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Long-term effects of maternal resveratrol intake during lactation on cholesterol metabolism in male rat offspring

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Abstract

Resveratrol (RSV) can protect against non-communicable diseases by improving cholesterol metabolism. However, it is unclear that effects of maternal RSV intake on health of adult offspring. In this study, we examined the effects of maternal RSV intake during lactation on cholesterol metabolism in adult male rat offspring. Female Wistar rats were fed a control diet (CON) supplemented with or without RSV (20 mg/kg body weight/day) during their lactation period. Male offspring were weaned onto a standard diet and maintained on this diet for 36 weeks. As a result, plasma cholesterol level significantly decreased in RSV offspring compared to CON offspring. Furthermore, a decrease in hepatic 3-hydroxy-3-methylglutaryl-CoA reductase level and an increase in hepatic LDL-receptor level were observed in the RSV offspring. These results indicate that maternal RSV intake causes long-term decrease in plasma cholesterol level in the offspring through suppression of hepatic cholesterol biosynthesis and promotion of hepatic cholesterol uptake.

Keywords: resveratrol; maternal; adult offspring; cholesterol

Introduction

It is a serious issue that the globally increased number of deaths due to non-communicable diseases (NCDs) especially cardiovascular diseases (CVDs). World Health Organization (2018) reported that the annual number of deaths from CVDs increased by more than 3 million between 2000 and 2016. Several studies have reported a relationship between hypercholesterolemia and deaths caused by CVDs, indicating that hypercholesterolemia is a risk factor of CVDs (Kannel et al. 1986; Chen et al. 1991; Stamler et al. 2000; Emberson et al. 2003; Grau et al. 2010; Imano et al. 2011; Sugiyama et al. 2015). Since cholesterol is an essential lipid found in hormones, bile, and the cell membrane, its homeostasis in the body is strictly regulated. However,

excess cholesterol is known to be a risk factor for CVDs as indicated in the above-mentioned studies. Hepatic cholesterol metabolism exhibits a close relationship with cholesterol homeostasis in the body since the liver is the main organ that regulates cholesterol homeostasis. In the liver, cholesterol homeostasis is controlled by four main routes: biosynthesis, uptake from blood, supply into blood, and conversion to bile acid. Statins, widely used as therapeutic agents for CVDs, reduce serum cholesterol levels by inhibition of hepatic cholesterol biosynthesis pathway (Reihner et al. 1990, Brown et al. 1978; Goldstein and Brown 2015).

It is already known that personal lifestyles, such as excessive nutritional intake or less exercise, play an essential role in dyslipidemia and the development of NCDs. On the other hand, recent studies have revealed that maternal nutrition during pregnancy and lactation affects the future health of children. Both epidemiological and animal studies have shown that maternal malnutrition or overnutrition increase the risk of NCDs in children. Epidemiological studies showed that fetal exposure to famine increases the risk of hyperglycemia in adulthood (Ravelli et al. 1998; Li et al. 2010). Animal studies have reported that maternal high-fat diet could lead to obesity, insulin resistance, and hyperlipidemia, including hypercholesterolemia, in their offspring (Samuelsson et al. 2008; Ribaroff et al. 2017). Furthermore, it has been reported that the consumption of high-fat diet by mothers during lactation is more responsible in affecting lipid metabolism in offspring than the consumption of such a diet during pregnancy (Sun et al. 2012). In other studies, the effects of long-term maternal treatment with peroxisome proliferator-activated receptor α (PPAR α) ligand during lactation were examined in mouse offspring (Ehara et al. 2015; Yuan et al. 2018).

Resveratrol (3,5,4-trihydroxystilbene; RSV) is a type of plant polyphenol found in red wine and grapes, and is known for its anti-oxidant, anti-inflammatory, and anti-

cancer properties (Brookins Danz et al. 2009). Furthermore, its preventive effect against diet-induced dyslipidemia has also been reported (Cho et al. 2008; Zhu et al. 2008). These biological effects of RSV depend on the activation of Sirtuin 1 (Sirt1) (Lagouge et al. 2006; Brookins Danz et al. 2009), which regulates the activation of AMP-activated protein kinase (AMPK) via deacetylation and activation of LKB1 (Hou et al. 2008; Lan et al. 2008). Numerous studies have already reported that activated-AMPK attenuates hepatic cholesterol biosynthesis (Henin et al. 1995; Liu et al. 2015), and along with RSV affects cholesterol metabolism. Li et al. (2011) reported that RSV attenuated hepatic cholesterol biosynthesis by the activation of AMPK. In addition, other studies have indicated that RSV could ameliorate hypercholesterolemia (Do et al. 2008; Xin et al. 2013; Yu et al. 2016).

Several studies have examined the effects of maternal RSV intake in offspring, but studies examining cholesterol metabolism with respect to RSV intake are limited. Ros et al. (2018) reported that maternal RSV treatment with high-fat or low-fat diet during pregnancy and lactation did not cause significant changes in serum cholesterol level in rat offspring on postnatal day 0 and 21. Similarly, Vega et al. (2016) showed that RSV exposure during gestation period did not significantly affect the serum cholesterol level in both male and female rat offspring on postnatal day 110. On the other hands, Sun et al. (2019) reported that maternal RSV intake during pregnancy and lactation increased HDL-cholesterol and LDL-cholesterol level in piglet offspring on postnatal day 21. The influence of maternal RSV intake on cholesterol metabolism in offspring cannot be concluded, because these studies were conducted under different experimental conditions and did not examine the underlying mechanisms in detail. Therefore, further investigations are needed to elucidate the effects of maternal RSV supplementation on cholesterol metabolism in the offspring. This study aimed to

examine the effects of maternal RSV ingestion during lactation on cholesterol metabolism in adult rat offspring and its underlying mechanism.

Materials and Methods

Animal treatments

All procedures were performed according to the Guidelines for Animal Experimentation, Aomori University of Health and Welfare. Pregnant Wistar rats were divided into two dietary groups and were fed a control diet (CON) during gestation and CON or CON supplemented with RSV (Sigma-Aldrich, Tokyo, Japan) during their lactation period. The mother rats in the CON supplemented with RSV group received RSV solution orally (20 mg/kg body weight) once a day by gavage during lactation, while the mother rats in the other group received a vehicle orally (0.05% carboxymethylcellulose) once a day by gavage during lactation. Six male offspring of each group were examined for this study. At 3 weeks of age, the male offspring were weaned onto a standard diet (MF diet; Oriental Yeast, Tokyo, Japan) and maintained on this diet for 36 weeks. The body weights and food intake of each offspring were recorded after weaning every 4 weeks. At 36 weeks, the offspring were fasted overnight and killed under ether anesthesia (Figure 1). Their blood and livers were immediately extracted, and the liver samples were stored at -80°C before further evaluation.

Blood chemistry analysis

Plasma samples were obtained after centrifugation ($800 \times g$ for 15 min at 4°C) and examined for levels of total cholesterol, blood glucose, blood urea nitrogen (BUN), and creatinine using an autoanalyser for blood chemistry analysis (Fuji Dri-Chem 3500 V; Fuji Film, Tokyo, Japan).

Hepatic cholesterol level

The liver sample was added to a solution of chloroform and ethanol (1:2, v/v) in an Eppendorf tube and homogenized. After incubation, chloroform was added to the mixture and blended. Next, distilled water was added and mixed again. The chloroform layer was separated from the samples after centrifugation. The lipid fraction was obtained by evaporating chloroform with an evaporator and was dissolved in isopropanol. Total cholesterol level was measured using LabAssay Cholesterol Kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) according to the manufacturer's instructions.

Western blot analysis

For western blot analysis, the liver samples were homogenized in a buffer using a Polytron (PCU Drehzahlregler; Kinematica, Luzern, Switzerland) on ice. The homogenate was centrifuged ($20000 \times g$ for 20 min at 4°C), and the supernatant was collected. Next, the obtained supernatant was heated to avoid denaturation of protein and the protein concentration in the sample was measured by Bradford assay (Protein Assay; BIO-RAD, Hercules, USA) (Marion M. Bradford 1976). Proteins in the sample were separated by SDS-PAGE by using biotinylated protein molecular weight markers (M&S TechnoSystems, Inc., Osaka, Japan) as protein standards. Proteins were then electrophoretically transferred onto a nitrocellulose membrane using the iBlot transfer system (Thermo Fisher Scientific K.K., Tokyo, Japan). The nitrocellulose membrane was incubated overnight at 4°C in a blocking solution containing 3% skim milk. The membrane was then washed and exposed to primary antibodies: CYP7A1 (bs-2399R; Bioss, Massachusetts, USA), HMGCR (ab180615, Abcam, Tokyo, Japan), Insig-1 (ab70784; Abcam, Tokyo, Japan), MTTP (ab186446, Abcam, Tokyo, Japan), LDL-

receptor (3839-30T; BioVision, California, USA), S1P (ab140592, Abcam, Tokyo, Japan), S2P (ab140594; Abcam, Tokyo, Japan), SREBP-2 (ab30682, Abcam, Tokyo, Japan), SOAT-2 (bs5020R; Bioss, Massachusetts, USA), and beta-Actin (ab8226, Abcam, Tokyo, Japan), in the presence of a 1% blocking solution. Next, the membrane was again washed and exposed to the secondary antibodies: anti-rabbit IgG IRDye 680 (926-68071; M&S TechnoSystems, Inc., Osaka, Japan) or anti-mouse IgG IRDye 800 (926-3221; M&S TechnoSystems, Inc., Osaka, Japan), in presence of a 1% blocking solution. Protein bands were quantitated using Odyssey infrared imaging system (M&S TechnoSystems, Inc., Osaka, Japan). Protein levels were normalized against those of beta-actin from the same sample.

Statistical analysis

Each value was expressed as mean \pm SEM. Statistical analyses were performed using Student's t-test. In all cases, $p < 0.05$ was considered as statistically significant.

Results

Body weight, food intake, blood chemistry parameters, and hepatic cholesterol levels.

Although the dietary RSV intake did not show any significant difference between the two groups, it was observed that the body weight of RSV offspring was significantly lower than that of the CON offspring from the age of 8 weeks to 32 weeks (Table 1 and Table 2). No significant change was observed in levels of blood glucose, BUN, and creatinine between the two dietary groups at 36 weeks (Table 3). Furthermore, there was no significant difference in the level of hepatic cholesterol between the two groups (Figure 2(A)). However, plasma cholesterol level in the RSV offspring was shown to be significantly lower than that in the CON offspring (Figure 2(B)). These results

suggested that maternal RSV ingestion alters cholesterol metabolism in offspring without alteration of the feeding behavior and glucose metabolism. And results in levels of plasma BUN and creatinine suggested that there was no significant kidney dysfunction.

Hepatic cholesterol metabolism

Cholesterol synthesis; the protein level of HMGCR

Hepatic cholesterol metabolism was investigated as a factor that affects cholesterol localization between liver and blood. During this investigation on hepatic cholesterol biosynthesis, a decrease in hepatic HMGCR level was observed in RSV offspring compared with that in CON offspring (Figure 3(A)). HMGCR is the enzyme that determines the rate of cholesterol biosynthesis (Geelen et al. 1986; Goldstein and Brown 1990). This result suggested that the rate of cholesterol biosynthesis is reduced in RSV offspring.

Cholesterol uptake; the protein level of LDL-receptor

Hepatic low-density lipoprotein (LDL)-receptor level was measured to evaluate hepatic cholesterol uptake from the blood. LDL-receptor translocates cholesterol-rich particles, LDL and very low-density lipoprotein (VLDL), from the blood into cells. In this study, a higher level of hepatic LDL-receptor was observed in RSV offspring compared to CON offspring (Figure 3(B)). Since hepatic LDL-receptor level is closely related to hepatic cholesterol uptake (Brown and Goldstein 1983; Spady 1992), this result suggested that hepatic cholesterol uptake from the blood is promoted in RSV offspring.

Cholesterol supply to blood; the protein levels of SOAT-2 and MTTP

Hepatic sterol O-acyltransferase (SOAT)-2 and microsomal triglyceride transfer protein (MTTP) levels were measured to investigate changes in the cholesterol transfer from liver to blood. SOAT-2 catalyses esterification of free cholesterol in hepatic lipid droplets with acyl-CoA (Chang et al. 2009; Marshall et al. 2014), while MTTP catalyses association of cholesterol ester and triglyceride with apo-B protein to form VLDL (Gordon and Jamil 2000; Hussain et al. 2012). These enzymes catalyse reactions at crucial steps to supply cholesterol into the blood and contribute in maintaining cholesterol homeostasis. In this study, there was no significant difference observed between the two groups in the levels of both SOAT-2 and MTTP, suggesting that there was no change in cholesterol supply from the liver to the blood (Figure 3(C) and Figure 3(D)).

Cholesterol excretion; the protein level of CYP7A1

To analyse cholesterol catabolizing pathways, hepatic cholesterol 7 alpha-hydroxylase (CYP7A1) level was measured. CYP7A1 is a crucial enzyme involved in the "classical pathway," which is the major pathway for bile acid synthesis and one of the few cholesterol excretion pathways (Chiang 2009). In this study, no significant difference in hepatic CYP7A1 level was found between the two groups, which suggested that there was no change in the bile acid synthesis through the cholesterol excretion pathway (Figure 3(E)).

The protein levels of SREBP-2, Insig-1, S1P and S2P

We also examined SREBP-2, a crucial transcription factor involved in cholesterol metabolism. SREBP-2 is retained on the endoplasmic reticulum (ER) by its association with insulin-induced gene protein (Insig) in the presence of cholesterol. However, as the

intracellular cholesterol level decreases, SREBP-2 is released from the ER and is transported to the Golgi apparatus (Yang et al. 2002). Furthermore, upon cleavage by the two proteases, Site-1 protease (S1P) and Site-2 protease (S2P), in the Golgi, the N-terminus of SREBP-2 which has transcriptional activity is transferred to the nucleus (Duncan et al. 1997; Sakai et al. 1996). SREBP-2 regulates the transcription of cholesterol-related proteins, such as HMGCR and LDL-receptor (Horton et al. 1998; Sakakura et al. 2001).

In this study, although higher precursor-SREBP-2 and Insig-1 levels were observed in RSV offspring (Figure 4(A), Figure 4(C) and Figure 4(F)), there was no significant difference in the levels of mature-SREBP-2 and the two proteases, S1P and S2P, between the two dietary groups (Figure 4(B), Figure 4(D) and Figure 4(E)).

Discussion

Our results showed that maternal RSV ingestion during lactation reduces plasma cholesterol in male rat offspring at 36 weeks of age. In addition, suppression of hepatic cholesterol biosynthesis and promotion of cholesterol uptake into the liver from the blood were speculated to contribute to the change in cholesterol localization, instead of altered cholesterol supply into blood or conversion into bile acid. To the best of our knowledge, this is the first report presenting the long-term effects of maternal RSV intake during lactation on cholesterol metabolism in male rat offspring and the first study to investigate the mechanisms underlying these effects.

The effects and mechanism observed in RSV offspring to lower plasma cholesterol resemble the mechanism mediated by statins. Although statins inhibit cholesterol synthesis by competitively inhibiting HMGCR (Reihner et al. 1990), the mechanism observed in the RSV offspring was different as only the level of HMGCR was decreased. However, it was similar in terms of reduction in blood cholesterol level

via suppression of hepatic biosynthesis and promotion of cholesterol uptake from the blood.

Since expressions of hepatic HMGCR and LDL-receptor are regulated by SREBP-2 (Horton et al. 1998; Sakakura et al. 2001), in this study, the expression and activation of SREBP-2 were examined to elucidate the mechanism responsible for changes in HMGCR and LDLR levels. This study showed a significant increase in hepatic precursor-SREBP-2 level in RSV offspring, but no significant difference was observed for hepatic mature-SREBP-2 level between the two groups. It has previously been reported that SREBP-2 expression is promoted during intracellular cholesterol depletion to increase cholesterol level by the expression of its target protein (Sato et al. 1996; Yang et al. 2002). Therefore, we hypothesized that although SREBP-2 expression in RSV offspring was promoted to increase hepatic cholesterol content, SREBP-2 was not activated and remained in its precursor, inactive state for some reason. We observed a result supporting this hypothesis, increased level of hepatic Insig-1 in RSV offspring. Insig-1 is known to anchor precursor-SREBP-2 to ER and inhibit its maturation (Sun et al. 2005). Therefore, it is suggested that hepatic SREBP-2 maturation in RSV offspring was inhibited by upregulation of Insig-1. On the other hands, previous studies have revealed that many mechanisms are involved in the activity of SREBP-2 and in the regulation of expression of its target protein (Sanchez et al. 1995; Sato et al. 1996; Walker et al. 2010; Hou et al. 2008; Liu et al. 2015), and RSV treatment was speculated to influence these mechanisms (De Amicis et al. 2011; Li et al. 2011; Zeng et al. 2017). Although no other detailed mechanism has been examined in this study, the results in this study suggested that inhibition of SREBP-2 activation was involved in the alteration of cholesterol metabolism in RSV offspring.

Our experimental design is similar to that employed by Vega et al. (2016) in terms of animal species used, dose of RSV, and offspring age. However, the results obtained in terms of cholesterol metabolism are different. Vega et al. did not observe significant difference in serum cholesterol level of offspring between different groups. This suggested that the duration of exposure to RSV is important in gauging its impacts on cholesterol metabolism in rat offspring. This hypothesis was supported by previous studies that reported that lactation period is more important for the regulation of lipid metabolism, including cholesterol, in the offspring (Sun et al. 2012; Ehara et al. 2015; Yuan et al. 2018).

Previous studies have reported that direct RSV treatment decreased the levels of plasma cholesterol and hepatic HMGCR in *in vivo*, and increased LDL-receptor in *in vitro* (Cho et al. 2008; Do et al. 2008; Zhu et al. 2008; Yashiro et al. 2012).

Interestingly, we observed similar effects in adult rat offspring at 36 weeks of age even though only the mothers were fed with RSV during lactation period, for 3 weeks.

Previous studies have reported that maternal nutrition causes long-lasting effects on health, including cholesterol metabolism, in offspring (Samuelsson et al. 2008; Sun et al. 2012; Ribaroff et al. 2017). In addition, previous studies have shown that dietary polyphenols transfer to offspring through breast milk (Franke et al. 2006; Romaszko et al. 2014; Khymenets et al. 2016). Therefore, it is considered that the effects of exposure to RSV via breast milk last through adulthood. To the best of our knowledge, this is the first study showing the long-term effects of maternal RSV supplementation during lactation on cholesterol metabolism in rat offspring. Although the mechanism behind such effects was not revealed in this study, previous studies have indicated that these long-lasting effects could be caused by epigenetic mechanisms such as DNA and histone modifications. Several studies have reported that the activation of PPAR α in

mother mice during lactation leads to long-term downregulation of lipogenesis in offspring through decreased DNA methylation (Ehara et al. 2015; Yuan et al. 2018). Other studies have indicated that maternal polyphenol intake could modify the expression of epigenetic modulators in offspring (Sun et al. 2013; Kataoka et al. 2018). Furthermore, Cong et al. (2012) indicated that maternal low-protein diet during pregnancy and lactation upregulated SREBP-2, HMGCR, and CYP7A1, and altered cholesterol metabolism in the offspring. Moreover, they also indicated changes in DNA methylation and histone modification status in the promoter regions of HMGCR and CYP7A1 in the offspring. These studies suggested that maternal RSV ingestion during lactation influences epigenetic mechanism in offspring. Thus, further investigations are needed since epigenetic modifications could be crucially involved in the changes observed during this study.

Conclusion

This study showed that maternal RSV intake during lactation decreases plasma cholesterol level while maintaining hepatic cholesterol content in adult male rat offspring. These results indicated inhibition of hepatic cholesterol biosynthesis and promotion of hepatic cholesterol uptake from the blood by RSV treatment. Additionally, these results indicated that maternal RSV intake during lactation period elicits long-lasting effects on cholesterol metabolism in the rat offspring.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Table 1. Body weight of male rat offspring after weaning from 4 weeks to 32 weeks.

Weeks	4	8	16	24	32
CON offspring	148.83 ± 4.25	407.97 ± 8.92	623.0 ± 23.87	721.25 ± 36.58	798.25 ± 49.51
RSV offspring	126.55 ± 8.49	354.95 ± 7.31*	532.85 ± 7.89*	597.12 ± 16.94*	652.65 ± 26.32*

CON offspring: a control diet during gestation and lactation, and a standard diet after weaning. RSV offspring: a control diet during gestation, 20 mg/body weight RSV added in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean ± SEM (n = 6). * p < 0.05 compared to CON offspring.

Table 2. Relative food intake of male rat offspring after weaning from 4 weeks to 32 weeks.

Weeks	8	16	24	32
CON offspring	7.10 ± 0.12	4.61 ± 0.15	3.68 ± 0.13	3.39 ± 0.14
RSV offspring	7.67 ± 0.30	4.77 ± 0.14	3.99 ± 0.14	3.81 ± 0.14

CON offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV offspring: a control diet during gestation, 20 mg/body weight RSV added in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean ± SEM (n = 6).

Table 3. Blood chemistry parameters in male rat offspring at 36 weeks: plasma glucose, BUN, and creatinine.

	Glucose	BUN	Creatinine
CON offspring	161.15 ± 6.44	17.00 ± 0.62	1.03 ± 0.05
RSV offspring	150.17 ± 2.63	15.62 ± 0.85	1.07 ± 0.03

CON offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV offspring: a control diet during gestation, 20 mg/body weight RSV added in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean ± SEM (n = 6).

Figure captions

Figure 1. Experimental design. CON offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV offspring: a control diet during gestation, 20 mg/body weight RSV added in the control diet during lactation, and a standard diet after weaning.

Figure 2. Hepatic cholesterol content (mg/g) (A) and plasma cholesterol level (mg/dl) (B) in male rat offspring at 36 weeks. The left column indicates CON offspring, and the right column indicates RSV offspring. CON offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV offspring: a control diet during gestation, 20 mg/body weight RSV added in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean \pm SEM (n = 6). ** p < 0.01 compared to CON offspring.

Figure 3. Protein expressions of HMGCR (A), LDLR (B), MTTP (C), SOAT-2 (D), and CYP7A1 (E) in the liver of male rat offspring at 36 weeks. For each condition densitometric analysis was conducted relative to beta-actin. The left column indicates CON offspring, and the right column indicates RSV offspring. CON offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV offspring: a control diet during gestation, 20 mg/body weight RSV added in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean \pm SEM (n = 6). * p < 0.05 compared to CON offspring. Each band in the figure is representative of the average behaviour of each group.

Figure 4. Protein expressions of precursor-SREBP-2 (A), mature-SREBP-2 (B), Insig-1 (C), S1P (D), and S2P (E) in the liver of male rat offspring at 36 weeks. For each condition densitometric analysis was conducted relative to beta-actin. The left column indicates CON offspring, and the right column indicates RSV offspring. CON offspring: a control diet during gestation and lactation, and a standard diet after weaning, RSV offspring: a control diet during gestation, 20 mg/body weight RSV added in the control diet during lactation, and a standard diet after weaning. Values are expressed as mean \pm SEM (n = 6). ** p < 0.01 compared to CON offspring. Each band in the figure is representative of the average behaviour of each group.

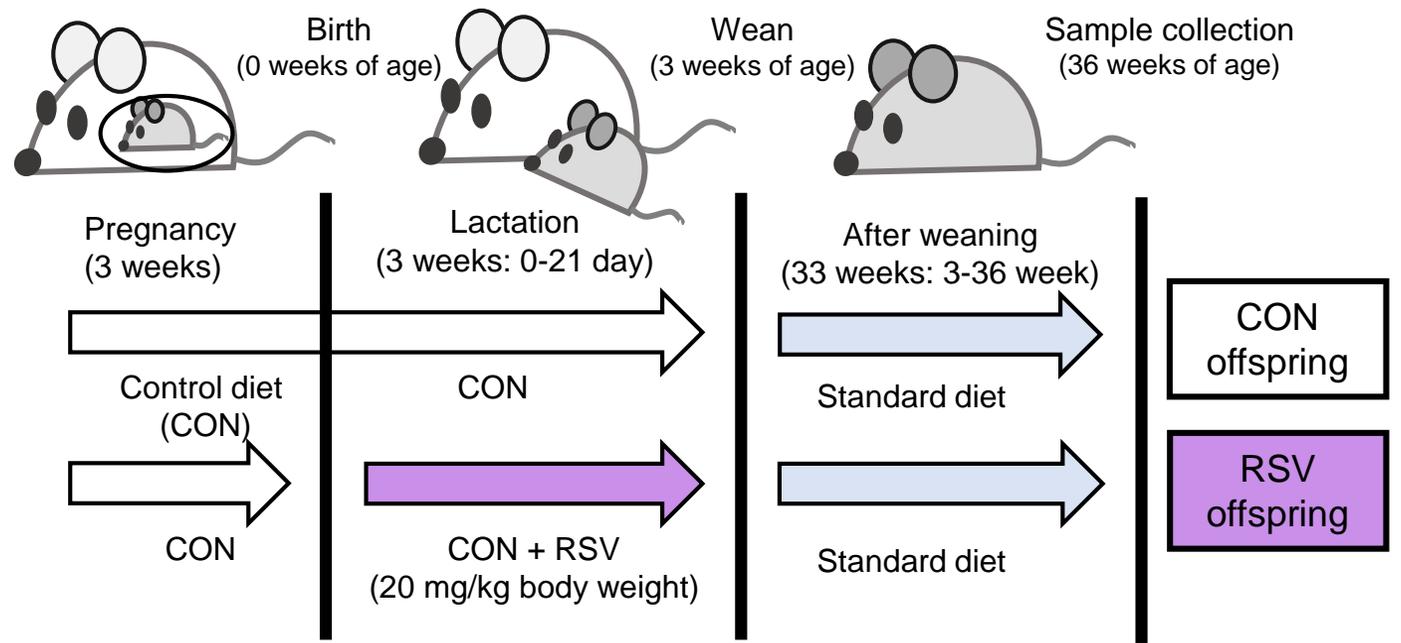


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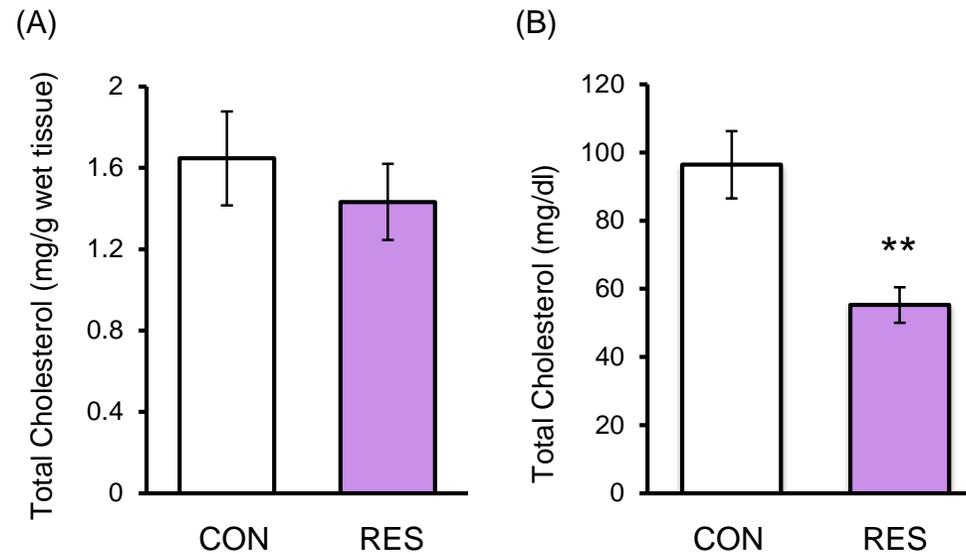


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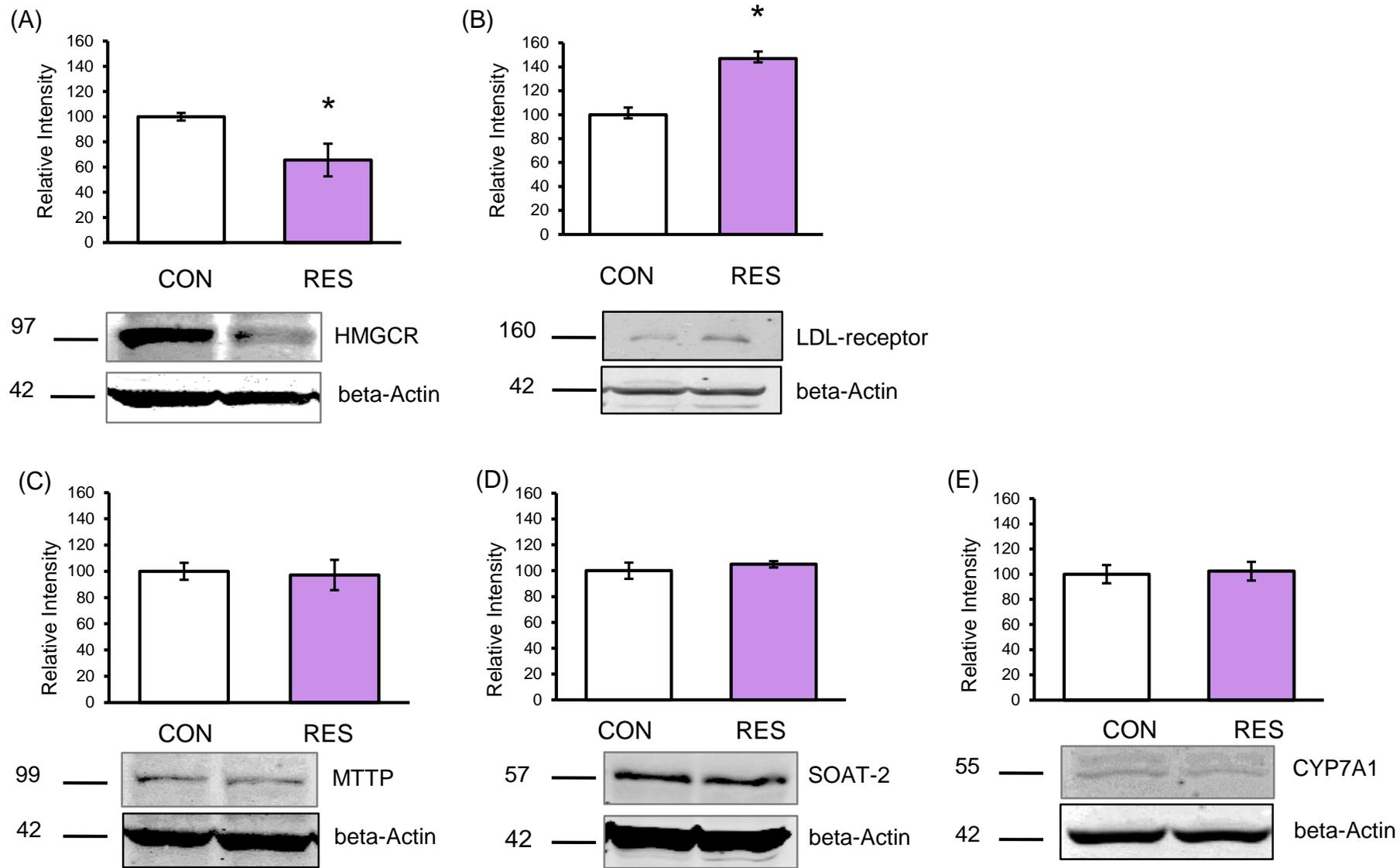


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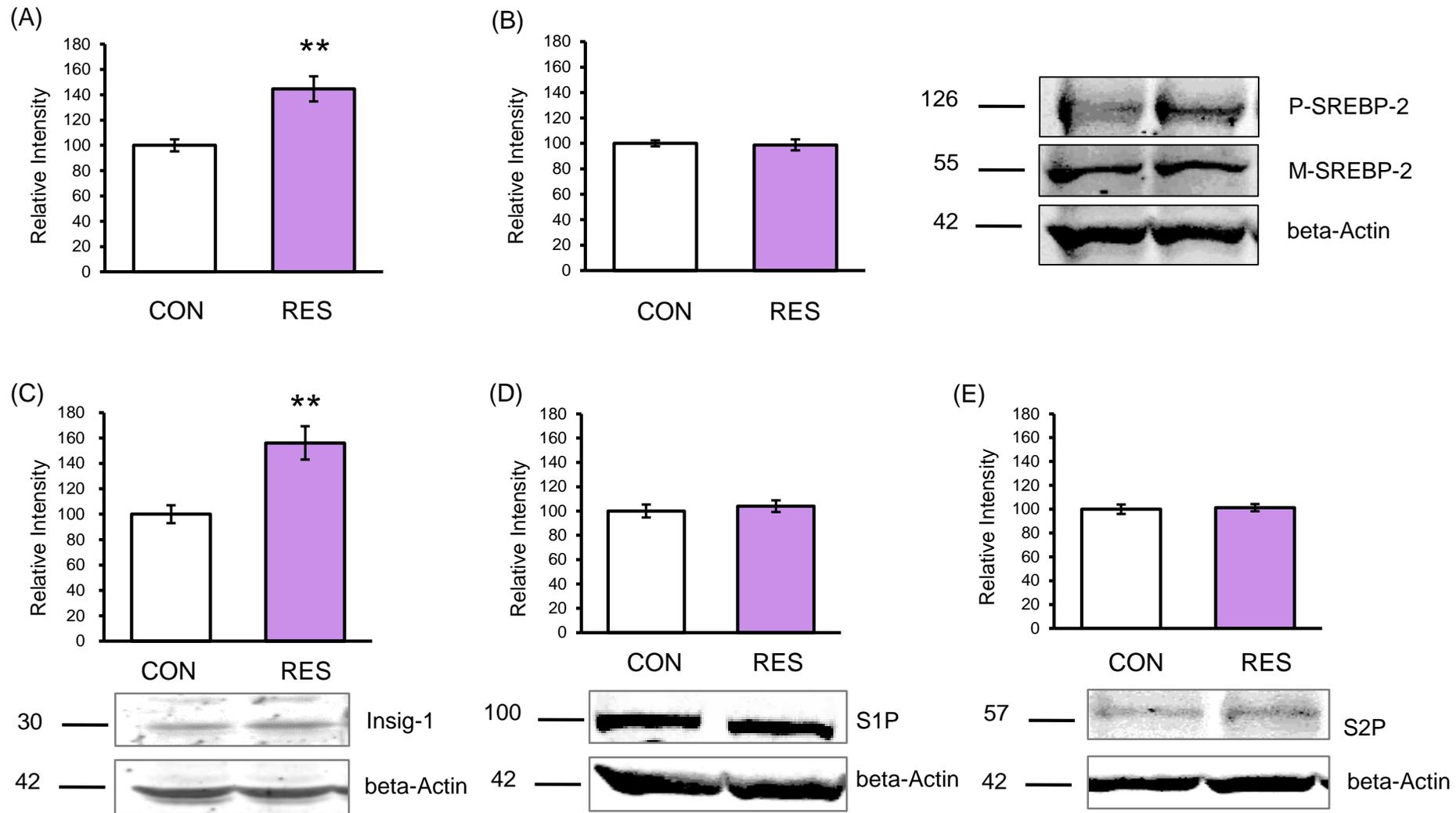


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