Telmisartan shows an equivalent effect of vitamin C in further improving endothelial dysfunction after glycemia normalization in type 1 diabetes.

Received for publication 15 February 2007 and accepted in revised form 10 April 2007.

Additional information for this article can be viewed in an online appendix at http://care.diabetesjournals.org.

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A. Ceriello: Telmisartan and long lasting endothelial dysfunction in diabetes.

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Abstract

Background: Long-lasting hyperglycemia in type 1 diabetic patients induces permanent alterations of endothelial function, by increased oxidative stress, even when glycemia is normalized.

Methods and Results: In this study 36 type 1 diabetic patients and 12 controls were enrolled. The diabetic patients were divided in three groups. The first group was treated for 24h with insulin, achieving a near-normalization of glycemia. At the 12 h of this treatment vitamin C was added for the remaining 12h.The second group was treated for 24h with vitamin C. At the 12 h of this treatment insulin was started, achieving a near-normalization of glycemia for the remaining12h.The third group was treated for 24h with both vitamin C and insulin, achieving near normalization of glycemia. The same protocols were run after 1 month of Telmisartan or placebo. Neither normalization of glycemia or vitamin C treatment alone was able to normalize endothelial dysfunction or oxidative stress. Combining insulin and vitamin C normalized endothelial dysfunction and decreased oxidative stress to normal level. Telmisartan significantly improved basal endothelial function and decreased nitrotyrosine plasma levels. In patients treated with Telmisartan a near normalization of both flow mediated vasodilation and oxidative stress was achieved when glycemia was normalized, while adding vitamin C infusion did not show further effect on endothelial function or nitrotyrosine plasma levels.

Conclusions: These data indicate that combining the normalization of glycemia with an antioxidant can normalize endothelial function in type 1 diabetic patients and that Telmisartan works as antioxidant as vitamin C.

INTRODUCTION

An increased incidence of macrovascular diseases in type 1 diabetes has long been recognized (1).The accelerated macrovascular disease is due partly to the increased incidence of classical risk factors, such as hypertension and dyslipidemia (2). However, recent evidence suggests that hyperglycemia also plays a significant role (3).

The endothelium is a major organ involved in the development of cardiovascular disease even in diabetes, and the presence of an endothelial dysfunction has often been reported in diabetes and been found as independent predictor of a future cardiovascular disease (4).

Several studies have shown that hyperglycemia induces an endothelial dysfunction in both diabetic and non-diabetic subjects (5-7), however, in type 1 diabetic patients endothelial dysfunction has been reported to present even when normoglycemia is achieved (8-9).

Evidences indicate that hyperglycemia induces endothelial dysfunction through the generation of oxidative stress (for review see 10), which has been suggested to be the key player in the generation of the diabetic complications, both micro and macrovascular (11). We have recently demonstrated that a near normalization of endothelial dysfunction can be achieved in type 1 diabetic patients combining an optimal control of glycemia with the infusion of the antioxidant vitamin C (12). Several compounds already in the clinical practice have the property of reducing oxidative stress generation (10). In particular, the AT-1 receptor blockers have been shown to be effective (13-14).

In this study we have evaluated the impact of the treatment with telmisartan, alone or in combination with normalization of glycemia, on the endothelial function and oxidative stress in type 1 diabetic patients. Furthermore, the effect of adding vitamin C has also been evaluated.

RESEARCH DESIGN AND METHODS

Subjects: Experiments were performed in 36 type 1 diabetic patients. 12 age and BMI matched healthy volunteers served as control group (Table 1).

The diabetic subjects were divided in three subgroups, matched for sex, age, BMI, metabolic control and duration of the disease (Table 1). None of the selected patients was hypertensive, microalbuminuric or on drug treatment, excluding insulin. Lipid levels were also normal.

The study was approved by the Local Ethical Committee and all subjects gave written informed consent.

Protocol: First Phase

All experiments were performed in a quiet, temperature-controlled room (24–25°C) after an overnight fast. Subjects were not allowed to smoke, drink (except for water), or eat for at least 10 h before the experiment. They were studied in the supine position and remained in bed during the experiments. On the morning of the experiments, the diabetic patients omitted their insulin injection.

In the diabetic patients, a catheter was inserted into an antecubital vein for the infusion of one of the three treatments described below.

The treatments consisted in:

A: insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) and/or 5% glucose to keep blood glucose levels between 4 and 6 mmol/l for 24 hours. The amount of insulin infused was based on the individual daily insulin dose. Blood glucose levels were determined every 5 min with adjustment of the intravenous insulin infusion, until steady-state glucose levels were between 4 and 6 mmol/l. At the steady state, venous glucose samples were drawn every 30 min. After 12 hours, vitamin C infusion, at the rate of 3 mg/min (15), was also started and continued for the remaining 12 hours;

B: vitamin C, at the rate of 3 mg/min (15), for 24 hours. After 12 hours, insulin and/ or 5% glucose infusion, aiming to maintain blood glucose levels between 4 and 6 mmol/l, as described above, for the remaining 12 hours;

C: insulin and/or 5% glucose to keep blood glucose levels between 4 and 6 mmol/l plus vitamin C infusion, at the rate of 3 mg/min (15), for 24 hours.

Second Phase: Telmisartan treatment
After the first phase of the study, Telmisartan (40 mg /day, six patients of each group) or placebo (six patients of each group) treatment was started for 1 month.
At the end of the treatment period, the protocols above described were repeated.

In all the experiments, of the first and the second phase, at baseline and after 12 and 24 hours, glycemia, endothelial function (flow mediated dilatation: FMD) and nitrotyrosine plasma level were evaluated.

Biochemical Measurements. Cholesterol and triglycerides were measured enzymatically (Roche Diagnostics, Basel, Switzerland). HDL-C was estimated after precipitation of apolipoprotein B with phosphotungstate/magnesium (16). LDL-C was calculated after lipoprotein separation (17).
Plasma glucose was measured by the glucose-oxidase method, HbA1c by HPLC.

Nitrotyrosine.
Nitrotyrosine plasma concentration was assayed by enzyme-linked immunosorbent assay (ELISA), as previously described (18).

Endothelial Function.
Endothelial function was evaluated measuring the flow-mediated vasodilation (FMD) of the brachial artery as previously reported. (19). At the end of each test, the subjects lay quietly for 15 min. Then, sublingual nitroglycerin (0.3 mg) was administered and 3 min later the last measurements were performed. Response to nitroglycerin was used as a measure of endothelium-independent vasodilation. All studies were performed in a quiet and temperature-controlled room (22° C to 23° C).

Statistical Analysis. As in previous studies (6, 20) The Kolmogorov-Smirnov algorithm was used to determine whether each variable had a normal distribution. Comparisons of baseline data among the groups were performed by use of unpaired Student's t test. Paired Student's t test was used to compare the various parameters before and after each treatment. Changes in variables during the tests were assessed by 2-way ANOVA with repeated measures. If differences reached statistical significance, post hoc analyses with a 2-tailed paired t test was used to assess differences at individual time periods in the study, with Bonferroni's correction for multiple comparisons. Statistical significance was defined as $P<0.05$.

RESULTS
Phase 1: Baseline nitrotyrosine was increased in diabetic patients, while FMD was reduced (table I).
As previously reported (12), in the treatment A and C, glycemia was almost normalized after 12 and 24 hours ($p< 0.001$ vs baseline; figure 1 and 3), while in the treatment B glycemia was normal at 24 h ($p< 0.001$ vs baseline; figure 2).
After 12 hours nitrotyrosine plasma levels significantly decreased in all the treatments ($p< 0.01$ vs baseline; figure 1, 2 and 3): more significantly in the treatment C compared to the two others ($p< 0.01$ vs A and $p<0.05$ vs B, respectively), and more in the treatment B compared to A ($p<0.05$). At 12 h, FMD increased ($p< 0.01$ vs baseline; figure 1, 2 and 3): again more significantly in the treatment C compared to the two others ($p< 0.01$ vs A and $p<0.05$ vs B, respectively), and more in the treatment B compared to A ($p<0.05$).
After 24 hours, the plasma levels of nitrotyrosine were significantly decreased compared to the levels of 12 h in the treatment A and B ($p<0.01$), but not in C. After 24 hours there were no differences between the plasma levels of nitrotyrosine reached in each of the three treatments.
FMD was significantly increased compared to the levels of 12 h in the treatment A and B ($p<0.01$), but not in C. After 24 hours also for FMD there were no differences between the three treatments.
Endothelium-independent vasodilation did not change during any of the tests (data not shown).

Second Phase: Telmisartan treatment.
One month of treatment with Telmisartan significantly improved baseline FMD and reduced nitrotyrosine plasma levels (Table 2).

In the protocol A, when glycemia was normalized after 12 h also FMD was almost normalized ($p<0.01$ vs baseline, $p<0.05$ vs placebo, figure 1), while nitrotyrosine was significantly reduced ($p<0.01$ vs baseline, $p<0.05$ vs placebo, figure 1), in the group treated with Telmisartan. Adding vitamin C did not change the results.
Similarly, in the protocol B, the infusion of vitamin C had not impact on both FMD and nitrotyrosine plasma level, while the normalization of glycemia at 24 h normalized FMD ($p<0.01$ vs baseline, $p<0.05$ vs placebo, figure 2), and reduced nitrotyrosine plasma levels ($p<0.01$ vs baseline, $p<0.05$ vs placebo, figure 2).
In the protocol C, the normalization of FMD ($p<0.01$ vs baseline, $p<0.05$ vs placebo at both 12 and 24 h, figure 3), and the reduction of nitrotyrosine plasma levels ($p<0.01$ vs baseline, $p<0.05$ vs placebo at both 12 and 24 h, figure 3), were achieved after 12 h and persisted until the 24 h.

Any variation of the endothelial independent vasodilation was found during the all the experiments.

DISCUSSION

This study confirms that in type 1 diabetic patients only the simultaneous normalization of glycemia and the use of an antioxidant, such as vitamin C, can normalize endothelial dysfunction (12). At the same time, the possibility of improving endothelial function in type 1 diabetes, as already reported for type 2 diabetic patients, using an AT-1 receptor blocker, in this case Telmisartan, is also reported (20). However, for the first time, we are able to show that Telmisartan in combination with the normalization of glycemia may lead to the almost normalization of the endothelial function. Furthermore, it is also shown that adding vitamin C does not influence the effect of the treatment with Telmisartan.
The role of oxidative stress in this phenomenon appears crucial: in the phase 1 of the study, when endothelial function is still altered after 12 h of normalization of glycemia or 12 h vitamin C treatment, nitrotyrosine, a good marker of peroxynitrite and nitrosative stress, is still increased, while when endothelial function is normalized, combining glycemia control and vitamin C, nitrotyrosine is also normalized. It has already been reported that Telmisartan reduces free radical production and oxidative stress (21-22). In this study, we show that Telmisartan influences the oxidative stress generation in diabetes. In the phase 2 of the study, as already reported for type 2 diabetic patients (23), the effect of the chronic treatment with an AT-1 blocker in decreasing nitrotyrosine plasma level is demonstrated, while the effect of Telmisartan on nitrotyrosine plasma levels in all the three protocols is equivalent to that of vitamin C. Interestingly, when vitamin C is added during the protocols in the patients already on Telmisartan treatment, it does not influence the results. These data, all together, suggest that Telmisartan reduces oxidative stress, achieving the same results of a well established antioxidant, such as vitamin C. This is particularly interesting, considering that hyperglycemia-induced PKC over-expression mediated the activation of the NADPH oxidase and may cause eNOS uncoupling (24), which can, in turn, favour peroxynitrite generation, which actually causes the increase in nitrotyrosine and is able to oxidize BH4, the eNOS cofactor, to the BH3 radical (25). In this case, vitamin C acts as a BH3 radical scavenger rather than an antioxidant, which reacts with superoxide (26).Because Telmisartan prevents NADPH activation, this property may contribute to explain our results (27).

Our data suggest that hyperglycemia convincingly induces an endothelial dysfunction trough the generation of an oxidative stress, because the administration of vitamin C (in the protocol B) in presence of hyperglycemia restores endothelial function and reduces nitrotyrosine plasma level, and because the normalization of glycemia (in the protocol A) is accompanied by an improvement of both endothelial function and nitrotyrosine. Therefore, considering the whole results, it seems reasonable that Telmisartan improves endothelial function because reduces oxidative stress.

Particularly for the protocol A, a possible role for insulin, more than for the reduction of hyperglycemia, in determining the improvement of endothelial dysfunction might be supposed. However, very recently, Ellger et al. have shown, in an animal model, that is the reduction of glucose toxicity, more than the action of insulin that improves endothelial function (28).

As above reported, previous studies have shown that in type 1 diabetic patients even normalizing glycemia endothelial dysfunction still persists (8-9), suggesting that long lasting hyperglycemia can induce several permanent alterations in endothelial cells leading to a permanent endothelial dysfunction. Our data suggest that this permanent alteration induces the persistence of the endothelial dysfunction through the generation of oxidative stress, because by adding vitamin C or Telmisartan to the insulin the endothelial function is almost normalized. Interestingly, these in vivo data are consistent with in vitro and animal studies: in endothelial cells in culture and in the retina of diabetic rats an overproduction of free radicals persists even after the normalization of the glucose and is accompanied by a prolongation of the induction of PKC-β, NAD(P)H oxidase, Bax, collagen and fibronectin, in addition to nitrotyrosine (29), suggesting that oxidative stress may be involved in this effect. In the animals the evidences are more consistent. The effect of reinstitution of good glucose control on hyperglycaemia-induced increased oxidative stress and nitrative stress has been evaluated in the retina of rats maintained in poor glucose control before initiation of good control (29). In diabetic rats, 2 or 6 months of poor control (GHb >11.0%) was followed by 7 months of good control (GHb <5.5%). Reinstitution of good control after 2 months of poor control inhibited elevations in retinal lipid peroxides and NO levels by approximately 50%, but failed to have any beneficial effects on nitrotyrosine formation. However, reversal of hyperglycaemia after 6 months of poor control had no significant effect on retinal oxidative stress and NO levels. In the same rats, inducible nitric oxide synthase expression and nitrotyrosine levels remained elevated by >80% compared with normal rats or rats kept in good control for the duration (29). In a similar study, caspase-3 activity in diabetic rats kept in poor control for 13 months was 175% that in normal rats (30). Re-institution of good glycaemic control after two months of poor control partially normalized the hyperglycaemia-induced activation of caspase-3 (to 140% of normal values) while re-institution of good control after six months of poor control had no significant effect on the activation of caspase-3. In the same study NF-kB activity was 2.5-fold higher in diabetic rats kept in poor control than in normal rats. Re-institution of good control after 2 months of poor control partially reversed this increase, but good control after 6 months of poor control had no effect. Initiation of good control soon after induction of diabetes in rats prevented activation of retinal caspase-3 and NF-kB (30). Similar results are available for the kidney. Diabetic rats were maintained in good glycaemic control (5% glycated hemoglobin, GHb) soon after or 6 months after induction of hyperglycaemia, and were sacrificed 13 months after induction of diabetes (31). For rats in which good control was initiated soon after induction of diabetes, oxidative stress [as measured by the levels of lipid peroxides (LPOs), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and reduced glutathione (GSH)] and NO in urine and renal cortex were not different from that observed in normal control rats, but when reinstitution of good control was delayed for 6 months after induction of diabetes, oxidative stress and NO remain elevated in both urine and renal cortex (31).
These data suggests that hyperglycaemia-induced oxidative stress and NO, as well as activation of apoptosis and of the NF-kB can be prevented if good glycaemic control is initiated very early, but are not easily reversed if poor is maintained for longer durations. Therefore, these findings suggest a persistence of the hyperglycaemia-induced damage in such organs even after its normalization.

Because in vitro evidences show that almost the same pathways are activated by hyperglycemia in endothelial cells (32), it is reasonable that the cardiovascular system

receives the same imprinting of the retina and kidney.

The finding that only the simultaneous control of glycemia and oxidative stress can normalize endothelial function in type 1 diabetic patients is clearly relevant. This evidence seems to suggest the existence of two different pathways working in the generation of endothelial dysfunction in type 1 diabetes: one directly related to hyperglycemia and one not. The explanation of this second pathway may be, at the moment, only speculative. It is well recognized that hyperglycemia, both chronic or acute, can induce an endothelial dysfunction through the generation of an oxidative stress, convincingly generated at the level of mitochondria (11,32). However, it is well recognized that chronic hyperglycemia induces the formation of the advanced end products (AGE) and chronic hyperglycemia is thought to alter mitochondrial function through glycation of mitochondrial proteins (33). This premise is important because a recent study, for the first time, has described a direct relationship between the formation of intracellular AGEs on mitochondrial proteins, the decline in mitochondrial function and the excess formation of reactive species (34): mitochondrial respiratory chain proteins that underwent glycation were prone to produce more $\bullet O_2^-$, independently from the level of hyperglycemia. Therefore, a possible explanation for the previous evidences and of our data is that two pathways are simultaneously working: one due to the actual level of glycemia and another one to the long lasting damage induced in the endothelial cells by chronic hyperglycemia, possibly through non-enzymatic glycation of mitochondria. This hypothesis has recently been reviewed (35).

It is also surprising that vitamin C or Telmisartan alone cannot normalize endothelial function if oxidative stress is the key convergent effectors of both hyperglycemia and of the long lasting damage (non-enzymatic glycation of mitochondria?). However, it may be hypothesized that when hyperglycemia is present it induces free radical generation which can be only partly counterbalanced by the antioxidant treatment: the persistence of increased levels of nitrotyrosine in the protocol B or after long-term Telmisartan treatment supports this hypothesis. Conversely, when hyperglycemia is normalized, the possible presence of a second pathway, still producing free radicals, also may explain the incomplete action of vitamin C or Telmisartan.

In any case, although the molecular basis for our findings is not clear, we believe that the clinical impact of our finding is important. Particularly, the evidence that Telmisartan has the same beneficial effect of vitamin C in improving the long-lasting effect of hyperglycemia, in our opinion deserves attention. In fact, the effectiveness of a chronic long-term with vitamin C is still an open debate (36), while Telmisartan, as others AT-1 receptor blockers, are already widely used for the prevention of diabetic complications, particularly nephropathy (37). It seems reasonable to suggest that AT-1 receptor blocker is a much better kind of therapy as compared to vitamin C also because a recent study indicates that vitamin C intake increases mortality in postmenopausal women with diabetes type II (38).

Moreover, the persistence of an endothelial dysfunction, which is a strong predictor of a cardiovascular event (4), even when glycemia is normalized, may contribute to explain the recent results from the EDIC/DCCT Study, showing, for the first time, the influence of the early glycemic control on the progression to macrovascular events (3). Of relevance, is also the evidence that in our study nitrotyosine is still elevated even in presence of near normal glycemia, since nitrotyrosine has also been shown to be an independent predictor of cardiovascular disease (39). Furthermore, our data seem to confirm that an early continuous aggressive treatment of glycemia is important to avoid future complications. Incidentally, in our opinion, the recent findings showing the existence of a continuum between the values of glycemia, endothelial dysfunction and the risk of a cardiovascular event, even in non diabetic patients, supports this concept (40-41).

The use of AT-1 blockers for the prevention of cardiovascular complications, particularly in diabetes is still an open question (42). Our study showing, for the first time, that a normalization of both endothelial dysfunction and oxidative stress can be achieved in type 1 diabetic patients combining the near normalization of glycemia and Telmisartan treatment, add a little piece in favor of the use of these compounds. Future long term controlled trials may help in understanding the possible clinical impact of this finding on the prognosis of cardiovascular disease in type 1 diabetic patients.

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