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CARDIOPROTECTIVE AND ANTIAPOPTOTIC EFFECTS OF AMINOGUANIDINE

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A b s t r a c t

Aminoguanidine (AG) has been shown to prevent diabetic complications including advanced glycation end-products (AGEs) and oxidative stress. The aim of this study was to evaluate the cardiac performance and the extent of programmed cell death in the model of experimental diabetes in rat. Diabetes was induced in male Wistar rats by a single dose of alloxan (60mg/kg; i.v.). Four experimental groups were designed: (a) group "diabetes" (DIA; n=6) received only alloxan, (b) group "diabetes+AG" (DIA+AG; n=6) received alloxan and 250mg.kg^{-1}/day of AG (p.o.), (c) group "controls"(n=6) without any treatment, and (d) group "controls+AG" (n=6) treated with 250mg.kg^{-1}/day of AG (p.o.). Standard haemodynamic parameters were studied using the technique of isolated working heart (cardiac output – CO, and heart rate – HR) 5 days after injection of alloxan. After 30 min of perfusion, the left coronary artery was igated for 10 min; the reperfusion followed for 20 min. In situ detection of apoptosis was also performed using the TUNEL technique. The values of CO and HR were significantly lower in the group DIA (*P<0.05 and **P<0.01); the pre-treatment with 250mg.kg^{-1} of AG led to a significantly better recovery during reperfusion (**P<0.01). The TUNEL techniques showed apoptotic cells in diabetic and control hearts [groups (a) and (c)]; in contrast, no signs of apoptosis were detected in diabetic and non-diabetic hearts pre-treated with AG [groups (a) and (c)]. The operating mechanisms in diabetic complications are not fully understood; however, the apoptotic process in this model of experimental diabetes could explain the impairment of cardiac performance. The presented cardioprotective and antiapoptotic effects of AG may have important implications for pharmacological use.

K e y w o r d s

Aminoguanidine, Cardioprotection, Diabetes mellitus, Heart, Ischaemia - reperfusion

A b b r e v i a t i o n s u s e d

Advanced glycation end-products (AGEs); aminoguanidine (AG); cardiac output (CO); controls (CON); diabetes (DIA); Krebs-Henseleit buffer (KHB); reactive oxygen species (ROS)

INTRODUCTION

A chronically increased plasmatic concentration of glucose in patients with diabetes mellitus initiates a variety of biochemical reactions responsible for a number of complications. Chronic hyperglycaemia leads, among other things, to an imbalance between pro- and antioxidant factors, including an increase of non-enzymatic glycosylation of protein amino groups (*1*). This process is accompanied by the production of reactive oxygen species which can have highly toxic effects on cellular homeostasis (*2*). Aminoguanidine (AG), a nucleophilic hydrazine compound, has been shown to prevent diabetic complications including advanced glycation end-product formation and free radical production in a variety of animal experiments (*3*). Since the close link between reactive oxygen species (ROS) production and apoptosis has now been widely accepted, the aim of this study was to evaluate the cardiac performance and the extent of programmed cell death in alloxan induced diabetes in rats using the model of isolated and perfused working heart.

MATERIAL AND METHODS

Four groups of male Wistar rats (mean body weight 290±15 g) were studied: (a) group "diabetes" (DIA, n=6) received alloxan in one single dose (60mg.kg^{-1}, i.v.), (b) group "diabetes+AG" (DIA+AG, n=6) received alloxan in a single dose (60mg.kg^{-1}, i.v.) and was treated with 250mg.kg^{-1} of AG (p.o.) for 5 days, (c) group "controls" (CON, n=6) without any treatment, and (d) group "controls+AG" (CON+AG, n=6) treated with 250mg.kg^{-1} (p.o.) of AG (Sigma-Chemicals, St. Louis, USA) for 5 days. Two other groups were studied for apoptosis evaluation: after the induction of diabetes by alloxan (60mg/kg) the group 1 (n=6) received 250mg/kg/day of AG (p.o.) for 5 days; group 2 (n=6) was untreated. The presence of diabetes was determined after 24 h by blood glucose measurement from the tail vein using an Ames Glucometer (Bayer Laboratories, Germany). Blood glucose level over 20 mmo.l^{-1} was considered as hyperglycaemia. The hearts were mounted on the Langendorff apparatus and perfused with oxygenated Krebs-Henseleit buffer (95% O_2 and 5%CO_2; pH 7.4; 37°C). The Krebs-Henseleit buffer (KHB) contained: glucose (11mM), NaCl 118 (mM), $MgSO_4$ (11mM), $NaHCO_3$ (25mM), KCl (4.5mM), KH_2PO_4 (1.2mM), $CaCl_2$ (3mM). The cardiac performance was tested by the technique of isolated working heart. After 30 min of perfusion (10 min stabilisation in Langendorff mode, 20 min in working heart mode), the ramus anterior of the left coronary artery was ligated for 10 min. Then, reperfusion followed for 20 min. Two basic haemodynamic parameters were registered throughout all the experiments: cardiac output (CO – expressed as a sum of aortic and coronary outputs; ml.min^{-1}), and heart rate (HR; beats per minute - bpm) using a physiographic recorder (Gould TA-240; Gould Instruments, Ohio, USA).

To evaluate the DNA damage, a method of TdT-mediated biotin-dUTP nick-end labelling was performed (TUNEL, In-situ Detection Kit, POD, Boehringer Mannheim, Germany). Frozen sections from the left ventricle were fixed by paraformaldehyde solution; the samples on the slides were incubated with proteinase (20µg/ml in 10mM TRIS-HCl) for 20 min. After several washes with PBS buffer the samples were incubated with TdT and the detection buffer conjugated with horse-radish peroxidase (Converter-POD) in a humid box at 37 °C for 60 min. DAB-chromogen (diamino-benzidine, Boehringer Mannheim, Germany) was used for visualisation and then counterstaining with haematoxylin was performed. Using the light microscopy (magnification

Scheme of the experimental protocol:

Pre-ischaemia			Reperfusion
Langendorff mode 10 min	Working heart mode 20 min	Ischaemia 10 min	Working heart mode 20 min

100x), a quantitative analysis was performed by counting cells in a randomly selected area of each sample (number of apoptotic cells/total number of cells counted x 100).

Statistical analysis of the collected data was performed using the analysis of variance (ANOVA) with the Tukey's test. All the values were expressed as mean ± SD. The *P value < 0.05 was considered as significant.

All the presented experiments conform to the "GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

RESULTS

The initial values of CO were significantly lower in the group DIA when compared with the other groups (expressed in ml.min^{-1}: group DIA **48.91.8 vs. group DIA+AG 71.61.6, group CON 67.7±1.5 and group CON+AG 69.1±1.3; **P<0.01). Also the pre-ischaemic values of HR were significantly decreased in the diabetic group (expressed in beats.min^{-1}: group DIA **22827 vs. group DIA+AG 29916, group CON 319±19 and group CON+AG 311±16; **P<0.01). At the end of reperfusion the final values of HR registered in the 60th minute were as follows: group DIA **19546 and group CON 21936 vs. group DIA+AG 274 22 and group CON+AG 25531; **P<0.01). Hearts treated with AG, and also the diabetic hearts in group DIA showed a significantly better recovery during reperfusion (expressed in ml.min^{-1}): group DIA+AG (47.35.3) and CON+AG (43.88.1) vs. group CON (22.41.9) - **P<0.01; and group DIA (39.71.5) vs. group CON - *P<0.05 (*Graph 1*). The incidence of arrhythmias in both diabetic groups and in the group CON+AG was limited; 1/3 of the hearts in group CON developed fatal arrhythmias at the beginning of reperfusion.

Infiltration of inflammatory cells and the presence of apoptotic endothelial cells were observed in the group DIA *(Fig. 1)* and group CON (29% 5 in group DIA and 33% 3 in group CON; without statistical significance between both groups). In contrast, no signs of programmed cell death were observed in groups DIA+AG (*Fig. 2*) and CON+AG pre-treated with AG.

DISCUSSION

Chronic hyperglycaemia alters cardiac excitation-contraction coupling (*4*) and develops important resistance against calcium overload (*5*) in diabetic rat hearts.

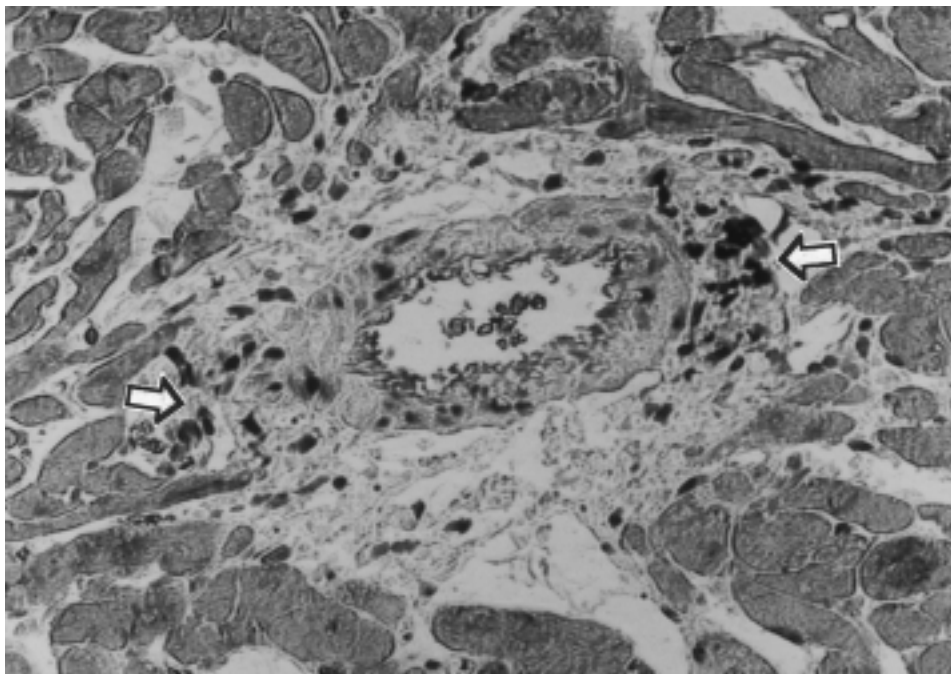


Fig. 1
Presence of apoptotic cells (ū - dark coloured) in the group DIA around the vessel wall (magnification 40x).

Impaired cardiac function in diabetic rats was seen also in our study and objectified by significantly lowered values of aortic and cardiac outputs, and also by decreased heart rate. The experimental model of an isolated and working heart of a rat is a suitable tool for the study of the heart work and contractile functions of myocardium, because in these conditions myocardium is free from all superior neurohumoral influences. The most important biological properties of AG have been discovered in the last decade and its therapeutic potential against diabetic complications was found out in numerous experiments (*6,7,8*). *Giardino et al.* (1988) demonstrated that AG acted as an antioxidant in vivo, preventing ROS formation and lipid peroxidation in cells and inhibiting oxidant-induced apoptosis (*9*). In vivo studies investigating the effects of diabetes on severe ischaemia are controversial; paradoxically, in vitro studies are more consistent in demonstrating that the diabetic heart is less susceptible to injury following severe periods of ischaemia (*10*); the rat hearts in experimentally STZ-induced diabetes develop considerable resistance against calcium overload (*11*). A negligible number of ventricular arrhythmias in early stages of reperfusion and a relatively quick

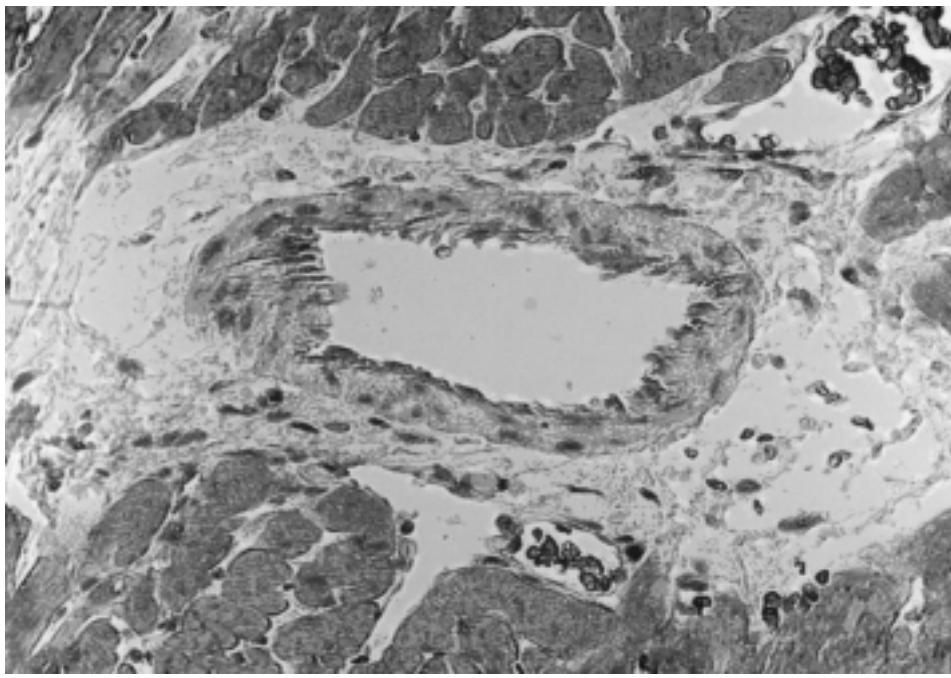
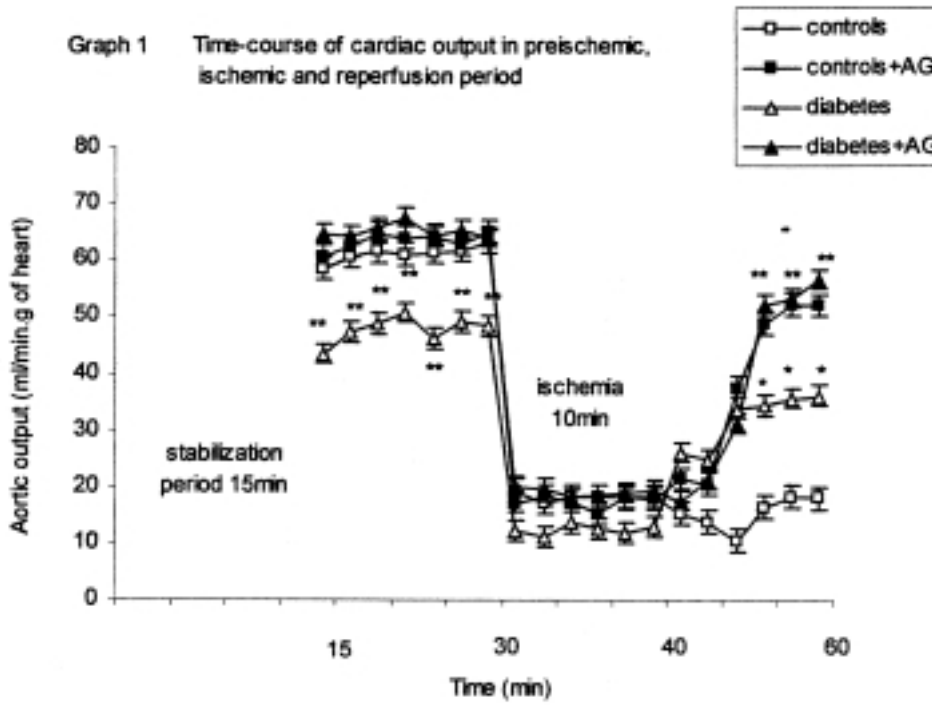


Fig. 2
Normal appearance of myocardial tissue from group DIA+AG (magnification 40x).

restoration of haemodynamic parameters were registered in the diabetic group of our study. The content of natural antioxidants (vitamin C and α-tocopherol) in the myocardium could participate in a considerable degree in this paradox (*12*). *Stanley et al.* (1997) described a high concentration of glucose-6-phosphate in the myocardium of diabetic rats. Glucose-6-phosphate (after being transformed to glucose-1-phosphate) can be transformed by mediation of glucose-1-phosphate of uridyl transferase to –uridine-diphosphate-glucose (UDP-glucose); UDP-glucose can then be further transformed (UDP-d-glucuronate and L-gulonolactone). By dehydrogenase of L-gulonolactone then L-ascorbate or vitamin C in the rat heart is formed. Due to this inborn metabolic pathway the diabetic myocardium could, theoretically, get a powerful antioxidant pool, and due to this it could more easily and more effectively resist to oxidative-reducing stress in ischaemia-reperfusion damage (*13*). Even if it cannot be stated definitely in what the protective influence of AG to diabetic myocardium precisely consists, several hypotheses on the most probable mechanisms can be expressed. AG is a powerful inhibitor of AGEs, but it is rather improbable that the AGEs would influence substantially cardiac



Graph 1
Evolution of cardiac output during pre-ischaemia, ischaemia, and in reperfusion. Data are expressed as mean _ SD of six experiments in each group (in pre-ischaemic period: group **DIA vs. groups DIA+AG, CON and CON+AG - **P<0.01; in reperfusion: group **DIA+AG and CON+AG vs. group CON - **P < 0.01; group *DIA vs. group CON - *P < 0.05).

performance in diabetic hearts in our experimental model, because they are formed relatively late, usually after several weeks (*14*). In the initial stages of diabetes a mighty production of $^{\bullet}NO$ occurs (a consequence of increased activity of macrophages and cytokines, especially tumour necrosis factor-α and interleukin 1), stimulating production of iNOS - inducible $^{\bullet}NO$-synthase (*15*). A toxic effect of nitric oxide consists apparently in its possible interaction with superoxide anion leading to the formation of peroxynitrite ($ONOO^{\bullet-}$), which could be decomposed to $NO_2^{\bullet}$ radical and the strongly toxic hydroxyl radical ($OH^{\bullet}$). As AG is a powerful inhibitor of iNOS, an assumption can be expressed about its possible participation in blocking production of $^{\bullet}NO$ (*16*). The selective inhibition of iNOS by AG could therefore be another important reason for the

resistance of diabetic (or non-diabetic) myocardium in rats treated by AG both in the pre-ischaemic stage and in the course of reperfusion. Finally it cannot be denied that also the fact that AG acts as a direct ROS scavenger can be a very important element of the cardioprotective potential of AG (*17*). In recent years, mitochondria have gained importance as a main site of ROS production and as a major factor in apoptosis (*18, 19*). ROS and the resulting cellular redox change can be a part of transduction pathways signalling in apoptosis (*20*). During ischaemia and reperfusion, a global shutdown of mitochondrial function occurs, and several essential proapoptotic challengers are released into the cytosol, i.e. pro-caspases, apoptosis-inducing factor (AIF), cytochrome C, etc., and could directly promo te the programmed cell death by the induction of expression of the responsible gene set (*21*). This study was intended to determine whether apoptosis occurred in our experimental conditions and if the pre-treatment with AG could be favourable; the results provided evidence that apoptosis is present in diabetic and control hearts subjected to ischaemia and reperfusion, and that the presence of AG exhibited a clear reduction of cell death in the myocardium. We assume that the significant cardioprotective effects of AG found in our study may be attributed to the effective prevention of oxidant-induced apoptosis and could have important implications for pharmacology. Further experiments are needed to provide more detailed information about the various types of cells involved in the apoptotic process in alloxan-induced diabetes. Nevertheless, the implication of the apoptotic process as demonstrated in this study could explain the impaired cardiac performance in the diabetic heart.

A c k n o w l e d g e m e n t s

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KARDIOPROTEKTIVNÍ A ANTIAPOPTOTICKÉ ÚČINKY AMINOGUANIDINU IN VITRO

S o u h r n

Aminoguanidin (AG) účinně potlačuje rozvoj diabetických komplikací, včetně vzniku tzv. produktů konečné glykace a oxidativního poškození. Cílem této studie bylo zhodnocení funkčních parametrů myokardu a rozsahu programované buněčné smrti pomocí modelu experimentálního diabetu u laboratorního potkana. Experimentální diabetes byl indukován pomocí jediné dávky alloxanu (60mg/kg; i.v.); celková doba trvání diabetu byla pět dní. Byly vytvořeny čtyři experimentální skupiny: a) skupině „diabetes" (DIA; n=6) byl aplikován pouze alloxan; b) skupině „diabetes+AG" (DIA+AG; n=6) byl aplikován alloxan a AG (250mg.kg-1/den; p.o.); c) skupině „controls"(n=6) nebyla aplikována žádná látka; d) skupině „controls+AG" (n=6) byl aplikován pouze

AG (250mg.kg-1/den; p.o.). Pro studium standardních hemodynamických parametrů byla použita technika izolovaného pracujícího srdce; hodnocen byl srdeční výdej (součet koronárního a aortálního průtoku) a srdeční frekvence. Po 30min perfuze byla podvázána levá koronární tepna na dobu 10min; následná reperfuze trvala 20min. Pro detekci apoptózy in situ byl použit TUNEL test. Výsledky ukázaly signifikantně nižší hodnoty srdečního výdeje a srdeční frekvence v neléčené diabetické skupině (**P<0.01); AG v dávce 250mg.kg-1/den signifikantně zlepšil fázi postischemického zotavení (**P<0.01). Pomocí TUNEL testu byla zjištěna přítomnost apoptotických buněk v myokardu diabetických a kontrolních potkanů [skupina a) a c)]; ani v jednom případě nebyly přítomny známky apoptózy v myokardech diabetických a kontrolních potkanů léčených AG [skupina b) a d)]. Podstata mechanismů diabetických komplikací není stále zcela vyřešena, avšak přítomnost apoptotického procesu v tomto modelu experimentálního diabetu by mohla být jedním z důvodů poruchy funkce myokardu. Zjištěné kardioprotektivní a antiapoptotické účinky AG mohou představovat významný impuls pro potenciální farmakologické využití.

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