Metformin, but Not Rosiglitazone, Attenuates the Increasing Plasma Levels of a New Cardiovascular Marker, Fibulin-1, in Patients With Type 2 Diabetes

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OBJECTIVE

The extracellular matrix protein fibulin-1 is upregulated in the arterial wall in type 2 diabetes (T2D) and circulates in increased concentrations in diabetes. Metformin is an antidiabetic drug with beneficial cardiovascular disease effects in diabetes. We hypothesized that metformin would influence the increased level of plasma fibulin-1 in diabetes.

RESEARCH DESIGN AND METHODS

After a 4-week run-in period, 371 eligible patients with T2D were randomized to treatment groups in a factorial design including insulin alone (control), +metformin, +rosiglitazone, or +both metformin and rosiglitazone. Plasma fibulin-1 was analyzed at the beginning of the study and after 18 and 24 months.

RESULTS

Plasma fibulin-1 increased in all groups throughout the 2-year period; however, the increase was strongly attenuated among patients treated with metformin. A highly significant difference was observed when the mean change in plasma fibulin-1 was compared between metformin- and non–metformin-treated individuals both at 18 and 24 months of treatment, but rosiglitazone had no effect. Metformin and rosiglitazone alone reduced the HbA_{1c} levels to comparable levels and in combination even further.

CONCLUSIONS

Metformin attenuates the increase in plasma fibulin-1 concentrations in T2D, independently of glycemic effects. Changes in fibulin-1 may reflect an important element in diabetic arteriopathy that can be influenced by metformin.

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Increased mortality and morbidity owing to a higher incidence of cardiovascular diseases (CVDs) is a major clinical challenge in type 2 diabetes (T2D) (1,2). Several studies have demonstrated that management of hypertension, LDL cholesterol, and other major CVD risk factors have reduced micro- and macrovascular complications in patients with T2D; however, optimal risk reduction is still not achieved when these important risk factors are controlled, indicating that further attempts are needed to control the CVD risk burden (3–5). One assumption is that improvement

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of the glycemic status will reduce risk; however, whether this is correct, and if different antidiabetic drugs perform differently, is still not clear. One obstacle for the judgment of treatment efficacies has been the lack of useful markers for the improvement of specific elements in the arterial damage induced by diabetes.

In the UK Prospective Diabetes Study (UKPDS), it has been shown that metformin significantly reduces macrovascular events in T2D (6) and, impressively, that this effect is sustained for 10 years (7). This result is assumed to be related to metformin's well-known ability to reduce plasma glucose due to increased insulin-stimulated glucose uptake in peripheral tissue, decreased glycogenolysis, and suppressed hepatic gluconeogenesis (8). However, it has also been suggested that the effect of metformin is at least partly independent of its blood glucose–lowering effect (9). This idea is compatible with a recent study that found beneficial effects on cardiovascular outcome in T2D patients with coronary artery disease on metformin compared with glipizide, despite similar effects on HbA_{1c} (10). The mechanisms by which metformin exerts such putative beneficial cardiovascular effects are unclear but have been suggested to include an ability to reduce inflammatory and coagulative activities and to reduce the expression of vascular adhesion molecules as well as improve endothelial function and fibrinolysis (11–15). These explanations are mainly based on data from experimental animal and in vitro studies. A few results from human studies have, however, indicated that there is an effect of metformin treatment on endothelial markers and this effect may be independent of the effects on the glycemic status (16).

The arterial pathology in diabetes comprises both preatherosclerotic, generalized vascular changes (endothelial dysfunction, extracellular matrix [ECM] modifications and accumulations, and calcifications) as well as increased occurrence of atherosclerotic plaques. We have recently identified the ECM gene product, fibulin-1 mRNA, as the most upregulated transcript in nonatherosclerotic arterial tissue from patients with T2D (17). In the initial presentation, we found higher amounts of fibulin-1 protein both in the arterial wall and in the circulation among patients with T2D. Moreover, we demonstrated an association between increased levels of plasma fibulin-1 and arterial stiffness, blood pressure, and glycemic status in a cohort of 305 well-described patients with T2D (18). These results, concerning relations between plasma fibulin-1, glycemic status, and hypertension, have been confirmed in three subsequent studies (19–21). In the initial paper, we also presented a 15-year follow-up study, showing that high plasma fibulin-1 concentrations among patients with T2D were independently predictive of mortality in T2D with a hazard ratio similar to that of plasma cholesterol (18). In the initially investigated diabetic patients, we observed that individuals treated with metformin had reduced fibulin-1 levels (18). Taken together, our previous results suggested that fibulin-1 is involved in arterial disease in T2D, and that the circulating concentration may serve as a biomarker for vascular ECM changes as part of diabetic arteriopathy. Fibulin-1 is a member of the fibulin family (22) and was identified by Argraves et al. (23) in 1989. It is an ECM glycoprotein found in high concentrations in blood vessels, lungs, and skin in association with elastic fibers, fibronectin microfibrils, and some basement membranes. Fibulin-1 is additionally present in high concentrations in plasma (24). Fibulin-1 knockout mice die early in utero with signs of endothelial and cardiovascular malformations (25,26); however, its exact molecular role is not known.

In the current study, we hypothesized that the vasculoprotective actions of metformin could include changes in the arterial cells and their surroundings, which may be reflected by alterations in the concentration of the new diabetes marker, fibulin-1. We therefore investigated plasma fibulin-1 concentrations in a previously published randomized trial, where a factorial design was used to explore effects of randomized treatments with metformin and rosiglitazone.

RESEARCH DESIGN AND METHODS

Subjects

Patients between 30 and 70 years of age with T2D were admitted to eight hospital centers participating in the South Danish Diabetes Study (SDDS) conducted between January 2003 and July 2006 (27). Eligibility criteria included BMI >25 kg/m^2, fasting plasma C-peptide >300 pmol/L, HbA_{1c} $>7\%$ (53 mmol/mol), diagnosis of T2D at least 2 years before participation, and treatment for at least 3 months with stable doses of oral antidiabetics and/or insulin. Exclusion criteria included impaired renal function, congestive heart failure, and known intolerance to metformin or rosiglitazone. The study was approved by the regional committee on biomedical research ethics (M-2417-02) and performed in accordance with the Declaration of Helsinki. All subjects provided written informed consent.

Study Design and Setting

The study is a prospective, randomized, and partly placebo-controlled trial with 450 patients included. After a 4-week run-in period, 371 eligible patients were randomized to one of eight treatment groups in a factorial design with NPH insulin versus insulin aspart, metformin versus placebo, or rosiglitazone versus placebo, as previously described (27). The factorial design made it possible, in the present paper, to present the combined results from groups treated with the two different insulins, since these treatment modalities did not influence plasma fibulin-1. Consequently, the results from four groups (presented in Table 1) are therefore reported as insulin alone (i.e., subjects only treated with insulin), +rosiglitazone (i.e., subjects treated with insulin and rosiglitazone), +metformin (i.e., individuals treated with insulin and metformin), and +Rosi +Met (i.e., subjects treated with insulin and both rosiglitazone and metformin in combination).

Intervention

Metformin or placebo was administered as one 500-mg tablet twice a day during the first 4 weeks succeeded by two tablets twice daily. Rosiglitazone or placebo was given as one 4-mg tablet

Table 1—Baseline values of the four study groups

	Insulin alone	+Rosiglitazone	+Metformin	+Rosi+Met
BMI (kg/m^2)	34 ± 0.6	33 ± 0.5	35 ± 0.7	34 ± 0.6
Body weight (kg)	100 ± 1.9	98 ± 1.6	103 ± 1.9	100 ± 1.8
Sex (female/male)	38/56	38/55	36/54	30/64
Age (years)	56 ± 0.8	57 ± 0.9	56 ± 0.9	56 ± 0.9
Diabetes duration (years)	8.5 ± 0.5	9.5 ± 0.7	8.6 ± 0.4	8.8 ± 0.6
HbA_{1c} (%) (mmol/mol)	8.6 ± 0.1 (70 ± 1.2)	8.6 ± 0.1 (70 ± 1.3)	8.6 ± 0.1 (70 ± 1.5)	8.7 ± 0.1 (72 ± 1.4)
Systolic blood pressure (mmHg)	145 ± 1.6	147 ± 2	145 ± 2	145 ± 2
Diastolic blood pressure (mmHg)	83 ± 0.9	85 ± 0.9	85 ± 1.1	84 ± 1
Pulse pressure (mmHg)	61 ± 1.4	62 ± 1.6	60 ± 1.5	60 ± 1.6
HDL (mmol/L)	1.3 ± 0.03	1.3 ± 0.05	1.3 ± 0.04	1.2 ± 0.03
LDL (mmol/L)	2.8 ± 0.1	2.8 ± 0.09	3 ± 0.1	2.7 ± 0.09
Total cholesterol (mmol/L)	5 ± 0.1	4.9 ± 0.1	5.2 ± 0.1	4.7 ± 0.1
Plasma triglycerides (mmol/L)	2.2 ± 0.1	2.2 ± 0.2	2.6 ± 0.3	2.1 ± 0.1
Plasma FFA (mmol/L)	0.58 ± 0.03	0.52 ± 0.03	0.54 ± 0.02	0.55 ± 0.03
Plasma fibulin-1 (μg/mL)	46 ± 1.3	47 ± 1.3	47 ± 1.3	45 ± 1.2

There were no significant differences between the insulin alone group and any treatment groups. Data are presented as means ± SEM. Student *t* test for nonpaired data was used.

per day for the first 4 weeks succeeded by one tablet twice a day.

Clinical and Biochemical Measurements

Blood was drawn for HbA_{1c} and plasma measurements at baseline and after 1 and 2 years. HbA_{1c} was measured every 3 months by cation exchange chromatography using Tosoh G7 (Medinor, Broendby, Denmark). Total cholesterol, LDL, HDL, and triglycerides were measured on a Modular Analytics P instrument (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Plasma fibulin-1 was measured with a sandwich immunoassay as previously described (18). The plasma concentration of metformin was determined by a validated high-performance liquid chromatography method (28). The limit of quantification for metformin in plasma was found to be 10 ng/mL. The limit of detection for metformin in plasma was found to be 5 ng/mL. The intra- and interday precision did not exceed 7.5% for plasma.

Statistical Analysis

Analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL), and GraphPad Prism 5 (GraphPad Software, La Jolla, CA) was applied to draw figures. Data from the groups of subjects receiving either insulin alone (control), metformin, rosiglitazone, or a combination of metformin and rosiglitazone were first analyzed by one-way ANOVA and then with unpaired Student *t* test. Bivariate linear correlations were calculated by determining the Pearson correlation coefficient. $P < 0.05$ was considered significant in all analyses. Data are presented as untransformed means ± SEM.

RESULTS

The HbA_{1c} results for all eight treatment groups have previously been reported (27). In Table 1, we present baseline patient characteristics for the four treatment groups, which are the main focus in this investigation, i.e., patients treated with insulin only (NPH or aspart, control), metformin only, rosiglitazone only, or a combination of metformin and rosiglitazone. We focus on these four groups and the effect of treatment with either NPH or aspart insulin is not considered, since we observed no differences between these two insulin treatment modalities in relation to plasma fibulin-1 and since no interference in the presented data occurred when insulin treatment was included (data not shown). There were no differences at baseline in clinical parameters between the four groups, as presented in Table 1. The percentage of patients receiving medications other than antidiabetics is reported in Table 2. There were no statistical differences between drug use in the four groups and no influence of any drug on plasma fibulin-1 concentration. No differences in any groups were observed when considering delta mean values (the difference between baseline and 2 years) of LDL, triglycerides, and free

Table 2—Percentage of patients treated with different medications

	Insulin alone	+Rosiglitazone	+Metformin	+Rosi+Met
Antihypertensives (RAAS-related)				
Angiotensin inhibitors	67	74	70	68
ACE inhibitors	51	58	64	53
Angiotensin II R inhibitors	30	34	26	23
Other antihypertensives				
CA antagonists	23	18	36	29
Diuretics	62	55	69	59
β-Blockers	23	25	23	22
Statins	70	68	68	70
Acetylsalicylic acids	44	37	33	46

RAAS, renin-angiotensin-aldosterone system.

fatty acid (FFA) (Table 3). HDL cholesterol increased in both groups receiving rosiglitazone (Table 3). No differences were observed in delta mean values of systolic and diastolic blood pressures, as well as pulse pressures. BMI and body weight were significantly increased in the rosiglitazone group compared with the control group (Table 3). Weight gain was reduced in the patients receiving the combined therapy compared with those receiving rosiglitazone alone (Table 3). The mean delta values of HbA_{1c} were significantly lowered in both the rosiglitazone alone and the metformin alone groups (~0.5%) and further reduced in the rosiglitazone and metformin combination group (~1%) (Table 3 and Fig. 1*B*).

There were no statistically significant differences in plasma fibulin-1 among the four groups at baseline. The concentration of fibulin-1 correlated weakly but significantly or borderline significantly to age, HbA_{1c}, and pulse pressure at baseline in the entire population (age: $r = 0.22$, $P < 0.001$; HbA_{1c}: $r = 0.10$, $P = 0.080$; pulse pressure: $r = 0.14$, $P = 0.006$). As can be seen in Fig. 1*A*, the plasma concentration of fibulin-1 increased significantly in the control group after 1 and 2 years and in the group treated with rosiglitazone only. In contrast, the plasma fibulin-1 concentration did not change with time in the two groups receiving metformin either alone or in combination with rosiglitazone. This difference resulted in significantly lower plasma fibulin-1 concentrations after 2 years in the two metformin-treated groups compared with the two groups not treated with metformin (Fig. 1*A*). There was no correlation between delta values of plasma fibulin-1 and delta values of HbA_{1c} in any treatment groups. Likewise, no correlations were observed between delta values of plasma fibulin-1 and baseline characteristics or delta values of lipid or blood pressure values.

Bivariate correlation analysis demonstrated a significant negative relationship between delta values of plasma fibulin-1 (2-year value minus baseline value) and the mean trough steady-state plasma concentration of all metformin-treated patients at the 1-year time point ($r = -0.26$, $P = 0.001$), i.e., high metformin concentration was associated with a low increase or even a decrease in plasma fibulin-1.

CONCLUSIONS

In this 2-year, investigator-driven, prospective, randomized, partly blinded trial (27), we tested the hypothesis that metformin and/or rosiglitazone modulates the level of plasma fibulin-1 in patients with T2D. We found a significant fibulin-1–lowering effect of metformin, but not of rosiglitazone. This finding is in accordance with results from a previous observational study where we observed lower plasma fibulin-1 concentrations among metformin-treated T2D patients (18). We find that the metformin-induced effect on plasma fibulin-1 is probably not mediated by improved glycemic status, since no effect on fibulin-1 was observed after rosiglitazone treatment even though HbA_{1c} was reduced to the same level in the metformin-only group, and no additional effect on plasma fibulin-1 was observed when HbA_{1c} was further reduced by rosiglitazone and metformin combination treatment. In addition, no association between delta HbA_{1c} and delta fibulin-1 values was observed. Further strengthening the direct connection between metformin treatment and fibulin-1, we observed an association between the change in plasma fibulin-1 level and the circulating concentration of metformin.

Using a hypothesis-free gene expression microarray experiment, we recently identified fibulin-1 as the most upregulated mRNA transcript in nonatherosclerotic arterial tissue from patients with T2D (17). The fibulin-1 protein amounts were also higher in arterial tissue from patients with T2D. These results are in agreement with previous findings of arterial ECM modulations in T2D (18). Fibulin-1 is also present at high levels in plasma, and in our previous study, we also found higher circulating amounts of fibulin-1 in patients with T2D. Moreover, the level of plasma fibulin-1 correlates with the glycemic status as well as blood

Table 3—Mean delta values of clinical parameters from baseline to 2 years

	Insulin alone	+Rosiglitazone	+Metformin	+Rosi+Met
BMI (kg/m^2)	1.89 ± 0.3	3.05 ± 0.4*	1.51 ± 0.3†	2.2 ± 0.4
Body weight (kg)	5.73 ± 0.8	9.27 ± 0.9**	4.42 ± 0.6††	5.77 ± 0.9§§
HbA_{1c} (%)	−0.56 ± 0.12	−1.26 ± 0.19**	−1.31 ± 0.15***	−1.83 ± 0.17***§
HbA_{1c} (mmol/mol)	−6.1 ± 1.4	−13.8 ± 2.0**	−14.3 ± 1.7***	−20 ± 1.8***§
Systolic blood pressure (mmHg)	−5.45 ± 2.4	−9.97 ± 1.7	−5.44 ± 2.2	−9.44 ± 2.3
Diastolic blood pressure (mmHg)	−5.35 ± 1.3	−5.69 ± 1.2	−6.99 ± 1.7	−6.69 ± 1.3
Pulse pressure (mmHg)	−0.61 ± 2.8	−4.83 ± 2	−6.69 ± 1.3	−4.25 ± 2.7
HDL (mmol/L)	0.09 ± 0.02	0.21 ± 0.07*	0.06 ± 0.04	0.25 ± 0.03***¶¶
LDL (mmol/L)	−0.6 ± 0.1	−0.31 ± 0.1	−0.73 ± 0.1	−0.47 ± 0.1
Total cholesterol (mmol/L)	−0.52 ± 1.23	−0.11 ± 0.14*	−0.7 ± 0.12†	−0.31 ± 0.12¶
Plasma triglycerides (mmol/L)	−0.18 ± 0.13	−0.35 ± 0.14	−0.55 ± 0.28	−0.4 ± 0.12
Plasma FFA (mmol/L)	−0.08 ± 0.04	0.07 ± 0.09	−0.03 ± 0.03	−0.07 ± 0.03
Plasma fibulin-1 (μg/mL)	7.32 ± 1.2	5.71 ± 1	1.28 ± 1.15***†	0.96 ± 0.93***§§§

Data are mean values at the first visit subtracted from means at the last visit. Data are presented as means ± SEM. Student *t* test for nonpaired data was used. ***$P < 0.001$. **$P < 0.01$. *$P < 0.05$ for insulin alone vs. treatment. ††$P < 0.001$. †$P < 0.01$ for rosiglitazone vs. metformin. §§§$P < 0.001$. §§$P < 0.01$. §$P < 0.05$ for rosiglitazone vs. +Rosi+Met. ¶¶$P < 0.001$. ¶$P < 0.05$ for metformin vs. +Rosi+Met.

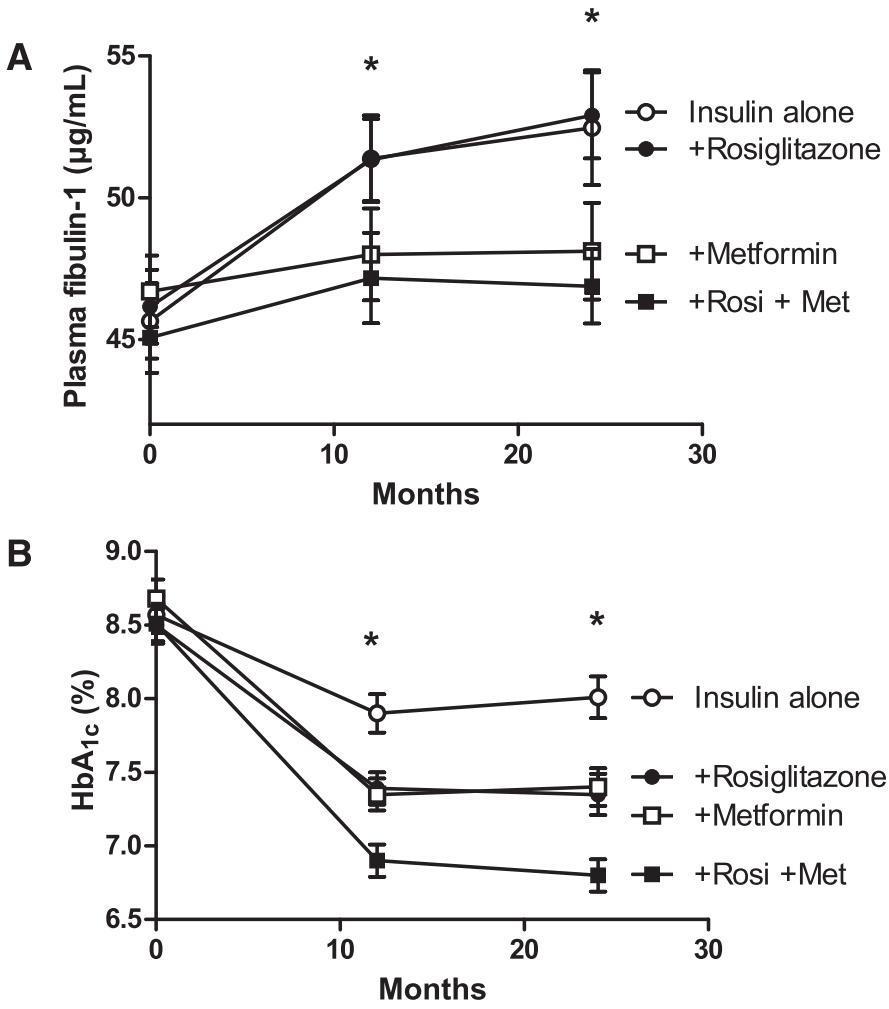

Figure 1—Plasma fibulin-1 and blood HbA_{1c} concentrations at baseline and after 1 and 2 years of antidiabetic treatments. The *top* panel (*A*) shows results for plasma fibulin-1 and the *bottom* panel (*B*) shows results for HbA_{1c}, both in the four treatment groups as indicated. Data are presented as mean ± SEM for the four treatment groups as indicated. *$P < 0.01$ compared with the control group.

pressure and carotid compliance, but not with lipids and CRP. In addition, plasma fibulin-1 concentrations independently predicted mortality in a 15-year follow-up period (18). Thus, plasma fibulin-1 appears as a new biomarker for vascular damage, which is in line with the results from four subsequent studies, where plasma fibulin-1 was found to be associated with diabetes, hypertension, and kidney damage (19–21). Interestingly, two of these studies used non–hypothesis-driven proteomic approaches to search for new markers (20,21). In the current study, we could confirm the previously observed associations between plasma fibulin-1 and age, glycemic status, and pulse pressure, although the correlations were not as strong as previously reported (18). Our current observation of increasing levels of plasma fibulin-1 during the 2-year study period may partly be explained, since we previously observed an association with age; however, the increase is rather large, and whether this is related to the effects of factors related to the diabetic condition is not known. We did not observe correlations to systolic blood pressure as previously seen (18); nevertheless, in our previous study, blood pressure measurements were obtained stringently after a period of rest, whereas in the current study, blood pressure measurements were not obtained as rigorously (18).

Metformin is recommended as the drug of choice in the treatment of T2D (29) and has been shown to provide a protective effect on the cardiovascular system (6,7,15,30,31), although its exact protective mechanism is not clarified. In the UKPDS, metformin treatment in obese patients with T2D has been shown to reduce the incidence of cardiovascular end points and mortality in the 10-year follow-up when compared with conventional treatment (8,9). Moreover, in the Danish nationwide study by Schramm et al. (31), metformin therapy was associated with lower mortality and cardiovascular risk when compared with the most common insulin secretagogues, in line with new findings by Hong et al. (10). These findings are partially confirmed by the Dutch randomized, placebo-controlled trial Hyperinsulinemia: the Outcome and its Metabolic Effect (HOME), where metformin treatment was found to be associated with a lower

incidence of macrovascular events (15). In contrast, the effect of rosiglitazone on macrovascular events is still questionable, despite the ability of this drug type to improve glycemic status (32–34).

It has previously been reported that metformin treatment improves endothelial function (13), and our findings are in agreement with one other human study, where metformin, in contrast to treatment with repaglinide, improved markers of endothelial function, such as von Willebrand factor, tissue plasminogen activator, plasminogen activator inhibitor-1, and intercellular adhesion molecule despite similar effects on the glycemic levels (16). Metformin has also been reported to improve endothelial function in normoinsulinemic, normal-weight polycystic ovary syndrome patients (35), suggesting a possible direct effect of the drug on arterial cells beside putative secondary effects due to the glucose-lowering properties. Furthermore, several animal studies show that metformin improves endothelial function and reduces remodeling; for example, in a rat model of induced obesity and insulin resistance, metformin has been shown to attenuate hypertrophic vascular remodeling (36). Likewise, in a rat model of nonobese T2D, metformin improved the levels of glycation, vascular oxidative stress, and nitric oxide availability and normalized endothelial function on aorta (37). It was suggested that metformin, through the activation of AMP-activated protein kinase, increases the expression of mitochondrial uncoupling protein-2, resulting in the inhibition of both O_2 and prostacyclin synthase nitration in diabetes (38). Studies indicate that metformin may exert direct effects on arterial cells. Thus, metformin appears to reduce the release of some proinflammatory cytokines through nuclear factor-κB (39), and, moreover, in vitro experiments on human aortic smooth muscle cells demonstrated that metformin inhibits leptin-induced nuclear factor-κB activation (40). Our findings fit well with the notion that metformin may exert direct beneficial effects in the arterial wall and expand this idea by pointing toward metformin-induced alterations in the metabolism of the ECM molecule fibulin-1. Somewhat surprising, we did not find that rosiglitazone changed plasma fibulin-1, despite the fact that previous publications have shown that glitazones may influence arterial functions and change vascular biomarkers (41,42). Nevertheless, it seems that even though rosiglitazone may reverse endothelial dysfunction, it did not change arterial remodeling in a study of Zucker diabetic fatty rats (43). Our findings are therefore compatible with the notion that metformin and rosiglitazone influence different pathophysiological pathways in the arterial wall, although they both improve glycemic status.

In conclusion, we find that plasma fibulin-1 increases over time in patients with T2D. Importantly, metformin treatment in T2D leads to a reduction of this increase, independent of effects on glycemic status. This is in agreement with the notion that metformin exerts direct beneficial effects on arterial cells and adds effects on the ECM to the targets of this drug. Rosiglitazone has no effect on fibulin-1 levels, suggesting that effects of metformin and glitazones follow different pathophysiological pathways in the cardiovascular system. Our results are compatible with the hypothesis that fibulin-1 may be an important molecular marker for changes in important pathways related to the development of arterial disease in T2D. We speculate that measurement of plasma fibulin-1 levels might have a potential application as an indicator of metformin efficacy and a marker of cardiovascular risk in diabetic patients. We suggest that further investigations on the physiology of fibulin-1 may unravel important mechanisms behind the development of macrovascular disease in T2D.

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