Metformin, but not rosiglitazone, attenuates the increasing plasma levels of a new cardiovascular marker, fibulin-1, in patients with type 2 diabetes

Running title: Metformin attenuates fibulin-1 levels

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Abstract

Objective

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

Research Design and Methods

After a four-week run-in period, 371 eligible patients with type 2 diabetes 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 two-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 not-metformin treated individuals both at 18 and 24 months of treatment, but rosiglitazone had no effect. Metformin and rosiglitazone alone reduced the HbA1c levels to comparable levels and in combination even further.

Conclusions

Metformin attenuate the increase in plasma fibulin-1 concentrations in type 2 diabetes, independently of glycemic effects. Changes in fibulin-1 may reflect an important element in diabetic arteriopathy which can be influenced by metformin.

Increased mortality and morbidity owing to a higher incidence of cardiovascular diseases (CVD) are a major clinical challenge in type 2 diabetes (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 type 2 diabetes (T2DM); 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 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 UKPDS study, it has been shown that metformin significantly reduces macrovascular events in T2DM (6), and, impressively, that this effect is sustained for ten years (7). This result is assumed to be related to metformin 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, which found beneficial effects on cardiovascular outcome in type 2 diabetic patients with coronary artery disease on metformin compared to glipizide, despite similar effects on HbA1c (10). The mechanisms by which metformin exert such putative beneficial cardiovascular effects are unclear, but has 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 that this effect may be independent of effects on the glycemic status (16).

The arterial pathology in diabetes comprises both pre-atherosclerotic, generalized vascular changes (endothelial dysfunction, extracellular matrix (ECM) modifications and accumulations, 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 non-atherosclerotic arterial tissue from patients with type 2 diabetes (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 type 2 diabetes. 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 type 2 diabetes (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 type 2 diabetes were independently predictive of mortality in type 2 diabetes 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 type 2 diabetes, and that the circulating concentration may serve as a biomarker for vascular ECM changes as part of a diabetic arteriopathy. Fibulin-1 is a member of the fibulin family (22) and was identified by Argraves et al. in 1989 (23). 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 in addition 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 present study, we hypothesized that the vasculo-protective 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 T2DM 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 $>$ 300pmol/l, HbA$_{1C}$ $> 7\%$ (53 mmol/mol), diagnosis of T2DM at least two years before participation, and treatment for at least three months with stable doses of oral antidiabetics and/or insulin. Exclusion criteria were impaired renal function, congestive heart failure, or 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 gave written informed consent.

Study design and setting

The study is a prospective, randomized, and partly placebo controlled trial with 450 patients included. After a four-week run-in period, 371 eligible patients were randomized to one of

eight treatment groups in a factorial design with NPH insulin vs. insulin aspart, metformin vs. placebo, or rosiglitazone vs. 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, results from four groups (presented in Table 1) are therefore reported: "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 tablet of 500 mg twice a day during the first four weeks succeeded by two tablets twice daily. Rosiglitazone or placebo was given as one tablet of 4 mg/day for the first four weeks succeeded by one tablet twice a day.

Clinical and biochemical measurements

Blood was drawn for HbA1c and plasma measurements at baseline and after one and two years. HbA_{1C} was measured every three 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 (HPLC) method (28). The limit of quantification (LOQ) for metformin in plasma was found to be 10 ng/mL. The limit of detection (LOD) 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 done using SPSS 20.0 (SPSS Inc., Chicago, USA), and GraphPad Prism 5 (GraphPad Software, La Jolla, USA) 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's 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 HbA1c 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 with 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 difference 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 two years) of LDL, triglycerides and 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 to the control group.(Table 3). Weight gain was reduced in the patients receiving the combined therapy compared to those receiving rosiglitazone alone (Table 3). The mean delta values of HbA1c were significantly lowered in both the rosiglitazone alone and the metformin alone groups (approximately 0.5 %) and further reduced in the rosiglitazone and metformin combination group (approximately 1 %) (Table 3 and Figure 1B).

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 significant to age, HbA1c and pulse pressure at baseline in the entire population (age: r=0.22, p<0.001; HbA1c: r=0.10, p=0.080; pulse pressure: r=0.14, p=0.006). As can be seen in Figure 1A, the plasma concentration of fibulin-1 increased significantly in the control group after one and two 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 two years in the two metformin treated groups compared to the two groups not treated with metformin (Fig. 1A). There was no correlation between delta values of plasma fibulin-1 and delta values of HbA1c 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 (two-year value minus baseline value) and the mean trough steady-state plasma concentration of all metformin treated patients at the one 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.

DISCUSSION

In this 2-year, investigator driven, prospective, randomized, and partly blinded trial (27), we tested the hypothesis that metformin and/or rosiglitazone modulate the level of plasma fibulin-1 in patients with T2DM. 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 T2DM 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 HbA1c was reduced to the same level in the metformin only group, and no additional effect on plasma fibulin-1 was seen when HbA1c was further reduced by rosiglitazone and metformin combination treatment. In addition, no association between delta HbA1c 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 non-atherosclerotic arterial tissue from patients with T2DM. The fibulin-1-protein amounts were also higher in arterial tissue from patients with T2DM. These results are in agreement with previous findings of arterial ECM modulations in T2DM (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 T2DM. Moreover, the level of plasma fibulin-1 correlates with the glycemic status as well as blood 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

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 present study, we could confirm the previously observed associations between plasma fibulin-1 and age, glycemic status, as well as 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 two year study period may partly be explained, since we previously observed association with age, however the increase is rather large and whether this is related to 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 drug of choice in the treatment of T2DM (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 UK Prospective Diabetes Study (UKPDS), metformin treatment in obese patients with T2DM has been shown to reduce the incidence of cardiovascular endpoints and mortality in the 10-year follow up when compared to conventional treatment (8; 9). Moreover, in the Danish nationwide study by Schramm *et al.* (31), metformin therapy was associated to lower mortality and cardiovascular risk when compared to 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 associated to 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 (vWF), tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and intercellular adhesion molecule (ICAM) 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 non-obese T2DM, metformin improved the levels of glycation, vascular oxidative stress, nitric oxide availability, and normalized endothelial function on aorta (37). It was suggested that metformin through the activation of AMPK increases the expression of mitochondrial uncoupling protein- 2 (UCP-2) resulting in inhibition of both O2- 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 pro-inflammatory cytokines through nuclear factor-kappa B [NF-kB] (39) and moreover, *in vitro* experiments on human aortic smooth muscle cells demonstrated that metformin inhibits leptin induced NF-kB 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 towards 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 (17473072, 21726411). 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 (20482873). Our findings are therefore compatible with the notion that metformin and rosiglitazone influences 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 type 2 diabetes. Importantly, metformin treatment in T2DM leads to a reduction of this increase, independent of effects on glycemic status. This is in agreement with the notion that metformin exert direct, beneficial effects on arterial cells and add effects of this drug on the arterial extracellular matrix to its putative targets. 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 T2DM. 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 type 2 diabetes.

Author contributions

V.S., C.C., and L.M.R. researched data, contributed to the discussion, wrote the manuscript. J.G. contributed to the discussion, reviewed/edited the manuscript. M.M.C. and D.S.

researched data. E.G. reviewed/edited the manuscript. W.S.A. and J.E.H. contributed to the discussion, reviewed/edited the manuscript.

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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
Gender (f/m)	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
Hemoglobin A1c (%) (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's t-test for non-paired data was used.

Table 2. Percentage of patients treated with different medications

	Insulin alone	**+Rosiglitazone**	**+Metformin**	**+Rosi + met**
Antihypertensives (RAAS)				
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
Beta-blockers	23	25	23	22
Statins	70	68	68	70
Acetylsalicylic acids	44	37	33	46

Table 3. Mean delta values of clinical parameters from baseline to two 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§§
Hemoglobin A1c (%)	-0.56 ± 0.12	-1.26 ± 0.19**	-1.31 ± 0.15***	-1.83 ± 0.17***§
Hemoglobin A1c (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. Students t-test for non-paired data was used. ***P<0.001, **P<0.01 and *P<0.05 for insulin alone vs. treatment; †††P<0.001 and ††P<0.01 for rosiglitazone vs. metformin; §§§P<0.001, §§P<0.01 and §P<0.05 for rosiglitazone vs. rosi + met; ¶¶¶P<0.001 and ¶P<0.05 for metformin vs. rosi + met.

Legends to figures

Figure 1
Plasma fibulin-1 and blood HbA1c concentrations at baseline and after one and two years of anti-diabetic treatments.
The upper panel (A) shows results for plasma fibulin-1 and the lower panel (B) shows results for HbA1c, both in the four treatment groups as indicated.
Data are presented as mean +/- SEM for the four treatment groups as indicated. * indicate $p < 0.01$ compared to the control group.

A

B