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Anti-Inflammatory Effects of β-Hydroxybutyrate on Broiler Chickens Induced by Lipopolysaccharide

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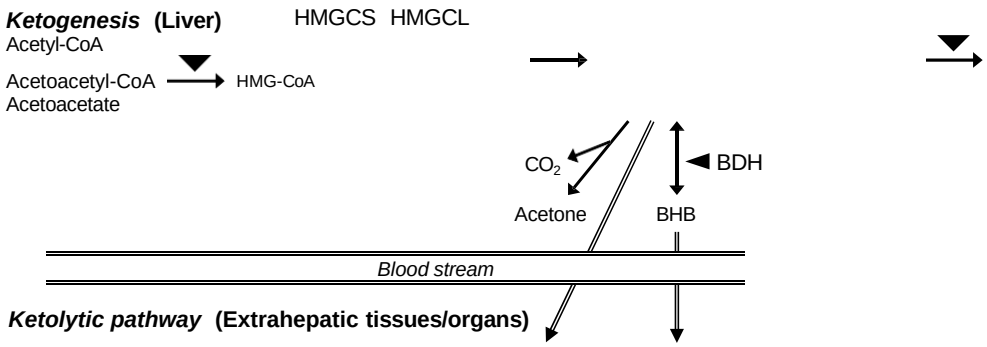
Abstract: Context: The purpose of this research was to determine whether or not broiler chickens were less prone to inflammation caused by lipopolysaccharide (LPS) when given β-hydroxybutyrate (BHB). Various techniques Each of the three groups of male broiler chickens, which were twenty days old, received either saline as a control, intraperitoneal injections of LPS [1.5 mg/kg BW], Escherichia coli O127:B8, or a combination of LPS and BHB (3 mmol/kg BW). Findings: LPS dose-dependently decreased plasma albumin and total protein concentrations, although BHB co-treatment only slightly mitigated these effects. Plasma aspartate and alanine aminotransferase activities, as well as interleukin (IL)-6 concentration, were elevated after LPS treatment, but these increases were reduced when BHB was co-treated ($p < 0.05$). In both the spleen and peripheral blood monocytes (PBMC), the gene expression levels of IL-1β, IL-6, and IL-18 were markedly elevated after LPS treatment. However, in the spleen, BHB somewhat reduced these increases. In contrast to PBMC and liver, spleen and skeletal muscle showed relatively greater amounts of succinyl-CoA:3-ketoacid CoA transferase and BHB dehydrogenase 1. Results show that BHB can reduce inflammation caused by LPS in broiler chickens, and this inflammation may be influenced by levels of the enzyme ketolytica.

Keywords: components: inflammatory cytokine, ketolytic enzyme, spleen, and peripheral blood monocytes

Introduction

Ketone bodies, acetoacetate, acetone, and β-hydroxybutyrate (BHB) are water-soluble substrates generated in the liver under fasted and hypoglycemic conditions. In the keto- genic process, acetyl-CoA derived from fatty acids is converted into acetoacetyl-CoA, from which acetoacetate is generated with 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS) and 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL) (Figure 1). Thereafter, BHB is gen- erated with BHB dehydrogenase (BDH), with acetone also generated by non-enzymatic decarboxylation. In extrahepatic tissues/organs, BHB is metabolized to acetoacetate, and this compound is subsequently re-converted into acetoacetyl-CoA, followed by acetyl-CoA formation. These ketolytic processes contribute to ATP production in mitochondria as an alternative energy source. In post-hatch chicks, plasma BHB concentration is higher as it is generated from residual egg yolk. The BHB levels are dramatically reduced with time after hatching [1,2]. It has also been reported that serum BHB concentration is not changed by 12 h of feed withdrawal, while the level is increased by 24 h treatment and maintained constantly up to the next 24 h in young chickens [3]. These reports suggest that BHB could be used as an energy substrate in response to physiological conditions.



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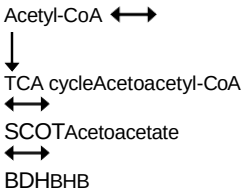

Figure 1. Schematic representation of ketogenic and ketolytic pathways.

Apart from the properties of BHB as energy fuel, recent studies have demonstrated that BHB exerts inflammatory effects due to a modulation of the signaling cascade [4,5]. It has been reported that BHB induces forkhead box protein O1 and its target gene, heme oxygenase-1 gene expression [6], reinforcing the anti-inflammatory effect of interleukin (IL)-10 [7]. Moreover, BHB has been reported to block the formation and activation of NLR family pyrin domain containing 3 (NLRP3) inflammasome [8–10] and stimulate GPR109A receptor [11], promoting anti-inflammation. Moreover, BHB administration has been reported to ameliorate renal inflammation, in which nuclear factor-erythroid 2 related factor 2 (Nrf2), a master regulator of antioxidative gene transcription [12], was activated with enhanced metabolic flux of TCA intermediates, acetoacetate, succinate, fumarate [13]. These lines of evidence suggest that BHB alleviates inflammation through various molecular signaling transduction pathways, and metabolic alterations could also be involved in the effects. The administration of lipopolysaccharide (LPS), a cell wall constituent of Gram- negative bacteria, is often used as a pathogenic inflammation model [14–17]. However, there is no available information on the effects of BHB on the innate immune response of LPS-treated chickens. Therefore, the present study aimed to investigate the therapeutic effects of BHB on LPS-treated chickens by measuring the plasma inflammatory parameters and cytokine expression. The study also examined the gene expression of ketolytic enzymes to seek a possible mechanism exhibiting the BHB effects.

Materials and Methods

Animals and Experimental Design

Twenty-day-old Ross 308 male broiler chickens (*Gallus gallus domesticus*) were obtained from a local commercial hatchery (Matsumoto Poultry Farms & Hatcheries Co., Ltd., Zao, Miyagi, Japan). The chicks were bred according to the breeding manuals and were provided ad libitum access to water and feed, a corn/soybean-based standard diet for broiler chickens at the grower phase (crude protein, 22%; metabolizable energy, 3200 kcal/kg) until they were 25 days old. The chickens were randomly allocated to the following treatment groups with similar average body weight (BW): sterile 0.9% (w/v) sodium chloride solution (saline, control, n = 7), LPS [1.5 mg/kg (BW), *Escherichia coli* O127:B8 (#L3129; Sigma-Aldrich, St. Louis, MO, USA), n = 6], or LPS plus BHB sodium [3 mmol/kg BW (#H0231; Tokyo Chemical industry, Co., Ltd., Tokyo, Japan), n = 7]. The LPS and BHB solutions were prepared using saline on the day of use. The chickens were intraperitoneally injected with BHB for 3 h, with LPS injected for 2 h before euthanasia. The same aliquot of saline was injected for treatments without LPS or BHB. Feed was withdrawn in all groups during the treatment. Chickens were euthanized by decapitation, and the spleen, liver, and *gastrocnemius* muscles were then excised and immediately frozen/powdered in liquid nitrogen. For the isolation of PBMC, whole blood was collected in a heparinized centrifuge tube from the wing vein. The blood was gently transferred onto Lymphoprep™ solution (#ST-07811; STEMCELL Technologies, Vancouver, BC, Canada) and thereafter centrifuged at 800 × g for 30 min at 20 °C. The organs, tissues, and PBMC were stored at −80 °C until use.

Analyses of Plasma Inflammation Markers

Plasma was obtained from heparinized-whole blood by centrifugation at 825 × g for 10 min at 4 °C. The following inflammation markers were measured using each commer- cial kit: aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities (#431-30901; FUJIFILM-Wako Pure Chemical Corporation, Osaka, Japan), and albumin and total protein concentration (#274-24301; FUJIFILM-Wako Pure Chemical Corporation, Osaka, Japan). The study also measured plasma interleukin (IL)-6 concentration using a commercial kit (#MBS2021018; MyBioSource, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Quantification of Gene Expression Levels

Real-time reverse transcript polymerase chain reaction (RT-PCR) was performed to quantify the

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gene expression levels of the inflammatory cytokines and ketogenic and ketolytic enzymes. Tissue RNA was isolated from the spleen, PBMC, skeletal muscle, and liver. Synthesis of complementary DNA and real-time RT-PCR analysis were conducted as previously described [18,19]. Inflammatory cytokines (IL-1β, IL-6, IL-18), and ketogenic and ketolytic enzymes (HMGCL, HMGCS2, BDH1, SCOT), were amplified using a specific primer (Table 1). Amplification was performed using CFX Connect® Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following cycling conditions: an initial denaturation step at 95 °C for 3 min, followed by 40 cycles consisting of 10 s at 95 °C for DNA denaturation, 10 s for primer annealing (see the temperatures in Table 1), and 30 s at 72 °C for DNA extension. The values were normalized to the expression levels of ribosomal protein S9 (RPS9) as an internal standard.

Accession ID			
BDH1	Sense GGAGGTCAAAGGGTCGTGTA Antisense CAGGTTGGTGGCAGCTATGA	56	NM_001006547.3
SCOT	Sense TCTACCAGCTGTCATCGCAA Antisense TCCAAAATTGTCAACGCCTGC	63	NP_001006578.2
RPS9	Sense TGCGAAGTTTTGTGACTGAAACA Antisense ATTCTTGGAGCATTCAGCCTTTC	60	NM_001277757

Abbreviations: IL, interleukin; HMGCL, 3-hydroxy-3-methylglutaryl-CoA lyase; HMGCS2, 3-hydroxy-3- methylglutaryl-CoA synthase 2; BDH1, β-hydroxybutyrate dehydrogenase 1; SCOT, succinyl-CoA:3-ketoacid CoA transferase; RPS9, ribosomal protein S9.

Statistical Analysis

Data are presented as the mean ± standard error (SE) of 6–7 individuals. Statistical analysis was performed using one-way analysis of variance with Bonferroni's method, with values of $p < 0.05$ indicating statistical significance in each test.

Results

Plasma Inflammatory Parameters

Plasma albumin and total protein concentration were significantly reduced by LPS administration, while the effects were partially inhibited in the BHB co-treated group ($p < 0.05$ in albumin; $p = 0.07$ in total protein) (Figure 2A,B). The LPS treatment significantly increased plasma AST and ALT activities, and the increases did not occur in the BHB co- treated group (Figure 2C,D), and similar results were obtained in plasma IL-6 concentration (Figure 2E). These results suggest that BHB intraperitoneal administration may have a suppressive effect on LPS-induced acute inflammation in broiler chickens.

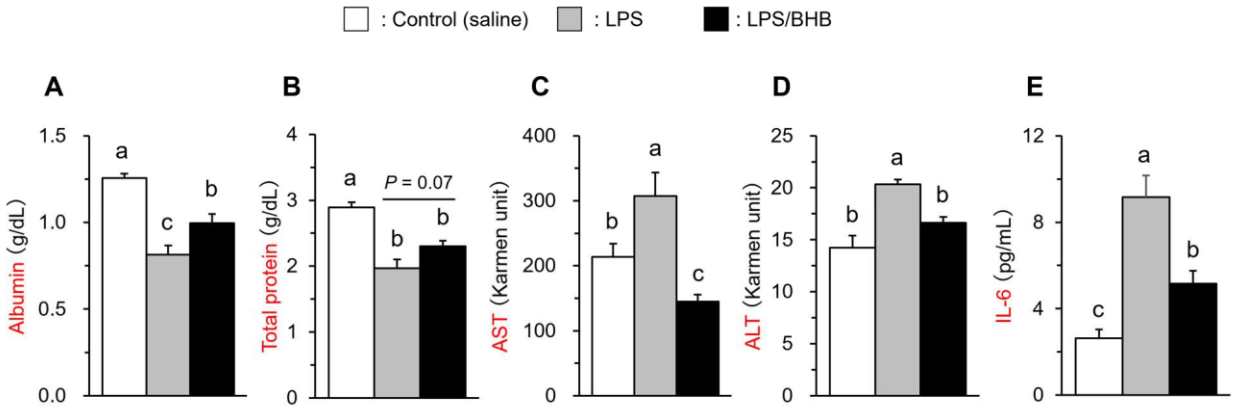

Figure 2. Effects of LPS and BHB on plasma albumin (**A**), total protein (**B**) concentrations, AST (**C**) and ALT (**D**) activities, IL-6 concentration (**E**) in broiler chickens. Data are expressed as means + SE, n = 6–7. ^{abc} $p < 0.05$ analyzed by Tukey–Kramer multiple comparison test, with different superscript letters indicating statistical difference.

The Effects of LPS and BHB Administration on Inflammatory Gene Expression

The gene expression levels of inflammatory cytokines were measured. As illustrated in Figure 3, the IL-1β, IL-6, and IL-18 gene expression levels were significantly increased by LPS administration in the spleen and PBMC, with greater up-regulation observed in the spleen than in PBMC. The above changes were partially inhibited in the BHB co-treated group in the spleen ($p < 0.05$); however, the suppression did not occur in PBMC (Figure 3).

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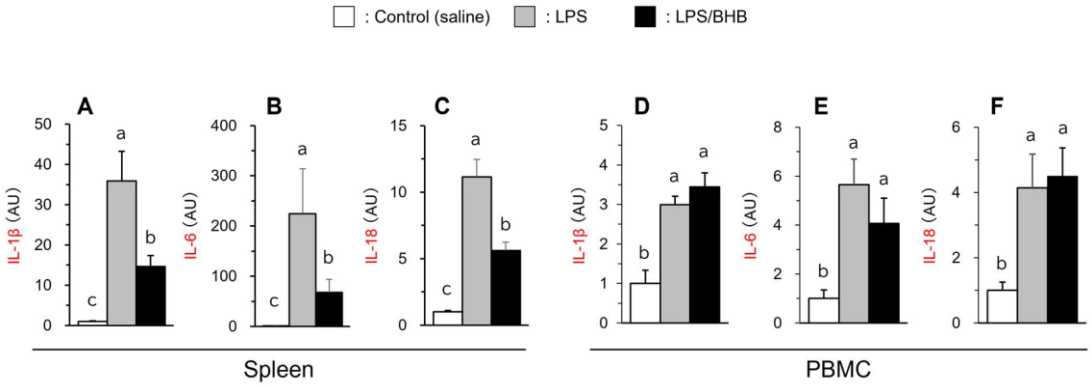

Figure 3. Effects of LPS and BHB on IL-1β (**A**,**D**), IL-6 (**B**,**E**), and IL-18 (**C**,**F**) in the spleen and PBMC of broiler chickens. Data are expressed as means + SE, n = 6–7. ^{abc} $p < 0.05$ analyzed by Tukey–Kramer multiple comparison test, with different superscript letters indicating statistical difference. Data are represented as fold changes relative to control values.

Different Gene Expression of Ketogenic and Ketolytic Enzymes in Peripheral Tissues/Organs Beta-hydroxybutyrate is metabolized to acetyl-CoA (Figure 1) and subsequently yields TCA-cycle intermediates. Therefore, the present study examined the gene expression levels of the enzymes of ketone body metabolism to seek possible machinery associated with the different responses of BHB effects on LPS-induced inflammation between the spleen and PBMC. The gene expression levels of HMGCS2 and HMGCL were investi- gated as a rate-limiting enzyme of ketone body synthesis, each of which catalyzes the formation of acetoacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA from acetyl-CoA and acetoacetyl-CoA, respectively. The study also measured the gene expression levels of BDH1 and SCOT, each of which catalyzes a reversible reaction of BHB to acetoacetate and acetoacetate to acetoacetyl-CoA, respectively. The above four gene expression levels were also measured in the liver and skeletal muscle tissue as typical ketogenetic and ketolytic tissues/organs, respectively.

The present study showed that both HMGCS2 and HMGCL gene levels were markedly higher in the liver compared to those of skeletal muscle, PBMC, and the spleen (^{ab} $p < 0.01$) (Figure 4A,B) since the liver is a major ketogenic organ. Next, as seen in Figure 4C,D, BDH1 and SCOT gene levels were relatively higher in skeletal muscle and the spleen because they use ketone bodies as an alternative energy substrate. Meanwhile, these gene expressions in PBMC were lower than the above extrahepatic tissues/organs and comparable to those observed in the liver. These results suggest that PBMC may be unable to utilize BHB, which could explain the little effect of BHB on the LPS-induced inflammatory response

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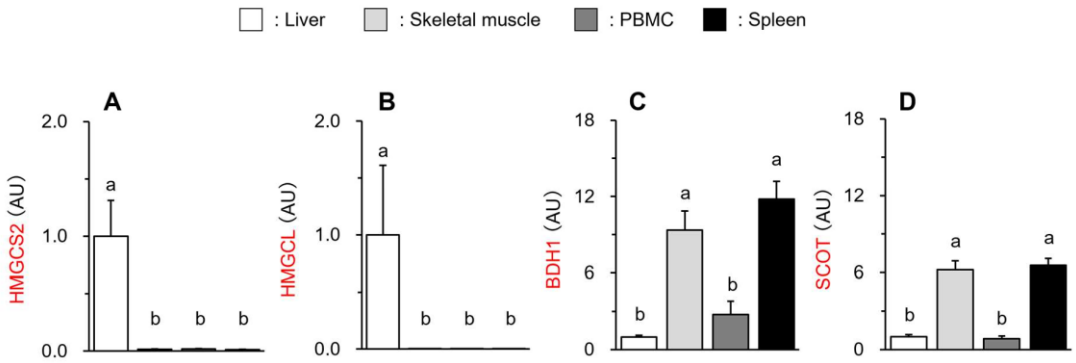

Figure 4. Different gene expression levels of HMGCS2 (**A**), HMGCL (**B**), BDH1 (**C**), and SCOT (**D**) of the liver, skeletal muscle, PBMC, and spleen of broiler chickens. Data are expressed as means + SE, n = 6–7. ^{ab} $p < 0.01$ analyzed by Tukey–Kramer multiple comparison test, with different superscript letters indicating statistical difference. Data are represented as fold changes relative to the values of the liver.

Discussion

There is little information regarding the effects of BHB on the innate immune response of chickens, to our knowledge. One study showed a possible involvement of BHB in chicken inflammation: serum BHB concentration

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was increased with ingestion of anti-inflammatory plant polysaccharides in pathogen-challenged laying hens