experimental Animal Center of the Academy of Military Medical Sciences of the Chinese People’s Liberation Army and maintained eight per cage in a climatic chamber at an ambient temperature of 23–25°C (range) with 40% relative humidity under a 12 h/12 h light/dark cycle (lights on from 08:00 h to 20:00 h). The mice were housed eight per cage, and had free access to food and water. All procedures involving animals were approved by the animal care committee of the Heilongjiang Bayi Agricultural University (Daqing, China), and all experimental protocols were performed in accordance with relevant guidelines and regulations of the Heilongjiang Bayi Agricultural University. Mice were allowed to acclimatize for at least 1 week before being subjected to experimental conditions.

**Cold exposure, core body temperature measurement and sample collection**

Mice were randomly divided into four groups (n=8/each group), the control group and the acute cold exposure groups which included exposure to an environmental temperature of 4°C for 2 h, 4 h, or 6 h (during 13:00 h to 19:00 h). In addition, during cold exposure the mice were fasted. Mice in the control group were kept in their usual conditions (25 ± 1°C) with fasting during the same period as the acute cold exposure protocol, as shown in Figure 1. After their respective treatments, core body temperature was measured, and the mice were euthanized by i.p. sodium pentobarbital (1.5%, 0.01 mL/g) injection. Blood was collected from an orbital vein and the serum was separated by centrifugation at 1000×g for 30 min at 4°C. Liver tissue was taken and quickly frozen at −80°C.

![Figure 1](image.png)  
**Figure 1.** Timeline of the acute cold exposure protocol. The control mice were left at room temperature undisturbed. Cold mice were exposed to 4°C. CET: cold exposure time.
**Periodic acid Schiff (PAS) stain and measurement of biochemical parameters**

PAS staining was performed using a Periodic Acid Schiff/PAS Stain Kit (Solarbio, Beijing, China). Serum and liver fasting glucose concentrations were measured using commercial cards (IDEXX Laboratories, Westbrook, ME; sensitivity 4.72–8.09 mmol/l, n = 8). Serum insulin and glucagon levels were measured by ELISA (Cloud-Clone Corp., Katy, TX; sensitivity 48.5–1000 pg/mL, intra-assay: CV < 10%, inter-assay: CV < 12%, n = 8). Fructose-1,6-diphosphate (FDP, sensitivity > 1.72 mg/L, n = 8) and pyruvic acid (PA, sensitivity > 1.56 μg/mL, n = 8) were measured by UV spectrophotometry (Solarbio, Beijing, China). ATP, AMP and ADP concentrations in the liver were determined by high-performance liquid chromatography (HPLC, sensitivity 1 mM, 200 Å) column, and UV detector at a wavelength of 254 nm (BW 16 nm).

**Western blot analysis**

Protein samples were homogenized in radio-immunoprecipitation assay buffer (Beyotime, Shanghai, China) with protease and phosphatase inhibitors (Beyotime, Shanghai, China). Proteins were separated on SDS-PAGE and transferred to poly(vinylidene difluoride) membranes (0.22 and 0.45 μm, Millipore, Billerica, MA). Membranes were blocked, probed with primary antibody which was incubated overnight at 4°C. The primary antibodies used in this study included AKT (1:1000, #9227), phospho-AKT (Ser473) (1:1000, #12655), phospho-p65 (Ser276) (1:1000, #183559), phosphorylation of p65 (1:1000, #8242), phospho-AKT (Ser473) (1:1000, #12655), phospho-p65 (Ser276) (1:1000, #8242), phosphorylation of p65 (1:1000, #8242), phospho-AKT (Ser473) (1:1000, #12655), phospho-p65 (Ser276) (1:1000, #8242), phosphorylation of p65 (1:1000, #8242). The secondary antibodies labeled with horseradish peroxidase goat anti-mouse IgG (1:20,000, #SA0001-1, Proteintech, Rosemont, IL) and goat anti-rabbit IgG (1:20,000, #SA0001-2, Proteintech, Rosemont, IL). Signals were detected with the Luminata Crescendo Western HRP substrate (Millipore, Billerica, MA). Blots were imaged with the ChemiDoc XRS (Bio Rad, Hercules, CA) and analyzed using Image J software (http://imagej.nih.gov/ij/). See Supplementary Material for illustrations of original western blots.

**Reverse transcription quantitative-PCR (RT-qPCR)**

Liver tissues were placed in a Retsch MM400 mill for grinding and then added to the ambion Trizol (#T9424; Sigma, St. Louis, MO) to extract total RNA from the tissue. The concentration of the extracted RNA was measured using a Nanodrop 2000 Nucleic Acid Protein Quantifier (Thermo, Waltham, MA). RT was performed using the TaKaRa reverse transcription kit (#RR047A, JP). Primer sequences of RBM3 and β-actin were synthesized by Jilin Comate Bioscience Co., Ltd. (Jilin, China). Primer sequences were as follows: RBM3 forward 5'-GATCATGAGGAAATGTCGC-3', RBM3 reverse 5'-ACTCCCATATCCTGGTCTCC-3', β-actin forward 5'-GTCCTATGTTGCTCTAGACTCT-3', and β-Actin reverse-5'-ATGGCACCAGGATCCATACC-3'. A fluorescence qPCR instrument was used (#CFX96; Bio-Rad, Hercules, CA). The conditions were set according to the TaKaRa kit instructions. The resulting data were calculated using the software included with the quantitative PCR instrument using the maximal second derivative method.

**Statistical analysis**

PAS staining was analyzed by image pro plus 6.0 (Media Cybernetics, Rockville, MD). All data were analyzed with GraphPad Prism software (La Jolla, CA) and expressed as the mean ± standard error of mean (SEM). One-way ANOVA was used to analyze all data from the western blotting. For all analyses, post hoc comparisons were made using Fisher’s LSD post hoc test. Statistical significance was considered at p < .05.
Results

Cold exposure induced changes in core body temperature and regulation of PGC1 expression in liver

Acute cold exposure (6 h) induced a decrease in core body temperature to \( \sim 35.8 \) °C in mice (DF = 3, \( F_{(2,208), 11.04} = 8.669, p = .0047, \) Figure 2(A)). The liver regulates expression of PGC1 for adaptive heat production. Western blot analysis revealed that production of PGC1 was significantly increased after cold exposure for 6 h (DF = 3, \( F_{(1,755), 8.775} = 8.201, p = .0112, \) Figure 2(B,C)).

Cold exposure increased serum insulin concentrations and the expression of HSP70/TLR4/MYD88/NF-κB signaling pathway in the liver

We investigated changes in insulin under acute cold exposure in plasma in the control and stressed groups. Insulin levels showed a fluctuation in regulation after acute cold stimulation (Figure 3(A)). The expression of HSP70 (DF = 3, \( F_{(1,345), 2.69} = 29.41, p = .0156, \) Figure 3(B,C)), TLR4 (DF = 3, \( F_{(1,793), 3.586} = 56.69, p = .0020, \) Figure 3(B,C)), and bone marrow differentiation factor 88 (MYD88) (DF = 3, \( F_{(1,431), 2.863} = 69.4, p = .0040, \) Figure 3(B,E)) was induced after exposure to cold for 2 h. Nuclear factor-κB (NF-κB) was found to be activated, and the phosphorylation of p65 when mice were exposed to the cold for 2 h (DF = 3, \( F_{(3, 12)} = 7.359, p = .0047, \) Figure 3(B,F)).

Cold exposure induced changes in serum glucose, FDP, PA and ATP concentrations

To investigate changes in glycolysis in the liver after acute cold exposure, we examined the serum glucose and glycogenolytic intermediate metabolites FDP and PA. Serum glucose concentrations at 6 h were significantly different between the control and stressed groups (DF = 3, \( F_{(3, 10)} = 5.871, p = .0141, \) Figure 4(A)). Acute cold exposure caused a significant change in the concentrations of FDP and PA in the liver of mice after 6 h (DF = 3, \( F_{(3, 9)} = 16.25, p = .0006, \) Figure 4(B)); DF = 3, \( F_{(3, 7)} = 13.81, p = .0025, \) Figure 4(C), respectively). Liver ATP concentrations and glycogen were rapidly depleted after cold exposure for 0–2 h (DF = 3, \( F_{(3, 10)} = 4.105, p = .0387, \) Figure 4(D,F), respectively).

Cold exposure promoted glycolysis, AKT, PFKFB2, and GSK3β phosphorylation

Since its initial discovery as a proto-oncogene, AKT has become a major focus of research due to its critical role in regulating a diverse range of cellular functions including metabolism, growth, proliferation, survival, transcription, and protein synthesis (Hers, Vincent, & Tavare, 2011). In this study, it was found that in the liver AKT was activated in response to cold stress after 6 h, as seen in Figure 5(A,B) (DF = 3, \( F_{(1,62), 4.239} = 68.71, p = .0024; \) DF = 3, \( F_{(1,024), 3.072} = 16.32, p = .0260, \) respectively). The bifunctional PFKFB2 catalyzes the synthesis and degradation of fructose 2, 6-bisphosphate and regulates its steady-state level (Atsumi et al., 2005; Kim, Manes, El-Maghrabi, & Lee, 2006). GS3 is a critical downstream effector of the PI3K/AKT cell survival and glycogen biosynthesis pathway whose activity can be inhibited by AKT-mediated phosphorylation at Ser\(^\beta\) (Cross et al., 1995). To detect changes in liver glucose metabolism, we examined the phosphorylation of GS3 and PFKFB2 and found that during cold exposure PFKFB2 was activated within 6 h (DF = 3, \( F_{(2,152), 6.455} = 48.65, p = .0001, \) Figure 5(A,B); DF = 3, \( F_{(1,284), 3.852} = 17.35, p = .0139, \) Figure 5(A,C)) and activity of GS3 was inhibited.

Cold exposure enhanced liver RBM3 expression and promoted cell survival

In response to cold exposure, the relative expression of RBM3 mRNA was observed to significantly increase after 2 h of cold exposure (DF = 5, \( p = .0053 < .01, \) Figure 6(A)). And western blot analysis revealed that the production of RBM3 was significantly increased after 2 h of cold exposure (DF = 10, \( p = .0216 < .05, \) Figure 6(B,C)). We found that the expression of Bcl-2/Bax was upregulated after cold exposure for 6 h (DF = 4, \( p = .0088 < .05, \) Figure 6(B,C)). In contrast, expression of Cyt C, cleaved-caspase 3, and cleaved-PARP-1 was not significantly altered (Figure 6(B,D–F)).

Discussion

When mammals are exposed to a cold environment, it can lead to enhanced glucose mobilization, improving their biological response to insulin (Shibata et al., 1989; Smith, 1984; Vallerand, Lupien, & Bukowiecki, 1983). Moreover, it has been reported that acute and chronic cold exposure markedly increase whole-body energy expenditure, which is accompanied by profound metabolic changes including hyperphagia, activation of WAT, enhanced hepatic glucose production, and increased glucose and fat utilization by peripheral tissues (Cunningham, Gulino, Meara, & Bode, 1985; Depocas & Masironi, 1960; Labbe et al., 2015; Smith & Davidson, 1982; Vallerand, Perusse, & Bukowiecki, 1990). Specifically, in rat BAT, WAT, and skeletal muscles, the molecular mechanisms that drive these adaptive metabolic changes, including glucose uptake, glycolysis, glycogen metabolism, and responses to cold, are controlled by the PI3K/AKT cell survival and glycogen biosynthesis pathway regulating the AKT and AMPK pathways (Sepa-Kishi, Katsnelson, Bikopoulos, & Ceddia, 2017; Wang & Wahl, 2014). In addition, it is known that the liver plays a major role in the maintenance of whole-body energy and glucose homeostasis by regulating the AKT and AMPK pathways (Sepa-Kishi, Sotoudeh-Nia, Iqbal, Bikopoulos, & Ceddia, 2017). In this study, we observed a change in glucose metabolism in the liver as well as phosphorylation of AKT, GSK, and PFKFB2 (Figure 5). It has been shown that AKT-dependent phosphorylation of GSK will activate glycogen synthase to up-regulate glycogen biosynthesis (Embly, Rylatt, & Cohen, 2005; McCubrey et al., 2014). In addition, it has been shown that GSK3β promotes apoptosis through numerous pathways, including acetylation of p53, the degradation of Bcl-2,
phosphorylation of Bax, and/or phosphorylation of heat shock factor 1 (HSF1) (Belkhiri, Dar, Zaika, Kelley, & El-Rifai, 2008; Linseman et al., 2004). According to these results, it may be hypothesized that the phosphorylation of GSK is related to the self-protection mechanism of GSK3β, which is induced by acute cold exposure (Figures 4(E) and 5(A,C)). It has been suggested that PFKFB2 is activated by AKT-dependent phosphorylation of PFKFB2 at Ser483 (Novellasdemunt et al., 2013). PFKFB2 activates phosphofructokinase, a critical enzyme in glycolysis, and does this by allosteric regulation.

Figure 3. Effect of acute cold exposure on the concentrations of serum insulin (A) and liver proteins. The expression of HSP70 (B and C), TLR4 (B and D), MYD88 (B and E), IkBα (B and F) and activation of p65 (B and G) in the liver under cold exposure. The data are presented as the mean ± SEM (n = 8). Significant differences were analyzed by one-way ANOVA. For all analyses, post hoc comparisons were made using Fisher’s LSD post hoc test. Statistically significant differences are indicated as *p < .05 and **p < .01.
with degradation of fructose 2, 6-bisphosphate (Atsumi et al., 2005; Kim et al., 2006). Fructose-2, 6-bisphosphate is the strongest allosteric activator of phosphofructokinase-1, which catalyzes fructose-6-phosphate to fructose-1, 6-bisphosphate, a key allosteric activator of pyruvate kinase in the glycolytic pathway. However, our results showed that PFKFB2 phosphorylation was enhanced during acute cold exposure (Figure 5(A,D)), but the liver glycolytic metabolic intermediate FDP, and PA level did not show continued accumulation after cold exposure (Figure 4(B,C)). This may have been caused by excessive intake of glucose by the peripheral tissues and maintenance of glucose homeostasis by the liver (Figure 4(A,F)), resulting in insufficient glycolytic substrates after complete decomposition of the liver glycogen. A similar reduction in core body temperature caused by cold exposure has been found in several studies. It has been suggested that, upon cold exposure, the liver maintains an intracellular energy balance by increasing circulating glucose through the processes of glycogenolysis and gluconeogenesis, including by activation of the AKT pathway to promote ATP generation and promote heat production via induced expression of PGC1α (Lemecha et al., 2018; Wang et al., 2013). Changes in concentration of ATP and expression of PGC1 were detected (Figures 2(B,C) and 4(D)), which may have been due to the ability of the liver to maintain body temperature for adaptive heat production. Hepatic insulin resistance has been demonstrated to prevent dramatic changes in hepatocyte homeostasis (Asea et al., 2002). It is well established that HSP70 activates TLR2/TLR4 to induce pro-inflammatory cytokine production via the MYD88/NF-κB signal transduction pathway.

Figure 4. Effect of acute cold exposure on the concentrations of serum glucose (A), liver glycolytic intermediates FDP (B) and PA (C) and ATP/(ADP + AMP) (D). Glycogen was counted by PAS staining (E and F, ×40). The data are presented as mean ± SEM (n = 8). Significant differences were analyzed by one-way ANOVA. For all analyses, post hoc comparisons were made using Fisher’s LSD post hoc test. Statistically significant differences are indicated, *p < .05.
HSP70, an important stress protein, plays a major role as a molecular chaperone and in stress protection, and acts as an important endogenous ligand (Asea et al., 2002; Zhang et al., 2018). In this study, cold exposure induced expression of HSP70 which can activate the TLR4/MYD88/NF-κB pathway (Figure 3). We also observed that insulin fluctuations were positively correlated with TLR4 signaling activation, and it has been shown that this is associated with insulin resistance, which affects lipid and glucose metabolism (Figure 3(A,B–G)) (Bartelt et al., 2018; Kim et al., 2007). This may be one of the reasons why AKT phosphorylation in the liver does not fluctuate with insulin, but this requires further in-depth study. Taken together, these results indicate that the liver activates the TLR4/MYD88/NF-κB signaling pathway with decreased core body temperature and glucose homeostasis by regulating the phosphorylation levels of AKT, GSK, PFKFB2, and expression of PGC1 under cold exposure.

In addition to the body’s regulation of metabolism, up-regulation of related cold shock protein expression is also one of the important mechanisms in response to cold exposure (Dresios et al., 2005). In cold environments, mammals have been shown to upregulate the expression of RBM3 to ensure the protection of cells. Previous studies have demonstrated that RBM3 is widely involved in cytoprotection. RBM3 confers clear effects from apoptosis induced by various changes to homeostasis, such as serum and glucose deprivation, ER stress, staurosporine, hydrogen peroxide, nitric oxide, retinoic acid, UV irradiation, hypoxia, and hypothermia (Wellmann et al., 2004). These protective mechanisms by promoting expression of Bcl-2 inhibit the caspase-3-induced apoptotic cascade and extensively involve the MAPK signaling pathway (Kim, Kim, Kim, & Jang, 2018; Yang et al., 2017; Zhuang et al., 2017). We found RBM3 expression was accelerated (Figure 6(A–C)) and its expression was enhanced with increased ratio of Bcl-2/Bax (Figure 6(A,D)). It was also observed that expression of the apoptosis markers (Monger et al., 2017) Cyt-c, cleaved-PARP-1 and cleaved-caspase-3 did not change (Figures 6(A,E,F) and 4(G)). This result is consistent with the protection of RBM3 in many other reports. Previously, it has been reported that RBM3 expression is regulated by NF-κB (Ushio & Eto, 2018). NF-κB proteins comprise a family of structurally related eukaryotic transcription factors, and are involved in the control of a large number of normal cellular processes (Baueuerle & Baltimore, 1996), including immune and inflammatory responses, developmental processes, cellular growth, and apoptosis (Gutierrez, Hale, Dolcet, & Davies, 2005). When a cell receives any number of extracellular signals, the phosphorylation of IκB leads to its ubiquitination and proteasomal degradation, freeing the NF-κB/Rel complexes (Karin & Ben-Neriah, 2000). NF-κB is then rapidly phosphorylated at Ser276 and activates RBM3 transcription (Ushio & Eto, 2018). In this study, our data showed cold exposure induces p65 phosphorylation at Ser276 and upregulates the expression of RBM3 (Figures 3(A,G) and 6(A–C)). Although phosphorylation of NF-κB Ser276 was
Figure 6. Effect of acute cold exposure on transcription of RBM3 mRNA (A) and protein levels of RBM3 (B and C), Bcl-2/Bax (B and D), Cyt-c (B and E), cleaved-caspase 3 (B and F), and cleaved-PARP-1 (B and G), in the liver. The data are presented as the mean ± SEM (n = 8). Significant differences were analyzed by one-way ANOVA. For all analyses, post hoc comparisons was made using Fisher’s LSD post hoc test. Statistically significant differences are indicated as *p < .05 and **p < .01.

Figure 7. A potential model for the promotion of the expression of RBM3 via the HSP70/NF-κB signaling pathway, and role in the AKT signaling pathway to promote glycolysis in the liver during cold exposure. HSP70: heat shock protein 70; TLR4: Toll-like receptor 4; MyD88: myeloid differentiation factor 88; IκBα: inhibitor of nuclear factor kappa-B alpha; NF-κB: nuclear factor κB; RBM3: RNA binding motif protein 3; Bax: Bcl-2-associated X protein; caspase 3; PARP1: poly(ADP-ribose)polymerase 1; Cyt-c: cytochrome c; AKT: protein kinase B; GSK-3β: glycogen synthase kinase-3 beta; PFKFB2: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PGC-1α: PPARγ coactivator-1α; : phosphorylation.
rapidly decreased after 2 h of cold exposure, we hypothesize that it may be affected by the special sequence of \textit{RBM3} mRNA. Previous studies have demonstrated the presence of an internal ribosome entry site in the 5' leader of \textit{RBM3}, which can recruit ribosomes to enhance expression (Chappell & Mauro, 2003). In addition, the mild-cold responsive element, which was found in the upstream sequence of the cold induced mRNA binding protein promoter, can enhance transcription (Sumitomo et al., 2012). We have detected similar sequences rich in cytosine deoxyribonucleotides and guanine deoxyribonucleotides in \textit{RBM3}, located in the upstream sequence of the mouse \textit{RBM3} gene. This means that \textit{RBM3} translational enhancement does not require a large number of transcripts. These results indicate that the liver inhibits apoptosis by up-regulating \textit{RBM3} by NF-κB under cold exposure.

In summary, we found that the liver promotes cell survival by modulating \textit{RBM3} expression and activates the TLR4 signaling pathway while promoting glycolysis in response to acute cold exposure. At the same time, we also infer that \textit{RBM3} may promote AKT phosphorylation (Sureban et al., 2008; Venugopal et al., 2016), and the TLR4 signaling pathway may be involved in the regulation of NF-κB activity, which provides a new way for studying the molecular response mechanism under cold exposure. We propose that mechanisms in the liver response to acute cold exposure may be represented as in Figure 7; these molecular mechanisms require further research.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**Notes on contributors**

Hongzhao Shi did the experiments and wrote the manuscript.

Ruizhi Yao contributed to study design.

Shuai Lian did the statistical work.

Peng Liu, Yuyinz Yang and Yang Liu analyzed the data and interpreted the results of experiments.

Huanmin Yang corrected the manuscript for publication.

Shize Li supervised the work, evaluated the data, and approved the final version. All authors read and approved the final manuscript.

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