Journal of Developmental Origins of Health and Disease (2017), **8**(2), 178–187.

doi:10.1017/S2040174416000647

ORIGINAL ARTICLE

Green tea extract intake during lactation modified cardiac macrophage infiltration and AMP-activated protein kinase phosphorylation in weanling rats from undernourished mother during gestation and lactation

E. Matsumoto[1], S. Kataoka[1], Y. Mukai[2], M. Sato[3] and S. Sato[1,3]*

[1]*Graduate School of Health Sciences, Aomori University of Health and Welfare, Aomori, Japan*
[2]*School of Nutrition and Dietetics, Faculty of Health and Social Work, Kanagawa University of Human Services, Kanagawa, Japan*
[3]*Department of Nutrition, Faculty of Health Sciences, Aomori University of Health and Welfare, Aomori, Japan*

Maternal dietary restriction is often associated with cardiovascular disease in offspring. The aim of this study was to investigate the effect of green tea extract (GTE) intake during lactation on macrophage infiltration, and activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) and serine-threonine kinase Akt (Akt) in the hearts of weanlings exposed to maternal dietary protein restriction. Pregnant Wistar rats were fed control (C) or low-protein diets (LP) throughout gestation. Following delivery, the dams received a control or a GTE-containing control diet during lactation: control diet during gestation and lactation (CC), low-protein diet during gestation and lactation (LPC), low-protein diet during gestation and 0.12% GTE-containing low-protein diet during lactation (LPL), and low-protein diet during gestation and 0.24% GTE-containing low-protein diet during lactation (LPH). The female offspring were sacrificed at day 22. Biochemical parameters in the plasma, macrophage infiltration, degree of fibrosis and expression levels of AMPK and Akt were examined. The plasma insulin level increased in LPH compared with LPC. Percentage of the fibrotic areas and the number of macrophages in LPC were higher than those in CC. Conversely, the fibrotic areas and the macrophage number in LPH were smaller (21 and 56%, respectively) than those in LPC. The levels of phosphorylated AMPK in LPL and LPH, and Akt in LPH were greater than those in LPC. In conclusion, maternal protein restriction may induce macrophage infiltration and the decrease of insulin levels. However, GTE intake during lactation may suppress macrophage infiltration and restore insulin secretion function via upregulation of AMPK and insulin signaling in weanlings.

Received 26 July 2016; Revised 11 September 2016; Accepted 1 November 2016; First published online 6 December 2016

Key words: Green tea extract, macrophage, heart, offspring, maternal protein restriction

Introduction

Cardiovascular disease is the most common cause of human morbidity and mortality. Although the clinical onset of cardiovascular disease is mainly acute, several studies have demonstrated the onset of cardiovascular pathologies by limiting fetal and neonatal growth through maternal nutrient restriction.[1,2] Epidemiological studies have shown that the association between low birth weight and the risk of ischemic heart disease in adulthood is mediated almost entirely by poor fetal growth.[3,4] In addition, fetuses with intrauterine growth restriction (IUGR) as infants showed aortic wall thickening progression, condensation of the elastic fibers, and infiltration of inflammatory cells, such as macrophages and fibroblastoid cells.[5] Larger cell sizes, macrophage-mediated inflammation, and marked fibrosis were observed in the pancreatic islets of adult rats with IUGR.[6] The number of alveolar macrophages in culture was reported to increase in 90-day-old rat offspring exposed to dietary restrictions during lactation.[7] Thus, an association exists between maternal malnutrition and inflammation in offspring.

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a serine/threonine protein kinase that plays a central role in regulating cellular metabolism and energy balance.[8] AMPK activation elicits various beneficial effects with a potential to ameliorate defects associated with metabolic disorders including obesity, type 2 diabetes and cardiac fibrosis.[9,10] Furthermore, the activation of AMPK may be associated with the suppression of a pro-inflammatory environment in adipose tissues.[11] High-fat diet-induced obesity was reported to reduce cardiac glucose metabolism and AMPK levels, which in turn was associated with increased levels of macrophages in the heart.[12] In mouse adipose tissue, the activation of AMPK reduced macrophage inflammation and insulin resistance in obesity.[13]

Green tea contains polyphenolic compounds, such as catechins, particularly (−)-epigallocatechin gallate (EGCG). EGCG is involved in the activation of AMPK signaling in cultured cells and in the liver of mice.[14] EGCG has been known to activate AMPK.[15] In an *in vivo* study, EGCG treatment ameliorated free fatty acid-induced peripheral insulin resistance through activation of the AMPK pathway.[16]

*Address for correspondence: Dr. S. Sato, Graduate School of Health Sciences, Aomori University of Health and Welfare, Mase 58-1, Hamadate, Aomori 030-8505, Japan.
(Email s_sato3@auhw.ac.jp)

Moreover, EGCG has been reported to attenuate cardiac hypertrophy and fibrosis in a model of pressure overload.[17,18] Furthermore, continuous ingestion of green tea catechin from an early age prevented the development of spontaneous stroke in stroke-prone spontaneously hypertensive rats.[19] In Japanese adults, green tea consumption was associated with a reduced risk of total stroke incidence, cerebral infarction and cerebral hemorrhage.[20] However, little is known about the effects of green tea intake during lactation on glucose metabolism and macrophage infiltration in the heart of young offspring subjected to malnutrition.

Here, we aimed to investigate whether green tea extract (GTE) intake during lactation could affect macrophage infiltration via AMPK activation in the heart of weanling rats from protein-restricted diet-fed dams during pregnancy and lactation. In addition, we assessed whether GTE intake modulated the levels of serine-threonine kinase Akt (Akt) phosphorylation, which is one of the steps in insulin signaling.

Methods

Animals

This study was approved by the Animal Research Committee, Aomori University of Health and Welfare, and all experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Aomori University of Health and Welfare (Permission number; 13,007). Seven-week-old virgin female Wistar rats were obtained from CLEA Japan Inc. (Tokyo, Japan). They were maintained at a constant temperature of 23 ± 1°C under a 12-h light/dark cycle with *ad libitum* access to a standard commercial laboratory diet (MF diet; supplied by Oriental Yeast Co. Ltd., Tokyo, Japan), and tap water. At 12–13 weeks of age, a vaginal impedance reader (Model MK-10C; Muromachi Kikai Co. Ltd., Osaka, Japan) was used to determine whether the female rats were in the appropriate stage of the estrus cycle for mating, this assessment was routinely performed in the afternoon. A reading of >3 kΩ indicated that the female was in pro-estrus and presumably in estrus. One appropriate female was mated with one male overnight. The next morning, the presence of a vaginal plug indicated successful mating; this day was taken as Day 0 of gestation. Pregnant rats were randomly allowed *ad libitum* access to a diet containing either 20% (control group: C, $n = 4$) or 8% (low-protein group: LP, $n = 12$) casein during gestation. Following delivery, dams, weighing 224–270 g, received a control or a GTE-containing control diet during lactation as follows: control diet during gestation and lactation (CC, $n = 4$), low-protein diet during gestation and lactation (LPC, $n = 6$); low-protein diet during gestation and 0.12% GTE-containing low-protein diet during lactation (LPL, $n = 3$), and low-protein diet during gestation and 0.24% GTE-containing low-protein diet during lactation (LPH, $n = 3$). The diets were isocaloric (Table 1). We administered diets containing GTE during lactation with the intention of delivering GTE to the newborn through the milk. The GTE, polyphenon E, contained 80–98% total catechins by weight (the main component was EGCG, composing ~65% of the material and 0.4% caffeine) and was kindly supplied by Mitsui-Norin Co. Ltd. (Shizuoka, Japan). In this study, we administered diets containing 0.12 and 0.24% GTE (polyphenon E), according to our previous study,[21] no observed adverse effect in dams and offspring was observed during lactation when 0.12 and 0.24% GTE-containing diets were used in the previous experiment. Although the ratio of male to female was not 1 to 1, eight pups per dam were randomly selected and housed together to ensure adequate nutrition during lactation, and the pups were weighed at postnatal day (PD) 4. At weaning (22-day-old), the female pups were separated from the CC ($n = 15$), LPC ($n = 13$), LPL ($n = 8$) and LPH ($n = 12$) groups and euthanized. The male pups were used in another experiment. The female pups were fasted overnight, weighed and blood samples were collected under pentobarbital (30–40 mg/kg, 1 ml/kg, i.p.) anesthesia. The hearts were removed immediately, rapidly rinsed with ice-cold saline and weighed. Subsequently, hearts were cut transversely close to the apex and fixed in 4% paraformaldehyde phosphate buffer solution for histopathology and immunohistochemistry. A portion of each heart was frozen in liquid nitrogen and stored at −80°C before evaluation.

Measurement of biochemical parameters in the plasma

Plasma samples were separated by centrifugation at 800 × ***g*** for 10 min at 4°C and tested for glucose and non-esterified fatty acids using a commercially available kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). Plasma levels of insulin were measured using a Rat Insulin ELISA kit (TMB; AKRIN-010T, Shibayagi, Gunma, Japan). According to the manufacturer's instructions, the detection limit of insulin is estimated to be 156 pg/ml.

Histopathology and Immunohistochemistry

For histological examination, paraformaldehyde-fixed heart tissues were embedded in paraffin, and sections 4 μm in thickness were stained with hematoxylin-eosin (HE). To assess the degree of fibrosis, staining of collagen fibrils was carried out using a Sirius red/fast green collagen staining kit (Chondrex Inc., Redmond, WA, USA). The area stained red by Sirius red was measured using a color image analyzer (WinROOF, Mitani Corp., Tokyo, Japan) in three randomly selected sections. The Sirius red-positive area was normalized to the total cross-sectional area and expressed as a percentage. Heart sections were stained for α-smooth muscle actin (α-SMA) and macrophages. Briefly, deparaffinized sections were heated in 1 mmol/l ethylenediaminetetraacetic acid solution pH 7.4 for 15 min at 120°C for α-SMA and were pretreated with 0.1% pepsin solution in phosphate-buffered saline (PBS), pH 7.4, for 15 min at 37°C for macrophages. After pre-incubation with 3% H_2O_2 for 10 min at room temperature, sections were incubated with 1.5% skim milk in PBS for 30 min at room temperature. They were then incubated for

https://doi.org/10.1017/S2040174416000647 Published online by Cambridge University Press

Table 1. *Composition of the diets*

Ingredients	Control diet	Low-protein diet	0.12% GTE-containing diet	0.24% GTE-containing diet
	g/100 g of diet			
Casein	20.000	8.000	8.000	8.000
L-cystine	0.300	0.300	0.300	0.300
Cornstarch	39.749	51.749	51.749	51.749
α-Cornstarch	13.200	13.200	13.200	13.200
Sucrose	10.000	10.000	10.000	10.000
Soybean oil	7.000	7.000	7.000	7.000
Cellulose	5.000	5.000	4.880	4.760
Mineral mixture[a]	3.500	3.500	3.500	3.500
Vitamin mixture[b]	1.000	1.000	1.000	1.000
Choline chlorhydrate	0.250	0.250	0.250	0.250
tert-Butylhydroquinone	0.001	0.001	0.001	0.001
GTE	–	–	0.120	0.240

GTE, green tea extract (polyphenon E).
[a]AIN-93G mineral mixture.
[b]AIN-93G vitamin mixture (Oriental Yeast Co. Ltd., Tokyo, Japan).

14 h at 4°C with α-SMA (1:50, Dako, Glostrup, Denmark) and ED1 antibodies (1:50, macrophages; AbD Serotec Ltd., Raleigh, NC, USA), which were diluted in PBS containing 1% bovine serum albumin. Thereafter, the sections were incubated with Histofine simple stain rat MAX PO detection reagent (Nichirei Biosciences Inc., Tokyo, Japan) for 30 min, and positive reactions were visualized with 3,3'-diaminobenzidine tetrahydrochloride in 50 mmol/l Tris-HCl buffer, pH 7.4, containing 3% H_2O_2. Sections were counterstained lightly with hematoxylin. Cells showing a distinct immunoreaction for ED1 were counted in 10 randomly selected areas (0.0625 mm^2 each) in sectioned hearts.

Western blot analysis

The hearts were homogenized in homogenizing buffer [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid 50 mmol/l, NaCl 150 mmol/l, dithiothreitol 1 mmol/l, and 0.5% (v/v) Tween-20; pH 7.4] containing protease inhibitor cocktail tablets (Roche Applied Science, Indianapolis, IN, USA). The homogenates were centrifuged at 5000 × ***g*** for 45 min at 4°C. Supernatants were collected, and the protein concentration was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). For Western blot analysis, the proteins were electrophoresed on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and subsequently electrotransferred onto polyvinylidene difluoride membranes (GE Healthcare UK Ltd., Buckinghamshire, UK). The membranes were then blocked in 5% nonfat dry milk in Tris-buffered saline containing 0.1% v/v Tween-20 (TBS-T) for 8 h and incubated overnight at 4°C with rabbit AMPKα, phospho-AMPKα-Thr^{172}, Akt and phospho-Akt-Ser^{473} polyclonal antibodies (1:1000; Cell Signaling Technology, Danvers, MA, USA) and endothelial nitric oxide synthase (eNOS) monoclonal antibody (1:1000; BD Tansduction Laboratories, San Jose, CA, USA). After washing, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized with ECL Western Blotting Detection Reagents (GE Healthcare UK Ltd.) on Hyperfilm (GE Healthcare UK Ltd.). Quantitative analysis of specific band density was performed using ATTO densitometry software (ATTO Corp., Tokyo, Japan). Protein levels were normalized to those of β-actin from the same sample.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by the Tukey test. Comparisons between C and LP groups were performed using the Student's *t*-test. All values are expressed as mean ± S.E.M. and $P < 0.05$ was considered statistically significant.

Results

Body/heart weights and food intake

The body weights of the C and LP groups did not differ significantly during pregnancy (Fig. 1a). The body weights of the female pups were measured at PD 10, 15 and 20 (Fig. 1b). The levels of maternal food intake of LP group per day during gestation were significantly higher than that of C group [C, 12.8 ± 1.2 g *v.* LP, 15.1 ± 0.4 at day 1–3 of gestation ($P < 0.05$) and 26.0 ± 1.3 *v.* 33.9 ± 1.6 g at day 20–21 ($P < 0.05$), respectively] (CC, $n = 4$; LP, $n = 12$). The levels of maternal food intake per day during lactation at PD 1–3 were 10.5 ± 1.6, 11.2 ± 0.5, 10.9 ± 0.9 and 13.2 ± 1.4 g in the CC, LPC, LPL and LPH groups, respectively; at PD 15–17, they were 44.7 ± 3.7, 22.6 ± 1.3, 26.2 ± 0.3 and 28.3 ± 0.9, respectively; and at PD 20–21, they were 43.9 ± 1.8, 28.1 ± 1.9, 26.8 ± 0.8

https://doi.org/10.1017/S2040174416000647 Published online by Cambridge University Press

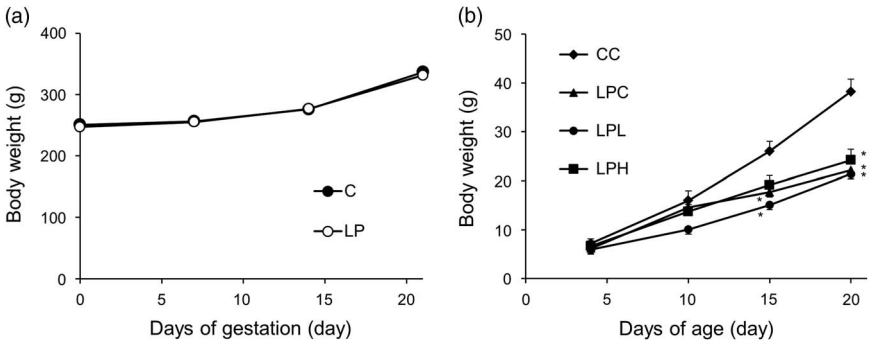

Fig. 1. Effect of green tea extract (GTE) intake on body weights of dams during the gestation period (*a*) and female offspring during the lactation period (*b*). C, control group; LP, low-protein group; CC, control diet during gestation and lactation; LPC, low-protein diet during gestation and lactation; LPL, 0.12% GTE-containing low-protein diet during gestation and lactation; LPH, 0.24% GTE-containing low-protein diet during gestation and lactation. Data are presented as mean ± S.E.M. (C; $n = 4$, LP; $n = 12$ during gestation, CC, $n = 4$; LPC, $n = 6$; LPL, $n = 3$; LPH, $n = 3$ during lactation). $^{*}P < 0.05$ compared with CC.

Table 2. *Morphological characteristics of female offspring*

		Low-protein diets		
	CC	LPC	LPL	LPH
BW[a] (g)	40.4 ± 1.9	22.0 ± 1.2*	20.9 ± 1.5*	24.3 ± 1.0*
Heart (g)	0.184 ± 0.007	0.118 ± 0.006*	0.114 ± 0.006*	0.114 ± 0.004*
H/BW (g/kg)	4.62 ± 0.11	5.36 ± 0.11*	5.51 ± 0.22*	4.72 ± 0.10$^{\#,\dagger}$

GTE, green tea extract (polyphenon E); BW, body weight; H, heart. CC, control diet during gestation and lactation; LPC, low-protein diet during gestation and lactation; LPL, low-protein diet during gestation and lactation, and 0.12% GTE-containing low-protein diet; LPH, low-protein diet during gestation and lactation, and 0.24% GTE-containing low-protein diet.

[a]At sacrifice at postnatal day 22. Values are means ± S.E.M. (CC, $n = 15$; LPC, $n = 13$; LPL, $n = 8$; LPH, $n = 12$). $^{*}P < 0.05$ compared with CC. $^{\#}P < 0.05$ compared with LPC. $^{\dagger}P < 0.05$ compared with LPL.

and 31.0 ± 1.4 g, respectively (CC, $n = 4$; LPC, $n = 6$; LPL, $n = 3$; LPH, $n = 3$ during lactation). Although there was no significant difference in the food intake levels among the four groups at PD 1–3, the levels of food intake of LPC, LPL and LPH groups were significantly lower than that of the CC group at PD 15–17 and PD 20–21 ($P < 0.05$). The absolute weights of hearts in the LPC, LPL and LPH offspring were lower than those in the CC offspring, although there were no significant differences among the three LP groups (Table 2). The relative weights of hearts in the LPC and LPL groups at PD 22 were significantly higher than those in the CC group (Table 2). Conversely, the relative weights in the LPH group were significantly lower than that in LPC and LPL groups.

Plasma parameters of offspring

Although there were no significant differences in plasma glucose levels between the CC and LPC groups at PD 22 ($P = 0.072$), the glucose level of the LPH group was significantly lower than that of the LPC offspring. A significantly lower level of plasma insulin was found in the LPC group compared with the CC group (Fig. 2). Conversely, the insulin level in the LPH group increased significantly compared with LPC group animals, indicating that GTE intake during lactation restored decreased insulin levels observed in the LPC offspring. There were no significant differences in the levels of non-esterified fatty acids among any of the four groups.

Effect of GTE diet during lactation on Sirius red-positive and α-SMA-positive areas, and ED1-positive macrophages in the hearts of offspring

Representative Sirius red stains of heart sections from offspring are shown in Fig. 3a–3c. The area that comprised interstitial collagen fibers in the hearts of the LPC group was expanded compared with the CC group. In contrast, the extent and magnitude of the expansion was reduced in the LPH group. The percentage of fibrous area per total cross-sectional area significantly increased in the hearts of the LPC group compared with controls (Fig. 3g). In the LPL and LPH groups from dams treated with the GTE diet during lactation, the percentage of the fibrotic areas was significantly lower than in the LPC group.

https://doi.org/10.1017/S2040174416000647 Published online by Cambridge University Press

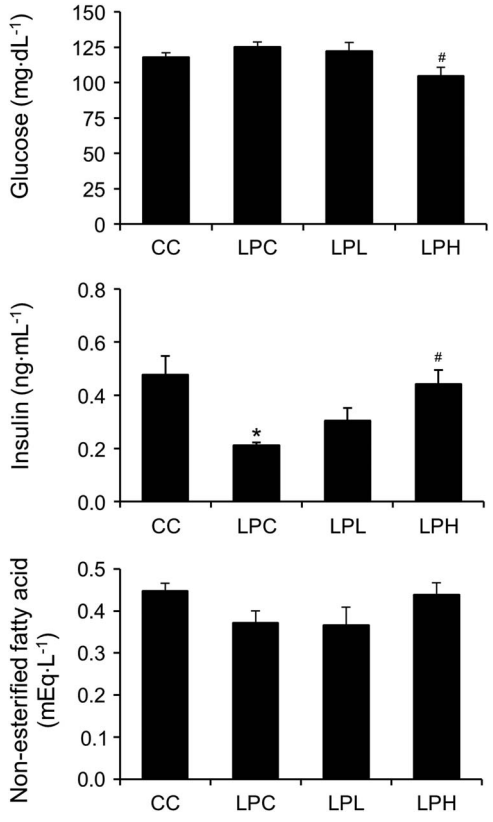

Fig. 2. Plasma parameters of 22-day-old weanling rats. CC, control diet during gestation and lactation; LPC, low-protein diet during gestation and lactation; LPL, 0.12% GTE-containing low-protein diet during gestation and lactation; LPH, 0.24% GTE-containing low-protein diet during gestation and lactation. Data are presented as mean ± S.E.M. (CC, $n = 14$; LPC, $n = 13$; LPL, $n = 8$; LPH, $n = 12$). $^{*}P < 0.05$ compared with CC. $^{\#}P < 0.05$ compared with LPC.

In immunohistochemical analyses, stronger α-SMA-immunoreactivity in the LPC group was clearly seen in the smooth muscle of the blood vessels and interstitial cells compared with the CC group (Fig. 3d–3f). In the LPH group, positive areas were observed weakly compared with the LPC group.

Immunohistochemical analysis revealed that ED1-positive macrophages were present in the heart in all groups (Fig. 4a–4c). The number of ED1-positive macrophages in the hearts of the LPC group was significantly higher than that of the CC group (Fig. 4d). Conversely, the number of macrophages in the LPL and LPH groups was significantly less than that in the LPC group, indicating that the GTE diet during lactation suppressed the infiltration of macrophages in the hearts of offspring.

Effect of the GTE diet on phosphorylated Akt levels

Because Akt phosphorylation is one of the steps in insulin signaling,[22] Akt expression and phosphorylation levels in the heart were determined. The total amount of Akt protein in the heart did not change in any group (Fig. 5a). The difference in the amount of phosphorylated Akt was not statistically significant between the CC and LPC groups. Akt phosphorylation in LPH offspring was significantly higher than that in LPC offspring and in CC offspring, as shown in Fig. 5a, indicating that GTE intake during lactation upregulated Akt signaling.

Effect of GTE diet on AMPK phosphorylation and eNOS levels

Because the activation of AMPK is associated with the suppression of cardiac hypertrophy,[23] the levels of total and phosphorylated AMPK in the heart were examined using Western blotting. The total amount of AMPK protein in the heart was not altered in any of the experimental groups (Fig. 5b). In contrast, the amount of phosphorylated AMPK in the hearts of offspring from the dams treated with 0.12 or 0.24% GTE diets during lactation were both 2.5-fold greater than that in LPC offspring (Fig. 5b). There were no significant differences in the levels of phosphorylated AMPK between the CC and LPC groups. The results indicated that the GTE diet during lactation increased the activity of AMPK in the heart.

AMPK phosphorylation is associated with an increase in the expression and activity of eNOS.[24] Here, the difference in the levels of eNOS expression was not statistically significant between the CC and LPC groups (Fig. 5c). Conversely, the eNOS levels in the LPH offspring were significantly higher than those in the LPC offspring.

Discussion

Maternal undernutrition and dietary protein restriction throughout pregnancy and lactation are thought to have metabolic consequences for offspring, such as obesity, diabetes and cardiovascular disease. In adult rats with IUGR, inflammation with macrophages and marked fibrosis was reported in pancreatic islets.[6] The beneficial effects of green tea on the prevention of human diseases, such as cardiovascular disease and type 2 diabetes, have been widely studied.[25] However, there is limited information about whether green tea intake during lactation could affect plasma insulin levels, macrophage infiltration and AMPK or Akt expression and/or phosphorylation in the hearts of weanling rats exposed to maternal dietary restriction. The main findings of this study were that (a) the number of macrophages in the LPH group was significantly lower than that in the LPC group, (b) the levels of plasma insulin and Akt phosphorylation were upregulated in the LPH group, and (c) AMPK phosphorylation and eNOS protein expression were upregulated in the hearts of female weanling rats from dams exposed to protein restriction throughout gestation and lactation.

Here, we found that the relative heart weights in the LPC group were significantly higher than those in the CC group, although the number of dams may be small (CC, $n = 4$; LPC, $n = 6$; LPL, $n = 3$; LPH, $n = 3$ during lactation). There was

https://doi.org/10.1017/S2040174416000647 Published online by Cambridge University Press

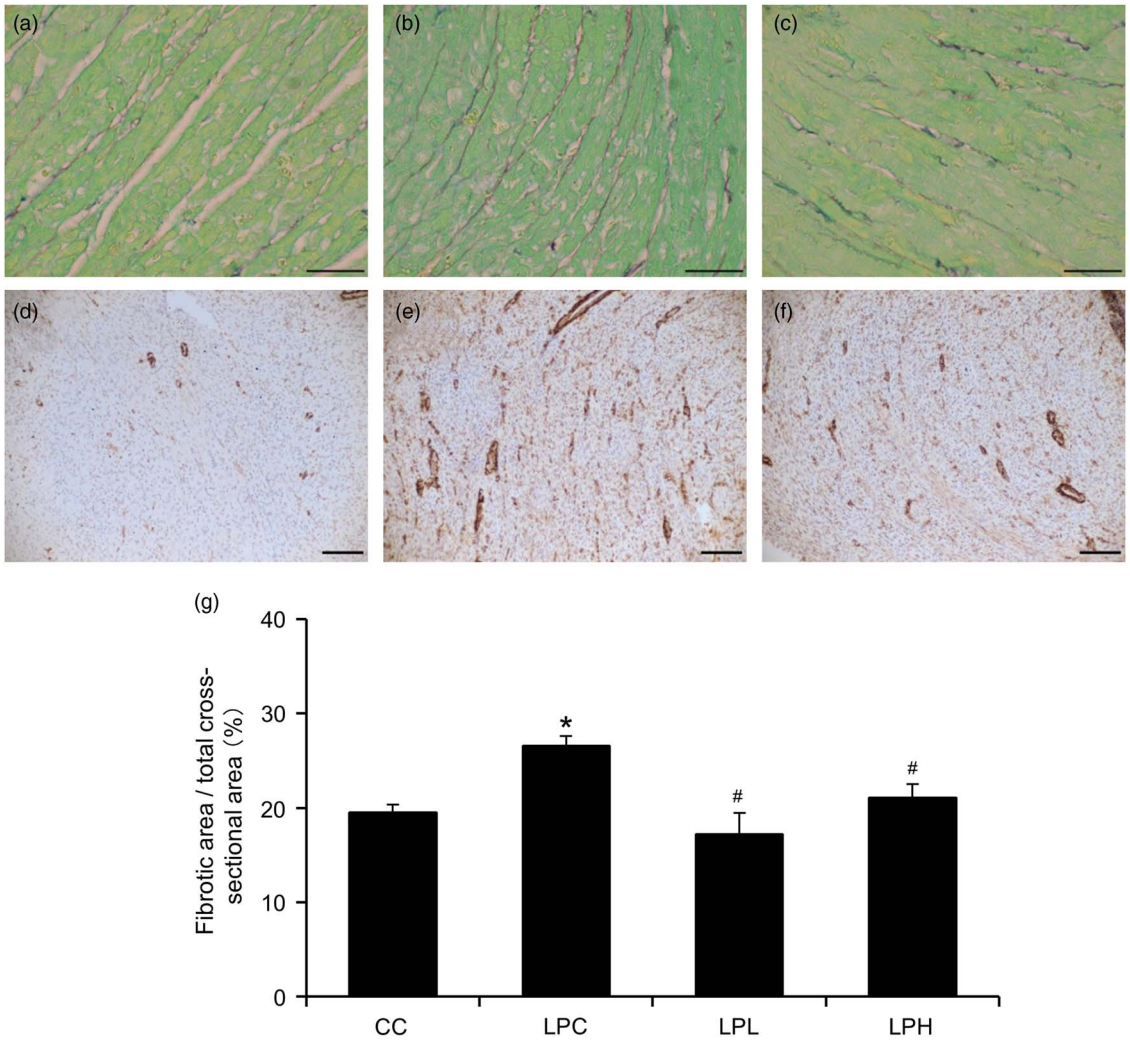

Fig. 3. Effects of green tea extract (GTE) intake during the lactation on fibrotic areas stained by Sirius red stain (*a–c*) and α-smooth muscle actin (α-SMA) positive cells (*d–f*) in the hearts of 22-day-old weanling rats. (*a*, *d*): CC, (*b*, *e*): LPC, and (*c*, *f*): LPH. (*g*): Sirius red-positive area was normalized to the total cross-sectional area and was expressed as a percentage (scale bar: 5 μm for all images for Sirius red-positive area, 10 μm for all images for α-SMA-positive area). CC, control diet during gestation and lactation; LPC, low-protein diet during gestation and lactation; LPL, 0.12% GTE-containing low-protein diet during gestation and lactation; LPH, 0.24% GTE-containing low-protein diet during gestation and lactation. Data are presented as mean ± S.E.M. (CC, $n = 13$; LPC, $n = 12$; LPL, $n = 7$; LPH, $n = 11$). $^{*}P < 0.05$ compared with CC. $^{\#}P < 0.05$ compared with LPC.

a similar report that the heart volume to body weight ratio was elevated in 4-week-old offspring from dams fed a low-protein diet (8.7% casein) during pregnancy and lactation.[26] Conversely, in the LPH group, lower relative heart weights were observed compared with the LPC group, suggesting that maternal GTE intake during lactation may moderate the progression of cardiac hypertrophy.

We demonstrated in this study that the percentage of the Sirius red-stained fibrous area and ED1-positive macrophage numbers in the heart of the LPH group were significantly lower than those in the LPC group. Previous studies reported that EGCG treatment inhibited peritoneal fibrosis and cardiac fibroblasts in rats with cardiac hypertrophy.[27,28] In cardiac muscle, α-SMA is a known marker of cardiac hypertrophy.[29] We also confirmed stronger α-SMA-immunoreactivity in the vascular smooth muscle and interstitial cells of the LPC group compared with CC group. In the pancreas of rats on a high-fat diet, EGCG prevented inflammation by reducing macrophage infiltration.[30] In addition, EGCG suppressed increased macrophage infiltration in aortic tissues in high-fat diet-induced mice and in rats with hepatic steatosis.[31,32] Therefore, it is likely that GTE intake during lactation suppressed the increased fibrous area and macrophage infiltration in the hearts of 22-day-old female offspring exposed to maternal protein restriction.

In this study, a significantly lower level of plasma insulin was found in the LPC group than that in the CC group; this concurs with several studies that have reported the impairment

https://doi.org/10.1017/S2040174416000647 Published online by Cambridge University Press

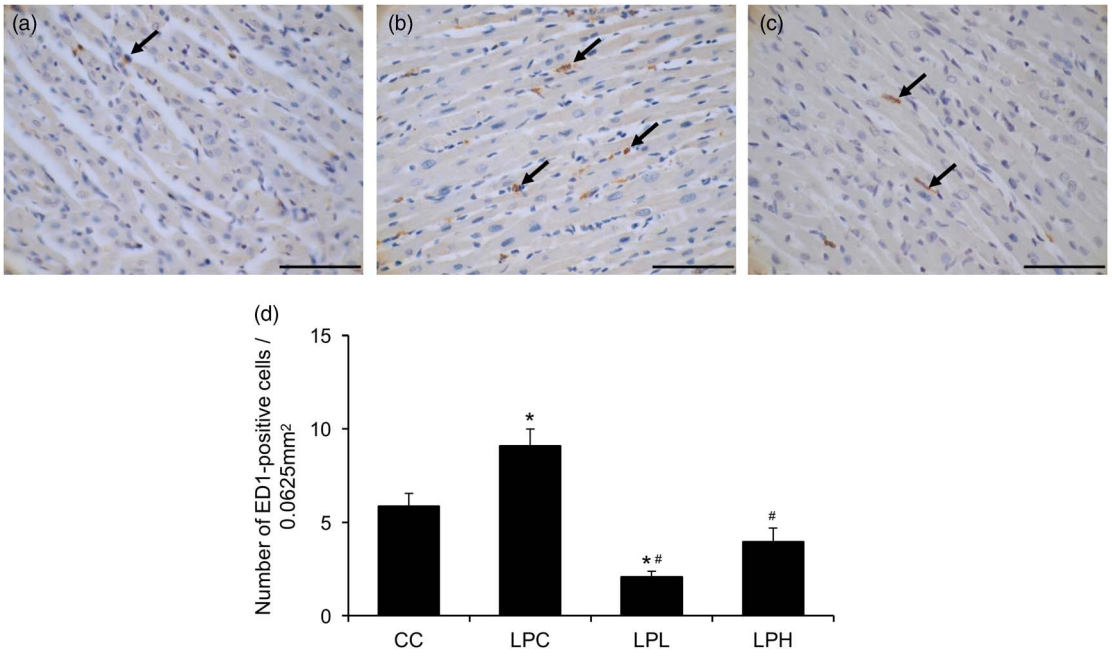

Fig. 4. Effect of green tea extract (GTE) intake during the lactation on the ED1-positive macrophages infiltration in the hearts of 22-day-old weanling rats. (*a*): CC, (*b*): LPC, and (*c*): LPH. (*d*): The number of ED1-positive macrophages was counted in 10 randomly selected regions of sections. Sections were counterstained lightly with hematoxylin (scale bar: 5 μm for all images). CC, control diet during gestation and lactation; LPC, low-protein diet during gestation and lactation; LPL, 0.12% GTE-containing low-protein diet during gestation and lactation; LPH, 0.24% GTE-containing low-protein diet during gestation and lactation. Data are presented as mean ± S.E.M. (CC, $n = 12$; LPC, $n = 10$; LPL, $n = 7$; LPH, $n = 11$). $^{*}P < 0.05$ compared with CC. $^{\#}P < 0.05$ compared with LPC.

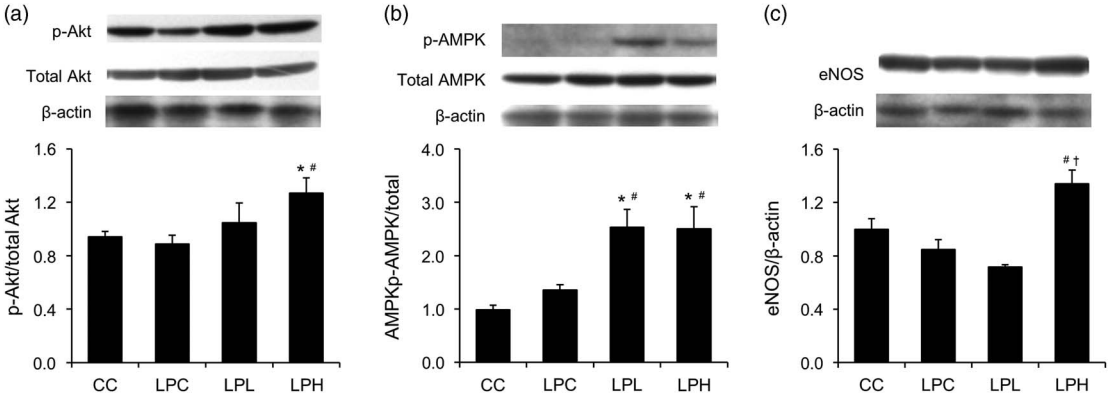

Fig. 5. Effect of green tea extract (GTE) intake during the lactation on (*a*) Akt, (*b*) AMPK, and (*c*) eNOS expression in the hearts of 22-day-old weanling rats. Protein levels were quantified as fold values of control levels after adjusting for endogenous β-actin. CC, control diet during gestation and lactation; LPC, low-protein diet during gestation and lactation; LPL, 0.12% GTE-containing low-protein diet during gestation and lactation; LPH, 0.24% GTE-containing low-protein diet during gestation and lactation. Data are presented as mean ± S.E.M. (CC, $n = 12$; LPC, $n = 12$; LPL, $n = 7$; LPH, $n = 11$). $^{*}P < 0.05$ compared with CC. $^{\#}P < 0.05$ compared with LPC. $^{\dagger}P < 0.05$ compared with LPL.

of pancreatic islet function in adulthood of offspring subjected to protein restriction during gestation and lactation.[33,34] Conversely, the LPH offspring showed higher plasma insulin levels than the LPC offspring. Although we did not examine the impairment of pancreatic islet function in this study, our results suggest that GTE intake during lactation may restore the insulin secretion function in the pancreatic islets of weanling rats. In addition, we showed that the plasma glucose levels in the LPH female offspring decreased compared with the LPC offspring. Green tea polyphenols has been reported to enhance glycogen synthesis by two-fold compared with insulin alone in a liver cell line,[35] suggesting that GTE intake during lactation may decrease plasma glucose levels through enhancement of glycogen synthesis in the liver. As shown in

https://doi.org/10.1017/S2040174416000647 Published online by Cambridge University Press

Fig. 5a, we demonstrated the upregulation of Akt phosphorylation in the heart of weanling rats from 0.24% GTE containing diet-fed dams. Akt is the main target of phosphatidyl inositol-3-kinase (PI3K), and the PI3K/Akt signaling pathway plays an important role in the metabolic effects of insulin and glucose.[22] In addition, EGCG enhanced the phosphorylation of Akt in myocardial ischemia/reperfusion injuries, suggesting that EGCG may have cardioprotective effects.[36] Therefore, we hypothesize that GTE intake during lactation may improve glucose metabolism in the heart by restoring the insulin signaling pathway.

The reason for the reduction of the infiltrated macrophage numbers and fibrosis areas in the heart of weanling rats from dams treated with GTE during lactation remains unclear. However, this phenomenon could be interpreted to mean that GTE during lactation is associated with AMPK activation in the heart. AMPK activation is known to exert anti-inflammatory effects in a variety of cell types and models of inflammatory/autoimmune disease.[37] Ko *et al.*[12] reported that diet-induced obesity reduced cardiac glucose metabolism and AMPK levels, and that this was associated with increased levels of macrophages in the heart. In addition, EGCG prevented inflammation by reducing macrophage infiltration in the pancreas of rats fed a high-fat diet and in hepatic steatosis in rats.[30,32] Green tea polyphenols supplementation attenuated lipopolysaccharide-induced myocardial fibrosis in vessels, tumor necrosis factor-α messenger RNA (mRNA) expression, and leukocytes in female rats.[38] Moreover, dietary quercetin, a flavonoid, suppressed adipose tissue macrophage infiltration and inflammation through upregulated AMPK phosphorylation in high-fat diet-fed mice.[39] Therefore, we hypothesize that GTE during lactation may play a role in reduction of macrophage infiltration through the upregulated AMPK activity that we observed in this study, leading to the suppression of extended fibrosis area formation in the heart.

AMPK phosphorylation is associated with the upregulation of expression and activity of eNOS.[24] Wang *et al.*[40] reported that activation of AMPK and eNOS, via pre-treatment with the AMPK activator metformin, improved cardiac function in a heart failure rat model. We found increased eNOS protein expression in the hearts of the LPH group. In human endothelial cell lines, EGCG treatment enhanced eNOS mRNA production and facilitated nitric oxide (NO) levels.[41] EGCG was reported to exert cardioprotective actions through induction of NO production.[27] In addition, Akt has been shown to phosphorylate eNOS and activate the enzyme, leading to NO production.[42] Furthermore, the activation of eNOS was increased through activation of the PI3K/Akt signaling pathway in human umbilical vein endothelial cells treated with α-lipoic acid.[43] We also showed the upregulation of Akt phosphorylation in the heart of weanling rats. Although we did not measure the amount of NO, it is possible that GTE during lactation may increase NO production through the activation of AMPK and/or PI3K/Akt signaling in the heart of weanling rats from dams exposed to protein restriction throughout gestation and lactation.

This study did have some limitations. First, although the main component of GTE was EGCG (composing ~65%), the GTE preparation is a crude extract and the component(s) have not been isolated. Second, because we did not examine the levels of the component(s) in the milk or in the pup plasma, it is not known if such component(s) could be passed onto the neonate through milk. Third, many animal models of developmental programming have shown the disparity between males and females in the timing of onset and severity of disease outcomes.[44–46] Although we reported the effects of GTE intake during lactation on the cardiac macrophage infiltration and AMPK phosphorylation in female weanling rats in this study, we did not examine the effects in male weanling rats.

In conclusion, we demonstrated that GTE intake during lactation inhibited the macrophage infiltration, and upregulated AMPK and Akt activation in the heart, and that GTE intake increased the plasma levels of insulin of 22-day-old female weanling rats from protein-restricted dams throughout gestation and lactation. Although further studies are required to elucidate the precise underlying mechanisms, the current findings are useful for better understanding the effects of maternal GTE intake during lactation on the attenuation of cardiovascular diseases in offspring programmed with maternal dietary protein restriction.

Acknowledgments

The authors thank Keiko Tamakuma for her technical assistance.

Financial Support

This study was supported by a Grant-in-Aid for Scientific Research C (No. 26350149) from the Japan Society for the Promotion of Science.

Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals.

References

1. Woodall SM, Johnston BM, Breier BH, Gluckman PD. Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure of offspring. *Pediatr Res*. 1996; 40, 438–443.
2. Langley-Evans SC. Fetal programming of cardiovascular function through exposure to maternal undernutrition. *Proc Nutr Soc*. 2001; 60, 505–513.
3. Kaijser M, Bonamy AK, Akre O, *et al*. Perinatal risk factors for ischemic heart disease: disentangling the roles of birth weight and preterm birth. *Circulation*. 2008; 117, 405–410.

4. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med*. 2008; 359, 61–73.
5. Lo Vasco VR, Salmaso R, Zanardo V, *et al*. Fetal aorta wall inflammation in ultrasound-detected aortic intima/media thickness and growth retardation. *J Reprod Immunol*. 2011; 91, 103–107.
6. Delghingaro-Augusto V, Madad L, Chandra A, *et al*. Islet inflammation, hemosiderosis, and fibrosis in intrauterine growth-restricted and high fat-fed Sprague-Dawley rats. *Am J Pathol*. 2014; 184, 1446–1457.
7. de Melo JF, da Costa TB, da Costa Lima TD, *et al*. Long-term effects of a neonatal low-protein diet in rats on the number of macrophages in culture and the expression/production of fusion proteins. *Eur J Nutr*. 2013; 52, 1475–1482.
8. Hardie DG. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. *Genes Dev*. 2011; 25, 1895–1908.
9. Zhou G, Sebhat IK, Zhang BB. AMPK activators – potential therapeutics for metabolic and other diseases. *Acta Physiol (Oxf)*. 2009; 196, 175–190.
10. Daskalopoulos EP, Dufeys C, Bertrand L, Beauloye C, Horman S. AMPK in cardiac fibrosis and repair: actions beyond metabolic regulation. *J Mol Cell Cardiol*. 2016; 91, 188–200.
11. Bijland S, Mancini SJ, Salt IP. Role of AMP-activated protein kinase in adipose tissue metabolism and inflammation. *Clin Sci (Lond)*. 2013; 124, 491–507.
12. Ko HJ, Zhang Z, Jung DY, *et al*. Nutrient stress activates inflammation and reduces glucose metabolism by suppressing AMP-activated protein kinase in the heart. *Diabetes*. 2009; 58, 2536–2546.
13. Galic S, Fullerton MD, Schertzer JD, *et al*. Hematopoietic AMPK beta1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. *J Clin Invest*. 2011; 121, 4903–4915.
14. Murase T, Misawa K, Haramizu S, Hase T. Catechin-induced activation of the LKB1/AMP-activated protein kinase pathway. *Biochem Pharmacol*. 2009; 78, 78–84.
15. Collins QF, Liu HY, Pi J, *et al*. Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5'-AMP-activated protein kinase. *J Biol Chem*. 2007; 282, 30143–30149.
16. Li Y, Zhao S, Zhang W, *et al*. Epigallocatechin-3-O-gallate (EGCG) attenuates FFAs-induced peripheral insulin resistance through AMPK pathway and insulin signaling pathway in vivo. *Diabetes Res Clin Pract*. 2011; 93, 205–214.
17. Hao J, Kim CH, Ha TS, Ahn HY. Epigallocatechin-3 gallate prevents cardiac hypertrophy induced by pressure overload in rats. *J Vet Sci*. 2007; 8, 121–129.
18. Khurana S, Venkataraman K, Hollingsworth A, Piche M, Tai TC. Polyphenols: benefits to the cardiovascular system in health and in aging. *Nutrients*. 2013; 5, 3779–3827.
19. Ikeda M, Suzuki C, Umegaki K, *et al*. Preventive effects of green tea catechins on spontaneous stroke in rats. *Med Sci Monit*. 2007; 13, BR40–BR45.
20. Tanabe N, Suzuki H, Aizawa Y, Seki N. Consumption of green and roasted teas and the risk of stroke incidence: results from the Tokamachi-Nakasato cohort study in Japan. *Int J Epidemiol*. 2008; 37, 1030–1040.
21. Sato S, Mukai Y, Hamaya M, Sun Y, Kurasaki M. Long-term effect of green tea extract during lactation on AMPK expression in rat offspring exposed to fetal malnutrition. *Nutrition*. 2013; 29, 1152–1158.
22. Shiojima I, Yefremashvili M, Luo Z, *et al*. Akt signaling mediates postnatal heart growth in response to insulin and nutritional status. *J Biol Chem*. 2002; 277, 37670–37677.
23. Beauloye C, Bertrand L, Horman S, Hue L. AMPK activation, a preventive therapeutic target in the transition from cardiac injury to heart failure. *Cardiovasc Res*. 2011; 90, 224–233.
24. Calvert JW, Gundewar S, Jha S, *et al*. Acute metformin therapy confers cardioprotection against myocardial infarction via AMPK-eNOS-mediated signaling. *Diabetes*. 2008; 57, 696–705.
25. Legeay S, Rodier M, Fillon L, Faure S, Clere N. Epigallocatechin Gallate: a review of its beneficial properties to prevent metabolic syndrome. *Nutrients*. 2015; 7, 5443–5468.
26. Lim K, Zimanyi MA, Black MJ. Effect of maternal protein restriction during pregnancy and lactation on the number of cardiomyocytes in the postproliferative weanling rat heart. *Anat Rec (Hoboken)*. 2010; 293, 431–437.
27. Sheng R, Gu ZL, Xie ML, Zhou WX, Guo CY. EGCG inhibits proliferation of cardiac fibroblasts in rats with cardiac hypertrophy. *Planta Med*. 2009; 75, 113–120.
28. Kitamura M, Nishino T, Obata Y, *et al*. Epigallocatechin gallate suppresses peritoneal fibrosis in mice. *Chem Biol Interact*. 2012; 195, 95–104.
29. Leslie KO, Taatjes DJ, Schwarz J, vonTurkovich M, Low RB. Cardiac myofibroblasts express alpha smooth muscle actin during right ventricular pressure overload in the rabbit. *Am J Pathol*. 1991; 139, 207–216.
30. Cao Y, Bao S, Yang W, *et al*. 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*. 2014; 34, 1066–1074.
31. Jang HJ, Ridgeway SD, Kim JA. Effects of the green tea polyphenol epigallocatechin-3-gallate on high-fat diet-induced insulin resistance and endothelial dysfunction. *Am J Physiol Endocrinol Metab*. 2013; 305, E1444–E1451.
32. Krishnan TR, Velusamy P, Srinivasan A, *et al*. EGCG mediated downregulation of NF-AT and macrophage infiltration in experimental hepatic steatosis. *Exp Gerontol*. 2014; 57, 96–103.
33. Merezak S, Reusens B, Renard A, *et al*. Effect of maternal low-protein diet and taurine on the vulnerability of adult Wistar rat islets to cytokines. *Diabetologia*. 2004; 47, 669–675.
34. Morimoto S, Calzada L, Sosa TC, *et al*. Emergence of ageing-related changes in insulin secretion by pancreatic islets of male rat offspring of mothers fed a low-protein diet. *Br J Nutr*. 2012; 107, 1562–1565.
35. Kim JJ, Tan Y, Xiao L, Sun YL, Qu X. Green tea polyphenol epigallocatechin-3-gallate enhance glycogen synthesis and inhibit lipogenesis in hepatocytes. *Biomed Res Int*. 2013; 2013, 920128.
36. Kim SJ, Li M, Jeong CW, *et al*. Epigallocatechin-3-gallate, a green tea catechin, protects the heart against regional ischemia-reperfusion injuries through activation of RISK survival pathways in rats. *Arch Pharm Res*. 2014; 37, 1079–1085.
37. Salt IP, Palmer TM. Exploiting the anti-inflammatory effects of AMP-activated protein kinase activation. *Expert Opin Investig Drugs*. 2012; 21, 1155–1167.

38. Shen CL, Samathanam C, Tatum OL, *et al*. Green tea polyphenols avert chronic inflammation-induced myocardial fibrosis of female rats. *Inflamm Res*. 2011; 60, 665–672.
39. Dong J, Zhang X, Zhang L, *et al*. Quercetin reduces obesity-associated ATM infiltration and inflammation in mice: a mechanism including AMPKalpha1/SIRT1. *J Lipid Res*. 2014; 55, 363–374.
40. Wang XF, Zhang JY, Li L, *et al*. Metformin improves cardiac function in rats via activation of AMP-activated protein kinase. *Clin Exp Pharmacol Physiol*. 2011; 38, 94–101.
41. Appeldoorn MM, Venema DP, Peters TH, *et al*. Some phenolic compounds increase the nitric oxide level in endothelial cells in vitro. *J Agric Food Chem*. 2009; 57, 7693–7699.
42. Fulton D, Gratton JP, McCabe TJ, *et al*. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*. 1999; 399, 597–601.
43. Ying Z, Xie X, Chen M, Yi K, Rajagopalan S. Alpha-lipoic acid activates eNOS through activation of PI3-kinase/Akt signaling pathway. *Vascul Pharmacol*. 2015; 64, 28–35.
44. Thone-Reineke C, Kalk P, Dorn M, *et al*. High-protein nutrition during pregnancy and lactation programs blood pressure, food efficiency, and body weight of the offspring in a sex-dependent manner. *Am J Physiol Regul Integr Comp Physiol*. 2006; 291, R1025–R1030.
45. Elmes MJ, Haase A, Gardner DS, Langley-Evans SC. Sex differences in sensitivity to beta-adrenergic agonist isoproterenol in the isolated adult rat heart following prenatal protein restriction. *Br J Nutr*. 2009; 101, 725–734.
46. Moritz KM, Cuffe JS, Wilson LB, *et al*. Review: sex specific programming: a critical role for the renal renin-angiotensin system. *Placenta*. 2010; 31(Suppl), S40–S46.

https://doi.org/10.1017/S2040174416000647 Published online by Cambridge University Press