BBA - Molecular Basis of Disease 1865 (2019) 1690–1700

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Dietary cholesterol is essential to mast cell activation and associated obesity and diabetes in mice

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ARTICLE INFO

Keywords:
Mast cell
Cholesterol
Obesity
Diabetes
DSCG
Ketotifen

ABSTRACT

Mast cell (MC) deficiency in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and inhibition with disodium chromoglycate (DSCG) or ketotifen reduced obesity and diabetes in mice on a high-cholesterol (1.25%) Western diet. Yet, Kit-independent MC-deficient mice and mice treated with DSCG disproved MC function in obesity and diabetes when mice are fed a high-fat diet (HFD) that contains no cholesterol. This study reproduced the obesity and diabetes inhibitory activities of DSCG and ketotifen from mice on a Western diet. Yet, such inhibitory effects were diminished in mice on the HFD. DSCG and ketotifen MC inhibitory activities were recovered from mice on the HFD supplemented with the same amount of cholesterol (1.25%) as that in the Western diet. DSCG and ketotifen effectively blunted the high-cholesterol diet-induced elevations of blood histamine and adipose tissue MC degranulation. Pearson's correlation test demonstrated significant and positive correlations between plasma histamine and total cholesterol or low-density lipoprotein-cholesterol (LDL). In cultured bone marrow-derived MCs, plasma from mice following a Western diet or a cholesterol-supplemented HFD, but not those from HFD-fed mice, induced MC degranulation and the release of β-hexosaminidase, histamine, and serotonin. IgE, LDL, very low-density lipoprotein, and high-density lipoprotein also induced MC activation, which can be inhibited by DSCG and ketotifen depending on the doses and types of MC inhibitors and cholesterol, and also the MC granule molecules of interest. DSCG or ketotifen lost their activities in inhibiting LDL-induced activation of MCs from LDL receptor-deficient mice. These results indicate that dietary cholesterol critically influences the function of mouse MCs.

1. Introduction

We reported previously that genetic deficiency of mast cells (MCs) in Kit-dependent *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, or pharmacologic inhibition of MCs in wild-type (WT) mice with the MC inhibitors disodium chromoglycate (DSCG) and ketotifen, reduced high cholesterol (1.25%), Western diet-induced body weight gain, and glucose tolerance, and increased insulin sensitivity [1]. Several studies showed that these *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice not only lack MCs but also show higher numbers of circulating neutrophils and elevated splenic and bone marrow Gr-1^{hi}CD11b^{hi} mature granulocytes, Gr-1^{int}CD11b^{int} mitotic granulocyte progenitors, and MC progenitor cells with reciprocal decreases of B and T cells, compared with those from WT control mice [2,3]. Therefore, reduced obesity and diabetes in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice may associate with alterations of these inflammatory cell contents rather than the lack of MCs. Consistent with this hypothesis, Gutierrez et al. recently used Kit-independent *Cpa3*$^{Cre/+}$ mice and their WT controls, and demonstrated similar levels of body weight gain, body adipose tissue content, and glucose tolerance when

Abbreviations: BAT, brown adipose tissues; BMMCs, bone marrow-derived mast cells; Cpa3, carboxypeptidase A3; DNP, 2,4-dinitrophenol; DSCG, disodium cromoglycate; EATs, epididymal adipose tissues; GTT, glucose tolerance test; Treg, regulatory T-cell; ILC2, type 2 innate lymphoid cells; ITT, insulin tolerance test; SAT, subcutaneous adipose tissues; HDL, high-density lipoprotein; HFD, high fat diet; LDL, low-density lipoprotein; MC, mast cell; Mcpt5, mast cell protease 5; PCA, passive cutaneous anaphylaxis; VLDL, very low-density lipoprotein; WT, wild-type

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https://doi.org/10.1016/j.bbadis.2019.04.006
Received 23 February 2018; Received in revised form 20 December 2018; Accepted 6 January 2019
Available online 09 April 2019
0925-4439/

mice were fed a cholesterol-free, high-fat diet (HFD) [4]. The same study also used DSCG to block MC activation, and found no effect of DSCG on HFD-induced bodyweight gain [4]. Unlike the *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, *Cpa3*$^{Cre/+}$ mice show complete deficiency of MCs and partial basophil ablation, yet these mice have normal numbers of splenic and bone marrow lymphocytes and myeloid cells [5,6]. Similar to the observations from the *Cpa3*$^{Cre/+}$ mice, Chmelar et al. used another line of Kit-independent, MC constitutive-deficient *Mcpt5-Cre R-DTA*$^{+}$ mice, and found that MC deficiency did not affect body weight gain, glucose tolerance, and insulin sensitivity when mice were fed the same cholesterol-free HFD [7]. In addition to MC-deficiency, *Mcpt5-Cre R-DTA*$^{+}$ mice have comparable numbers of adipose tissue macrophages and T cells with those from *Mcpt5-Cre-negative R-DTA*$^{+}$ control mice [7]. Therefore, authors from these studies concluded that "Hematopoietic Kit Deficiency, rather than Lack of Mast Cells, Protects Mice from Obesity and Insulin Resistance" and "No Role for Mast Cells in Obesity-Related Metabolic Dysregulation" [4,7].

Upon careful analysis and comparison between our study [1] and those from Gutierrez et al. and Chmelar et al. [4,7], we noticed dietary differences among these studies, in addition to the fact that we used Kit-dependent MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and Gutierrez et al. and Chmelar et al. used Kit-independent *Cpa3*$^{Cre/+}$ and *Mcpt5-Cre R-DTA*$^{+}$ mice. In our study, we fed mice a high-cholesterol Western diet (Cat# D12108C, Research Diets, Inc., New Brunswick, NJ), whereas all mice in Gutierrez's and Chmelar's studies consumed a HFD with 60% kcal% fat (Cat# D12942, Research Diets, Inc.) that contained no cholesterol (Table 1). The dietary cholesterol content differences might have yielded the conflicting obesity and diabetes phenotypes among these different studies [1,4,7]. Several lines of unexplained observations support this hypothesis. First, if reduced obesity and diabetes in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice from our prior study did not result from the loss of MCs but from alterations in other immune cells [2,3], we cannot explain the restored obesity and diabetes phenotypes in *Kit*$^{W\text{-}sh/W\text{-}sh}$-recipient mice after adoptive transfer of donor MCs from WT mice [1]. Although we detected only partial phenotype recovery in WT MC-reconstituted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, this can be explained by the partial recovery of adipose tissue MCs after donor MC engraftment [1]. Criticism of donor MC reconstitution reports that it may show potential differences in both numbers and anatomical distribution in recipient mice. Therefore, donor MCs may never act the same as native endogenous MCs [4,8]. Second, we showed that MC inhibition with either DSCG or ketotifen reduced obesity and insulin resistance in Western diet-fed WT mice [1]. Yet, using the same doses of DSCG, Gutierrez et al. did not show any inhibitory activity of DSCG in HFD-induced body weight gain from WT mice (Fig. 2R, ref. [4]). These two experiments used the same mice, same mast cell inhibitor, and same dose, but yielded different conclusions.

All these prior observations point to a possibility that dietary cholesterol may have made the metabolic phenotype discrepancies among these different studies [1,4,7]. In this study, we induced obesity and insulin resistance in 6-week-old male WT mice with a cholesterol-free HFD, a high-cholesterol (1.25%) Western diet, or the same HFD but supplemented with 1.25% cholesterol (HFD + Cho, Cat# D08063002, Research Diets, Inc.) (Table 1), while mice received the same doses of DSCG and ketotifen as previously used by us and Gutierrez et al. [1,4].

Table 1
Diet compositions.

Product code	D12492 (HFD)		D12108C (Western diet)		D08063002 (HFD + Cho)	
	g%	kcal%	g%	kcal%	g%	kcal%
Protein	26	20	23	20	26	20
Carbohydrate	26	20	45	40	26	20
Fat	35	60	20	40	34	60
kcal/g	5.2		4.5		5.2	

Ingredient	g	kcal	g	kcal	g	kcal
Casein, 80 mesh	200	800	200	800	200	800
L-cystine	3	12	3	12	3	12
Corn starch	0	0	212	848	0	0
Maltodextrin 10	125	500	71	284	125	500
Sucrose	68.8	275	113	452	68.8	275
Cellulose, BW200	50	0	50	0	50	0
Soybean oil	25	225	25	225	25	225
Lard	245	2205	155	1395	245	2205
Mineral Mix S10026	10	0	10	0	10	0
Dicalcium phosphate	13	0	13	0	13	0
Calcium carbonate	5.5	0	5.5	0	5.5	0
Potassium citrate·H_2O	16.5	0	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40	10	40
Choline bitartrate	2	0	2	0	2	0
Cholesterol	0	0	11.25	0	9.8	0
FD&C Yellow Dye #5	0	0	0.05	0	0.025	0
FD&C Red Dye #40	0	0	0.05	0	0.025	0
FD&C Blue Dye #1	0.05	0	0	0	0	0
Total	773.85	4057	897.35	4056	783.65	4057

2. Materials and methods

2.1. Mice and diets

Six-week-old male C57BL/6 mice (Cat# 000664, Jackson Laboratory, Bar Harbor, ME) were randomly divided into 10 dietary groups:

1) Chow (Research Diets, Inc., Cat# D12450B containing 3.85 kcal/g and 10% of energy from fat) (n = 10);
2) High-fat diet (HFD) (Research Diets, Inc., Cat# D12492 containing 5.2 kcal/g and 60% of energy from fat without cholesterol) (n = 15) with daily intraperitoneal (i.p.) injection of saline (n = 15);
3) HFD with daily i.p. injection of disodium cromoglycate (DSCG) (25 mg/kg, Cat# C0399, Sigma-Aldrich, Saint Louis, MO) (n = 15);
4) HFD with daily i.p. injection of ketotifen (20 mg/kg, Cat# sc-201094, Santa Cruz Biotechnology, Dallas, TX) (n = 15);
5) Western diet (Research Diets, Inc., Cat# D12108C containing 4.5 kcal/g, 40% of energy from fat and 1.25% cholesterol) (n = 15) with daily i.p. injection of saline (n = 15);
6) Western diet with daily i.p. injection of DSCG (n = 15);
7) Western diet with daily i.p. injection of ketotifen (n = 15);
8) HFD supplemented with cholesterol (HFD + Cho) (Research Diets, Inc., Cat# D08063002 containing 5.2 kcal/g, 60% of energy from fat and 1.25% cholesterol) with daily intraperitoneally injected by saline (n = 15);
9) HFD + Cho with daily i.p. injection of DSCG (25 mg/kg) (n = 15);
10) HFD + Cho with daily i.p. injection of ketotifen (20 mg/kg) (n = 15) for 16 weeks, respectively.

Equal amounts of non-modified cholesterol were added to both the Western diet and HFD + Cho during manufacture (Table 1, Research Diets, Inc.). Mice were housed in ventilated cages in a pathogen-free barrier facility that was maintained at 22 ± 2 °C and with a 12-hour light/12-hour dark cycle, and free to access autoclaved water and irradiated food throughout the study. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Harvard Medical School Standing Committee on Animals (protocol #03759). Their body weight was monitored weekly. After 16 weeks, mice were euthanized by CO_2,and the plasma, liver, and adipose tissue were collected, including epididymal adipose tissue (EAT) from the epididymal region depots from the testes, epididymides, and vasa deferentia regions, subcutaneous adipose tissue (SAT) from the inguinal region

beneath the skin, and brown adipose tissues (BAT) from the interscapular region around the neck and shoulders. Tissue was weighed on ice.

2.2. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

At 21-weeks-old, GTT and ITT were performed after overnight fasting. In brief, mice were i.p. injected with D-glucose (1 g/kg bodyweight, Cat# G7021, Sigma-Aldrich) for GTT and with insulin (1.5 IU/kg bodyweight, NDC 0169-1833-11, NOVOLIN, Wellington, FL) for ITT. Blood glucose levels were measured from tail veins using a blood glucose meter (Bayer Healthcare LLC, Mishawaka, IN) at 0, 15, 30, 45, 60, 90, and 120 min after injection.

2.3. Plasma lipoprotein and adipose tissue lipid measurements

Plasma triglyceride, total cholesterol, and high-density lipoprotein (HDL) cholesterol levels were determined by enzymatic methods using the triglyceride (Cat# T7532, Pointe scientific, Canton, MI), total cholesterol reagents (Cat# C7510, Pointe scientific), or HDL cholesterol-precipitating reagent (Cat# H7511, Pointe scientific) according to the manufacturer's protocols, as previously reported [9–12]. Plasma low-density lipolipoprotein (LDL) cholesterol was calculated using the Friedewald's formula: Plasma LDL cholesterol concentration (mg/dL) = total cholesterol − HDL cholesterol − (triglycerides / 5) [13,14]. To quantify the triglyceride and total cholesterol levels in adipose tissue, we homogenized each 100 mg of adipose tissue in 1 mL saline, followed by two rounds of freezing and thawing. Tissue homogenate was centrifuged at 5000*g* for 5 min at 4 °C to separate the protein lysate from the up-layer fat cake. We removed 5 μl of protein lysate to determine the total adipose tissue protein concentration using a Pierce's BCA protein assay kit according to the manufacturer's recommendation (Cat# PI23223, Fisher Scientific). The remaining fat and tissue homogenate was mixed again. We measured the triglyceride and total cholesterol levels in this homogenate using the same enzymatic lipid reagents as we used for the plasma samples and then normalized the triglyceride and cholesterol levels to adipose tissue total protein concentrations as previously described [15,16].

Mouse serum lipoprotein concentrations were also determined using size-exclusion chromatography. In brief, mouse serum lipoproteins were fractionated by fast-performance liquid chromatography (FPLC; Merck-HPLC System) using the Superose 6 HR 10/30 size-exclusion chromatography column (GE Healthcare, Buckinghamshire, UK). The column was first equilibrated with 10 mM sodium phosphate buffer, pH 7.4 containing 140 mM NaCl; 400 μL of serum pool was then applied to the column with a flow rate of 0.5 ml/min at room temperature, and fractions of 0.5 ml were collected and analyzed for cholesterol. For each group of animals, serum pools were prepared from a combination of 5 mice and used for the FPLC-based lipoprotein profiling. Analyses of lipids in the elution fractions were performed using the total cholesterol enzymatic method (CHOD-PAP 1489232 kit, Roche Diagnostics), as we reported previously [17].

2.4. Histology

Adipose tissue was fixed in 4% formalin, embedded in paraffin, and serially sliced into 6 μm thickness. To detect degranulated MC, adipose tissue paraffin sections were stained with toluidine blue (Cat# 89640, Sigma-Aldrich) according to the standard protocols [1].

2.5. Cell culture

BMMCs were obtained by *in vitro* differentiation of bone marrow cells taken from mouse femurs as described [1]. After 5 weeks, 1×10^6 BMMCs per well were treated with 10 μg/mL, 100 μg/mL, and 1 mg/mL LDL (Cat# 360-10), very low-density lipoprotein (VLDL, Cat# 365-10) or HDL (Cat# 361-12) (all from Lee Biosolutions, Inc., Maryland Heights, MO), or plasma from mice fed a chow diet, HFD, Western diet, or HFD + cho for 24 h with and without 100 nM, 1 μM, 10 μM DSCG, or ketotifen in 48-well plates based on prior reported doses [18,19]. All experiments were performed with at least three individual BMMC cultures.

2.6. Antigen-induced BMMC activation

Antigen-induced BMMC activation was performed by culturing BMMCs (1×10^6 per well) with 50 μg/ml anti-DNP IgE (Cat# D8406, Sigma-Aldrich) with and without DSCG or ketotifen for 24 h prior to stimulation with 100 ng/ml antigen (DNP-HAS, Cat# A6661, Sigma-Aldrich) for 1 h, followed by measuring the supernatant β-hexosaminidase, histamine, and serotonin levels.

2.7. Measurement of MC granule molecules histamine, serotonin, β-hexosaminidase

MC activation releases the granule molecules histamine, serotonin, and β-hexosaminidase, although a minor portion of β-hexosaminidase may be found in the lysosomes [20–22]. To assess MC activation in mice and cultured BMMCs, we measured these MC granule mediators in both the BMMC culture supernatant and mouse plasma samples. Histamine (Cat# RE59221, IBL, Hamburg, Germany) and serotonin (Cat# BA E-8900, LDN, Nordhorn, Germany) were assessed using the ELISA kits according to manufacturer protocols. For plasma-treated BMMCs, histamine and serotonin from the blank media with the same amount of plasma were subtracted from those of BMMC supernatant measurements. We measured the release of β-hexosaminidase from BMMCs using a previously described method [23]. After different treatments, cells were placed on ice and immediately centrifuged to pellet cells. The supernatants were removed, and 0.5% Triton X-100 solution was then added to the cell pellets. The enzymatic activities of β-hexosaminidase in supernatants and the cell pellets were measured with 1.3 mg/ml *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Cat# N9376, Sigma-Aldrich) in 0.1 M sodium citrate (pH 4.5, Cat# S4641, Sigma-Aldrich) for 60 min at 37 °C. The reaction was stopped by the addition of 0.2 M glycine (pH 10.7, Cat# G7126, Sigma-Aldrich). The release of the product 4-*p*-nitrophenol was detected by absorbance at 405 nm. The percentage of degranulation was calculated using the following formula: % Degranulation = $OD_{supernatant} / (OD_{supernatant} + OD_{Triton\ X\text{-}100}) \times 100$. For plasma-treated BMMCs, absorbance at 405 nm from the blank media with the same amount of plasma were subtracted from those of BMMC supernatant measurements. The percentage of degranulation was calculated using the following formula: % Degranulation = $(OD_{supernatant} - OD_{blank}) / (OD_{supernatant} - OD_{blank} + OD_{Triton\ X\text{-}100}) \times 100$.

2.8. Statistical analyses

All data from mice and cell cultures were expressed as means ± SEM. Pearson's correlation test was used to detect the correlation between plasma histamine and triglyceride, cholesterol, or LDL concentrations. One-way ANOVA with post-hoc Bonferroni test was used for comparison among multiple groups. SPSS 22.0 was used for analysis, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. DSCG does not reduce cholesterol-free, HFD-induced obesity and diabetes

Results from Gutierrez et al. showed that the MC inhibitor DSCG did not affect body weight gain in WT mice fed a HFD that contains no cholesterol [4]. To avoid any possible inter-laboratory error, we

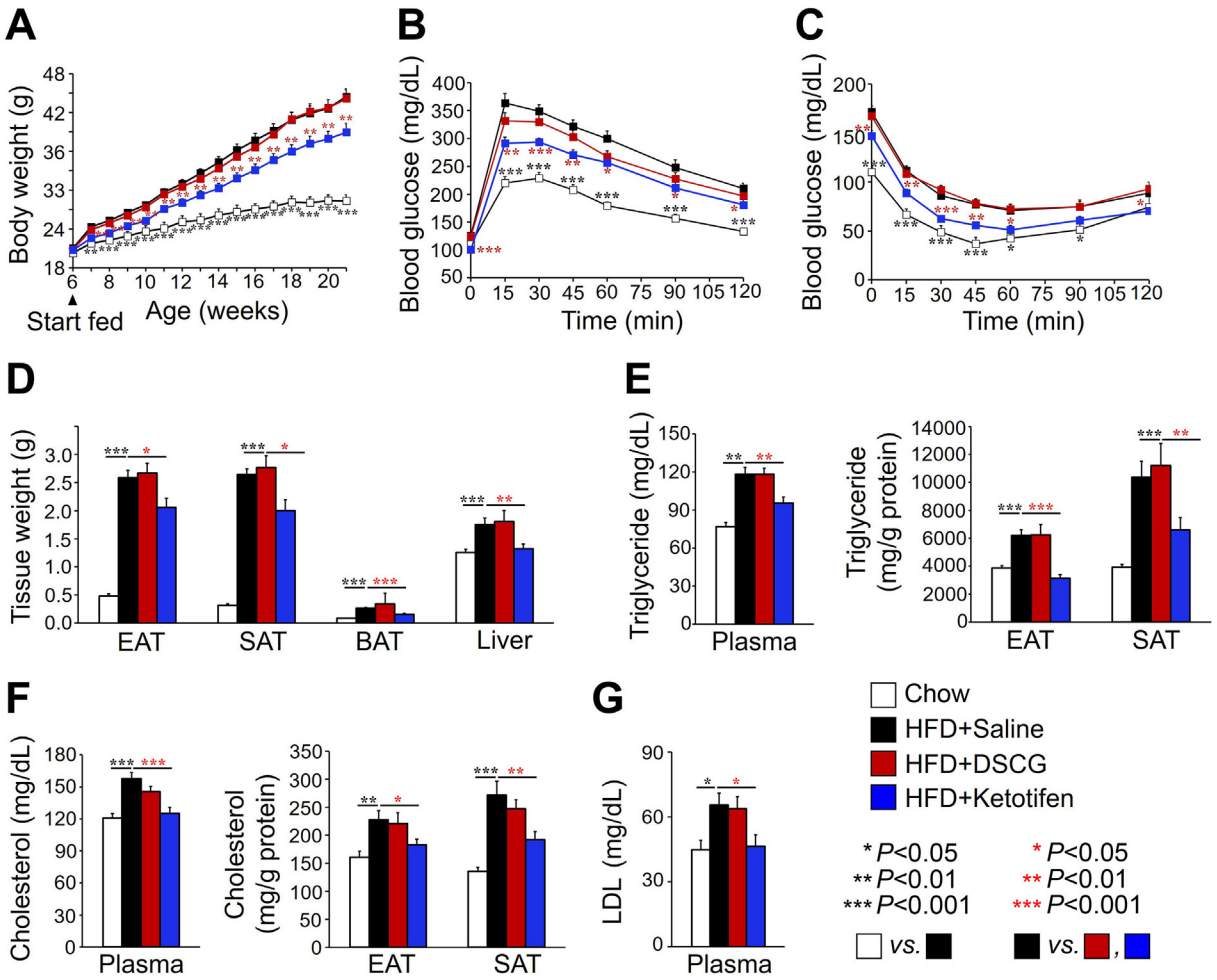

Fig. 1. Effect of HFD on DSCG- and ketotifen-mediated suppression of obesity and diabetes. Six-week-old male WT C57BL/6 mice consumed a chow diet and a cholesterol-free high fat diet (HFD) for 16 weeks, with daily i.p. administration of saline, DSCG (25 mg/kg/day) or ketotifen (20 mg/kg/day). Bodyweight gain (*A*), GTT (*B*) and ITT (*C*), EAT, SAT, BAT, and liver weight (*D*), plasma triglyceride levels, and EAT and SAT triglyceride levels (*E*), plasma total cholesterol levels, and EAT and SAT total cholesterol levels (*F*), and plasma LDL levels from chow- and HFD-fed mice (*G*). Data are mean ± SEM, n = 15 mice per group.

repeated these experiments and obtained the same results. When mice consumed a HFD, DSCG-treated mice showed no difference from saline-treated mice in body weight gain (Fig. 1A). Further, we also showed that DSCG- and saline-treated mice did not differ significantly in glucose tolerance (Fig. 1B), insulin sensitivity (Fig. 1C), EAT, SAT, BAT, and liver tissue weight (Fig. 1D), total triglyceride and cholesterol levels in plasma, EAT, SAT (Fig. 1E and F), and plasma levels of LDL (Fig. 1G). In contrast, mice that received ketotifen showed improved bodyweight gain (Fig. 1A), glucose tolerance (Fig. 1B), insulin sensitivity (Fig. 1C), and reduction of plasma and adipose tissue triglyceride and cholesterol (Fig. 1E–G).

3.2. DSCG and ketotifen reduce high cholesterol Western diet-induced obesity and diabetes

Differing from the study by Gutierrez et al. [4], we reported previously that both DSCG and ketotifen effectively prevented mice from obesity and diabetes and reduced disease progression in mice with pre-established diseases when mice were on a high-cholesterol Western diet [1]. To eliminate any possible inter-laboratory and reagent lot-to-lot variations, we repeated our earlier studies by using the WT mice from the Jackson Laboratory and the Western diet from Research Diets, Inc., and treated the mice with the exact same dose of DSCG and ketotifen, as shown in Fig. 1. Consistent with our prior study, when mice consumed a Western diet that contained 1.25% cholesterol, either DSCG or ketotifen reduced bodyweight gain (Fig. 2A), glucose intolerance (Fig. 2B), insulin insensitivity (Fig. 2C), EAT, SAT, BAT, and liver tissue weight (Fig. 2D), triglyceride and total cholesterol levels in plasma, EAT, SAT (Fig. 2E and F), and plasma LDL (Fig. 2G). These observations suggest that the discrepant results from Gutierrez et al. [4] and our earlier study [1] related to the different diets.

3.3. DSCG and ketotifen reduce cholesterol-supplied HFD-induced obesity and diabetes

HFD contains no cholesterol, whereas a Western diet contains 1.25% cholesterol. We hypothesized that such a cholesterol difference in diets accounted for the different results from HFD- (Fig. 1) and Western diet-fed mice (Fig. 2). We also hypothesized that DSCG and ketotifen both would show effective inhibitory activities in obesity and diabetes in mice fed a HFD if we increased the cholesterol levels in the HFD to the same levels as seen in a Western diet. To test this possibility, we modified the HFD by adding the same amount of non-modified cholesterol (1.25%) as in the Western diet without changing any other components (Table 1). In mice fed this modified diet (HFD + Cho), either DSCG or ketotifen reduced body weight gain (Fig. 3A), glucose intolerance (Fig. 3B), insulin insensitivity (Fig. 3C), EAT, SAT, BAT, and liver tissue weight (Fig. 3D), triglyceride and total cholesterol levels in plasma, EAT, SAT (Fig. 3E and F), and plasma LDL (Fig. 3G). The results from the mice fed a HFD, 1.25% cholesterol Western diet, and HFD

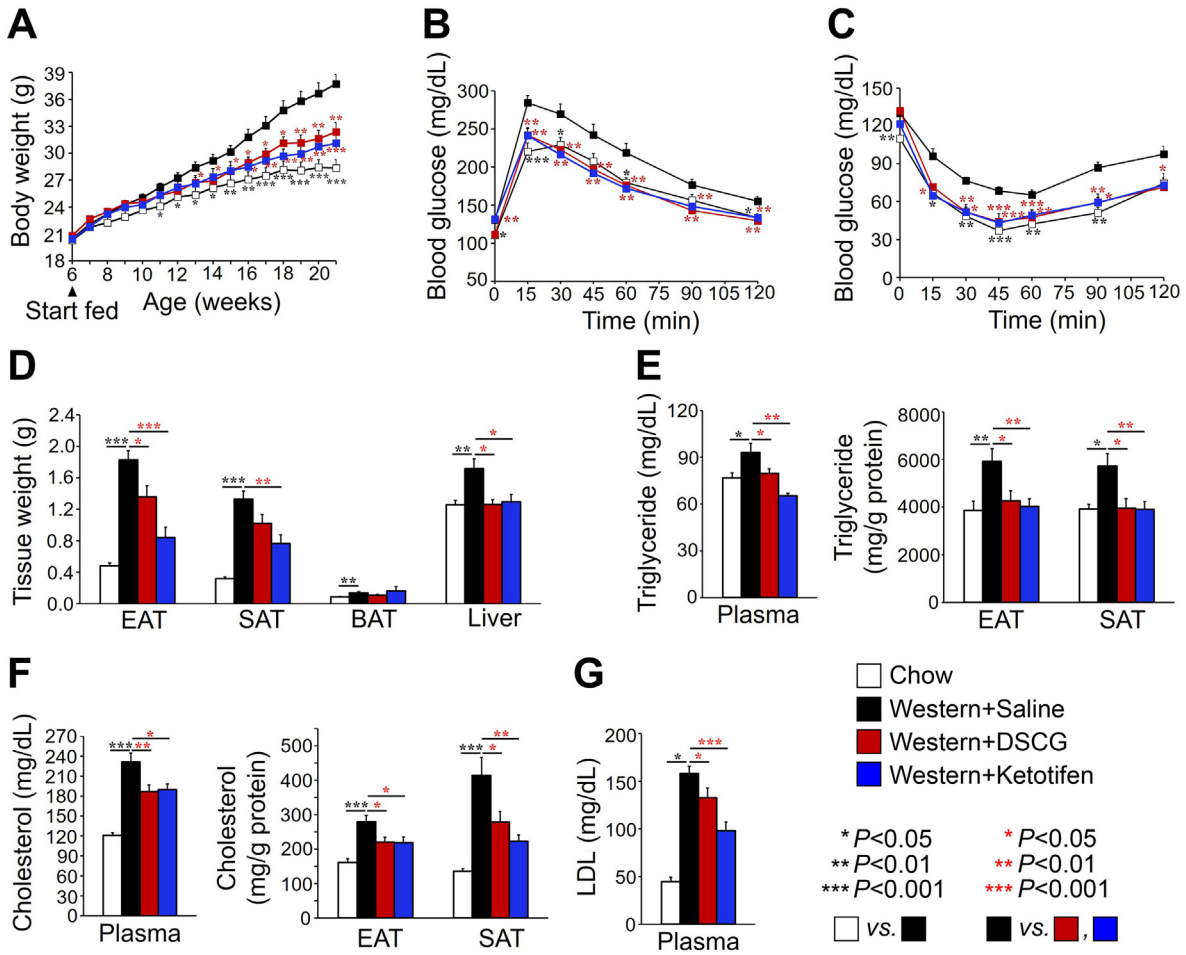

Fig. 2. Effect of Western diet on DSCG- and ketotifen-mediated suppression of obesity and diabetes. Six-week-old male WT C57BL/6 mice consumed a chow diet and a Western diet containing 1.25% cholesterol for 16 weeks, with daily i.p. administration of saline, DSCG (25 mg/kg/day) or ketotifen (20 mg/kg/day). Bodyweight gain (*A*), GTT (*B*) and ITT (*C*), EAT, SAT, BAT, and liver weight (*D*), plasma triglyceride levels, and EAT and SAT triglyceride levels (*E*), plasma total cholesterol levels, and EAT and SAT total cholesterol levels (*F*), and plasma LDL levels from chow- and Western diet-fed mice (*G*). Data are mean ± SEM, n = 15 mice per group.

supplemented with 1.25% cholesterol support the role of dietary cholesterol in regulating MC functions during diet-induced obesity and insulin resistance. Plasma FPLC analysis revealed similar observations. In HFD-fed mice, DSCG and ketotifen did not affect plasma LDL levels, although both inhibitors increased plasma HDL levels (Fig. 4A). In contrast, in mice fed a Western diet or the modified HFD + Cho, both DSCG and ketotifen reduced plasma LDL levels, although these inhibitors showed no effect on plasma HDL (Fig. 4B and C). Over the past two years, our groups located in Boston, USA, and Hefei, China independently made the same observations, although the data from Hefei, China, were not included.

3.4. The dietary differences affect MC activation differently

Data presented in Figs. 1 to 4 indicate that the dietary cholesterol contents affected the inhibitory activities of both DSCG and ketotifen. It is possible that the increased dietary cholesterol in both the Western and HFD + Cho diets increased MC activation in mice fed these diets, which may help contrast the inhibitory activities of DSCG and ketotifen. We performed toluidine blue staining to count the numbers of degranulated MCs in EAT. Mice fed a Western diet or HFD + Cho diet demonstrated many more degranulated MCs in EAT than those in mice fed a HFD (Fig. 5A–D). In EAT from HFD-fed mice, DSCG showed moderate activity likely because of the low levels of baseline MC activation, although ketotifen remained effective (Fig. 5A and D). In contrast, in EAT from Western diet- and HFD + Cho diet-fed mice, both DSCG and ketotifen were effective (Fig. 5B–D). Measurement of plasma histamine levels yielded a similar conclusion. Dietary cholesterol enhanced systemic MC activation as reflected by increased plasma histamine levels. The inhibitory activities of DSCG and ketotifen in Western diet- and HFD + Cho diet-fed mice were much more potent than those in HFD-fed mice, likely because of increased baseline MC activations in these mice (Fig. 5E).

Plasma cholesterol levels correlated with systemic MC activation. Pearson's correlation test revealed significant and positive correlations between plasma histamine and total cholesterol or LDL (Fig. 5F and G), although plasma triglyceride levels showed no correlation with histamine (Fig. 5H). This insignificance may be caused by the low level of MC activation in HFD-fed mice (Fig. 5A and E) and our limited number of mice in each group. Indeed, this association became stronger when only the Western diet-fed and HFD + Cho diet-fed mice were used ($r = 0.219$, $P = 0.058$).

3.5. The MC inhibitory activities of DSCG and ketotifen vary depending on the experimental conditions

The activities of DSCG in blocking the development of obesity and diabetes in mice fed a cholesterol-rich Western diet (Fig. 2) or a cholesterol-supplemented HFD + Cho diet (Fig. 3), but not in those fed a cholesterol-deficient HFD (Fig. 1), support a role of dietary cholesterol in MC activation. To test this hypothesis, we treated mouse BMMCs with plasma from mice fed varying diets, followed by measuring cell

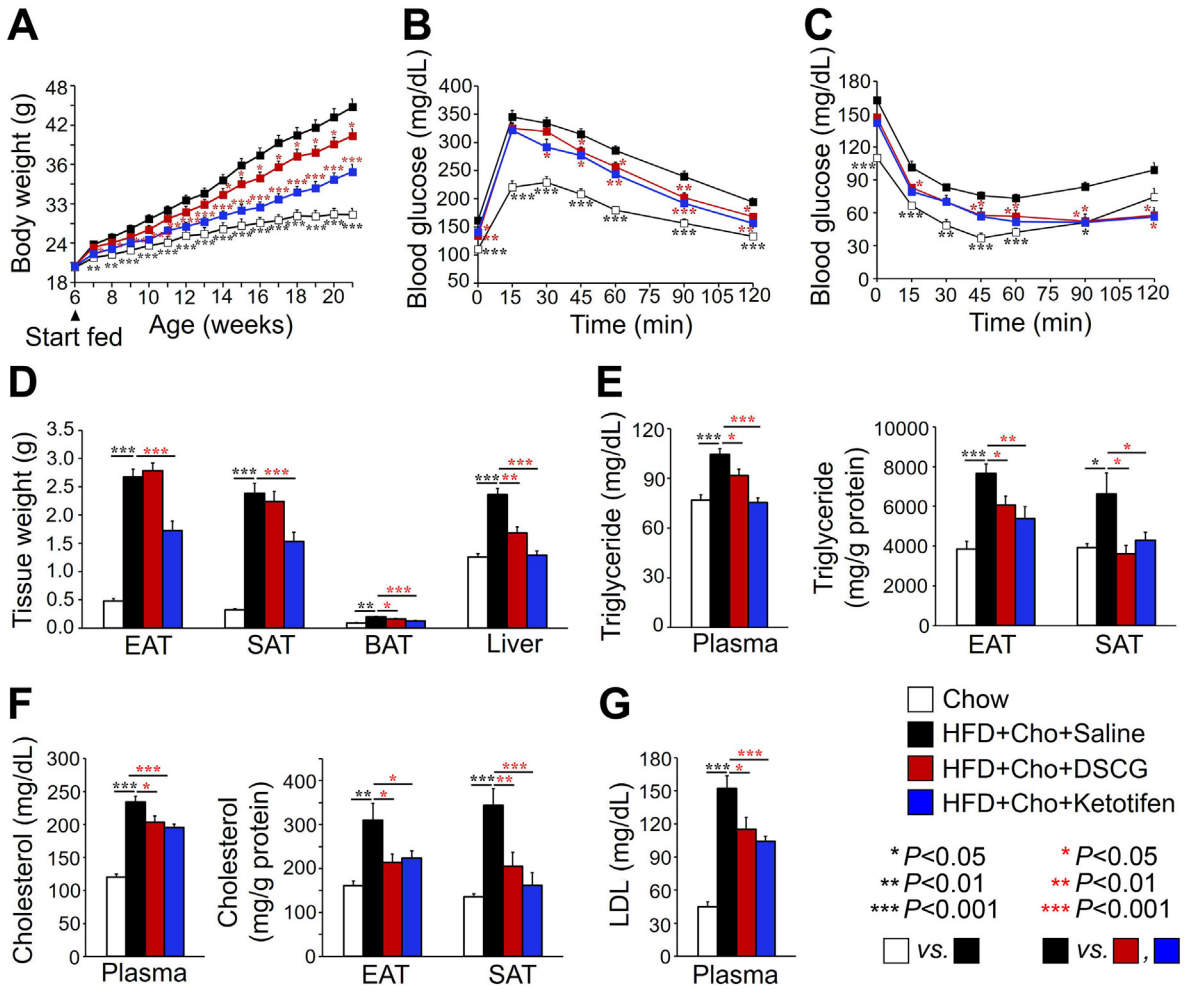

Fig. 3. Effect of cholesterol supplemented HFD on DSCG- and ketotifen-mediated suppression of obesity and diabetes. Six-week-old male WT C57BL/6 mice consumed a chow diet and HFD supplemented with 1.25% cholesterol (HFD + Cho) for 16 weeks, with daily i.p. administration of saline, DSCG (25 mg/kg/day) or ketotifen (20 mg/kg/day). Bodyweight gain (*A*), GTT (*B*) and ITT (*C*), EAT, SAT, BAT, and liver weight (*D*), plasma triglyceride levels, and EAT and SAT triglyceride levels (*E*), plasma total cholesterol levels, and EAT and SAT total cholesterol levels (*F*), and plasma LDL levels from chow- and cholesterol supplemented HFD-fed mice (*G*). Data are mean ± SEM, n = 15 mice per group.

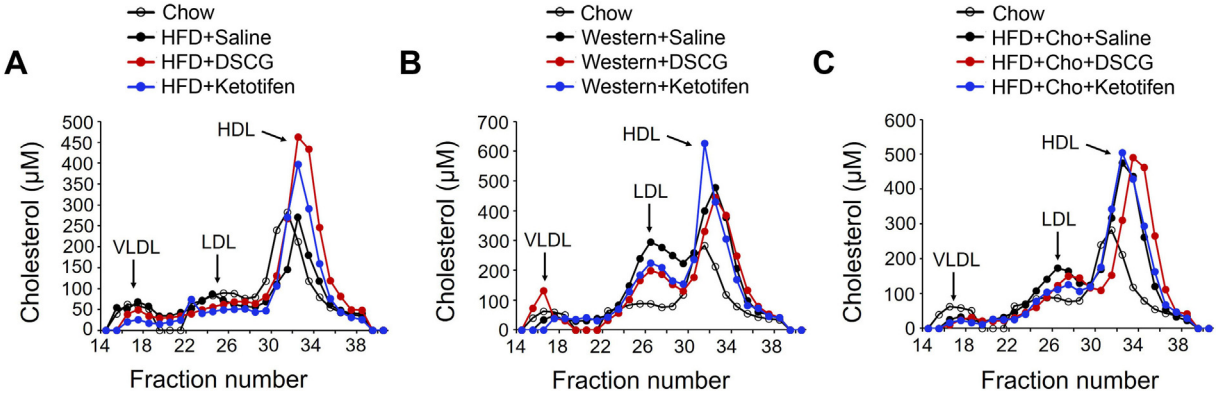

Fig. 4. Distribution of cholesterol in lipoprotein fractions based on particle size using FPLC from chow- and HFD-fed mice (A), chow- and Western diet-fed mice (B), and chow- and HFD + Cho diet-fed mice (C).

culture supernatant MC granule mediators β-hexosaminidase, histamine, and serotonin as the readouts of MC activation [24]. Among all these tested MC granule mediators, only plasma from mice fed a Western diet or a HFD + Cho diet increased BMMC release of these mediators, compared with the BMMCs treated with plasma from chow diet-fed mice. Under the same test conditions, however, plasma from HFD-fed mice behaved the same as that from chow diet-fed mice in BMMC activation (Fig. 6A–C).

Plasma LDL levels in chow diet-fed mice were around 45 mg/dL (450 μg/mL). These levels reached 700 μg/mL in HFD-fed mice (Fig. 1G) and 1500 μg/mL in Western diet- and HFD + Cho diet-fed mice (Figs. 2G and 3G). To test further the role of cholesterol in MC

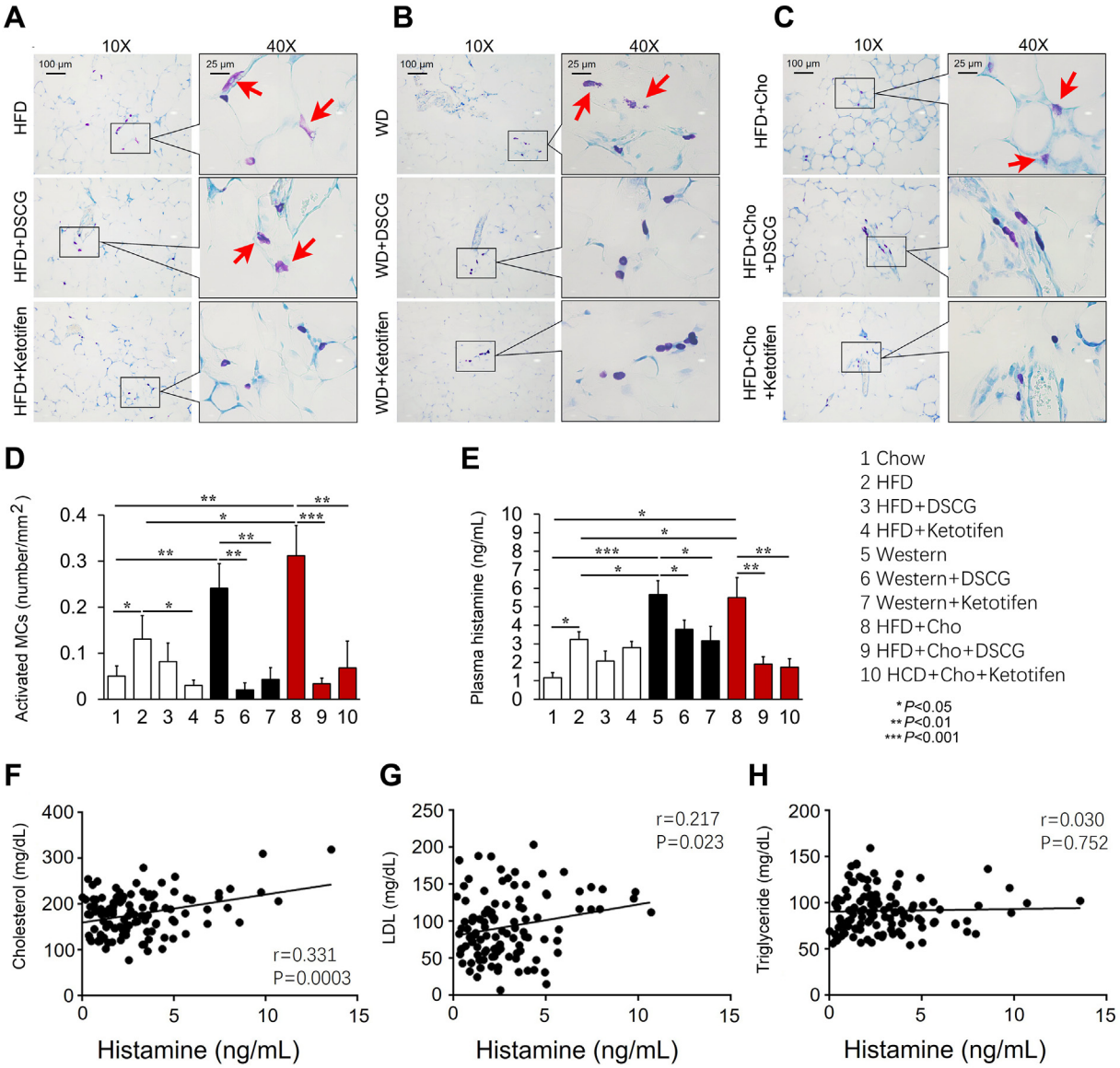

Fig. 5. Effect of different diets with or without mast cell stabilizers on mast cell activation. Toluidine blue staining for MCs in EAT from HFD with or without DSCG or ketotifen (A), western diet (WD) with or without DSCG or ketotifen (B), and HFD + Cho with or without DSCG or ketotifen (C) and activated (degranulated) MC number quantification (D). Plasma histamine level from different groups of mice as indicated (E). Pearson's correlation tests between plasma histamine and cholesterol (F), LDL (G), and triglyceride (H). Data are mean ± SEM, n = 10–15 mice per group.

activation, we exposed mouse BMMCs to graded amounts of commercial non-modified LDL according to the levels in mouse plasma from 10 to 1000 μg/mL with and without DSCG or ketotifen. LDL dose dependently induced the secretion of the MC granule β-hexosaminidase, histamine, and serotonin. LDL at 1000 μg/mL showed the highest activity in releasing all three tested granule mediators (Fig. 6D–F). Under 1000 μg/mL of LDL, both DSCG (100 nM) and ketotifen (100 nM) significantly reduced the LDL-induced secretion of these MC granule contents from WT BMMCs (Fig. 6G–I). In contrast, when BMMCs from LDL receptor-deficient ($Ldlr^{-/-}$) mice received the same stimuli, we did not detect LDL-induced MC activation or modulation by DSCG or ketotifen (Fig. 6J–L).

To compare the relative potency of MC activation by cholesterol and a reference MC activator IgE, we treated mouse BMMCs with 50 μg/mL of IgE as we have used previously [25], which was much higher than what we detected in the plasma from Western diet-fed mice (~300 ng/mL) [25,26]. DSCG and ketotifen from 0.1 to 10 μM displayed dose-dependent inhibition of IgE-induced BMMC secretion of β-hexosaminidase and histamine (Fig. 7A). DSCG also showed dose-dependent inhibition of IgE-induced MC release of serotonin. Yet, only a low dose of ketotifen (0.1 μM) showed significant inhibition of IgE-induced MC release of serotonin, likely because of the weak IgE activity in release MC serotonin (Fig. 7A, right panel), about ten-fold weaker than that of 1000 μg/mL LDL (Fig. 6F). Not only LDL (Fig. 6D–F), but also VLDL and HDL dose-dependently (10–1000 μg/mL) induced BMMC secretion of β-hexosaminidase, histamine, and serotonin (Fig. 7B), although such activities of VLDL and HDL were weaker than those of LDL (Fig. 6D–E). DSCG and ketotifen from 0.1 to 10 μM showed different levels of inhibition of β-hexosaminidase, histamine, and serotonin secretion from LDL-activated BMMCs, but negligible activities from VLDL- and HDL-activated BMMCs (Fig. 7C–E). Therefore, the MC inhibitory activities of DSCG and ketotifen depended on not only the inhibitor types and concentrations (DSCG and ketotifen), but also the types and concentrations of MC stimuli (IgE, LDL, VLDL, and HDL) and the types of MC granule molecules to be tested (β-hexosaminidase, histamine, and serotonin).

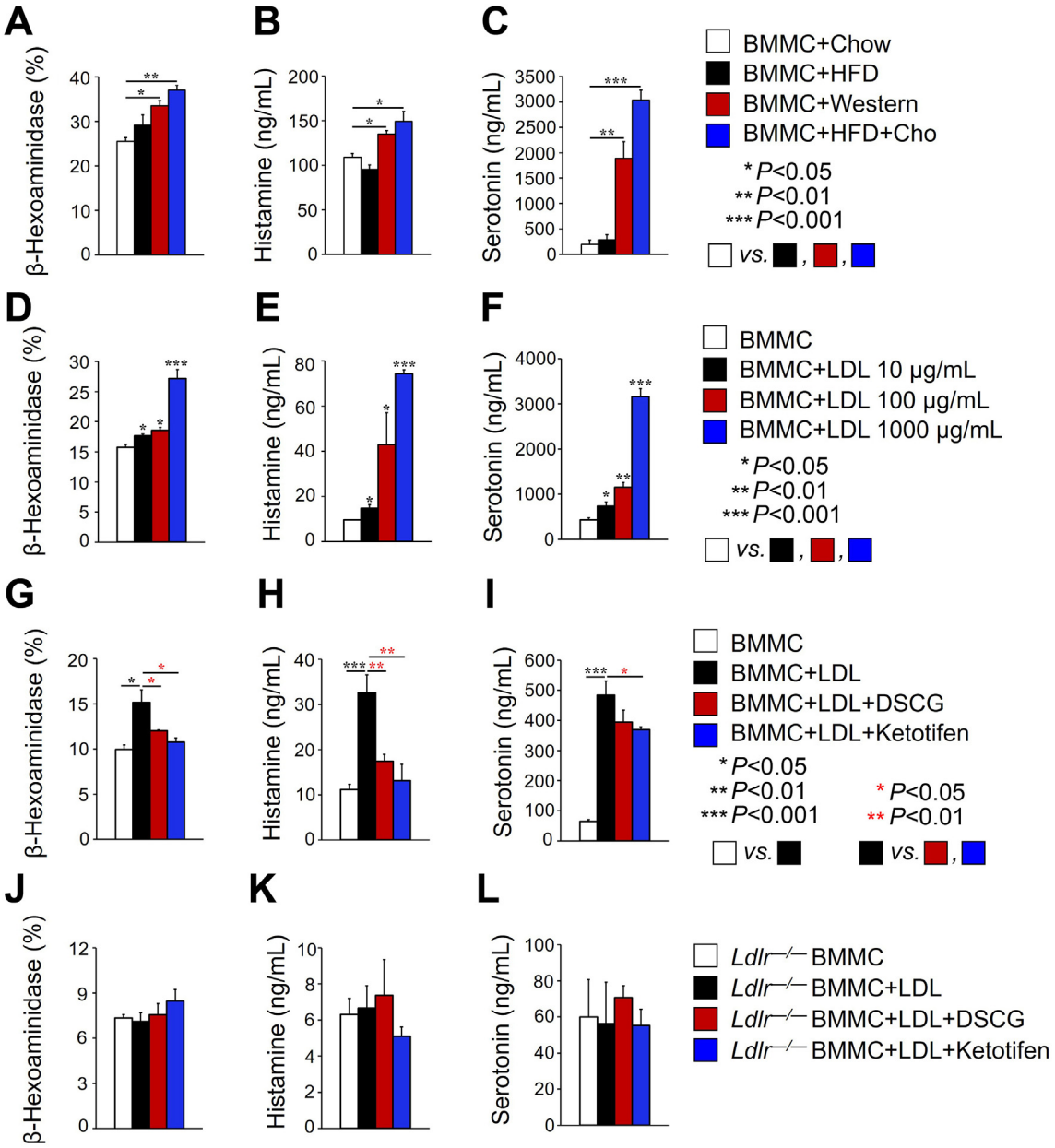

Fig. 6. Mast cell stabilizers inhibited LDL-induced MC activation. Release of β-hexosaminidase (A), histamine (B), and serotonin (C) from WT BMMCs treated with plasma from mice fed with different diets. Release of β-hexosaminidase (D), histamine (E), and serotonin (F) from WT BMMCs treated with different doses of LDL. Secretion of β-hexosaminidase, histamine and serotonin from WT (G–H) or *Ldlr*$^{-/-}$ (J–L) BMMCs after activation with LDL (1000 μg/mL) with and without DSCG (100 nM) or ketotifen (100 nM). Data are mean ± SEM of four data sets. *In vitro* data were a representative of at least three independent experiments.

4. Discussion

Published studies have consistently demonstrated increased MCs in adipose tissue from obese humans and mice [1,27,28]. Such accumulation of MCs in adipose tissue may not just serve as a signature of tissue inflammation. MCs are known to interact with macrophages [1,29], regulatory T cells (Tregs) [30,31], and group 2 innate lymphoid cells (ILC2) [32], suggesting other potential MC effects through cross-interactions with these immune cells when considering obesity and diabetes [33–35]. We report here that mice that consumed a cholesterol-free HFD demonstrated much weaker activity in local (adipose tissue) and systemic MC activation than those that consumed a high-cholesterol Western diet or a high cholesterol-supplemented HFD + Cho diet (Fig. 5A–G). We demonstrated that the plasma from mice that consumed a Western diet or the HFD + Cho diet was much more potent in MC activation than the plasma from HFD-fed mice (Fig. 6A). Such differences in dietary cholesterol intake may explain why MC-deficiency or MC inhibition by DSCG did not affect HFD-induced obesity and insulin resistance in some studies [4,7]. Low degree of MC activation may cause minimal contribution of MCs to HFD-induced obesity and diabetes. Diet-induced obesity and diabetes in HFD-fed mice may be MC-independent. In contrast, high cholesterol diet-fed mice showed much high levels of MC activation. The high activity of plasma from these mice in MC activation may affect the pathobiology of other immune cells, such as macrophages, Tregs, and ILC2 [1,29–35]. Therefore, MC function in these mice may become significant.

Adipose tissue is a major site for cholesterol storage. Over half of total body cholesterol resides within the adipose tissues in obesity [36–38]. In EAT and SAT from high cholesterol diet (Western or HFD + Cho)-fed mice, we detected about 300–400 mg of cholesterol

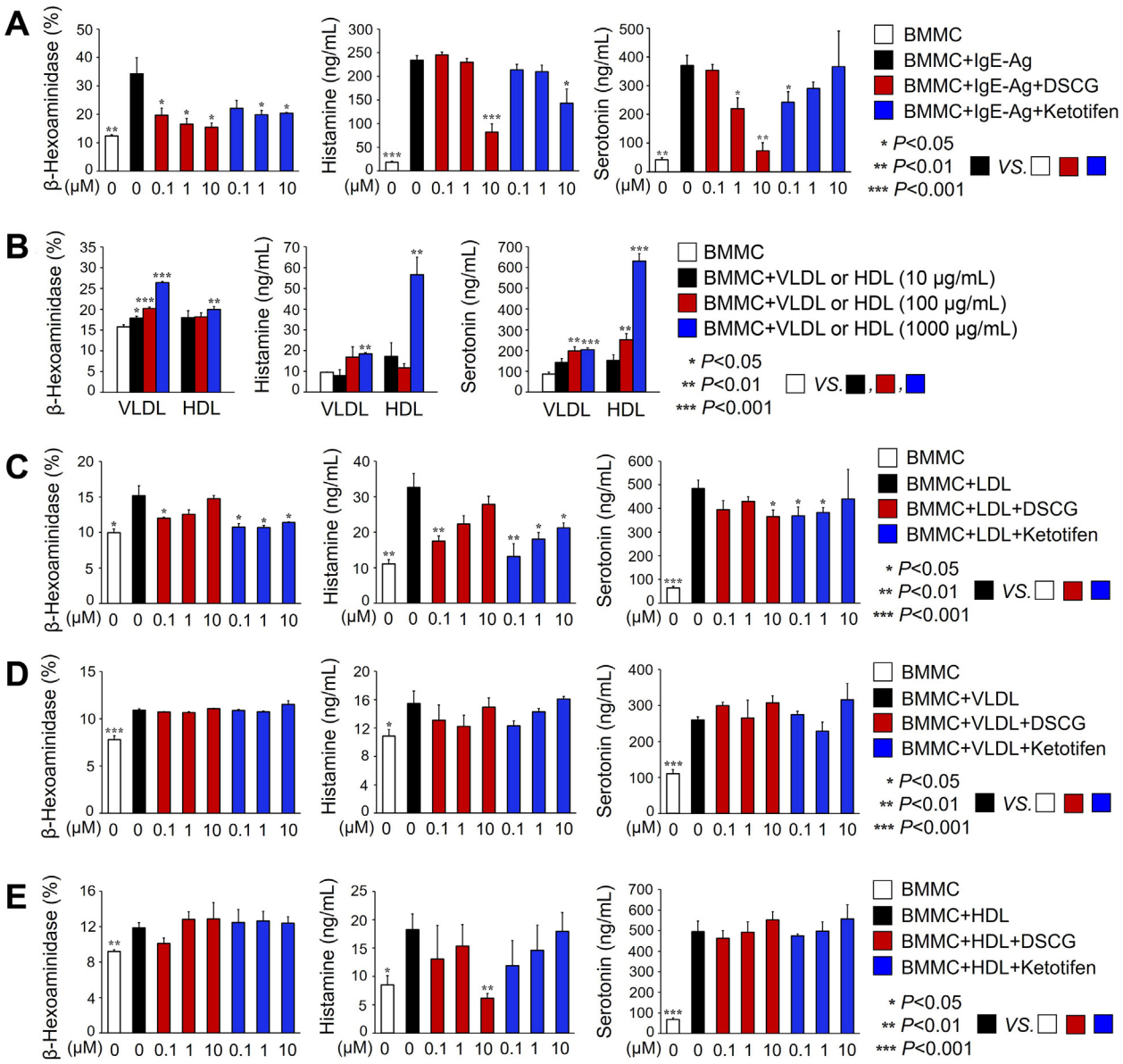

Fig. 7. Mast cell stabilizers inhibited IgE- and cholesterol-induced MC activation. Measurement of MC granule mediators (β-hexosaminidase, histamine, and serotonin) from WT BMMCs treated with 50 μg/mL of IgE (A), different doses of VLDL and HDL as indicated (B), 1000 μg/mL LDL (C), VLDL (D), and HDL (E) with and without different doses (0.1, 1, and 10 μM) of DSCG or ketotifen. Data are mean ± SEM of four data points. All data were a representative of at least three independent experiments.

per gram of tissue, whereas in the same tissue from HFD-fed mice, we detected about 100 mg cholesterol less (Figs. 1F, 2F, and 3F). Dietary cholesterol also affects adipocyte cholesterol uptake [15,39]. Although not tested in this study, adipocytes from high cholesterol diet (Western or HFD + Cho)-fed mice may contain much more intracellular cholesterol and become more potent in activating MCs than those from cholesterol-free HFD-fed mice. In addition to this possibility, dietary cholesterol may also aggravate adipose tissue macrophage accumulation in obese mice [40] or directly activate MCs [41]. Here, we showed that MCs produced similar amounts of β-hexosaminidase and serotonin in response to 1000 μg/mL LDL, HDL, and VLDL, but the same dose of LDL produced five-fold more serotonin than HDL and VLDL did (Figs. 6D–F and 7B). IgE is one of the best-known endogenous MC activators [42,43]. LDL at 1000 μg/mL produced similar levels of β-hexosaminidase from BMMCs to those treated with 50 μg/mL of IgE antibody (Figs. 6D and 7A), although IgE induced histamine production from BMMCs twice as much as those from LDL (Figs. 6E and 7A). However, 1000 μg/mL LDL produced nearly ten-fold levels of serotonin than 50 μg/mL IgE did (Figs. 6F and 7A). In Western diet- and HFD + Cho diet-fed mice, the plasma LDL levels reached about 1500 μg/mL (Figs. 2G and 3G), whereas plasma IgE levels reached about 300 ng/mL after following a Western diet [25,26], more than 150-fold lower than what we used here. Together, our observations from this study and those from earlier work indicate that, in high cholesterol diet-fed mice, cholesterol may be the major MC activator. Relative to the high plasma cholesterol, the IgE activity in MC activation may become negligible.

Both DSCG and ketotifen are MC inhibitors. From mice fed with all three types of diets, ketotifen showed much stronger inhibitory activities than DSCG in bodyweight gain, glucose tolerance, insulin insensitivity, and all other tested variables (Figs. 1–3). Yet, in cultured BMMCs, both DSCG and ketotifen showed comparable inhibitory activities on IgE- and LDL-induced releases of all tested MC mediators (β-hexosaminidase, histamine, and serotonin) (Figs. 6G–I, 7A, and C).

These observations suggest that ketotifen acts as more than just MC inhibitor. It may target other components in HFD-fed mice (Fig. 1). For example, ketotifen is a non-competitive histamine antagonist (H1-receptor) [44] and it may also target monocytes [45] and other untested inflammatory cells.

The recent development of Kit-independent MC-deficient models may advance the studies of MC pathobiology because these mice showed no significant impact of most other immune cells [7], unlike the Kit-dependent MC-deficient mice [2]. Based on our observations presented in this study, it appears that many studies from Kit-dependent models may remain valid. It is possible that Kit-independent MC-deficient *Cpa3*$^{Cre/+}$ mice [4] and *Mcpt5-Cre R-DTA*$^{+}$ mice [7] will be protected from cholesterol-rich, diet-induced obesity and diabetes, a hypothesis that merits careful investigation using different diets described in this study. Therefore, same experimental conditions remain essential to fairly evaluate results from different experimental models [46]. Together, this study revealed an essential role of dietary cholesterol in MC activation. High cholesterol diet increases the cholesterol level in adipose tissues, plasma, and possibly adipocytes and other cell types, and turns them into potent MC activators that may obscure the activities from other endogenous MC activators. Demonstrated MC activation is required in order to evaluate MC functions in disease models.

Author contributions

X.Z., Q.H., X.W., Z.D., J.L. (Jie Li), and X.Y. performed all mouse and cell culture experiments. M.J., J.M., and P.T.K. helped plasma cholesterol FPLC analysis. P.L., J.L., and G.P.S. were involved in experimental design, data discussion, and manuscript preparation. The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

The authors thank Ms. Chelsea Swallom for her editorial assistance. This work is supported by grants from the National Natural Science Foundation of China (31471320 and 31671485 to JL), the National Institutes of Health [HL080472 to PL, HL123568, HL60942, and AG058670 to GPS], and the RRM Foundations Charitable Fund to PL. Dr. Xian Zhang is supported by the American Heart Association Postdoctoral Fellowship # 18POST34050043.

Conflict of interest

None declared.

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