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Effect of irbesartan on nitrotyrosine generation in non-hypertensive diabetic patients

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Abstract

Aims/hypothesis. Oxidative stress is involved in the pathogenesis of microangiopathic and macroangiopathic diabetic complications. The results of recent trials suggest that type 1 angiotensin II (AT-1) receptor blockers may prevent or delay nephropathy and cardiovascular disease in diabetic patients, independently of their anti-hypertensive action. There is evidence that AT-1 receptor blockers can work as intracellular antioxidants. This study investigated whether the AT-1 receptor blocker irbesartan is able to reduce nitrotyrosine formation in non-hypertensive diabetic patients under fasting conditions and during acute hyperglycaemia.
Methods. A total of 40 non-hypertensive, non-microalbuminuric Type 2 diabetic patients and 20 healthy, normotensive subjects were recruited for this study. Diabetic patients followed a randomised, double-blind, placebo-controlled, crossover protocol, taking either irbesartan (150 mg orally, twice daily) or placebo for 60 days. Fasting glucose and nitrotyrosine were measured at baseline and at the end of each treatment period. An OGTT was also performed at the same time intervals, during which plasma glucose and nitrotyrosine levels were monitored.
Results. Compared with baseline measurements, treatment with irbesartan (0.57±0.4 vs 0.35±0.3 μmol/l, $p<0.01$) but not placebo (0.58±0.3 vs 0.59±0.2 μmol/l) significantly reduced fasting nitrotyrosine levels. Irbesartan also significantly reduced nitrotyrosine formation during the OGTT.
Conclusions/interpretation. This study demonstrates that irbesartan reduces plasma levels of nitrotyrosine in diabetic patients and is effective in counterbalancing nitrotyrosine formation during acute hyperglycaemia. Our results may help to elucidate how AT-1 receptor blockers exert their beneficial effect independently of their BP-lowering activity.

Keywords Acute hyperglycaemia · Diabetic complications · Irbesartan · Nitrotyrosine

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Abbreviations: AT-1, type 1 angiotensin II receptor · NO, nitric oxide

Introduction

Hyperglycaemia plays a central role in the development of microvascular complications [1, 2] and significantly contributes to macrovascular complications in patients with diabetes mellitus [3]. It is therefore not surprising that glycated HbA_1c is a strong predictor of the development of microvascular and macrovascular diabetic complications [1, 2, 4]. However, the acute toxic effects of short-term hyperglycaemia, which are not fully reflected by the HbA_1c level, may also be involved. For example, post-prandial plasma glucose levels are independently correlated with the risk of developing cardiovascular disease [5], nephropathy and retinopathy [6].

Hyperglycaemia-derived oxygen free-radicals have been implicated as mediators of diabetic complications [7]. According to recent studies, a single hyperglycaemia-induced process of superoxide overproduction by the mitochondrial electron-transport chain seems to be the first and key event in the activation of all other pathways involved in the pathogenesis of diabetic complications [8]. In hyperglycaemia, superoxide overproduction is accompanied by increased nitric oxide (NO) generation [9]. NO and superoxide react to produce peroxynitrite, a potent long-lived oxidant [10]. The peroxynitrite anion is cytotoxic because it oxidises sulphydryl groups on proteins, initiates lipid peroxidation and nitrates amino acids such as tyrosine, which affects many signal transduction pathways [10]. Peroxynitrite production can be indirectly inferred by the presence of nitrotyrosine [11], and increased levels of nitrotyrosine have been found in the plasma of diabetic patients [12, 13]. There is also evidence that an acute increase in glycaemia induces an increase in nitrotyrosine [14, 15]. Interestingly, increased nitrotyrosine staining has been reported in kidneys from diabetic patients with nephropathy [16].

Three recently published trials have reported a protective effect of two type 1 angiotensin II (AT-1) receptor antagonists, losartan and irbesartan, in diabetic nephropathy [17, 18, 19]. In all three studies, the protection afforded by these two treatment agents was independent of their BP-lowering effect. AT-1 receptor blockade does not appear to be effective in reducing cardiovascular events in Type 2 diabetic patients with overt nephropathy [20]. Conversely, the results of the Losartan Intervention for Endpoint Reduction in Hypertension (LIFE) study showed that AT-1 receptor blockade is more effective than conventional therapy in reducing cardiovascular morbidity and mortality in diabetic patients with hypertension and left ventricular hypertrophy [21]. These data suggest that AT-1 receptor blockade may reduce the cardiovascular risk in at least a subset of diabetic patients.

It has been reported that AT-1 receptor blockers can reduce oxidative stress [22, 23]. Specifically, it has been shown that irbesartan can reduce oxidative stress by modulating superoxide and NO production [24, 25]. Oxidative stress, particularly nitrosative stress, produced during chronic and acute hyperglycaemia seems to play an important role in the pathogenesis of microvascular and macrovascular complications of diabetes mellitus [7, 8]. Based on this observation, the aim of this study was to evaluate whether irbesartan therapy is effective in reducing nitrotyrosine production in Type 2 diabetic patients even during an acute increase in glycaemia.

Subjects and methods

The study was approved by the ethics committee of our institution. All patients were seen at the Outpatients Clinic of Metabolic Disease (Institute of Internal Medicine, University of Udine, Italy) and were recruited over a 6-month period.

A total of 40 non-hypertensive, non-microalbuminuric Type 2 diabetic patients were recruited for this study. Patients had plasma creatinine levels within the normal range and had the disease under good metabolic control using oral hypoglycaemic agents (glybenclamide [*n*=20], metformin [*n*=3] or combination therapy [*n*=17]) as judged by HbA_1c levels. A group of 20 healthy, normotensive subjects who were matched to the patients in terms of age, sex and BMI served as controls. All subjects were non-smoking and were not taking antioxidant supplements, ACE inhibitors or statins.

The clinical characteristics of the two groups are reported in Table 1.

Table 1. Baseline characteristics of the control subjects and diabetic subjects

Characteristic	Control subjects (*n*=20)	Diabetic subjects (*n*=40)
Sex (men/women)	12/8	32/18
Age (years)	52.4±2.2	53.3±2.5
BMI (kg/m^2)	27.4±2.1	28.7±2.3
Fasting glucose (mmol/l)	4.8±0.2	11.2±2.3[a]
HbA_1c (%)	5.8±0.2	7.5±0.3[a]
Diabetes duration (years)		4.4±0.8
Resting systolic BP (mm Hg)	110.3±5.5	112.4±6.5
Resting diastolic BP (mm Hg)	76.4±2.1	75.2±2.1
Fasting nitrotyrosine (μmol/l)	0.24±0.7 0.09–0.43[b]	0.59±0.4[a] 0.33–0.88[b]

Data are expressed as means ± SEM.
[a] $p<0.001$ vs control subjects; [b] range of values

After written informed consent had been obtained, the subjects followed a randomised, double-blind, placebo-controlled, cross-over protocol. After an initial 15-day placebo run-in period, patients were randomised to receive either irbesartan (150 mg orally, twice daily) or placebo, and entered a treatment period of 60 days. After a 15-day wash-out period, the patients were crossed over to the alternate regimen for a further 60 days. Fasting glucose, HbA_1c level, serum potassium, microalbuminuria and plasma nitrotyrosine were measured at the end of the run-in period, after the first treatment phase, at the end of the wash-out period and at the end of the second treatment phase. An OGTT was also performed, with plasma glucose and nitrotyrosine levels measured at 0, 30, 60, 90 and 120 min. Every 2 weeks from the beginning of the run-in period until the end of the study, 24-h ambulatory BP monitoring was performed by the same expert physician.

During the study the patients followed a normocaloric diet (30 kcal/kg) with a normal sodium level (~150 mmol/day); lifestyle and oral hypoglycaemic therapy remained unchanged. The investigation was performed in accordance with the principles of the Declaration of Helsinki.

Plasma glucose was measured by the glucose-oxidase method and HbA_1c was assessed using HPLC. Plasma nitrotyrosine concentration was determined by ELISA as previously described [12]. The 24-h ambulatory BP monitoring was performed according to the Holter method with measurements taken every 30 min using a system from Welch Allyn/Tycos (Arden, N.C., USA).

Statistical analysis. The Kolmogorov–Smirnov algorithm was used to determine whether each variable had a normal distribution. Comparisons of baseline data between the groups were

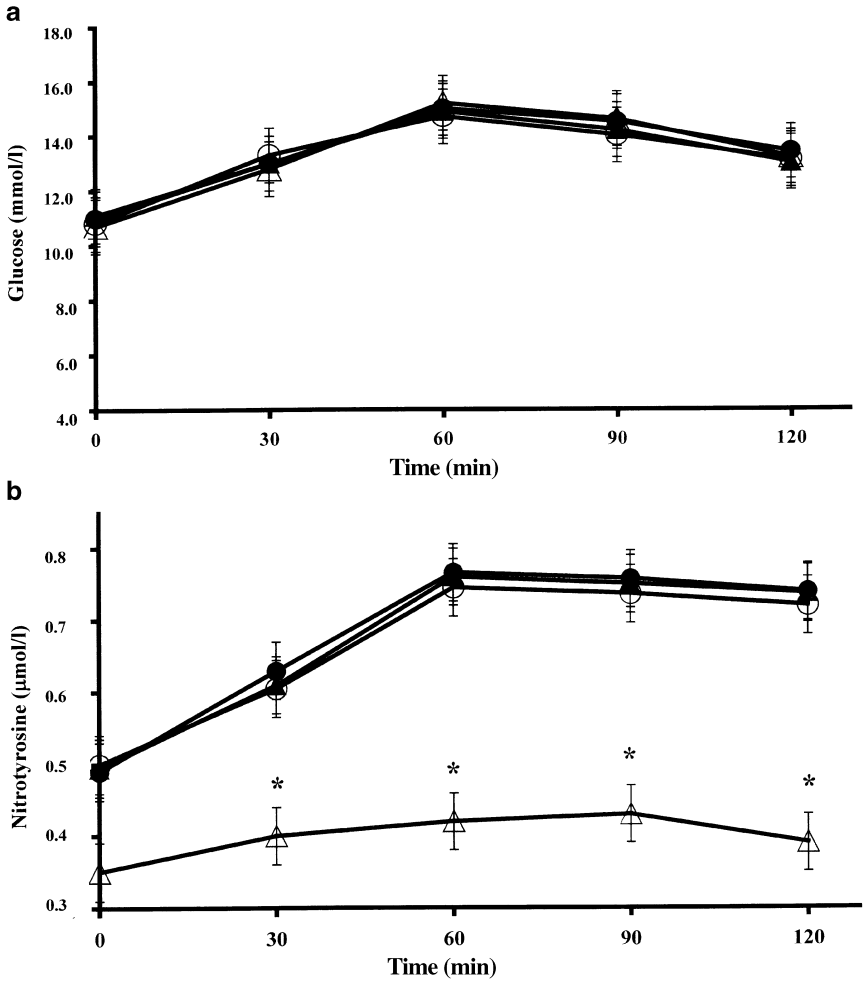

Fig. 1. Changes in plasma glucose (**a**) and nitrotyrosine (**b**) levels during the OGTT at baseline (placebo, filled circles; irbesartan, filled triangles) and after treatment (placebo, empty circles; irbesartan, empty triangles). Bars indicate SEM. * $p<0.01$ vs placebo

Table 2. Effects of irbesartan treatment in diabetic patients

Characteristic	Baseline prior to placebo treatment	After placebo treatment	Baseline prior to irbesartan treatment	After irbesartan treatment
BMI (kg/m^2)	27.4±2.1	27.7±2.2	27.5±2.2	27.4±2.3
Fasting glucose (mmol/l)	11.3±2.3	11.4±2.3	11.2±3.2	11.1±2.4
HbA_1c (%)	7.5±0.2	7.7±0.3	7.6±0.5	7.6±0.5
24-h mean systolic BP (mm Hg)	119.1±7.0	120.4±6.5	115.3±7.1	118.2±6.5
24-h mean diastolic BP (mm Hg)	76.4±4.1	75.2±5.1	76.6±4.1	75.4±3.7
Fasting nitrotyrosine (µmol/l)	0.58±0.3 0.35–0.79[b]	0.59±0.2 0.36–0.75[b]	0.57±0.4 0.36–0.82[b]	0.35±0.3[a] 0.20–0.61[b]

Data are expressed as means ± SEM. [a] $p<0.01$ vs baseline; [b] range of values

performed using the unpaired Student's *t* test. The paired Student's *t* test was used for comparison of the various parameters before and after treatment with irbesartan or placebo. The changes in variables during the OGTT were assessed by two-way ANOVA with repeated measures. If differences reached statistical significance, secondary analyses with the two-tailed paired *t* test was used to assess differences at individual time periods in the study, using the Bonferroni correction for multiple comparisons. Regression analysis was also performed to evaluate possible significant correlations between the various parameters. Statistical significance was defined as a *p* value of less than 0.05.

Results

Baseline fasting glucose and nitrotyrosine were higher in diabetic patients than in controls (Table 1, Fig. 1). In the diabetic group, the HbA_1c level did not change throughout the study (Table 2). No significant correla-

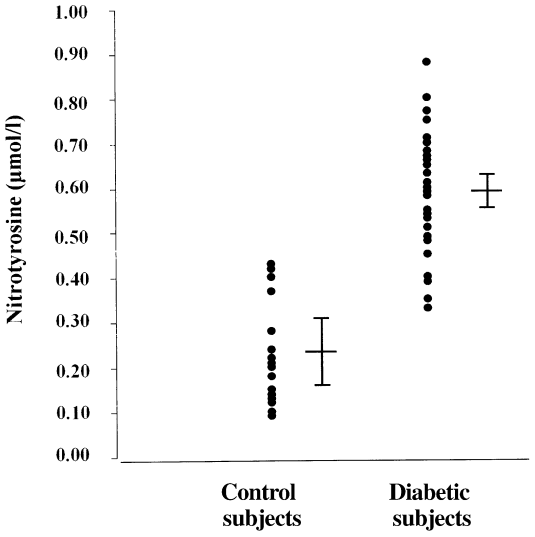

Fig. 2. Individual values of nitrotyrosine in control subjects and diabetic patients. Vertical bars indicate means ± SEM

tion was found between HbA_1c and plasma nitrotyrosine level at any point during the study.

The 24-h mean systolic and diastolic BP was not affected by irbesartan treatment. Fasting nitrotyrosine was significantly reduced after irbesartan treatment, whereas it remained unchanged after placebo (Table 2).

An increase in plasma glucose was observed during the OGTTs performed at baseline and after the washout period. This was accompanied by a significant and similar increase in plasma nitrotyrosine concentration ($p<0.01$ by ANOVA; Fig. 2). Irbesartan treatment reduced the increase in nitrotyrosine during the OGTT, whereas placebo did not (Fig. 2). No correlation was found between peak blood glucose values and peak plasma nitrotyrosine values in the untreated patients during the OGTT.

Both irbesartan and placebo were well tolerated. The most frequent adverse event was headache, which was experienced by three subjects on irbesartan and one on placebo. All patients completed the study.

Discussion

This study confirms that fasting plasma nitrotyrosine levels are increased in diabetic patients and that an acute increase in glycaemia is accompanied by an increase in nitrotyrosine [12, 13, 14, 15]. We have shown, for the first time, that plasma nitrotyrosine concentrations can be reduced by treatment with irbesartan and that this agent reduces nitrotyrosine generation during acute hyperglycaemia.

Three recent, large trials have demonstrated that AT-1 receptor blockers prevent the development of clinical proteinuria and the progression of nephropathy in Type 2 diabetic patients and that these beneficial results are independent of their BP-lowering effect [17, 18, 19]. Interestingly, the ability of irbesartan to reduce proteinuria independently of its BP-lowering action has also been confirmed [26], which suggests that AT-1 receptor blockers may have a protective effect on diabetes-related complications through some ancillary properties.

Since oxidative stress has been confirmed to play a key and pivotal role in the development of diabetic complications [7, 8], it has been suggested that the protection afforded by AT-1 receptor blockers against the development of diabetic nephropathy may be related to their "antioxidant" activity [27]. This study supports this hypothesis by demonstrating that irbesartan prevents oxidative stress in diabetic patients. Furthermore, in line with previous observations [26], our results indicate that irbesartan treatment does not affect BP in normotensive patients.

Recent insights into oxidative stress and diabetic complications suggest that nitrotyrosine formation and deposition in tissues may be involved in their development [28]. Increased nitrotyrosine staining has been reported in tissues that have either been exposed to high concentrations of glucose [29, 30, 31] or obtained from diabetic patients, including heart [32] and kidney [16]. Our study is the first to show that an AT-1 receptor blocker can reduce in vivo nitrotyrosine formation in diabetic patients. Interestingly, irbesartan not only reduces fasting nitrotyrosine levels, but also attenuates the pro-oxidant effect of acute hyperglycaemia. This effect is of particular relevance as recent evidence suggests that both chronic stable and acute hyperglycaemia may play a distinct and important role in the pathogenesis of diabetic complications, including nephropathy [1, 2, 6].

The effect of irbesartan on nitrotyrosine formation may rationally be explained by its "preventive" antioxidant activity [28]. Enhanced renin–angiotensin activity has been demonstrated in several pathological conditions, including diabetes [33], and the effects of angiotensin II are mediated primarily through its AT-1 receptor [33]. There is accumulating evidence that angiotensin II increases vascular oxidative stress [34], partly through the activation of the membrane-bound NADPH oxidase enzyme [34], which is linked to AT-1 receptor activity [34]. This is consistent with the finding that AT-1 receptor stimulation increases superoxide anion production in endothelial cells [35]. Since hyperglycaemia enhances AT-1 receptor expression, this is considered to be the mechanism by which it amplifies oxidative stress generation [36]. Interestingly, AT-1 blockade prevents the oxidative stress induced by hyperglycaemia [36].

It has been reported that AT-1 receptor activation plays an important role in nitrotyrosine production [37], and that AT-1 receptor blockade reduces nitrotyrosine deposition in animal models of diabetes [31, 38].

Although the data are not conclusive [20, 21], it has been suggested that AT-1 receptor blockade may also be used for the prevention of cardiovascular disease in diabetic patients [39]. Importantly, nitrotyrosine has recently been identified as an independent predictor of cardiovascular disease [13]. Our data on the effect of AT-1 receptor blockade on plasma nitrotyrosine levels contribute to our understanding of the possible beneficial effects of AT-1 receptor blockers in the prevention of cardiovascular disease in diabetes.

It is of note that similar effects, mediated through different pathways, are exhibited by statins, and the protective effects of these compounds on cardiovascular disease are well established [13, 40, 41].

In conclusion, our study shows that irbesartan can reduce plasma nitrotyrosine levels in diabetic patients in vivo and can counterbalance the effects of acute hyperglycaemia on nitrotyrosine formation. Since nitrotyrosine is a strong marker of oxidative stress [28], is frequently associated with tissue damage in diabetic patients [16, 29, 30, 31, 32] and is possibly a predictor of vascular disease [13], the nitrotyrosine-lowering effect of irbesartan has implications for the control of diabetic complications. Moreover, our results may help to explain how AT-1 receptor blockers exert their beneficial effect independently of their BP-lowering activity.

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