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Effect of Nateglinide and Glibenclamide on Endothelial Cells and Smooth Muscle Cells from Human Coronary Arteries

H. Seeger, C. Abletshauser, A. O. Mueck

Abstract: In the present work the effect of nateglinide and glibenclamide, two different substances used for therapy of diabetes mellitus type 2, were investigated on the synthesis of markers of endothelial function and on the proliferation of smooth muscle cells *in vitro*. As cell models endothelial and smooth muscle cells from human coronary arteries were used. Both substances were tested at concentrations of 0.1, 1 and 10 µmol/l. As markers of endothelial function prostacyclin, endothelin and plasminogen-activator-inhibitor-1 (PAI-1) were tested. Nateglinide and glibenclamide were similarly able to inhibit endothelial endothelin and PAI-1 synthesis, but only at the highest concentration tested. Endothelial prostacyclin synthesis and proliferation of smooth muscle cells were not significantly changed by both substances.

These results indicate that both nateglinide and glibenclamide may have potential in reducing negative long-term effects of diabetes such as atherogenesis.

Kurzfassung: Effekt von Nateglinid und Glibenclamid auf Endothel- und Muskelzellen humaner Koronararterien. In der vorliegenden Arbeit wurde die Wirkung von Nateglinid und Glibenclamid, zweier unterschiedlicher Substanzen zur Behandlung des Diabetes mellitus Typ 2, auf die Synthese von Markern der Endothelfunktion und auf die Proliferation glatter Muskelzellen untersucht. Als Zellmodell dienten Endothelzellen und glatte Muskelzellen menschlicher Koronararterien. Beide Substanzen wurden in den Konzentrationen 0,1, 1 und 10 µmol/l getestet. Als Marker der Endothelfunktion dienten Prostazyklin, Endothelin und Plasminogen-Aktivator-Inhibitor-1 (PAI-1). Sowohl Nateglinid als auch Glibenclamid konnten die endotheliale Endothelin- und PAI-1-Produktion in ähnlichem Ausmaß senken, allerdings nur in der höchsten Konzentration. Die Prostazyklinsynthese und die Muskelzellproliferation wurden nicht signifikant beeinflußt.

Diese Ergebnisse deuten daraufhin, daß sowohl Nateglinid als auch Glibenclamid in der Lage sein könnten, negative Langzeitwirkungen des Diabetes, wie die Atherogenese, zu verzögern. **J Kardiol 2004; 11: 35–7.**

■ Introduction

Diabetes mellitus type 2 appears to be one of the major human health concerns in the near future. In the recent years the pathophysiology of this disease has been partly evaluated. Insulin resistance seems to be responsible for hyperglycaemia and a number of cardiovascular risk factors such as hypertension, endothelial dysfunction and disturbances in the fibrinolytic/coagulation system [1]. Apart from early diagnosis, prevention, diet and physical activity an effective therapy is important. In addition to the older class of antidiabetics such as sulfonylureas (e.g. glibenclamide) and metformin new substance classes such as the "glinides" (e.g. nateglinide, repaglinide) are now available. The aim of medical therapy is not only to reduce the hyperglycaemic status but also to prevent long-term sequelae of the insulin resistance syndrome such as atherosclerosis. Endothelial dysfunction and remodelling of the vasculature is one of the major risk factors for developing atherosclerosis [2]. In the present *in vitro* study we have investigated the effect of nateglinide on the synthesis of markers of endothelial function and on smooth muscle cell proliferation for the first time and compared it to the effect of glibenclamide.

■ Material and Methods

Nateglinide (Nat) was kindly provided by Novartis Pharma, Nuremberg (Germany). Glibenclamide (Glib) was purchased from Sigma, Munich, Germany. The substances were dissolved in ethanol and tested at the concentrations 0.1, 1 and 10 µmol/l. Endothelial cells from human male coronary arteries were purchased from PromoCell, Germany, and the experiments were conducted at passages 3–4 with two different cell pools. The cells were placed in culture dishes and allowed to replicate to confluence in medium containing 5 % fetal calf serum, 0.4 % endothelial cell growth factor/heparin, 10 ng/ml epidermal growth factor, 1 µg/ml hydrocortisone, 50 µg/ml gentamicin sulfate and 50 ng/ml amphotericin B. Cell cultures were maintained in an atmosphere of 5 % CO_2 in air. The cells were transferred into 24-well dishes and maintained in growth medium. After confluence, medium was changed to a serum-free one, the test substances were added and the cells incubated for 24 h. Control values were determined by addition of ethanol alone; the final ethanol concentration per well was 1 %. No significant effects on the parameters investigated were found for this solvent concentration. Cell viability was determined by the trypan blue exclusion method.

As markers of endothelial function, we chose prostacyclin, endothelin and plasminogen-activator-inhibitor-1 (PAI-1). Prostacyclin production was monitored by measuring its stable metabolite 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) in the cell medium by enzyme immunoassay (Biotrend, Cologne, Germany). The sensitivity of the assay was 10 pg/ml. Inter- and intraassay variation coefficients were 10.5 % and 7.8 %, respectively. Accuracy of the assay was 107 % (range 98–123 %), the cross-reactivity with thromboxane and thromboxane metabolites was < 0.1 % and with other prostacyclin metabolites < 5 %. Endothelin was measured in the cell medium by enzyme immunoassay (Biotrend, Germany). The sensitivity of the assay was 13 pg/ml. Interassay and intraassay variation coefficients were 10.3 % and 7.9 %, respectively. Accuracy of the assay was 99.5 % (range 96–104 %), the cross-reactivity with endothelin 1 and endothelin 2 was < 4 %.

PAI-1 was measured directly in the cell medium by enzyme immunoassay (American Diagnostics, Germany). The sensitivity of the assay was 10 pg/ml. Interassay and intraassay variation coefficients were 10.5 % and 6.4 %, respectively. Accuracy of

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the assay was 106 % (range 95–110 %); the cross-reactivity with tPA and other possible interfering substances was < 0.1 %.

The experiments on smooth muscle cells were conducted with human male smooth muscle cells from coronary arteries (Promocell, Germany) at passages 5–8. The cells were cultured in MDCB 131, 10 % FCS, 1 % amphotericin B and 1 % penicillin/streptomycin. After confluence, 30,000–50,000 cells were transferred in standard medium into 6-well plates. Twenty-four hours later the medium was changed for one containing only 5 % FCS. The test solutions as well as the control solutions contained ethanol in a final concentration of 1 %. Medium and test substances were changed every 48 h. After incubation for 7 days the cells were lysed by trypsination and counted using a Coulter counter ZM1.

Statistical analyses of the absolute values were performed by Student's t-test. Statistics of the relative changes were calculated after logarithmic transformation of the values by ANOVA and Dunnett test (two-sided) from triplicates of two different experiments.

■ Results

For all endothelial markers time-dependency experiments were conducted prior to the final experiments. The results indicate that an incubation time of 18–24 h is optimal (data not shown). In Figure 1 the results of 6-keto-$PGF_{1\alpha}$ synthesis in endothelial cells are given. Basal values of this prostacyclin metabolite were 420 ± 24 pg/ml. Neither Nat nor Glib significantly changed the synthesis in the tested concentrations of 0.1, 1 and 10 µmol/l.

In Figure 2 the changes of endothelial endothelin synthesis after addition of Nat and Glib are depicted. The basal values of endothelin were 17.1 ± 2.7 pg/ml. Both substances were able to significantly reduce these basal endothelin concentrations. The reductions were 36 and 64 % for Glib at the concentration of 1 and 10 µmol/l. The corresponding values for Nat were 20 and 60 %. There was a significant difference between Glib and Nat at 1 µmol/l.

In Figure 3 the changes in PAI-1 concentrations in the supernatant of endothelial cell cultures are shown. The basal values of PAI-1 were 1132 ± 106 pg/ml. Both Glib and Nat significantly reduced PAI-1 synthesis at a concentration of 10 µmol/l. The values were 15 and 20 %. No significant difference between Glib and Nat was found.

In Figure 4 the changes in cell numbers of smooth muscle cells is depicted. Neither Glib nor Nat significantly changed cell number after incubation for 7 days.
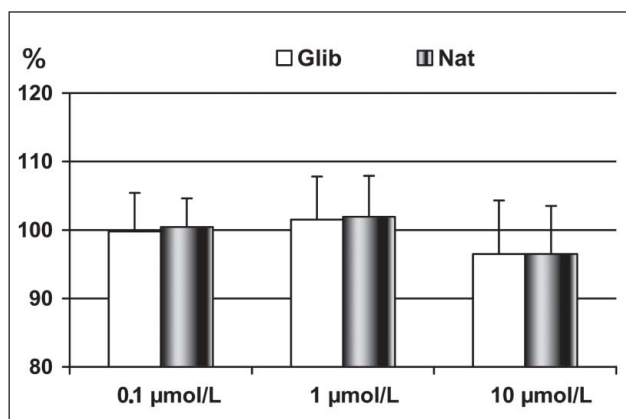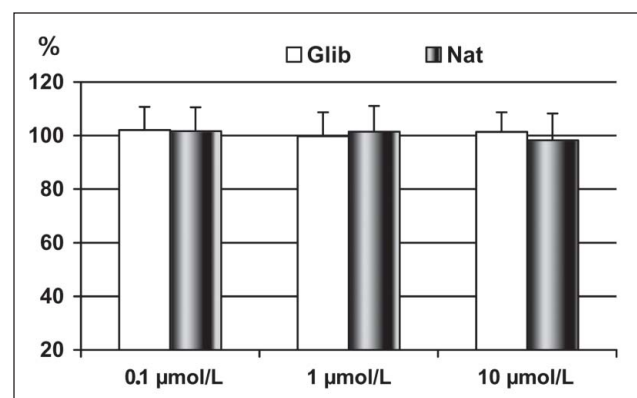

Figure 1: Changes in prostacyclin concentration in endothelial cell cultures from human coronary artery after addition of nateglinide (Nat) and glibenclamide (Glib) as percentage of control value (= 100 %); means ± SD, triplicates from 2 different experiments
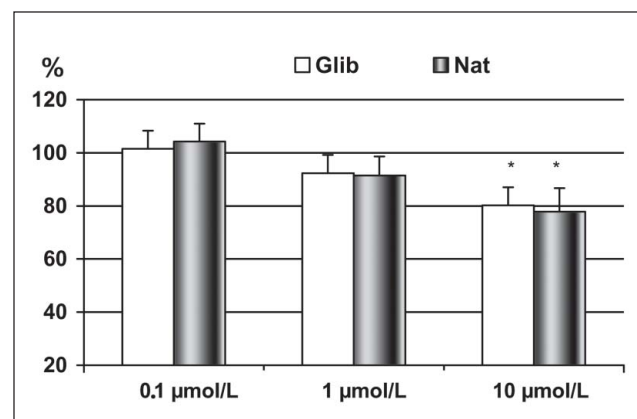
Figure 3: Changes in PAI-1 concentration in endothelial cell cultures from human coronary artery after addition of nateglinide (Nat) and glibenclamide (Glib) as percentage of control value (= 100 %); means ± SD, triplicates from 2 different experiments (*p < 0.05 vs. control)
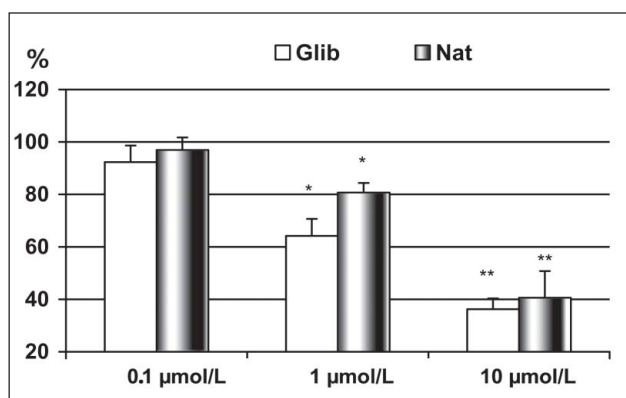
Figure 2: Changes in endothelin concentration in endothelial cell cultures from human coronary artery after addition of nateglinide (Nat) and glibenclamide (Glib) as percentage of control value (= 100 %); means ± SD, triplicates from 2 different experiments (*p < 0.05, **p < 0.01 vs. control)

Figure 4: Changes in cell number of smooth muscle cells from human coronary artery after addition of nateglinide (Nat) and glibenclamide (Glib) as percentage of control value (= 100 %); means ± SD, triplicates from 2 different experiments

■ Discussion

The pathophysiology of atherosclerosis is not completely understood, but recent research claims that it is a fibroproliferative-antiinflammatory process being manifested over several years [3]. Endothelial dysfunction is believed to be a critical step in atherosclerosis, coronary heart disease and congestive heart failure. The vascular endothelium is well recognized as an important organ with multifactorial properties. The endothelium synthesizes a range of substances which play a decisive role in regulating vascular tone and hemostasis and modulate inflammatory and proliferative actions. A variety of markers of endothelial function such as prostacyclin, endothelin and PAI-1 were investigated in the present study. In addition, measurement of smooth muscle cell proliferation which represents a crucial step in the pathogenesis of atherosclerosis was included in the experiments.

Prostacyclin is a prostanoid with strong vasodilatory and antiaggregatory properties [4]. Moreover, prostacyclin acts antiatherogenic by reversing intracellularly cholesterol esterification and by facilitating removal of cholesterol via interaction with HDL [4]. Our results show that neither nateglinide nor glibenclamide seem to be able to significantly influence prostacyclin synthesis by the vascular endothelium. Endothelin is one of the strongest vasoconstrictory substances known so far. It is mainly synthesized in the vascular endothelium and acts abluminally, i.e. influencing the smooth muscle cells [5]. Endothelin is believed to be an important counter part to vasodilatory compounds such as nitric oxide or prostacyclin. Nateglinide as well as glibenclamide were able to significantly suppress endothelial endothelin synthesis. This mechanism may contribute to long-term cardiovascular protection.

Plasminogen-activator-inhibitor-1 (PAI-1) is an important compound within the hemostatic system. Increased serum concentrations of PAI-1 may shift the fibrinolytic/coagulatory balance towards an increased risk of arterial thrombosis. PAI-1 has been detected in high concentrations in atherosclerotic plaques and therefore may accelerate arterial thrombosis following plaque rupturing [6]. Evidence is accumulating that PAI-1 may be an independent risk factor for cardiovascular diseases [6]. In the present study both nateglinide and gliben-clamide significantly reduced endothelial synthesis of PAI-1. This effect may contribute to an improvement of the fibrinolytic/coagulation balance and furthermore to delaying of atherogenesis. However, the real contribution of endothelial cells to serum PAI-1 levels remains to be determined. Evidence is accumulating that smooth muscle cells within the arterial plaque are an important source of PAI-1 synthesis. However, since the smooth muscle cells found within the atherosclerotic plaque are of a different phenotype (synthetic) than that one available for cell cultures (contractile), investigations with the latter phenotype may not be representative for the smooth muscle cells presented in plaques and have therefore not been conducted in the present work.

The migration of vascular smooth muscle cells seems to be a crucial step in atherogenesis. Subsequent modulation renders smooth muscle cells into a proliferating phenotype which is involved in remodelling of the vascular wall [3]. In our experiment both substances did not elicit any significant effect on the proliferation of vascular smooth muscles. Thus glibenclamide and nateglinide seem not to support the development of atherosclerosis by negatively influencing the proliferation of smooth muscle cells. However, it should be mentioned that smooth muscle cell proliferation could be a positive event that stabilizes the lesion as well as a negative event leading to restenosis. Further experiments on the effect of both substances on smooth muscle cells may be necessary to evaluate a possible role of these compounds on plaque stability.

The present data indicate that nateglinide and glibenclamide seem to have beneficial effects on the synthesis of certain markers of endothelial function. This property of both substances may contribute to the delay of atherogenesis in the course of long-term insulin resistance syndrome.

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