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ORIGINAL ARTICLE

The pattern of plasma BCAA concentration and liver *Bckdha* gene expression in GK rats during T2D progression

Wenlu Zhang[1] | **Yu'e Wu**[2] | **Wei Fan**[1] | **Hongmei Chen**[3] | **Hongli Du**[1] | **Junhua Rao**[4]

[1]School of Biological and Biological Engineering, South China University of Technology, Guangzhou, China

[2]Guangdong Key Laboratory of Laboratory Animals, Guangzhou, China

[3]Guangdong General Hospital, Guangzhou, China

[4]Guangdong Key Laboratory of Animal Conservation and Resource Utilization, Guangdong Public Laboratory of Wild Animal Conservation and Utilization, Guangdong Institute of Applied Biological Resources, Guangzhou, China

Correspondence
Hongli Du, School of Biological and Biological Engineering, South China University of Technology, Guangzhou, China.
Email: hldu@scut.edu.cn
and
Junhua Rao, Guangdong Key Laboratory of Animal Conservation and Resource Utilization, Guangdong Public Laboratory of Wild Animal Conservation and Utilization, Guangdong Institute of Applied Biological Resources, Guangzhou, China.
Email: junhuar919@163.com

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Abstract

Background: This study was conducted to measure the concentration of branched chain amino acid (BCAA) in different species and detect the expression pattern of the liver *Bckdha* gene in Goto-Kakizaki (GK) rats during type 2 diabetes (T2D) progression.

Methods: We measured the concentration of BCAA in GK rats, induced T2D cynomolgus monkeys and T2D humans by liquid chromatography tandem mass spectrometry, and used real-time quantitative PCR to analyze the gene expression of *Bckdha* and *Bckdk*, which encode the rate-limiting enzymes in catabolism of, respectively, branched chain amino acids and branched chain α-keto acid dehydrogenase kinase.

Results: In this study, we showed that GK rat BCAA concentrations were significantly reduced at 4 and 8 weeks ($P < 0.05$ and $P < 0.01$, respectively), while the expression of *Bckdha* in GK rat liver was increased at 4 and 8 weeks (1.62-fold and 1.93-fold, respectively). The BCAA concentrations were significantly reduced in diet-induced T2D cynomolgus monkeys ($P < 0.01$), but significantly increased in T2D humans ($P < 0.001$).

Conclusions: Our results showed that BCAA concentrations changed at different times and by different amounts in different species and during different periods of T2D progress, and the significant changes of BCAA concentration in the three species indicated that BCAA might participate in the progress of T2D. The results suggested that the increased expression of *Bckdha* in GK rat liver might partially explain the reduced plasma BCAA concentration at 4 and 8 weeks. Further studies are required to investigate the exact mechanism of BCAA changes in non-obese T2D.

KEYWORDS
GK rat, liquid chromatography tandem mass spectrometry, liver, non-obese

Wenlu Zhang and Yu'e Wu contributed equally to this work.

1 | INTRODUCTION

Type 2 diabetes (T2D), the most prevalent form of diabetes, has affected more than 425 million people in the world.[1] Increased concentrations of branched-chain amino acid (BCAA), including leucine (Leu), isoleucine (Ile) and valine (Val) have been detected in obese and T2D rodent models, such as Zucker diabetic fatty rat *ob/ob* mice and high-fat diet-induced diabetic mice.[2-6] Furthermore, metabolomics demonstrate that elevated BCAA concentrations are highly associated with the development of insulin resistance.[7-9] It has been reported that BCAA could stimulate the mammalian target of rapamycin and S6 kinase and phosphorylate insulin receptor substrate 1 on serine residues, impairing the insulin signaling pathway.[10-12] Decreased BCAA catabolism could contribute to insulin resistance and glucose intolerance, ultimately leading to T2D.[7] Increasing evidence has indicated that elevated BCAA concentrations could serve as useful markers for T2D.[13-17]

The first step of BCAA catabolism is transamination catalyzed by branched-chain aminotransferase, which is expressed mainly in muscle, kidney, and heart, yielding corresponding branched-chain α-ketoacids (BCKAs).[18] BCKAs are then catalyzed by the branched-chain a-ketoacid dehydrogenase complex (BCKDC), which is the rate-limiting enzyme of BCAA catabolism.[19-21] Agus and colleagues report that the activity of Bckdc in liver comprises up to 60%-83% of the whole body Bckdc activity in rats.[22] The BCKDC is a multienzyme complex, consisting of E1 (with α and β components), E2, and E3,[23] and its activity is regulated by phosphorylation (inactivation) and dephosphorylation (activation) of the E1α component by BCKDC kinase (BCKDK) and BCKDC phosphatase, respectively.[24] Moreover, the gene *BCKDHA* encoding the BCKDC E1α component has been identified as a candidate gene associated with obesity and T2D.[25] It has been reported that knock out of *Bckdk* led to a significantly reduced blood BCAA concentration.[20]

Increased BCAA concentrations have been observed in obesity and T2D rodent models, such as Zucker diabetic fatty rat,[3,4,6,26] *ob/ob* mice[4] and high-fat diet (HFD)-induced mice[2] and T2D human.[13] In contrast, the BCAA concentrations in spontaneous T2D Otsuka Long-Evans Tokushima Fatty (OLETF) rats are lower than in controls, although the difference was not significant,[26] and BCAA concentrations in T2D young humans are significantly lower than in controls.[27] Together, these findings suggest that differences in BCAA concentrations occur between species and at different stages of life. However, BCAA concentrations in non-obese T2D GK rats have not been reported. Thus, we measured BCAA concentrations in three species—spontaneous T2D Goto-Kakizaki (GK) rats, induced T2D cynomolgus monkeys and T2D humans - using liquid chromatography tandem mass spectrometry (LC/MS).

Furthermore, it has been reported that Bckdha protein expression is decreased in the liver of obese T2D Zucker rats,[4] and liver BCKDC activity is significantly decreased in T2D rats.[26] It has also been reported that the enzymes in BCAA catabolism are decreased in adipose tissue and liver in *ob/ob* mice and *fa/fa* rats.[4] As the pattern of change in plasma BCAA concentrations and liver *Bckdha* and *Bckdk* expression in non-obese T2D GK rats has not yet been reported, we measured plasma BCAA concentrations and liver *Bckdha* and *Bckdk* gene expression in these rats.

2 | METHODS

2.1 | Experimental animals and blood collection

This study involved 50 T2D GK male rats and 50 control Wistar male rats obtained from SLAC Laboratory Animal Co., Ltd (Shanghai, China). The feeding regime was as previously described.[28] Rats were anesthetized using pentobarbital sodium (3%, 0.2 ml/100 g) and sacrificed by abdominal aortic exsanguination. Five control cynomolgus monkeys, five STZ-induced T2D cynomolgus monkeys and three diet-induced T2D cynomolgus monkeys were involved in this study. Diet-induced T2D was achieved as previously described in the diet-induced group.[29] STZ-induced T2D was achieved by intravenous injection of STZ (10 mg/kg) in the STZ-induced group, with a second injection of STZ (10 mg/kg) given at 2 months. The T2D model was formed within a month, when the blood sugar was stable at 7.0 mmol/L. The blood samples of cynomolgus monkeys were collected from the brachial vein after anesthezing the animals with zoletil-50 (5 mg/kg, intramuscular injection). The blood samples of rats and cynomolgus monkeys were extracted using EDTA anticoagulant tubes. The plasma samples were prepared from blood samples by centrifugation (2000×*g*, 4°C, 15 minutes), and stored at −80°C. Twenty healthy humans, 10 prediabetic humans and 20 T2D humans from Guangdong General Hospital participated in this study. Serum samples from those humans were prepared from blood samples extracted from the elbow vein by centrifugation (2000×*g*, 4°C, 15 minutes), and stored at −80°C. All rat experiments were approved by the institutional review board of the Guangdong Key Laboratory of Laboratory Animals, and all protocols were carried out in accordance with the approved guidelines of the Institutional Animal Care and Use Committee (IACUC) (Ethics certificate No. IACUC2014029). All cynomolgus monkey experiments were subjected to approval and surveillance by the Institutional Animal Care and Use Committee of Guangdong Landao Biotechnology Co., Ltd (Ethics certificate No. LD20150518). The study of humans was approved by the Research Ethics Committee of Guangdong General Hospital, Guangdong Academy of Medical Sciences (Ethics certificate No. GDREC2012067H (R1)). All human subjects involved in this study provided their written informed consent for this study.

2.2 | Plasma glucose concentration measurements

A GLU Assay Kit (KOFA, China) and an automatic biochemistry analyzer (Hitachi 7020, Tokyo, Japan) were used to measure glucose concentration. Assays were carried out according to manufacturer's directions, with standards run in duplicate and experimental samples run in triplicate.

2.3 | Plasma insulin concentration measurements

Plasma insulin was measured with a Luminex MAGPIX using a Milliplex MAG Rat Metabolic Magnetic Bead Panel Kit (insulin kit,

Milliplex, Germany). Assays were carried out according to manufacturer's directions, with standards run in duplicate and experimental samples run in triplicate.

2.4 | BCAA concentration measurements

The BCAA concentration of each plasma and serum sample was measured by liquid chromatography-tandem mass spectrometry (LC/MS, Agilent 1260-6460 Triple Quadrupole), using an ACQUITY UPLC® BEH Amide column (50 mm × 2.1 mm, 1.7 μm; Waters, USA) and a VanGuard column (5 mm × 2.1 mm, 1.7 μm; Waters, USA). The protocol for measurement of BCAA concentrations was based on previously reports.[13,30,31] L-2-Aminobutyric acid (Sigma, St Louis, MO, USA) diluted to a concentration of 50 μg/ml was used as an internal standard. The Leu, Ile, and Val reference solutions were diluted first to a concentration of 2 mg/ml, and then to 4, 4, and 200 μg/ml, respectively, for stock standard mixtures. The stock standard mixture was then diluted with acetonitrile 2, 4, 8, 16, 32, 64, 128, 256, 1024 times to give different standard solutions. Then, 5 μl prepared samples were injected into an ACQUITY UPLC® BEH Amide column maintained at 35°C and eluted with mobile phase solutions: mobile phase A was 20 mmol formic acid, mobile phase B was acetonitrile-20 mmol formic acid, and the flow rate was set to 0.5 ml/min. Detection was performed using positive jet stream electronic spray ionization (ESI) in multiple reaction monitoring (MRM) mode at a source temperature of 300°C and a voltage of 3500 V. Nitrogen was used as the curtain, nebulizer and collision gas and the nebulizer pressure was 45 psi.

2.5 | RNA extraction, reverse transcription and real-time PCR quantification

Total RNA was extracted using TRIzol Reagent (Cat#15596-018, Life Technologies, USA) following the manufacturer's instructions. Real time quantitative PCR (M-MLV Reverse Transcriptase kit, Life Technologies, USA) was performed using a SYBR Green qPCR SuperMix-UDG kit (Life Technologies, USA) with a LightCycler 96 system (Roche, Switzerland). Wistar rats were used as the controls and β-actin (*actb*) was used as a an internal reference (measured with the $2^{-\Delta\Delta Ct}$ method).

2.6 | Statistical analysis

All data were expressed as means ± SD. Significant differences were assessed using a two-tailed Student's *t* test. $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Cynomolgus monkey plasma glucose and BCAA concentrations

The plasma glucose concentrations in STZ-induced and diet-induced T2D cynomolgus monkeys were both significantly increased compared with those in healthy cynomolgus monkeys ($P < 0.001$ and $P < 0.01$, respectively) (Figure 1A). The plasma BCAA concentrations in STZ-induced T2D cynomolgus monkeys were lower than those in controls, though the differences were not significant (Figure 1B). However, the plasma BCAA concentrations in diet-induced T2D cynomolgus monkeys were significantly lower than in controls ($P < 0.01$) (Figure 1B), in contrast to previous reports of significant increases in BCCA concentrations in this T2D model.[3,5,6] This difference might be due to differences in experimental species.

3.2 | Human serum glucose and BCAA concentrations

The serum glucose concentrations in prediabetic humans and T2D humans were both significantly elevated compared to those in healthy humans ($P < 0.001$); the serum glucose concentrations in T2D humans were significantly higher than those in prediabetic humans ($P < 0.01$) (Figure 2A). The BCAA concentrations in prediabetic humans were higher than in healthy humans, but not significantly (Figure 2B). T2D human BCAA concentrations were significantly increased in comparison with healthy humans ($P < 0.001$) (Figure 2B). These results in prediabetic humans and T2D humans indicate that differences in the trends and levels of BCAA exist at different periods during the progress of T2D. The rise in BCAA concentrations in T2D humans was in accordance with previous reports of significant increases in BCAA concentration.[32,33]

3.3 | GK rat plasma glucose and insulin concentrations

The plasma glucose concentration in GK rats increased between 4 and 12 weeks, and then it began to decrease. In Wistar rats, the plasma glucose concentration also increased between 4 and 12 weeks (Figure 3A). The plasma glucose concentrations in GK rats were significantly increased compared to Wistar rats between 4 and 20 weeks ($P < 0.001$) (Figure 3A). The plasma insulin concentration in GK rats increased at 4 weeks and reached a maximum at 8 weeks, and then began to decline (Figure 3B). In contrast, the plasma insulin concentration in Wistar rats also rose from 4 weeks and reached a maximum at 8 weeks, but then the concentration remained steady until 16 weeks. The insulin concentration in GK rats was higher than in Wistar rats, although not significantly, at 8 weeks, but significantly lower than in Wistar rats at 4 ($P < 0.01$), 12 ($P < 0.01$), 16 ($P < 0.001$), and 20 weeks ($P < 0.001$).

3.4 | BCAA concentrations in GK and Wistar rats

GK rat BCAA concentrations were significantly lower than in Wistar rats at 4 and 8 weeks ($P < 0.05$ and $P < 0.01$, respectively), and remained lower at 12 and 16 weeks, though not significantly (Figure 4A). The concentrations were above control levels, but not

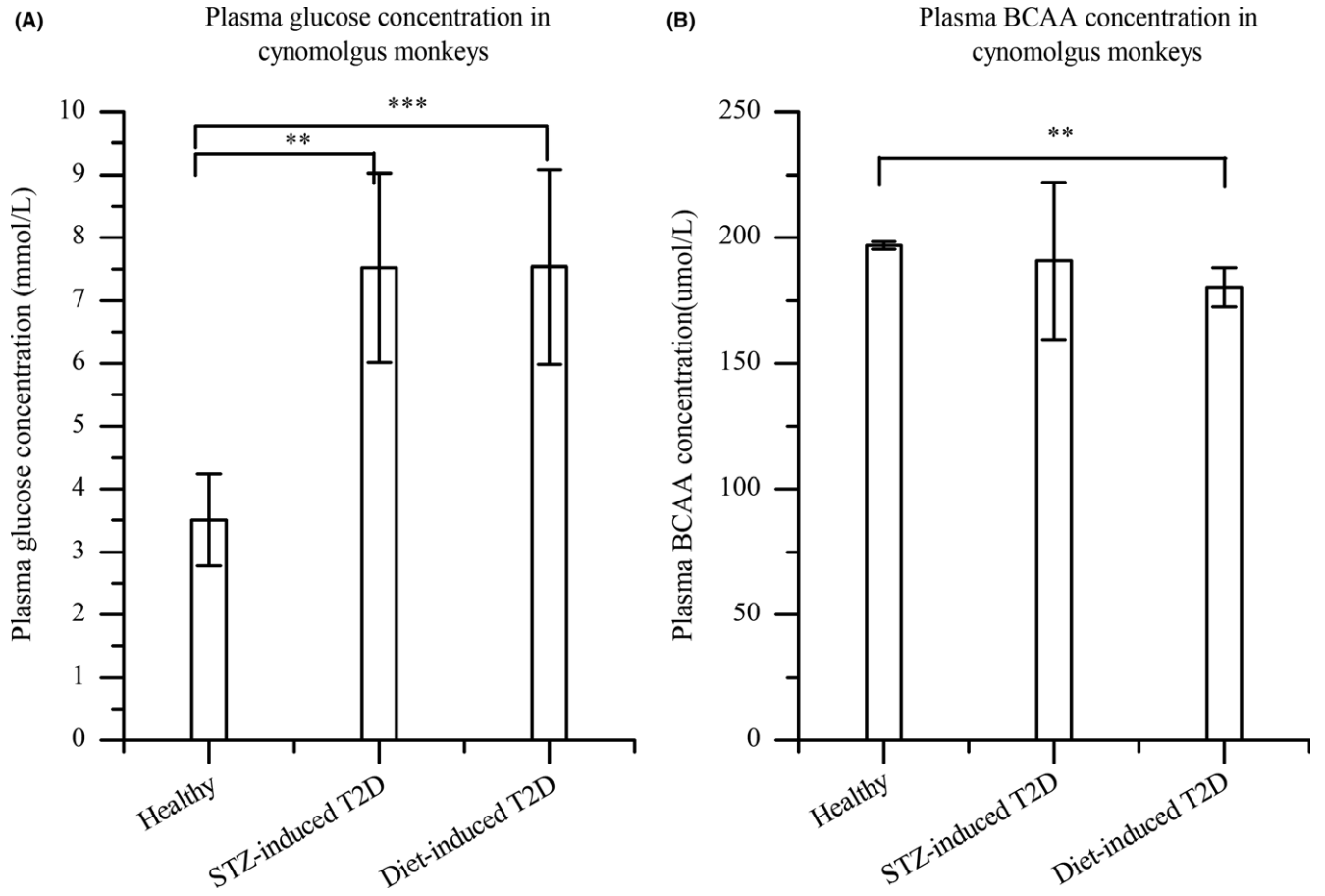

FIGURE 1 Plasma glucose and BCAA concentrations in cynomolgus monkeys. A, Plasma glucose concentration. B, Plasma BCAA concentration. Data are shown as means ± SD. Healthy, n = 5. STZ-induced T2D, n = 5. Diet-induced T2D, n = 3. $^{**}P < 0.01$, $^{***}P < 0.001$

significantly, at 20 weeks (Figure 4A). The plasma Leu concentrations in the GK rats were significantly reduced compared to controls between 4 and 12 weeks ($P < 0.001$), remained lower than in controls, but not significantly, at 16 weeks, and were higher than controls, though not significantly, at 20 weeks (Figure 4B). The plasma Ile concentrations in the GK rats were significantly lower than in control between 4 and 12 weeks ($P < 0.01$, $P < 0.001$, $P < 0.01$, at 4, 8, and 12 weeks, respectively) and lower than controls, though not significantly, at 16 weeks and 20 weeks (Figure 4C). GK rat plasma Val concentrations were lower than controls, but not significantly, at 4 and 8 weeks, and higher than controls, but not significantly, from 12 weeks to 20 weeks (Figure 4D). The results indicated that GK rat BCAA concentrations were different at different stages of life. These GK rat concentrations are not in accordance with previous reports of significantly increased BCAA concentrations in rat models.[3,5,6]

3.5 | Expression of *Bckdha* and *Bckdk* genes

Previous reports indicated that BCAA concentrations significantly increase and the expression of *Bckdha* encoding the rate-limiting enzyme BCKDHA is significantly reduced in T2D animal models.[5,6] Based on our results showing significantly elevated GK BCAA concentrations, we performed real time PCR to quantify the expression of *Bckdha*, and *Bckdk*, which encodes the Bckdha activity inhibiter Bckdk, aimed at exploring the mechanism responsible for the elevated BCAA concentration in GK rats. The expression of *Bckdha* in GK rats was higher than in Wistar rats, though not significantly, between 4 and 12 weeks (1.62-fold, 1.93-fold and 1.33-fold, at 4, 8, and 12 weeks, respectively), lower than in Wistar rats, though not significantly, at 16 weeks, and higher than in Wistar rats, though not significantly, at 20 weeks (Figure 5A). Likewise, the expression of GK rat *Bckdk* was lower than in Wistar rat, though not significantly, at 8 and 16 weeks, and higher than in Wistar rat, though not significantly, at 4, 12, and 20 weeks (Figure 5B). The GK plasma Leu and Val concentrations were significantly reduced between 4 and 12 weeks, compared to controls, and the expression of GK *Bckdha* was increased between 4 and 12 weeks, suggesting that the increase in GK *Bckdha* expression was related to the significantly reduced GK Leu and Val concentrations during that period.

4 | DISCUSSION

In this study, BCAA concentrations were significantly reduced in diet-induced T2D cynomolgus monkeys ($P < 0.01$) and in GK rats at

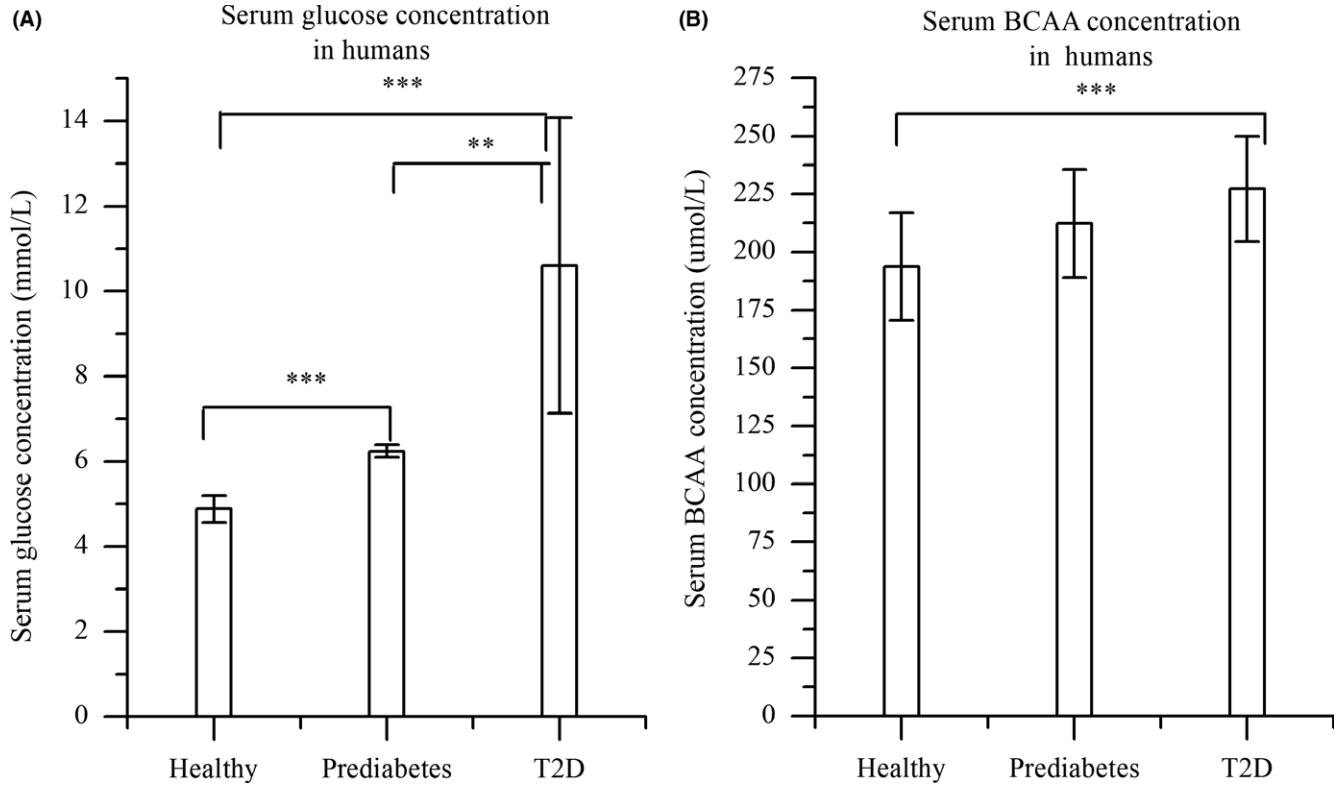

FIGURE 2 Human serum glucose and BCAA concentrations. A, Glucose concentration. B, BCAA concentration. Data are shown as means ± SD. Healthy, n = 20. Prediabetic, n = 10. T2D, n = 20. $^{**}P < 0.01$, $^{***}P < 0.001$

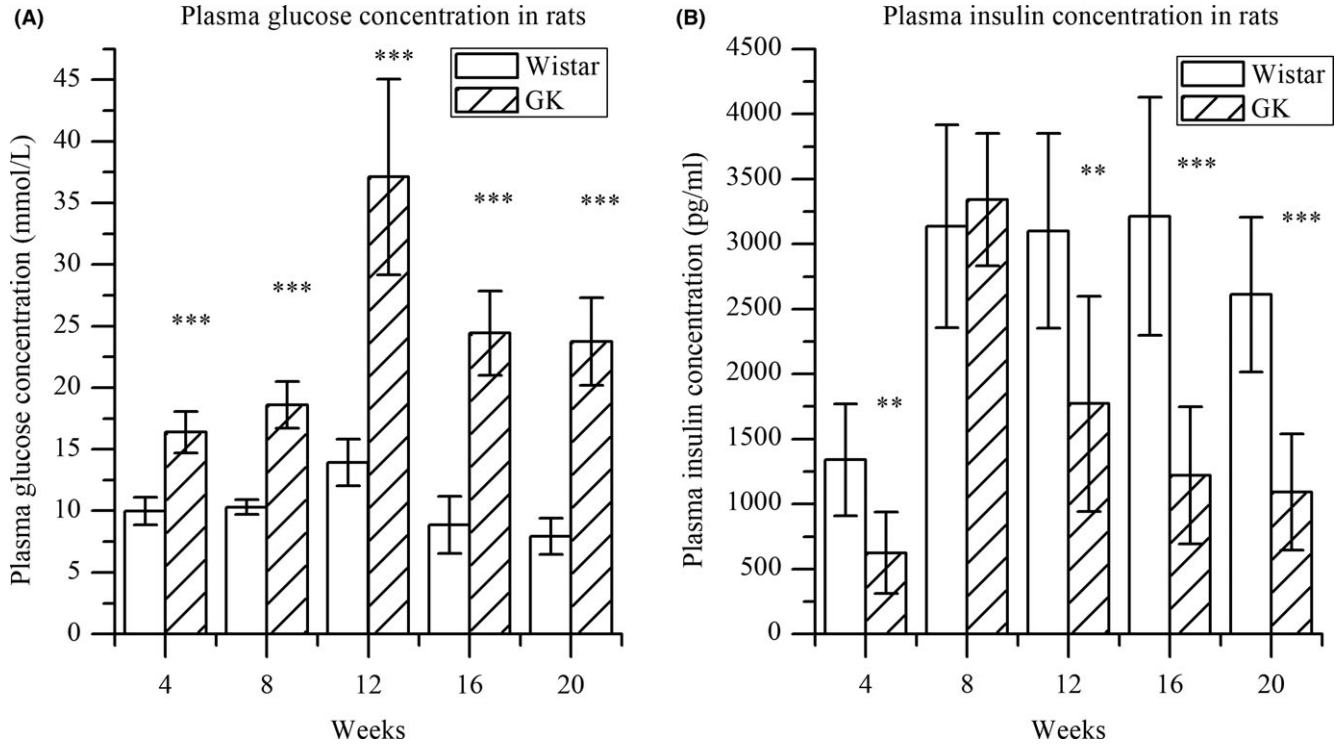

FIGURE 3 Plasma glucose and insulin concentrations in GK and Wistar rats. A, Plasma glucose concentrations. B, Plasma insulin concentrations. Data are shown as means ± SD. n = 10. $^{**}P < 0.01$, $^{***}P < 0.001$

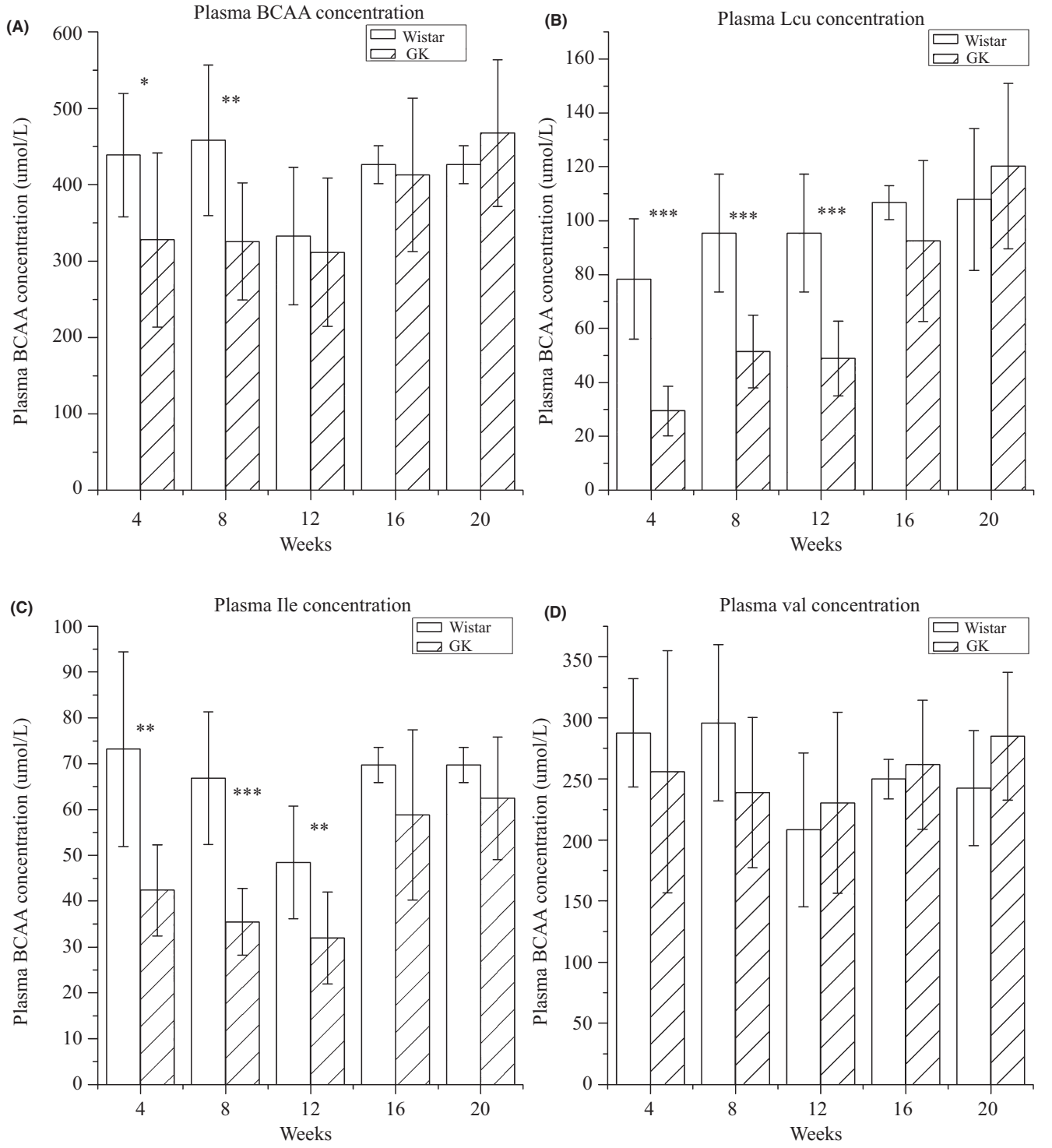

FIGURE 4 Plasma BCAA concentrations in GK and Wistar rats. A, Plasma BCAA concentrations. B, Plasma Leu concentrations. C, Plasma Ile concentrations. D, Plasma Val concentrations. Data are shown as means ± SD. n = 10. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

4 weeks ($P < 0.05$) and 8 weeks ($P < 0.01$); however, they were significantly increased in T2D humans ($P < 0.001$). Plasma Leu and Ile concentrations were significantly reduced between 4 and 12 weeks in GK rats, but the reduction in plasma Val concentration was not statistically significant. In addition, the expression of *Bckdha* encoding the rate-limiting enzyme in catabolism of BCAA in GK rats was higher than in Wistar rats at 4 and 8 weeks. The increase in *Bckdha* expression could partially explain the decrease in BCAA concentrations in GK rats at 4 and 8 weeks. However, the BCAA concentrations in our diet-induced T2D cynomolgus monkeys and GK rats were different from previous studies, which demonstrated that BCAA concentrations were increased,[5,6] and that *Bckdha* expression

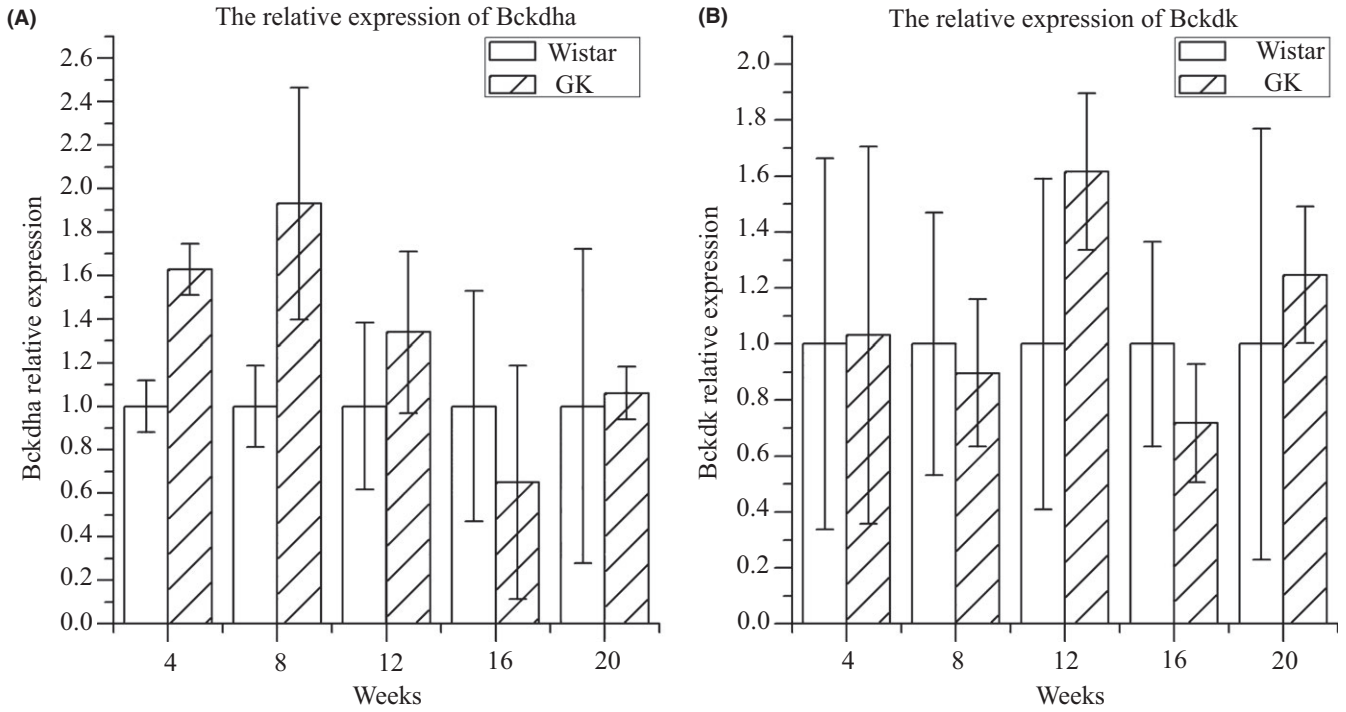

FIGURE 5 The relative expression of *Bckdha* and *Bckdk*. A, Relative expression of *Bckdha*. B, Relative expression of *Bckdk*. Data are shown as means ± SD. n = 10

was reduced in diabetes and obesity.[34,35] Our results show that BCAA concentrations change in direction and level in different species, and during different periods of T2D development, and the significant changes in BCAA concentration seen in the three species used in this study indicate that BCAA may participate in the progress of T2D.

It has been reported that BCAA can increase glucose uptake and act as insulin secretagogues.[36,37] Leu could stimulate glucose transport in skeletal muscle via phosphatidylinositol 3-kinase and the protein kinase C pathway.[38] Leu has been identified as an insulin secretagogue, as it can induce and enhance pancreatic β-cell insulin secretion through its oxidative decarboxylation, as well as allosterically activate glutamate dehydrogenase.[37,39,40] In addition, Leu supplementation can improve glucose metabolism, and reduce diet-induced insulin resistance in mice.[41] Moreover, it has been reported that BCAA reduce oxidative stress in pancreatic islets and ameliorate β-cell dysfunction, involving c-Jun N-terminal kinase, protein kinase D1 and pancreatic/duodenal homeobox-1[42] in streptozotocin-induced insulin-deficient rats. Therefore, the significantly reduced Leu concentration seen in our GK rats might partially reduce insulin-independent glucose uptake in skeletal muscle, and reduce whole body glucose oxidation and insulin secretion, leading to the increase of plasma glucose shown in Figure 3A.

It has been reported that Ile administration can stimulate muscle glucose uptake and whole body glucose oxidation, and depress gluconeogenesis in the liver, leading to the hypoglycemia.[36,43] Ile stimulates insulin-independent glucose uptake in skeletal muscle, which might contribute to the plasma glucose-lowering effect seen in SD rats.[44] In our study, GK rat Ile concentrations were significantly reduced between 4 and 12 weeks, suggesting that the stimulating effect of Ile on insulin-independent glucose uptake in skeletal muscle might partially be weakened. Thus, a significantly reduced plasma Ile concentration could contribute to the elevated plasma glucose concentration in the GK rats.

The expression of GK rat *Bckdha* was higher than control between 4 and 12 weeks (1.62-fold, 1.93-fold and 1.33-fold, at 4, 8, and 12 weeks, respectively), but the difference did not reach significance. As *Bckdha* encodes the rate-limiting enzyme in catabolism of BCAA the increase in its expression might partially lead to the reduced plasma BCAA concentrations seen in the GK rats. The levels of *Bckdha* gene expression and Bckdha protein expression, and the activities of *Bckdh* and *Bckdk* could all affect the concentration of BCAA. However, we did not measure the levels of Bckdha protein expression or the activities of *Bckdha* and *Bckdk* in GK rat liver, and this, along with the mechanism responsible for the reduced GK plasma BCAA concentrations, requires further investigation.

Plasma BCAA concentrations and liver *Bckdha* gene expression were different in different T2D humans and different T2D models, suggesting that the mechanism of BCAA in T2D is complex and the further study is needed to investigate the exact mechanism of BCAA actions in T2D.

5 | CONCLUSION

In summary, the changes in the trends and levels of BCAA concentrations were different among different species and different periods of T2D progress. The increase in *Bckdha* gene expression might

partially lead to the increased BCAA catabolism and might partially explain the reduced plasma BCAA concentrations in GK rats at 4 weeks and 8 weeks. The plasma BCAA concentrations and liver *Bckdha* gene expression were different in prediabetic and T2D humans and different T2D rat models, suggesting that the role of BCAA in T2D is complex. Elucidation of the mechanism by which BCAA contribute to T2D development in of GK rats needs further study.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

All authors contributed significantly to the work and the preparation of the manuscript, in accordance with the latest guidelines of the International Committee of Medical Journal Editors. WLZ and YEW performed the study, analyzed the data and wrote the manuscript. WF and HMC analyzed the data, and reviewed the manuscript. HLD and JHR conceived and supervised the studies, analyzed the data, and wrote the manuscript. All authors revised the manuscript.

ORCID

Hongli Du http://orcid.org/0000-0002-6419-0160

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