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Resorcylidene aminoguanidine induces antithrombotic action that is not dependent on its antiglycation activity

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ABSTRACT

There is good evidence supporting the notion that aminoguanidine(AG)-derived compounds prevent glycation/glycooxidation-dependent processes and therefore inhibit late diabetic complications. The aim of the present work was to analyse the antithrombotic action and antiglycation activity of β-resorcylidene aminoguanidine (RAG) in comparison with another commonly used aminoguanidine (AG)-derived compound, pyridoxal aminoguanidine (PAG).

In vitro RAG and PAG prevented exhaustive glycation and glycooxidation of BSA to a similar extent. However, merely RAG showed almost complete binding to sepharose-immobilized heparin, while PAG and other AG derivatives had much poorer affinities. In the model of *in vivo* thrombosis in Wistar rats with extracorporeal circulation RAG (i.v. 30 mg/kg), but not PAG, produced sustained (2 h) antithrombotic effect, which was abrogated by indomethacin (5 mg/kg) and rofecoxib (1 mg/kg). The 60-day treatment of streptozotocin-diabetic animals with RAG (p.o. 4 mg/kg) significantly decreased plasma concentration of a thromboxane B_2 and reduced whole blood platelet aggregability triggered by ADP or collagen.

In conclusion, although RAG and PAG displayed similar antiglycation and antioxidation activities *in vitro*, only RAG showed antithrombotic activity *in vivo* that involved activation of COX-2/PGI_2 pathway. Our results indicate that designing novel RAG derivatives with optimal antithrombotic and antiglycation activities may prove useful to treat diabetic complications.

1. Introduction

The evidence accumulated for years points that both protein non-enzymatic modification by glucose (non-enzymatic glycosylation or glycation) and a persisting oxidative stress (Ceriello, 1999; Januszewski et al., 2003; Kennedy and Lyons, 1997; Vlassara and Palace, 2002) may be crucial in the development of long-term vascular complications in the course of diabetes mellitus (DM). The enhanced protein glycation and formation of early glycation products in diabetes is intimately associated with the processes of glycation and "glycoxidation", the formation of advanced glycation end-products and AGE-derived protein crosslinks (Jakus and Rietbrock, 2004; Miyata et al., 2003; Vlassara and Palace, 2002, 2003).

In turn, aminoguanidine (AG) — the prototype therapeutic agent preventing the formation of advanced glycation end-products (AGEs) and the inhibitor of "carbonyl stress" have proven efficient to inhibit various diabetic sequelae in experimental animal models of diabetes mellitus (Cameron et al., 2005; Ceriello, 1999; Jakus and Rietbrock, 2004; Miyata, 2002; Nilsson, 1999; Thornalley, 2003; Vlassara and Palace, 2002). AG is a highly reactive nucleophilic reagent that reacts with α or β-dicarbonyl compounds in numerous biological molecules (pyridoxal phosphate, pyruvate, glucose, malondialdehyde, and others) to form the substituted 3-amino-1,2,4-triazine derivatives (Nilsson, 1999; Thornalley, 2003). However, AG has been also shown to have other undesired pharmacological activities, like inhibition of inducible nitric oxide synthase and semicarbazide-sensitive amine oxidase, at high pharmacological concentrations achieved *in vivo* (Nilsson, 1999; Thornalley, 2003), and therefore, several novel AG derivatives were invented.

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The Schiff base adducts formed from aminoguanidine derivatives containing aromatic moieties, like pyridoxal or resorcylidene, have been suggested to be more safe and equally or even more efficient antiglycation compounds and carbonyl stress inhibitors. Indeed, they are effective at significantly lower doses than aminoguanidine. Therefore they appear more promising antidiabetic agents than aminoguanidine itself (Jakus et al., 1999; Korytar et al., 2003; Taguchi et al., 1999; Waczulikova et al., 2002a). Quite surprisingly, despite the plethora of experimental evidence showing efficacy of AG-derived compounds in prevention of glycation- and/or oxidative stress-dependent late diabetic complications, there is no evidence on antithrombotic properties of these compounds that may contribute to their therapeutic efficacy.

Therefore, in the present work we compared the antithrombotic actions of aminoguanidine (AG) and its two derivatives, β-resorcylidene aminoguanidine (RAG) and its analogue — pyridoxal aminoguanidine (PAG), using *in vivo* animal model of thrombosis. The early antiglycation and antioxidant activities, as well as endothelial-binding properties of the studies compounds were also explored in order to elucidate the possible mechanisms of the antithrombotic action of RAG. Our rationale in the present study was to show that the antithrombotic effects of RAG remain largely independent of antiglycation. To argue in favour of this hypothesis we monitored the effects of AG derivatives on early glycation (model experiments with BSA glycation and oxidation) and on antithrombotic activity *in vivo*. In addition, to explore whether the preventive effects of RAG towards endothelium may be an important contributor in the pathophysiology of diabetic complications, we verified our leading hypothesis in an *in vivo* model with chronically diabetic rats.

Our present study clearly demonstrates that RAG and PAG displayed similar antiglycation and antioxidation activities *in vitro*, but merely RAG possessed antithrombotic activity *in vivo*. Accordingly, RAG derivatives with optimal antithrombotic and antiglycation activities may prove more efficient to treat diabetic complications than prototype AG derivatives.

2. Materials and methods

2.1. Synthesis of aminoguanidine derivatives

The resorcylidine aminoguanidine (RAG, $C_8H_{10}O_2N_4$.HCl), 2,5-dihydroxybenzylidene aminoguanidine (BAG, $C_8H_{10}O_2N_4$.HCl) have been prepared by the direct condensation of equimolar amounts of resorcylaldehyde or 2,5-dihydroxybenzaldehyde with aminoguanidine hydrogen carbonate ($CH_6N_4\,H_2CO_3$) (Hovorka et al., 1953; Carsky et al., 1978). The salicylidene semicarbazone (SAS-on, $C_8H_9N_3O_2$) and resorcylidene semicarbasone (RAS-on, $C_8H_9N_3O_2S$) have been prepared by general methods of the synthesis of thiosemicarbazone aldehydes and ketones (Hovorka and Holzbecher, 1951; Stankoviansky and Carsky, 1961). Pyridoxylidene aminoguanidine (pyridoxal aminoguanidine, PAG, $C_8H_{13}O_2N_5$.HCl) hydrochloride has been prepared by the method described by Taguchi et al. (1999).

2.2. Other chemicals

All chemicals were of analytical grade and were purchased in Sigma (St. Louis, MO, USA), unless otherwise stated. Arachidonic acid and equine tendon collagen type I for platelet aggregation were from Chrono-Log Corp. (Havertown, USA). 2,4-dinitrophenyl hydrazine (DNPH) was from Lachema (Brno, Czech Republic). L-Glutamic acid and L-lysine were from Fluka GmbH (Buchs, Switzerland). Fungizone and gentamycin were from Gibco (Invitrogen Ltd., Paisley, UK). Vacutainer™ CTAD tubes and hirudin (Refludan® (lepirudin (rDNA) for injection) for blood anticoagulation were respectively from Becton Dickinson (Plymouth, UK) and Aventis (Aventis Pharma Deutschland GmbH, Bad Soden a. Ts., Germany). Aspisol® (DL-lysinmonoacetylsalicylate) was a kind gift from Bayer AG (Leverkusen, Germany). Cartridges for HbA_{1c} determination with the use of DCA 2000 analyser were also from Bayer AG and kit for creatinine was from bioMérieux (Marcy L'Etoile, France). Commercial kits for determination of plasma TXB_2 and urinary 11-dehydro-thromboxane B_2 (11-dehydro-TXB_2) were purchased from Cayman Chemical Company (Ann Arbour, MI, USA). Heparin Sepharose CL-6B was from Amersham (Biosciences AB, Sweden). The chemicals for the synthesis of aminoguanidine derivatives were purchased from Fluka (Buchs, Switzerland). Thiopental and unfractionated heparin were from Polfa (Tarchomin, Poland).

Standard biochemical kits for the Hitachi 911 analyser (Hitachi Ltd., Tokyo, Japan) were from Roche (Basel, Switzerland) and urine DiaPhan® strips were from Pliva-Lachema (Brno, Czech Republic). Linco's Rat C-Peptide Radioimmunoasay Kit was from DRG International Inc. (Mountainside, NJ, USA, sensitivity 25 pM). Pentobarbitone (Morbital®) was from Biowet (Pulawy Ltd., Poland). Insulin Ultratard® HM was from NovoNordisk (Bagsværd, Denmark). 1-Deoxy-1-*p*-toluidine-D-fructose (DTF, calibration standard for the Amadori form of glycated proteins) was synthesised according to the procedure described by Hanai et al. (2000). The structure and purity of synthesised DFT was verified by infrared and nuclear magnetic resonance spectrometry and elemental analysis (Center for Molecular and Macromolecular Research, Polish Academy of Science, Lodz).

Water used for solution preparation and glassware washing was passed through an Easy Pure UF water purification unit (Thermolyne Barnstead, USA).

2.3. In vitro non-enzymatic glycosylation and oxidation of bovine serum albumin

In order to evaluate the effect of RAG and PAG on early non-enzymatic protein glycosylation the aliquots of 0.8% BSA in PBS were supplemented with glucose (0–16.7–27.8 mmol/l), PMSF (1 mmol/l), Fungizone (2 μg/ml), Gentamycin (5 μg/ml), sodium azide (0.02%) and optionally AG derivative (RAG or PAG, 100 μmol/l), and the deaerated samples were kept at 37 °C in the dark for 5 days (Watala et al., 2005). The extent of the short-term BSA glycation was determined as fructosamine concentration and expressed as the absorbance of the adduct with nitrotetrazolium blue reagent read at 530 nm and using 1-deoxy-1-*p*-toluidine-D-fructose (DTF) as a standard (Johnson et al., 1983). Free amino groups in BSA were determined as previously described (Sashidhar et al., 1994). Protein concentration was assayed with bicinchoninic acid (BCA) reagent (Smith et al., 1985). Protein carbonyls were determined using ELISA assay according to Buss et al. (1997) with slight modifications (Sitte et al., 1998). Determination of advanced glycation end-products (AGEs) in blood plasma (fluorescence intensity expressed in arbitrary units per g of total plasma protein) was based on spectrofluorometric detection according to Henle et al. (1999) and Munch et al. (1997) with modifications (Kalousova et al., 2002). Noteworthy, this assay gives us some approximation of AGEs content, as far as they may contain several adducts, including pentosidine and carboxymethyllysine CLM), of which not all are fluorescent (f.i. CLM).

2.4. Determination of binding of aminoguanidine derivatives to Sepharose-immobilized heparin

The degree of binding (DB) of the AG-derived compounds with heparin was determined based on the absorption measurements of water solutions of the investigated compounds before and after a contact with heparin. The solutions of the investigated aminoguanidines were incubated (5 min) with Sepharose-immobilized heparin (25 mg/ml), and centrifuged (13000 g, 4 min) to measure the absorption spectrum of the clear solution collected from a space over the sediment (Fig. 1A, B).