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Metformin Attenuates Early-Stage Atherosclerosis in Mildly Hyperglycemic Oikawa-Nagao Mice

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Aim: Although metformin treatment has been reported to reduce the risk of cardiovascular events in patients with type 2 diabetes, the underlying mechanisms have not been elucidated fully. Here we assessed atherosclerotic lesion formation in newly established 2 mouse lines with different blood glucose levels (Oikawa-Nagao Diabetes-Prone [ON-DP] and -Resistant [ON-DR]) to evaluate the effect of metformin on early-stage atherosclerosis.

Methods: Mildly hyperglycemic ON-DP and normoglycemic ON-DR female mice fed an atherogenic diet for 20 weeks (8–28 weeks of age). During the feeding period, one group of each mouse line received metformin in drinking water (0.1%), while another group received water alone as control. Atherosclerotic lesion formation in the aortic sinus was quantitively analyzed from the oil red O-stained area of the serial sections.

Results: Metformin treatment did not affect food intake, body weight, and casual blood glucose levels within each mouse line during the 20-week feeding period. Nevertheless, metformin treatment significantly reduced atherosclerotic lesion formation in the ON-DP mice (59% of control), whereas no significant effect of metformin was observed in the lesion size of the ON-DR mice.

Conclusion: Metformin can attenuate early-stage atherogenesis in mildly hyperglycemic ON-DP mice. Pleiotropic effects of metformin, beyond its glucose-lowering action, may contribute to the antiatherogenic property in the early-stage atherosclerosis.

Key words: Atherosclerosis, Diabetes, Impaired glucose tolerance, Metformin, Oikawa-Nagao mouse

Introduction

Epidemiological evidence has demonstrated consistently that patients with diabetes are more likely to suffer from cardiovascular disease[1)]. In addition to the patients with established diabetes, recent reports have pointed out increased cardiovascular risk in individuals with newly diagnosed type 2 diabetes or even those with prediabetic states (e.g., impaired glucose tolerance)[2, 3)]. Thus, expert committees state the necessity for the management of the patients with diabetes regarding the increased risk of macrovascular atherosclerotic events, as well as that of microvascular complications[4-8)].

Metformin (1,1-dimethylbiguanide hydrochloride) is now prescribed worldwide as a first-line oral hypoglycemic agent to manage type 2 diabetes. Given that the near-linear relationship between blood glucose level and cardiovascular event risk[9)], hypoglycemic effect *per se* of metformin should have a benefit for cardiovascular risk reduction. Besides the glycemic control-dependent benefit, laboratory investigations have suggested that glycemic control-independent "pleiotropic" action of metformin may also contribute to the cardiovascular risk reduction[10)]. However, the antiatherosclerotic potential of metformin has not been fully confirmed *in vivo*, as well as the uncertainty in the epidemiological results[11)].

Researchers have used several animal models for the study of atherosclerosis[12-15)]. Among them, geneti-

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cally modified hypercholesterolemic mice (e.g., apolipoprotein E- or LDL receptor-deficient mice) have been used predominantly in recent decade. However, extremely atherosclerosis-prone features of these mice, owing to their abnormally severe hypercholesterolemia, can mask the effect of blood glucose levels on the atherosclerosis process[16, 17]. Recently, we established 2 mouse lines with different susceptibilities to glucose intolerance: i.e., Oikawa-Nagao Diabetes-Prone (ON-DP) and -Resistant (ON-DR), formerly referred to as SDG-Prone and -Resistant, respectively[18-20]. The ON-DP mice have mild hyperglycemia (~40 mg/dL higher than the normoglycemic ON-DR mice in casual measurements). Additionally, compared to ON-DR, ON-DP showed accelerated atherosclerotic lesion formation without overt hypercholesterolemia[21].

Considering that intensive glycemic control with metformin early in the course of diabetes reduced subsequent cardiovascular events in the long-term follow-up of the United Kingdom Prospective Diabetes Study[22], the mildly hyperglycemic ON-DP mice would be a suitable model for understanding the effects of glycemic control (and also other interventions) on early-stage atherosclerosis in individuals with pre- and early-stage of type 2 diabetes. In this study, we assessed the effect of metformin on the early-stage atherosclerotic lesion formation in the ON-DP/DR mice.

Methods

Animals

Female ON-DP/DR mice (selectively bred from three inbred strains of C57BL/6J, C3H/HeJ, and AKR/N; formerly referred to as SDG-Prone/Resistant, respectively), bred at the Institute for Animal Reproduction (Kasumigaura, Japan), were used[20]. After they arrived at our animal facility at 5 weeks of age, the mice were fed an MF standard chow (Oriental Yeast, Tokyo, Japan) until 8 weeks of age. The mice of each line were assigned into two groups to avoid group differences within a line in body weight and single nucleotide polymorphisms in *Tlr4* and *Vcam1*; both of the polymorphisms are reported to affect atherosclerotic lesion formation in apolipoprotein E-deficient mice[23-25]. From 8 weeks of age, one group of each line received 0.1% metformin in drinking water (MET), while another received water alone as control (CON). The diet was also changed to an atherogenic diet (AD), containing 1.25% cholesterol, 0.5% sodium cholate, and 36% energy as fat (F2HFD1; Oriental Yeast), and maintained on the AD for 20 weeks. Every 4 weeks, food and drink intake, body weight, and random-fed blood glucose levels (at 16:00) were measured. Daily intake of food and drink per mouse was calculated from the 1-week consumption per cage. The mice were housed in standard plastic cages with paper chip bedding (3–5 mice/cage) and maintained in a temperature-controlled room (23 ± 1℃) with 14-h light (06:00–20:00)/10-h dark cycle. The Animal Care and Use Committee of Nippon Medical School reviewed and approved the study protocol (27-059).

Oral Glucose Tolerance Test (OGTT)

Glucose tolerance was evaluated by OGTT. Overnight-fasted mice were given a 20% glucose solution by oral gavage at 1 week before, 10 weeks after, and 19 weeks after the start of AD feeding (40, 50, and 60 mg glucose/mouse, respectively). Blood glucose levels were measured with a glucose sensor (Glutest Neo Super; Sanwa Kagaku Kenkyusho, Nagoya, Japan) before (0 min) and at 15, 30, 60, and 120 min after the glucose administration. An Ultra Sensitive Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Yokohama, Japan) was used to measure the plasma insulin levels at 0, 15, and 30 min.

Insulin Tolerance Test (ITT)

ITT was performed in the last week of AD feeding. After 6-h fast, insulin (Humulin R; Eli Lilly Japan, Tokyo, Japan) was injected intraperitoneally at 0.25 U/kg of body weight. Blood glucose levels before and at 15, 30, 60, and 90 min after the insulin injection were measured as described above.

Plasma Lipid and Cytokine Analyses

After the 20-week AD feeding, blood was collected from the inferior vena cava of each mouse under anesthesia. Total cholesterol, HDL cholesterol (sodium phosphotungstate-magnesium chloride precipitation method), and triacylglycerols in the blood plasma were measured with commercial kits (Wako Pure Chemical, Osaka, Japan). Non-HDL cholesterol level was calculated from the total and HDL cholesterol levels. Plasma insulin was measured by ELISA as described above. Concentrations of plasma adiponectin and monocyte chemoattractant protein-1 (MCP-1) were measured using a Mouse/Rat Adiponectin ELISA Kit (Otsuka Pharmaceutical, Tokyo, Japan) and a Mouse CCL2/JE/MCP-1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), respectively.

Quantification of Atherosclerotic Lesion Size

Atherosclerotic lesion in aortic sinus was quantitatively analyzed based on the method of Paigen *et al.*[26] with modifications[27]. After the heart and aorta were perfused *in situ* with saline, the thoracic aorta

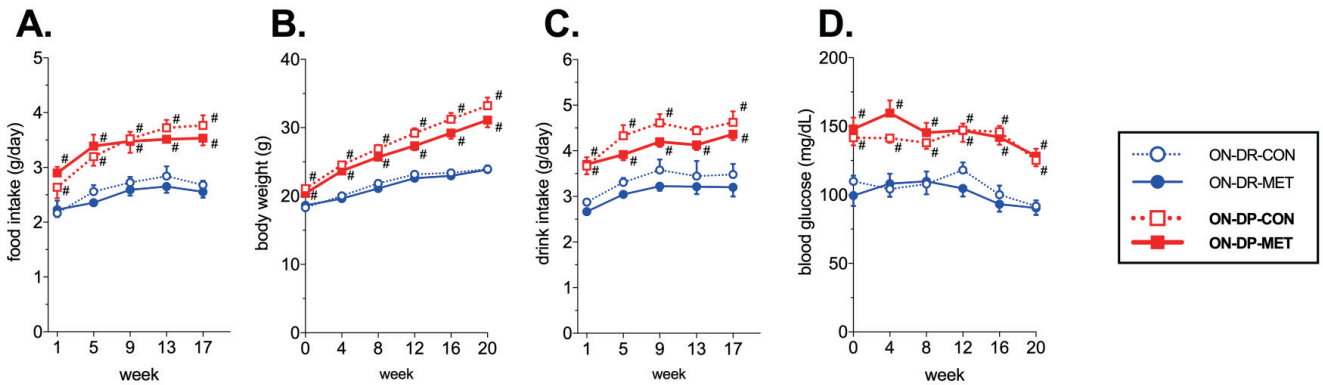

Fig. 1. Food intake (A), body weight gain (B), drink intake (C), and random-fed blood glucose levels (D)

ON-DR and -DP mice were given a drinking water without (CON) or with metformin (MET) during 20-week AD feeding. Values are expressed as mean ± SEM (A and C, n=5–7 cages; B and D, n=16–24 mice). $^{\#}P<0.05$ vs. ON-DR given the same drink.

was collected for gene expression analysis[27]. The heart was fixed with 4% formaldehyde in PBS and immersed in 30% sucrose/PBS. The upper part of the heart including aortic root was embedded in an OCT compound (Sakura Finetek, Tokyo, Japan). Frozen sections were cut from the left ventricular outflow tract until aortic valve cusps were observed. Thereafter, 45 serial cross sections (10-μm thick) were prepared. Every 5 of the sections (9 sections, each separated by 50 μm) were stained with oil red O, followed by hematoxylin counter stain. An observer who was not aware of the group allocation used Photoshop Elements software (Adobe Systems, San Jose, CA) to determine the oil red O-stained atherosclerotic lesion area from the photomicrograph images. The lesion area size of the nine sections for each mouse was averaged and expressed as the mean lesion size.

Statistical Analysis

Values are expressed as mean ± SEM. Values of $P<0.05$ by Student's t-test between CON and MET within a mouse line or between two mouse lines given the same drink (CON or MET) were considered statistically different.

Results

Food and Drink Intake, Body and Tissue Weight, and Plasma Lipid Profiles

The ON-DP mice showed higher food intake and greater body weight than the ON-DR mice as reported previously[21] (**Fig. 1A** and **B**). Drink intake was also higher at almost all time points in ON-DP compared to ON-DR (**Fig. 1C**). From the drink consumption and body weight, daily metformin intake in ON-DP-MET and ON-DR-MET was calculated to ~190 and ~150 mg/kg of body weight, respectively.

Table 1 shows tissue weight, plasma lipid profile, and plasma concentrations of insulin, adiponectin, and MCP-1 after the 20-week AD feeding. Compared to CON, MET had lower gonadal fat weight in ON-DP, whereas had higher liver weight in both mouse lines (**Table 1**). Drug-induced liver hypertrophy is often observed in rodents and considered an adoptive non-adverse response[28, 29]. However, further attention should be paid to the possible harmful effects of metformin related to the liver weight gain. No significant differences were observed in HDL cholesterol, non-HDL cholesterol, and plasma triacylglycerol concentrations between CON and MET in each mouse line (**Table 1**). Metformin treatment did not also affect plasma concentrations of insulin, adiponectin, and MCP-1 in each mouse line (**Table 1**).

Random-Fed Blood Glucose, Glucose Tolerance, and Insulin Sensitivity

Compared to the ON-DR mice, the ON-DP mice showed approximately 40 mg/dL higher random-fed blood glucose levels, whereas no significant differences were observed between CON and MET in each mouse line (**Fig. 1D**). In OGTT, ON-DP showed inferior glucose tolerance compared to ON-DR; however, no significant differences were observed in the blood glucose levels between CON and MET at any time point within a mouse line (**Fig. 2A–C**). Compared to the ON-DR mice, plasma insulin response to the glucose challenge tended to be lower in the ON-DP mice (**Fig. 2D–F**), most likely due to their inferior pancreatic islet insulin secretion capacity[19]. No significant differences were seen in the blood glucose levels in ITT between CON and MET in each mouse line (**Fig. 2G**).

Table 1. Relative tissue weight, plasma lipid profile, and plasma concentrations of insulin, adiponectin and MCP-1 after 20-week atherogenic diet feeding

	ON-DR		ON-DP	
	CON	MET	CON	MET
Tissue weight (mg/g body weight)				
Liver	74.1 ± 1.7	87.2 ± 3.4*	70.1 ± 1.4	82.1 ± 2.7*
Gonadal fat	12.7 ± 1.0	12.0 ± 1.0	34.4 ± 2.8#	26.0 ± 2.7*, #
Plasma lipids (mg/dL)				
HDL cholesterol	37 ± 4	39 ± 3	29 ± 2	27 ± 2#
Non-HDL cholesterol	196 ± 10	239 ± 19	230 ± 16	280 ± 21
Triacylglycerols	26 ± 2	26 ± 2	37 ± 3#	33 ± 3
Insulin (ng/mL)	0.57 ± 0.13	0.54 ± 0.09	0.63 ± 0.09	0.71 ± 0.11
Adiponectin (μg/mL)	18.1 ± 1.3	15.4 ± 0.8	24.6 ± 1.2#	23.4 ± 1.7#
MCP-1 (pg/mL)	112 ± 15	91 ± 9	96 ± 11	77 ± 5

Values are expressed as mean ± SEM (n = 16–24 mice for tissue weight and plasma lipids; n = 7–16 for Insulin, adiponectin and MCP-1). *$P < 0.05$ vs. CON within a mouse line. #$P < 0.05$ vs. ON-DR mice given the same drink.

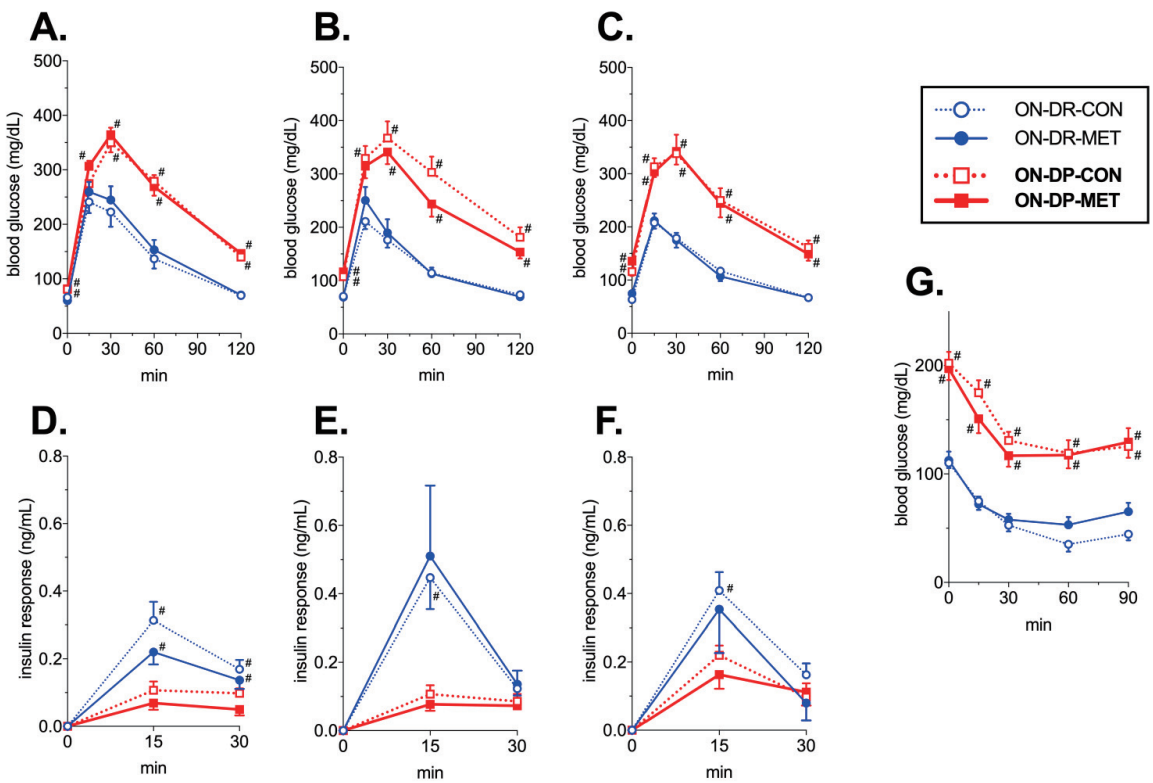

Fig. 2. Results of OGTT and ITT

Blood glucose levels (A–C) and insulin response (changes from baseline; D–F) in OGTTs at 1 week before (A and D), 10 weeks after (B and E), and 19 weeks (C and F) after the start of AD feeding. An ITT was performed at the last week of the feeding period (G). ON-DR and -DP mice were given a drinking water without (CON) or with metformin (MET) during the 20-week AD feeding. Values are expressed as mean ± SEM (n = 9–12 mice). #$P < 0.05$ vs. ON-DR given the same drink.

Atherosclerotic Lesion Formation

Lipid-laden plaque formation was observed in aortic sinus after the 20-week AD feeding (Fig. 3A–D). Consistent with our previous report[22], the early-stage atherosclerotic lesions of plaque formation were confined to the aortic sinus area but the lesion size in the ON-DP mice was approximately 4-fold greater than that in the ON-DR mice (Fig. 3E). The athero-

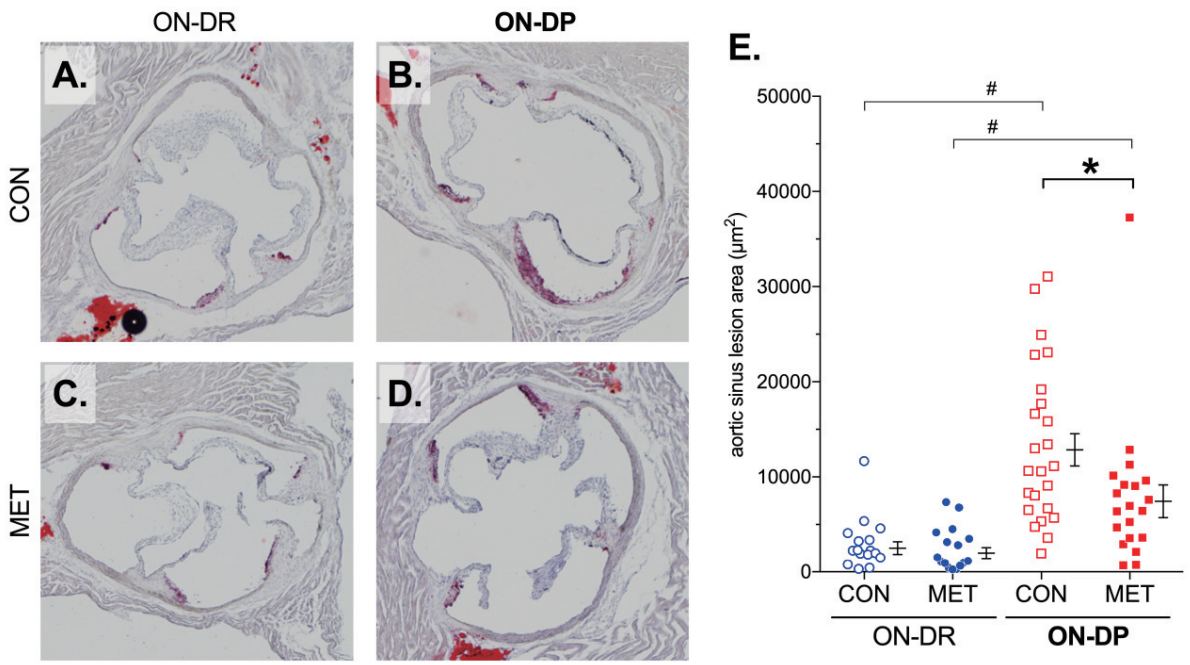

Fig. 3. Atherosclerotic lesion formation in aortic sinus

Representative photomicrograph images of oil red O-stained aortic sinus sections from ON-DR (A and B) and -DP (C and D) mice given a drinking water without (CON; A and C) or with metformin (MET; B and D). Quantitative analysis of the lesion size (E). Each dot indicates the mean of oil red O-stained lesion area in each mouse. Bars indicate mean ± SEM for each group of mice (n = 16–24 mice). *P < 0.05 within a mouse line. $^{\#}P$ < 0.05 between two mouse lines given the same drink.

sclerotic lesion size in MET was significantly decreased to be 59% of that in CON in the ON-DP mice, whereas no significant difference was shown in the lesion size between CON and MET in the ON-DR mice (**Fig. 3E**).

Discussion

Considering that metformin is a hypoglycemic agent, no differences in blood glucose levels between CON and MET suggest that the metformin treatment was insufficient at least for improving glycemic control in the present study. There have been inconsistent results in animal experiments regarding the hypoglycemic effect of metformin; however, most of previous studies in mice actually reported that chronic doses of metformin (in drinking water or diet) did not lower blood glucose levels at similar dose ranges to the present study (100–1000 mg/kg of body weight per day)[30-35]. In spite of the lack of significant differences in blood glucose levels, metformin treatment significantly attenuated atherosclerotic lesion formation in the ON-DP mice. The result suggests that orally administered metformin can exert its antiatherogenic effects *in vivo*, independent of its hypoglycemic property. From a clinical perspective, the attenuation of lesion formation in the mildly hyperglycemic ON-DP mice would be intriguing because the long-term follow-up of the United Kingdom Prospective Diabetes Study demonstrated a long-standing cardiovascular benefit after the end of intensive glycemic therapy with metformin[22]. The so-called "legacy effect" highlights the clinical benefit of metformin therapy for newly diagnosed patients with early-stage type 2 diabetes.

In the present study, we clearly demonstrated the antiatherogenic effect of metformin in mildly hyperglycemic ON-DP mice. However, as a limitation of the present study, the underlying mechanisms still remain elusive. To date, several mechanisms have been postulated to the "pleiotropic" antiatherosclerotic potential of metformin[10]. We have also tried to reveal the differences between CON and MET, e.g., plasma cytokine levels (**Fig. 1**) and several gene expression levels in thoracic aorta (**Supplementary Fig. 1**); however, we have not found any convincing differences yet. Since the present study focused on the early-stage of atherosclerosis development, the differences might be subtle compared to those shown in developed atherosclerosis (e.g., in apolipoprotein E-deficient mice)[35-37]. More detailed analysis, in sub-organ or cellular levels, may therefore be needed to reveal the underlying

mechanisms in the arterial wall and other tissues.

In addition to the possible pleiotropic effects, metformin might improve glycemic control in the present study at an undetectable level. As **Table 1** shows, metformin treatment reduced gonadal fat weight in ON-DP mice. Correspondingly, acute insulin sensitivity (within 30 min in ITT) in ON-DP-MET tended to be slightly improved compared to ON-DP-CON, though not reached significant difference (**Fig. 2G**). Additionally, post-challenge blood glucose levels were actually decreased by a single oral gavage of metformin (100 mg/kg of body weight) before a glucose challenge (**Supplementary Fig. 2**), whereas metformin treatment did not affect blood glucose levels in OGTT performed after an overnight fast (**Fig. 2A–C**). Since mice take water chiefly with food, metformin intake in the fasting period should be lower than that in *ad libitum* feeding (and drinking) conditions in MET. Therefore, metformin in the drinking water might attenuate postprandial (and post-drink) blood glucose fluctuations in *ad libitum*-fed MET. Taken together, considering the postulated involvement of insulin resistance and blood glucose fluctuations in atherosclerosis process[2, 3, 38, 39], improved glycemic control (at an undetectable level but for longer duration) might contribute to the attenuated lesion formation in ON-DP-MET. To reach conclusive evidence for the possible subtle effect on glycemic control for longer duration, more sophisticated approaches, e.g., continuous blood glucose monitoring, may be required.

In conclusion, the present results demonstrate that metformin can attenuate early-stage atherosclerosis in mildly hyperglycemic conditions *in vivo*. In spite of the over 60 years of clinical use with established efficacy and safety, the molecular mechanisms of metformin, not only for its pleiotropic antiatherosclerotic effects but also for its hypoglycemic actions *per se*, are still under debate[10, 40, 41]. Additionally, although a number of etiological factors have been postulated in diabetes-accelerated atherosclerosis, the underlying mechanisms have not been fully elucidated *in vivo*[42]. Thus, the present results warrant further study in ON-DP mice on the underlying mechanisms and the efficacy of therapeutic strategies, including metformin treatment, for preventing atherosclerotic cardiovascular disorders in pre- and early-stage of type 2 diabetes.

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None of the authors have any conflicts of interest associated with this manuscript.

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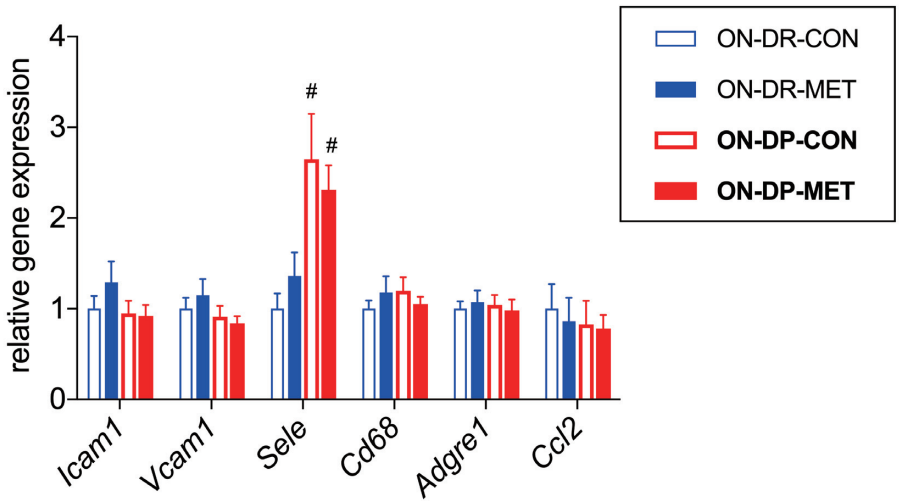
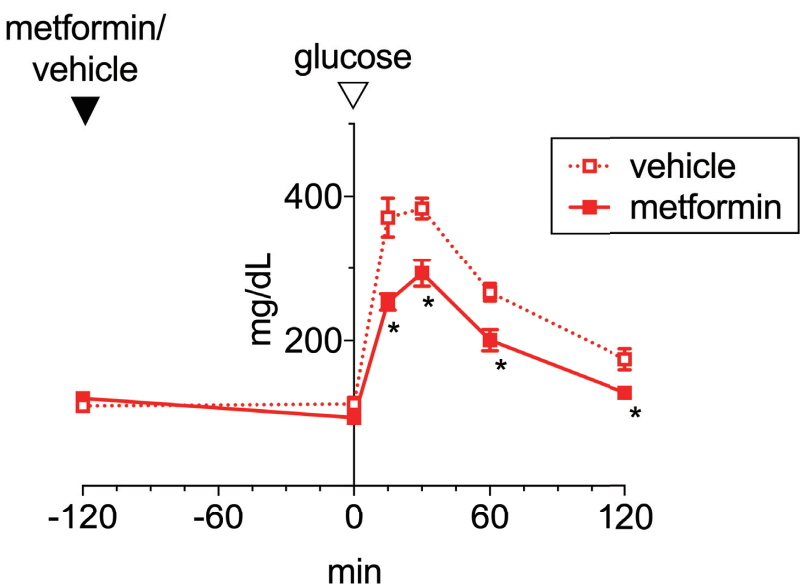


Supplementary Fig. 1. Relative gene expression levels in thoracic aorta

ON-DR and -DP mice were given a drinking water without (CON) or with metformin (MET) during 20-week AD feeding. Gene expression levels were calculated by the ΔΔCt method with *Actb* (β-actin) used as an internal control. Values are expressed as mean ± SEM (n=16–24 mice). *Icam1*, intracellular adhesion molecule-1; *Vcam1*, vascular cell adhesion molecule-1, *Sele*, E-selectin; *Adgre1*, adhesion G protein-coupled receptor E1 (F4/80); *Ccl2*, C-C motif chemokine ligand 2 (MCP-1). No significant differences were observed between CON and MET within a mouse line. $^{\#}P<0.05$ vs. ON-DR given the same drink.



Supplementary Fig. 2. Effect of a single oral dose of metformin on post-glucose challenge blood glucose levels in ON-DP mice

Metformin (100 mg/kg body weight) or vehicle (water) alone were administered to female ON-DP (29–30 weeks of age) by oral gavage at 120 min before an oral glucose challenge (2 g/kg body weight). Blood glucose levels were measured before the metformin (–120 min) and glucose (0 min) doses, and 15, 30, 60, and 120 min after the glucose dose. Values are expressed as mean ± SEM (n=8 mice of a crossover trial performed with 1-week interval). $^{*}P<0.05$ vs. vehicle (paired t-test).