Epigallocatechin Gallate Suppresses Inflammatory Responses by Inhibiting Toll-like Receptor 4 Signaling and Alleviates Insulin Resistance in the Livers of High-fat-diet Rats

Huimin Hou, Wanli Yang, Suqing Bao, and Yanli Cao*

Department of Endocrinology and Metabolism, Institute of Endocrinology, Liaoning Provincial Key Laboratory of Endocrine Diseases, The First Affiliated Hospital of China Medical University, China Medical University, Shenyang, Liaoning, CHINA

Abstract: EGCG is a major pharmacological compound in green tea. Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide. Inflammation and insulin resistance are involved in the development of the disease. In this study, we investigated the beneficial effect of EGCG on the liver tissue of NAFLD rats induced by a high-fat diet and its underlying mechanism. Thirty Sprague-Dawley rats received a normal diet, a HFD and a HFD+EGCG. The expression levels of inflammatory signaling pathway genes (e.g., TLR4, TRAF6, IKKβ, NF-κB, TNF-α) and insulin signaling transduction pathway genes (e.g., PI3K, AKT, IRS-1, IRS-2) were detected in the liver. We observed that EGCG decreased the triglyceride (TG) concentration in rat livers and suppressed TLR4, TRAF6, IKKβ, p-IKKβ, p-NF-κB, and TNF-α levels compared with those in the HFD group, whereas PI3K, AKT, IRS-1, and IRS-2 indicators were improved. EGCG improves obesity-associated subacute hepatic inflammation states, probably through the TLR4 signaling pathway. Furthermore, EGCG also alleviated hepatic insulin resistance. These data indicate that EGCG improves NAFLD from two ways: inhibition of inflammation and improvement of insulin resistance in liver tissues.

Key words: epigallocatechin gallate, inflammation, insulin sensitivity, liver tissue, toll-like receptor 4

1 Introduction

Obesity is a worldwide problem, and its morbidity has increased each year. Obesity affects the function and metabolism of many organ systems and eventually leads to a variety of chronic diseases. Non-alcoholic fatty liver disease (NAFLD) is closely linked to obesity, insulin resistance, and other factors associated with metabolic syndrome. Among these factors, insulin resistance is the key pathogenic abnormality associated with obesity-associated NAFLD.

Toll-like receptors (TLRs) are a class of proteins that play an essential role in recognizing different pathogen-associated molecular patterns. Previous studies have shown that TLR4 is a key mediator of the liver inflammation associated with other chronic diseases such as obesity-related insulin resistance. TLR4, the receptor for lipopolysaccharide, endotoxin, free fatty acids (FFAs) and other ligands, is activated by ligands and transduces signals via two pathways in the liver. Both pathways mediate the upregulation of pro-inflammatory regulators through the activation of nuclear factor kappa B (NF-κB). Chronic inflammation impairs insulin signal transduction and decreases insulin sensitivity.

Previous studies have shown the potential health benefits of green tea, including its antiproliferative, antimutagenesis, antioxidant and anti-obesity effects. EGCG accounts for 50% to 80% of the catechin combination in green tea. EGCG, epigallocatechin gallate; FFA, free fatty acid; TLR4, toll-like receptor 4; FINS, fasting insulin; HOMA-IR, homeostasis model assessment-insulin resistance index; GIR, glucose infusion rate; HFD, high fat diet; TG, triglyceride; TLR4, Toll-like receptor 4; TRAF-6, TNF receptor-associated factor 6; IKKβ, IKappaB kinaseβ; p-IKKβ, phosphorylation IKappaB kinaseβ; p-NF-κB, phosphorylation nuclear factor B; TNF-α, tumor necrosis factorα; IRS-1, insulin receptor substrate 1; IRS-2, insulin receptor substrate 2; PI3K, phosphoinositide-3-kinase; AKT (PKB), protein kinase B

*Correspondence to: Yanli Cao, Department of Endocrinology and Metabolism, Institute of Endocrinology, Liaoning Provincial Key Laboratory of Endocrine Diseases, The First Affiliated Hospital of China Medical University, No. 155, North Nanjing Street, Heping District, Shenyang, Liaoning 110001, People’s Republic of CHINA
E-mail: vanilla421@163.com
Accepted January 28, 2020 (received for review November 26, 2019)
tea\textsuperscript{17–19}. Our previous study indicated that EGCG decreases inflammation by affecting the TLR4 signaling pathway and improving insulin signaling in adipose tissue\textsuperscript{20}. In this study, we aimed to observe whether EGCG prevents NAFLD by affecting the TLR4 signaling pathway and whether EGCG reduces insulin resistance in liver tissue.

2 Experimental

2.1 Chemicals and reagents

Antibodies for TLR4 and Akt were purchased from Abcam (Hong Kong, China), and anti-TNF-\(\alpha\) was purchased from Santa Cruz (CA, USA). Other antibodies were obtained from Cell Signaling Technology (MA, USA). The primers were from TaKaRa (Dalian, China). EGCG with purity \(\geq 95\%\) was purchased from Sigma-Aldrich (MO, USA).

2.2 Animal experiments permission

The Animal Research Committee of China Medical University (Shenyang, China) approved the study protocol, and we carried out this study according to the Guide for the Care and Use of Laboratory Animals, as mandated by the National Institutes of Health (Approval No. 14061R).

2.3 Animal models

Thirty male Sprague-Dawley rats at 4 weeks of age were acquired from Beijing HFK Bioscience Co. Ltd. (Beijing, China). All of the rats in this study were housed individually under 12-h light-dark cycle at 24±2°C and 45%–55% humidity. Rats were fed a normal diet (NC, \(n=10\), 10\% energy as fat) or a high-fat diet (HFD, \(n=20\), 60\% energy as fat). After 16 weeks, the latter continued to receive a HFD (\(n=10\)) or were changed to a high-fat diet + EGCG diet (HFD + 0.32\% EGCG, \(n=10\)) for 16 weeks. The specific components and quantity of the diet composition and the choice of the EGCG dose can be found in our previous literature\textsuperscript{21}. The rats were weighed twice per week and food consumption was determined every day during the feeding period. After being fed an EGCG diet for 16 weeks, a hyperinsulinemic-euglycemic clamp test was performed. Glucose and insulin were infused from two sides of femoral veins and blood samples were collected from the femoral arteries of anesthetized rats after overnight fasting. After the rats were kept quiet for 30 min, insulin was infused (1.67mIU/kg per min) to ensure the hyperinsulinemic conditions.

The rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital, and the blood was then collected and centrifuged (3,000 g*15 min). Serum and liver tissues were stored at \(-80^\circ\text{C}\).

2.4 Biochemical analysis

Liver tissue (100 mg) was homogenized in 900 \(\mu\)L of absolute ethanol alcohol with disruption. The triglycerides (TGs) in the collected supernatant were measured with a TG assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The FFA concentration in the serum and fasting blood glucose (FBG) and fasting insulin (FINS) levels were tested as previously described\textsuperscript{22}. Insulin sensitivity was assessed using the glucose infusion rate (GIR) and the homeostasis model assessment-insulin resistance index (HOMA-IR). GIR was tested in anesthetized rats using a hyperinsulinemic-euglycemic clamp technique. HOMA-IR was determined by FBG (mmol/L) and FINS (mIU/L), HOMA-IR = FBG (mmol/L)×FINS (mIU/L)/22.5.

2.5 Detection of lipid accumulation via Oil Red O stain

TRizol reagent was used to extract total RNA. After reverse transcription of RNA into cDNA, PCR was performed. Primer sequences are shown in Table 1. The PCR values were calculated using Light Cycler 480 Software version 1.5.

2.6 Real-time quantitative PCR

Liver tissues were stained in fresh Oil Red O for 10 min, followed by incubation in 60\% isopropanol for 2 min. The liver samples were stained in fresh Oil Red O for 10 min, followed by washing in 50\% isopropanol for 3 min. Then, the slides were stained with hematoxylin for 30 s and rinsed in water. The slides were then viewed under a light microscope.

2.7 Protein analysis via Western blot

Liver proteins were extracted using lysis buffer containing protease and phosphatase inhibitors. The samples were homogenized and incubated for 30 min at 4°C. After centrifugation at 12,000 rpm for 15 minutes at 4°C, the supernatants were collected. Twenty micrograms of protein per well was separated by 10\% SDS-PAGE gel electrophoresis,

| Gene name | Forward primer | Reverse primer |
|-----------|----------------|----------------|
| TLR4      | CTCACAACATTCAGTGGCTGGATTTA | GTCTCCACAGCACCACGATTCTC |
| TRAF6     | TTTGGGCTCGGAGACACTTG | TCGCTTGAAGACTGGCTGGA |
| GAPDH     | GCAAGTTCACCGGACAG | GCCAGTAGACTCCACGACAT |
EGCG Suppresses TLR4 in the Liver

J. Oleo Sci.

3 Results
3.1 Effects of EGCG intervention on metabolic indices

The weight of rats in the high-fat diet group before EGCG intervention was higher than that in the normal diet group (p < 0.05), without significant changes in food consumption. After EGCG intervention, the weight of rats in the EGCG group decreased. However, no significant differences were found between the HFD and EGCG groups with regard to body weight. Metabolic indices, such as FFAs and FINS, were significantly increased after a 16-week HFD and were significantly reduced after EGCG feeding.

Table 2  Effects of EGCG intervention on metabolic indices.

| Indices          | NC group     | HFD group     | EGCG group  |
|------------------|--------------|---------------|-------------|
| Weight (1) (g)   | 468 ± 9 (n=10) | 533 ± 8* (n=20) | –           |
| Weight (2) (g)   | 566 ± 7 (n=10) | 654 ± 19* (n=10) | 597 ± 12 (n=10) |
| Food intake (g·rat⁻¹·week⁻¹) | 149 ± 9 | 138 ± 11 | 142 ± 10 |
| FFAs (mmol/L)   | 0.32 ± 0.09 | 0.83 ± 0.12* | 0.36 ± 0.13* |
| FINS (mIU/L)    | 13.9 ± 0.6 | 32.5 ± 2.9* | 12.7 ± 1.8* |
| FBG (mmol/L)    | 5.18 ± 0.24 | 5.25 ± 0.54 | 5.31 ± 0.25 |
| GIR (mg/kg/min) | 4.46 ± 0.75 | 2.11 ± 0.41* | 4.42 ± 1.21* |
| HOMA-IR (n=10)  | 3.32 ± 0.20 | 7.61 ± 0.82* | 3.23 ± 0.39* |

Weight (1): body weight before EGCG intervention, Weight (2): body weight after EGCG intervention; *p < 0.05, HFD vs. NC; †p < 0.05, HFD vs. EGCG

3.2 Effect of EGCG intervention on hepatic lipid accumulation

Intrahepatic TG concentrations were notably increased in the HFD group vs the NC group and were much decreased in the EGCG group vs the HFD group. EGCG administration eliminated excess hepatic fat accumulation, as detected via hematoxylin and Oil Red O staining (Fig. 1).

3.3 Effects of EGCG treatment on the TLR4 signaling pathway in liver tissue

After the experiment, we tested the expression levels of certain fundamental elements of the TLR4 signaling pathway. HFD led to elevated protein expression levels of TLR4, IKKβ, p-IKKβ, p-NF-κB and TNF-α, while EGCG treatment decreased these effects considerably (Fig. 2). The mRNA levels of TLR4 and TRAF6 displayed similar trends as the protein results (Fig. 3).

3.4 Effects of the EGCG treatment on key modulators in the insulin signaling pathway in liver tissue

The protein levels of certain key modulators in the insulin signaling pathway were detected via Western blot. HFD reduced the protein expression levels of IRS-1, PI-3K and Akt, and the differences were statistically significant. IRS-2 protein expression also decreased, but the difference was not statistically significant. However, EGCG obviously improved the expression levels of all of these markers (Fig. 4).

and the target proteins were transferred onto PVDF membrane in transfer buffer of 50 V for an appropriate amount of time. Then, the membranes were blocked with 5% BSA for 2 h at room temperature, incubated with following primary antibody: rabbit antibody against IRS-1 (1:1000); rabbit antibody against IRS-2 (1:1000); rabbit antibody against PI-3K (1:1000); rabbit antibody against Akt (1:1000); mouse antibody against TLR4 (1:500); rabbit antibody against IKKβ (1:1000); rabbit antibody against p-IKKβ (1:1000); rabbit antibody against p-NF-κB (1:1000); goat antibody against TNF-α (1:200); mouse antibody against β-actin (1:2000) at 4°C overnight, and then anti-rabbit, anti-mouse or anti-goat IgG (1:1000–3000) for 2 h. All of the bands were detected by an ECL chemiluminescence kit. ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA) was used to quantify band intensities.

2.8 Statistical analysis

The data (means ± SD) were processed using SPSS 19.0 statistical software. The variance of three sets of data was determined by one-way analysis of variance (one-way ANOVA), and statistical analysis of two sets of data was performed by Student’s t-test. The difference was statistically significant if p < 0.05.
Discussion

Large amounts of inflammatory factors in the liver might accelerate the development of NAFLD and simultaneously impair insulin sensitivity. This study provided evidence that a 16-week dietary EGCG treatment protected against the development of NAFLD by affecting the TLR4 signaling pathway and reduced the release of inflammatory cytokines such as TNF-α in liver tissue. EGCG might also improve the expression levels of PI3K, Akt, IRS-1, and IRS-2, all of which are vital during insulin secretion. (Fig. 2)
The results from our study demonstrate the beneficial effects that EGCG has on metabolic parameters including decreased body weight (BW), FFA, and FINS without affecting food intake; these data are similar to those of previous research\(^2^4\)\(^{2^7}\). Unlike previous studies, FBG did not noticeably change among the three groups during the experiment. The number of rats or the amount of food intake might explain these different results\(^2^8\),\(^2^9\). Previous research suggests that EGCG improves glucose homeostasis\(^2^5\),\(^2^6\). The beneficial effects of EGCG against insulin resistance have also been confirmed in experiments using dogs\(^3^0\). Consistent with those findings, the results of our study indicate that EGCG intervention notably reduces HOMA-IR and increases GIR in rats fed a HFD.

A major function of the liver is to regulate lipid metabolism. Overloaded FFA accumulation in the liver increases TG synthesis and aggravates insulin resistance\(^3^1\). NAFLD, one kind of metabolic disease, can be caused by obesity, insulin resistance, and hypertriglyceridemia\(^3^2\),\(^3^3\). Sayama et
provided persuasive evidence regarding the protective role that EGCG plays in reducing liver TG levels. Our study further shows that EGCG reduces TG levels in liver tissues and improves the degree of hepatic steatosis in rats fed a HFD.

Activation of the TLR4 signaling pathway is closely associated with NAFLD. TLR4 is activated by its ligands and triggers its downstream adaptors, which eventually leads to the phosphorylation of NF-κB and the production and release of inflammatory factors. Studies have shown that EGCG can inhibit the TLR4 signaling pathway in adipose tissue and macrophages. However, the role of the TLR4 pathway in liver with a HFD and the intervention of green tea have not been studied. Our results indicated that EGCG suppressed the expression of TLR4, the phosphorylation of IKKβ, and the activation of NF-κB, which ultimately reduced TNF-α transcription, thereby alleviating NAFLD after the EGCG intervention. A study suggested that EGCG can attenuate inflammation in non-alcoholic fatty liver disease rat model through NF-κB pathways, which is consistent with our findings. Many inflammatory stimuli damage insulin receptor signaling, thereby leading to insulin resistance. FFAs activated the TLR4/NF-κB pathway and promoted NF-κB translocation into the nucleus. NF-κB is also involved in oxidative stress and insulin resistance in patients with non-alcoholic steatohepatitis (NASH). However, these studies did not answer questions regarding how HFDs cause hepatic insulin resistance. Our study found that EGCG improved certain key components in the insulin signaling pathway that were inhibited by an HFD. EGCG might improve obesity-associated hepatic insulin resistance and subacute hepatic inflammation by inhibiting the TLR4 signaling pathway. However, we were not able to prove the relationship between the TLR inflammatory signaling pathway and insulin signaling, further evidence is needed. More cell experiments to demonstrate the roles of signaling pathways will also be required. Our study also needs to establish a dose gradient of EGCG groups to further improve our experimental findings.

### 5 Conclusion
In conclusion, EGCG may prevent NAFLD by alleviating inflammation in the liver tissue of HFD rats and improve insulin signaling. EGCG administration might offer new strategies for the treatment of NAFLD.

### Acknowledgement
This project was supported by the National Natural Science Foundation of China (grant no. 81000327), the Department of Education of Liaoning Province (grant no. L2013300) and the Science and Technology Department of Liaoning Province (grant no. 2015020487).

### Notes
The authors have declared no conflicts of interest.
EGCG Suppresses TLR4 in the Liver

References

1) Malik, V.S.; Willett, W.C.; Hu, F.B. Global obesity: Trends, risk factors and policy implications. *Nat. Rev. Endocrinol.* **9**, 13-27 (2013).

2) Corey, K.E.; Kaplan, L.M. Obesity and liver disease: The epidemic of the twenty-first century. *Clin. Liver Dis.* **18**, 1-18 (2014).

3) Moore, J.B. Non-alcoholic fatty liver disease: the hepatic consequence of obesity and the metabolic syndrome. *Proc. Nutr. Soc.* **69**, 211-220 (2010).

4) Tuyama, A.C.; Chang, C.Y. Non-alcoholic fatty liver disease. *J. Diabetes** 4**, 266-280 (2012).

5) Kawai, T.; Akira, S. The role of pattern-recognition receptors in innate immunity: Update on toll-like receptors. *J. Immunol.* **181**, 373-384 (2010).

6) Kesar, V.; Odin, J.A. Toll-like receptors and liver disease. *Liver Int.* **34**, 184-196 (2014).

7) Soares, J.B.; Pimentel-Nunes, P.; Roncon-Albuquerque, R.; Leite-Moreira, A. The role of lipopolysaccharide/toll-like receptor 4 signaling in chronic liver diseases. *Hepatol. Int.* **4**, 659-672 (2010).

8) Shi, H.; Kokoeva, M.V.; Inouye, K.; Tzameli, I.; Yin, H.; Fliser, J.S. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J. Clin. Invest.* **116**, 3015-3025 (2006).

9) Ferreira, D.F.; Fiamoncini, J.; Pimentel-Nunes, P.; Roncon-Albuquerque, R.; Leite-Moreira, A. The role of lipopolysaccharide/toll-like receptor 4 signaling in chronic liver diseases. *Hepatol. Int.* **4**, 659-672 (2010).

10) Kim, J.J.; Sears, D.D. TLR4 and insulin resistance. *Gastroenterol. Res. Pract.* **2010**, 212563 (2010).

11) Mencin, A.; Khwe, J.; Schwabe, R.F. Toll-like receptors as targets in chronic liver diseases. *Gut* **58**, 704-720 (2009).

12) Potamisgil, G.S.; Erbay, E. Nutrient sensing and inflammation in metabolic diseases. *Nat. Rev. Immunol.* **8**, 923-934 (2008).

13) Higdon, J.V.; Frei, B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit. Rev. Food Sci. Nutr.* **43**, 89-143 (2003).

14) Meydani, M.; Hasen, S.T. Dietary polyphenols and obesity. *Nutrients* **2**, 737-751 (2010).

15) Crespy, V.; Williamson, G. A review of the health effects of green tea catechins in *in vivo* animal models. *J. Nutr.* **134**, 3431s-3440s (2004).

16) Gupta, S.; Saha, B.; Giri, A.K. Comparative antimutagenic and anticlastogenic effects of green tea and black tea: A review. *Mutat. Res.* **512**, 37-65 (2002).

17) Schraunm, L. Going green: The role of the green tea component EGCG in chemoprevention. *J. Carcinog. Mutagen.* **4**, 1000142 (2013).

18) Singh, B.N.; Shankar, S.; Srivastava, R.K. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. *Biochem. Pharmacol.* **82**, 1807-1821 (2011).

19) Nagle, D.G.; Ferreira, D.; Zhou, Y.D. Epigallocatechin-3-gallate (EGCG): Chemical and biomedical perspectives. *Phytochemistry* **67**, 1849-1855 (2006).

20) Bao, S.; Cao, Y.; Fan, C.; Fan, Y.; Bai, S.; Teng, W.; Shao, Z. Epigallocatechin gallate improves insulin signaling by decreasing toll-like receptor 4 (TLR4) activity in adipose tissues of high-fat diet rats. *Mol. Nutr. Food Res.* **58**, 677-686 (2014).

21) Cao, Y.; Bao, S.; Yang, W.; Zhang, J.; Li, L.; Shao, Z.; Teng, W. Epigallocatechin gallate prevents inflammation by reducing macrophage infiltration and inhibiting tumor necrosis factor-alpha signaling in the pancreas of rats on a high-fat diet. *Nutr. Res.* **34**, 1066-1074 (2014).

22) Li, X.; Yuan, L.; Li, J.; Li, H.; Cheng, S. Blockade of renin angiotensin system increased resistance to STZ-induced diabetes in rats with long-term high-fat diet. *Exp. Diabetes Res.* **2012**, 618923 (2012).

23) Farrell, G.C.; Van Rooyen, D.; Gan, L.; Chitturi, S. NASH is an inflammatory disorder: Pathogenic, prognostic and therapeutic implications. *Gut Liver* **6**, 149-171 (2012).

24) Bose, M.; Lambert, J.D.; Ju, J.; Reuhl, K.R.; Shapses, S.A.; Yang, C.S. The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. *J. Nutr.* **138**, 1677-1683 (2008).

25) Chen, N.; Bezzina, R.; Hinch, E.; Lewandowski, P.A.; Cameron-Smith, D.; Mathai, M.L.; Jois, M.; Sinclair, A.J.; Begg, D.P.; Wark, J.D.; Weisinger, H.S.; Weisinger, R.S. Green tea, black tea, and epigallocatechin modify body composition, improve glucose tolerance, and differentially alter metabolic gene expression in rats fed a high-fat diet. *Nutr. Res.* **29**, 784-793 (2009).

26) Ramadan, G.; El-Beih, N.M.; Abd El-Ghffar, E.A. Modulatory effects of black v. green tea aqueous extract on hyperglycaemia, hyperlipidaemia and liver dysfunction in diabetic and obese rat models. *Br. J. Nutr.* **102**, 1611-1619 (2009).

27) Tian, C.; Ye, X.; Zhang, R.; Long, J.; Ren, W.; Ding, S.; Liao, D.; Jin, X.; Wu, H.; Xu, S.; Ying, C. Green tea polyphenols reduced fat deposits in high-fat-fed rats via erk1/2-PPARgamma-adiponectin pathway. *PLoS One* **8**, e53796 (2013).

28) Klaus, S.; Pultz, S.; Thone-Reinecke, C.; Wolfram, S. Epigallocatechin gallate attenuates diet-induced obesity in mice by decreasing energy absorption and increasing fat oxidation. *Int. J. Obes. (Lond.)* **29**, 615-623 (2005).

29) Ueda, M.; Ashida, H. Green tea prevents obesity by increasing expression of insulin-like growth factor binding protein-1 in adipose tissue of high-fat diet-fed mice. *J. Oleo Sci.*
mice. *J. Agric. Food Chem.* **60**, 8917-8923 (2012).
30) Serisier, S.; Leray, V.; Poudroux, W.; Magot, T.; Ouguerram, K.; Nguyen, P. Effects of green tea on insulin sensitivity, lipid profile and expression of PPARalpha and PPARgamma and their target genes in obese dogs. *Br. J. Nutr.* **99**, 1208-1216 (2008).
31) Koppe, S.W. Obesity and the liver: Nonalcoholic fatty liver disease. *Transl. Res.* **164**, 312-322 (2014).
32) Salgado, A.L.; Carvalho, L.; Oliveira, A.C.; Santos, V.N.; Vieira, J.G.; Parise, E.R. Insulin resistance index (HOMA-IR) in the differentiation of patients with non-alcoholic fatty liver disease and healthy individuals. *Arq. Gastroenterol.* **47**, 165-169 (2010).
33) Farrell, G.C.; Larfer, C.Z. Nonalcoholic fatty liver disease: From steatosis to cirrhosis. *Hepatology* **43**, S99-s112 (2006).
34) Sayama, K.; Lin, S.; Zheng, G.; Oguni, I. Effects of green tea on growth, food utilization and lipid metabolism in mice. *In Vivo* **14**, 481-484 (2000).
35) Spruss, A.; Kanuri, G.; Wagnerberger, S.; Haub, S.; Bischoff, S.C.; Berghiem, I. Toll-like receptor 4 is involved in the development of fructose-induced hepatic steatosis in mice. *Hepatology* **50**, 1094-1104 (2009).
36) Rivera, C.A.; Adegboyega, P.; Van Rooijen, N.; Tagalicud, A.; Allman, M.; Wallace, M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. *J. Hepatol.* **47**, 571-579 (2007).
37) Kumazoe, M.; Nakamura, Y.; Yamashita, M.; Suzuki, T.; Takamatsu, K.; Huang, Y.; Bae, J.; Yamashita, S.; Murata, M.; Yamada, S.; Shinoda, Y.; Yamaguchi, W.; Toyoda, Y.; Tachibana, H. Green tea polyphenol epigallocatechin-3-gallate suppresses toll-like receptor 4 expression via up-regulation of E3 ubiquitin-protein ligase RNF216. *J. Biol. Chem.* **292**, 4077-4088 (2017).
38) Hong Byun, E.; Fujimura, Y.; Yamada, K.; Tachibana, H. TLR4 signaling inhibitory pathway induced by green tea polyphenol epigallocatechin-3-gallate through 67-kDa laminin receptor. *J. Immunol.* **185**, 33-45 (2010).
39) Xiao, J.; Ho, C.T.; Liong, E.C.; Nanji, A.A.; Leung, T.M.; Lau, T.Y.; Fung, M.L.; Tipoe, G.L. Epigallocatechin gallate attenuates fibrosis, oxidative stress, and inflammation in non-alcoholic fatty liver disease rat model through TGF/SMAD, PI3 K/Akt/FoxO1, and NF-kappaB pathways. *Eur. J. Nutr.* **53**, 187-199 (2014).
40) Zhao, L.; Lee, J.Y.; Hwang, D.H. Inhibition of pattern recognition receptor-mediated inflammation by bioactive phytochemicals. *Nutr. Rev.* **69**, 310-320 (2011).
41) Boden, G.; She, P.; Mozzoli, M.; Cheung, P.; Gumireddy, K.; Reddy, P.; Xiang, X.; Luo, Z.; Ruderman, N. Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-kappaB pathway in rat liver. *Diabetes* **54**, 3458-3465 (2005).
42) Cai, D.; Yuan, M.; Frantz, D.F.; Melendez, P.A.; Hansen, L.; Lee, J.; Shoelson, S.E. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat. Med.* **11**, 183-190 (2005).
43) Videla, L.A.; Tapia, G.; Rodrigo, R.; Pettinelli, P.; Haim, D.; Santibanez, C.; Araya, A.V.; Smok, G.; Csendes, A.; Gutierrez, L.; Rojas, J.; Castillo, J.; Korn, O.; Maluenda, P.; Diaz, J.C.; Rencoret, G.; Poniachik, J. Liver NF-kappaB and AP-1 DNA binding in obese patients. *Obesity (Silver Spring)* **17**, 973-979 (2009).