The effect of aldose reductase inhibition by JMC-2004 on hyperglycemia-induced endothelial dysfunction

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Abstract

OBJECTIVES: An increased glucose utilization by aldose reductase (ALR-2) has been implicated in the pathogenesis of diabetic vascular complications. In this process, several mechanisms are involved, including the depletion of cofactors required for the action of antioxidant enzymes or endothelial NO synthase. In this study, the effect of a novel ALR-2 inhibitor JMC-2004 on hyperglycemia-induced endothelial dysfunction was studied.

METHODS: Bovine aortic endothelial cells (BAEC) were treated with glucose (30 mM), JMC-2004 (0.01mM), or glucose and JMC-2004 for 24 h. The cells were then stimulated with calcium ionophore A23187 after which NO production was measured electrochemically using a porphyrine-coated carbon NO electrode. Nitrite concentrations were determined in the cell supernatants. The peroxyl and hydroxyl radical-scavenging activity of JMC-2004 was measured with luminol–enhanced chemiluminescence. The expression of eNOS was determined by Western blotting. JMC-2004 IC50 for ALR-2 was determined colorimetrically with D-glyceraldehyde as a substrate.

RESULTS: Incubating the cells with 30 mM glucose strongly diminished A23187-induced NO production. Treatment with JMC-2004 restored NO production by 40% without affecting eNOS expression. This effect was probably antioxidant-independent, since JMC-2004 did not have any antioxidant capacity. JMC-2004 exerted high selectivity towards ALR-2.

CONCLUSIONS: ALR-2 inhibition with JMC-2004 was able to abolish hyperglycemia-induced endothelial dysfunction in bovine aortic endothelial cells.

ORIGINAL ARTICLE

INTRODUCTION

Increased glucose utilization by ALR-2, a rate-limiting enzyme of the polyol pathway, that catalyzes NADPH-dependent reduction of glucose to sorbitol, has been implicated in the pathogenesis of chronic diabetic complications. Under normoglycemic conditions, ALR-2 has a low affinity for glucose. However, under hyperglycemia glucose flux through polyol pathway strongly accelerates (Farhangkhoee *et al*., 2006) which results in an accumulation of polyol pathway products as well as in the depletion of the cofactors consumed by the pathway. In the tissues with an insulin-independent glucose uptake, this leads to serious metabolic disturbances, which clinically manifest as diabetic neuropathy, retinopathy, nephropathy or cataract (Yabe-Nishimura, 1998). These chronic diabetic complications are largely responsible for the high morbidity and mortality of diabetic patients (Nicolaou *et al*., 2004).

As it is very difficult to maintain normoglycemia in diabetic patients, research in recent decades has focused on ALR-2 as a potential target of pharmacological intervention. Several aldose reductase inhibitors (ARIs) have been developed with promising results both *in vitro* and in animal models, however, most of them have been ruled out in the stage of clinical trials due to their adverse effects or low efficiency (El-Kabbani *et al*., 2004). Recently, the attention has been focused to discover ARIs of distinct chemical structures other than hydantoin or carboxylic acid derivatives, which exert toxicity or narrow spectrum of tissue activity (Nicolaou *et al*., 2004).

Vascular endothelium with an insulin-independent glucose uptake is one of the primary targets of glucose-induced damage (Zúrová-Nedelčevová *et al*., 2006; De Vriese *et al*., 2000; Farhangkhoee *et al*., 2006). Although the molecular basis of endothelial dysfunction in diabetes is not fully elucidated, an increased polyol pathway activity is known to be one of the important contributing factors. NADPH depletion affects the activity of endothelial NO synthase (eNOS) and glutathione reductase, which leads to an increased susceptibility to oxidative stress along with decreased NO bioavailability (El-Kabbani *et al*., 2004). This results in the disturbed vasomotoric functions as well as in a loss of antiadhesive, antithrombotic and antiinflammatory properties of vascular endothelium.

In this study, the effect of ALR-2 inhibitor JMC-2004 was tested on bovine aortic endothelial cells (BAEC). JMC-2004 ((1-(3,5-difluoro-4-hydroxyphenyl)-1H-pyrrol-3-yl)(phenyl)methanone) was synthesized in the Department of Pharmaceutical Chemistry, School of Pharmacy, Thessaloniki as a putative ARI, based on the concept of bioisosterism between the carboxylic acid and the 2,6-difluorophenol group. 2,6-difluorophenol retains the acidic properties required for potent ARIs, while at the same time has improved lipophilicity in comparison to the carboxylic acid moiety. This property could add to a better pharmacokinetic profile of the aforementioned compound when compared to carboxylic acid derivatives. Also, JMC-2004 was found to inhibit the *in vitro* glycation of proteins (Nicolaou *et al*., 2004), a common attribute in hyperglycaemic conditions.

MATERIALS AND METHODS

Assessment of IC_{50} for ALR-1 and antioxidant activity of JMC-2004

IC_{50} was tested on ALR-1 from rat kidney as described previously by Stefek *et al* (2008). The scavenging activity against the hydroxyl radical and peroxyl radical were measured as described previously by Papežíková *et al*. (2007) and Číž *et al*. (2008).

Cell culture and toxicity test

BAEC (ECACC, UK) were cultivated in gelatine-coated plates (TPP, Switzerland) in Dulbecco's Modified Eagle Medium (DMEM, PAN, Germany) containing 5 mM glucose. DMEM was supplemented with 5% of fetal calf serum and 5% of newborn calf serum (PAN, Germany). Confluent cells at passage 6 – 9 were used for the experiments.

First, toxicity of JMC-2004 was tested. BAEC were treated with 10 nM – 100 μM of JMC-2004 for 24 h. Cell viability was then measured luminometrically with the use of ATP Cell Viability test (BioThema, Sweden). For further experiments, 10 μM concentration of JMC-2004 was chosen.

Direct electrochemical measurement with NO-sensitive microelectrode

For the measurement of NO production, the cells were cultivated in gelatine-coated glass vials. When the cells reached the confluence, the medium was changed and the cells were treated with 25 mM of glucose (reaching a final concentration of 30 mM) or 25 mM of glucose (Sigma, Germany) + 10 μM of JMC-2004. After 24 h, the vials were connected to a three-electrode system (porphyrinic microsensor working electrode, a platinum wire counter electrode and a miniature saturated silver/silver chloride reference electrode) and placed in a Faraday chamber. After the signal stabilized, 1μg/ml of calcium ionophore A23187 (Sigma, Germany) was injected to the vial and nitric oxide production was measured for 15 minutes. Then the cell supernatants were collected for measuring their nitrite concentrations, which was done using the Griess method (Číž *et al*., 2008). The cells were lysed with a SDS lysing buffer and protein concentration was estimated using the BCA protein assay (Číž *et al*., 2008).

eNOS expression

Electrophoresis and western blotting were performed in the standard manner (Pečivová *et al*., 2006). Polyclonal rabbit anti-eNOS antibody (Cell Signaling, USA; 1/1000)

and donkey anti-rabbit IgG horseradish peroxidase – conjugated antibody (GE Healthcare, UK; 1:2000) were used. The immunoreactive bands were detected using ECL detection reagent kit (Pierce, USA) and exposed to radiographic film (AGFA, Belgium). Relative protein levels were quantified by scanning densitometry using the ImageJ™ program and the individual band density value was expressed in arbitary units.

Statistical analysis

Data were evaluated using the Student t-test. Statistical significance was assumed when $p<0.05$.

RESULTS

IC_{50} for ALR-1 was 13.4 ± 1.0 μM, which is a 33.8 fold higher than IC_{50} for ALR-2 (IC_{50} 0.4 ± 1.2 μM as shown previously by Nicolaou *et al*., 2004). JMC-2004 did not exert signicifant antioxidant activity towards any of the tested radicals (data not shown).

The test of toxicity revealed that applied concentrations of JMC-2004 did not significantly decrease cell viability after 24h incubation (Figure 1). Only 100 μM concentration of JMC-2004 exerted some slight toxicity for BAEC.

Glucose (30 mM) strongly decreased A23187-induced NO production by BAEC. JMC-2004 restored NO production by approximately 40% (Figure 2). Changes in nitrite concentrations in the cell supernatants corresponded to changes in NO production (Figure 3). In contrast, western blot analysis of eNOS protein expression did not reveal any significant changes.

DISCUSSION

Chronic diabetic complications affect a huge number of diabetic patients and strongly decrease their quality of life (Vojtaššák *et al*., 2006). ALR-2 inhibition appears to be a very promising strategy. However, clinical trials with ARIs gave rather disappointing results. Poor pharmacokinetic properties, lack of specificity and unsatisfactory therapeutic effects of tested compounds were the main problems. Nevertheless, diabetic complications are such a serious problem with limited chances of treatment, that the development of more effective and less toxic inhibitors appears highly desirable (Nicolaou *et al*., 2004).

One problem associated with ALR-2 inhibition is the co-inhibition of other oxidoreductases, which leads to adverse side effects. So the testing for selectivity should be a criterion for pharmacologically applicable ARIs. IC_{50} of JMC-2004 for ALR-1, an enzyme with the highest homology to ALR-2, was considerably higher than to IC_{50} for ALR-2. This is in contrast to some of the previously tested compounds that inhibited both enzymes with approximately the same IC_{50} (Yabe-Nishimura, 1998).

JMC-2004 has proved to be nontoxic at much higher concentrations than its IC_{50} for aldose reductase and probably higher than the concentrations reachable *in vivo* in body fluids. However, the toxicity was tested only on BAEC. The effect on human cells is yet to be determined.

In the most of target tissues including vascular endothelium, the main problem is considered to be the depletion of NADPH consumed by polyol pathway (Yabe-Nishimura, 1998). This leads to changes in the activity of other NADPH-dependent enzymes, mainly glutathione reductase and NO synthases, and results in a decrease in NO production and increased susceptibility to oxidative stress. In BAEC, cultivation with 30 mM glucose caused a strong decrease in NO production. Concomitant treatment with JMC-2004 partially restored NO production as measured directly electrochemically and confirmed by the measurement of nitrite concentrations in the cell supernatants. This effect was independent on changes in eNOS expression as no significant changes in the enzyme expression were found. Also, JMC-2004 probably did not act as an antioxidant as no antioxidant activity towards any of the tested ROS was observed.

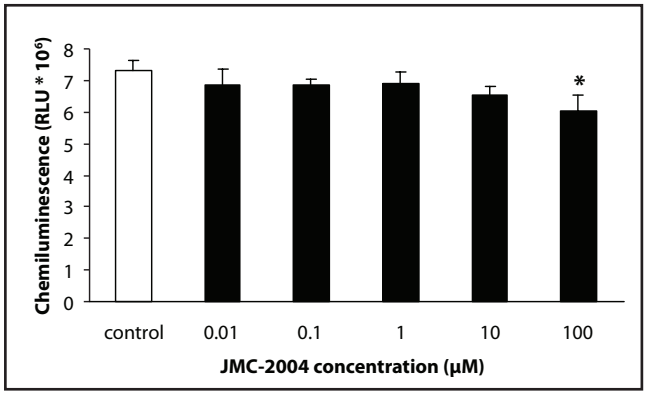

Figure 1. BAEC viability after 24 h incubation with JMC-2004. The data represent integrals of obtained kinetic curves (means ± SEM, n=4). Significant differences are marked with asterisks.

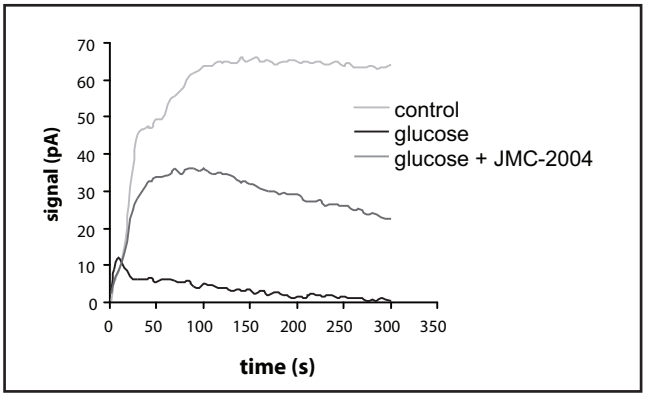

Figure 2. NO production measured electrochemically, n=4. Kinetic curves.

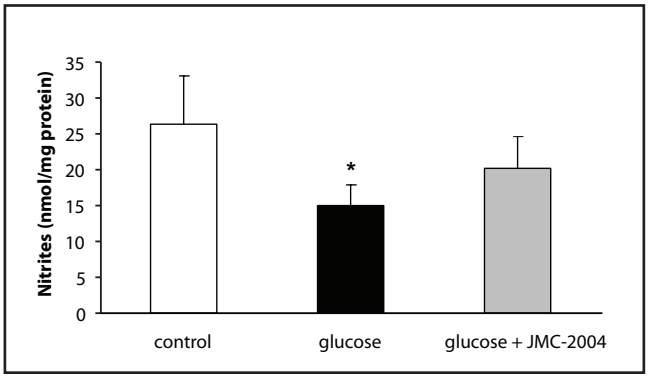

Figure 3. Nitrites in the cell supernatants from A23187-stimulated cells. Data represent mean ± SEM, n=4. Statistically significant differences from the control are marked with asterisks.

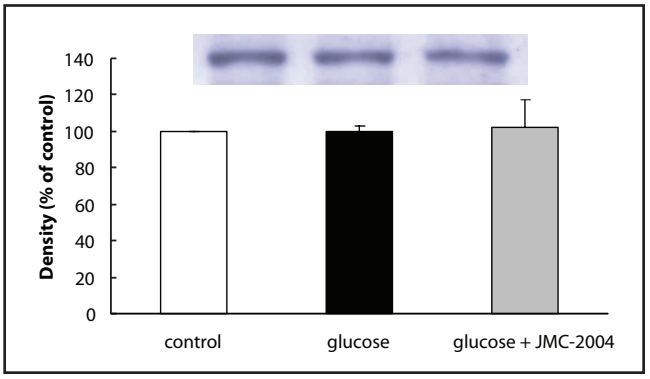

Figure 4. eNOS expression – density (n= 4) and a representative picture.

In conclusion, JMC-2004 exerted low toxicity and high effectivity in endothelial cells *in vitro*. Moreover, JMC-2004 has a significant selectivity factor regarding the inhibition of ALR-1, so it is expected that adverse effects and toxicity due to the inhibition of oxidoreductases apart from ALR-2 will not manifest. Thus, JMC-2004 appears to be a promising molecule with the potential to become a useful lead compound in the field of ARIs.

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