ANTIMUTAGENIC 1,4-DIHYDROPYRIDINE AV-153 NORMALIZES EXPRESSION OF GLUT1, GLUT4, INOS, PARP1, AND GAMMA H2AX HISTONE IN MYOCARDIUM OF RATS WITH STREPTOZOTOCIN MODEL OF DIABETES MELLITUS

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Diabetic cardiomyopathy increases the risk of heart failure and worsens prognosis for diabetes mellitus (DM) patients. Its development depends on many factors, including modification of nitric oxide production and impaired DNA repair. The goal of the present work was to study in vivo effects of a 1,4-dihydropyridine AV-153, known as antimutagen and DNA-binder, on DNA integrity, and on the expression of several proteins involved in glucose transport, nitric oxide metabolism, and DNA repair in myocardium in diabetic rats. DM was induced in rats by streptozotocin (STZ) injection. Expression of proteins was studied by means of immunohistochemistry. Development of the STZ-induced DM significantly induced PARP1 and gamma H2AX histone, markers of DNA breakage, protein expression in heart tissue, while AV-153 administration decreased PARP1 and H2AX protein expression. In this model of diabetes, myocardial expression of iNOS was also significantly increased, but administration of AV-153 reduced it to normal levels. AV-153 also upregulated the expression of insulin-dependent GLUT4 and insulin-independent GLUT1 glucose transporters up to normal level in diabetic rats. Thus, AV-153 appears to be prospective for creation of a remedy for prevention of diabetic cardiomyopathy.

Keywords: diabetic cardiomyopathy, nitric oxide, DNA damage, glucose transporters.

INTRODUCTION

Diabetes mellitus and its complications cause numerous health and social problems throughout the world. Diabetic cardiomyopathy (DCM) represents an important complication of diabetes mellitus, with silent development in its earlier stages, involving intricate pathophysiological mechanisms and leading to heart failure (Gilca et al., 2017). Pathogenesis of the DCM involves several metabolic and epigenetic processes (Khullar et al., 2017; Pepin and Wende, 2019) leading to dysregulation of numerous signaling axes and abnormal expression of various groups of enzymes. These include enzymes regulating production and scavenging of the free radicals, including uncoupling of the endothelial NO synthase (eNOS) and overexpression of the inducible NO synthase (iNOS) (Faria, Persaud, 2016). Oxidative stress leads to DNA damage in cardiac tissue (Ivanović-Matić et al., 2014), with subsequent over-expression of the DNA repair enzymes including poly(ADP)ribose polymerases (PARP), and inhibition of PARP1 produces benevolent effect on the diabetic heart (Qin et al., 2016; Waldman et al., 2018). Hyperglycaemia and a drop of the insulin level itself lead to decrease of expression and transfer to the cell surface of the insulin dependent glucose transporters (GLUT4) (Faramoushi et al., 2016; Maria et al., 2016) and even insulin-independent transporters (GLUT1) (Chen et al., 2015; Szablewski, 2017), leading to disturbances of heart metabolism. Numerous possible remedies
preventing development of the DCM were proposed in recent years including inhibitors of the histone deacetylase (Bagchi and Weeks 2019; Chen et al., 2015), PARP1 (Waldman et al., 2018), catalase (Ivanović-Matić et al., 2014).

In the present study, we tried to assess the effects of AV-153, a compound known as an antimutagen and enhancer of the DNA repair, on expression of the DNA damage and repair markers (PARP1 and histone gamma H2AX), NO synthases (iNOS and eNOS) and glucose transporters (GLUT1 and GLUT4) in myocardium of the rats with the streptozotocin model of diabetes mellitus. It inhibits chemical mutagenesis due to modulation of DNA repair, and reduces the number of DNA strand breaks in cells exposed to several genotoxic agents (Ryabokon et al. 2005; 2009; Leonova et al., 2018). We recently reported the ability of the compound to bind DNA (Buraka et al., 2014) and to increase the activity of the DNA repair enzymes (Leonova et al., 2018). The compound induced some benevolent effects in kidneys of diabetic rats (Osinà et al., 2016). The present paper extends the study to myocardium.

MATERIALS AND METHODS

Animals. Animals were obtained from the Laboratory of Experimental Animals, Riga Stradiņš University, Riga, Latvia. Wistar male rats were fed with a standard laboratory diet with free access to food and water, each weighing 215.0 ± 5.6 g at the beginning of the experiments. They were maintained at a temperature of 22 ± 0.5 °C with a 12-h light/dark cycle.

Chemicals. AV-153 was synthesised at the Latvian Institute of Organic Synthesis and was fully characterised by usual physic-chemical methods. Glucose (anhydrous) was purchased from Chempur (Karlsruhe, Germany). Streptozotocin and other chemicals were purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany). All antibodies were ordered from Abcam (Cambridge, United Kingdom).

Induction of diabetes. Diabetes mellitus was induced in rats by a single injection of STZ (50 mg/kg, freshly dissolved in 10 mM citrate buffer, pH 4.5) via tail vein (injection volume 0.2–0.25 ml). Control animals received 0.2 ml of 0.9 % NaCl via tail vein. Diabetes was confirmed via tail vein bleeding 48 hours after induction by measuring the fed state blood glucose level. Rats with blood glucose 13.89 mmol/l (250 mg/dl) were included in the study. Blood glucose was determined using a portable glucometer MedSense OptiumXceed (Abbott Diagnostics Ltd, Maidenhead, UK).

Experiment design and drug administration. Animals were divided in four groups: controls (n = 10), controls treated with AV-153 (n = 5), diabetic non-treated animals (STZ-group) (n = 8), and diabetic animals treated with AV-153, (STZ + AV-153) (n = 6). Treatment of animals with AV-153 started nine days after the DM induction and was performed per os (0.5 mg/kg for three days). After finishing the protocol, blood glucose was measured again, animals were euthanised by decapitating with a four-knife guillotine, and tissue samples of the hearts were fixed for immunohistochemistry.

Tissue processing and immunohistochemistry. Tissue processing. Rat heart tissues were cut into several vertical consecutive slices of 0.5 mm thickness with systematic sampling. Then the tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. Specimens were cut in 4-µm-thick sections on a rotary microtome and mounted on poly-lysine coated glass slides. The slides were stained with haematoxylin and eosin to evaluate histopathological changes in the heart tissue.

Immunohistochemical examination of the tissues. Heart tissue sections were immunostained to visualise cells that were positive for iNOS, eNOS, PARP1, and Histone gamma-H2AX, GLUT-1, GLUT-4. Endogenous peroxidase activity was blocked with 3.0% H2O2 for 10 minutes. Non-specific primary antibody binding was blocked with normal horse serum for 10 min prior to secondary antibody incubation. The slides were incubated overnight at 4 °C with rabbit polyclonal iNOS antibody (Abcam, ab 3523; dilution 1 : 200), rabbit polyclonal eNOS antibody (ab66127; dilution 1 : 100), rabbit polyclonal PARP1 (ab6079, dilution 1 : 200), rabbit polyclonal histone gamma H2AX (ab11175, dilution 1 : 500), rabbit polyclonal GLUT-1 antibody (ab652, dilution 1 : 250) and rabbit polyclonal GLUT-4 antibody (ab33780, dilution 1 : 200). Bound antibodies were detected using the EnVision reagent (DAKO, Glostrup, Denmark) for 30 min at room temperature. The immunoperoxidase reaction colour was developed by incubating the slides with 3,3′-diaminobenzidine for 7 min following a previously described protocol (Choi et al., 2012). A negative control omitting the primary antibody was included for each experiment. Each experiment included positive controls — lung and liver tissues for iNOS, liver and aorta tissues for eNOS and thyroid papillary carcinoma tissue for PARP1 and histone H2AX.

To obtain similar staining, the sections from all groups were always stained simultaneously in the same tray. All experiments were done in duplicate.

Imaging and quantitation of cells. For each experimental group, immunopositive cells were counted in all tissue sections from at least ten independent sections at 20 high powered fields at magnification × 400. Slides were counted by two pathologists in blinded fashion. Regions of interest were captured using a Motic digital camera (Motic, Xyamen, China) mounted on a microscope (Motic BA400) using Motic Image Advanced 3.2 software. The results were expressed as the number of immunopositive cells per mm2.

Statistics. The results are given as the means ± SD. Data were analysed by two-way ANOVA followed by the Bonferroni post hoc test for comparison between groups. In all tests, a p value of < 0.05 was considered statistically signifi-
significant. GraphPad Prism 6 (GraphPad Inc.) software was used for the statistical analysis.

RESULTS

Expression of two proteins involved in DNA repair (PARP1 and histone gamma-H2AX), two NO synthases (iNOS and eNOS) and two glucose transporters (GLUT1 and GLUT4) was monitored in the myocardium.

Expression of the marker of DNA breakage histone H2AX was strongly up-regulated in myocardial cells of diabetic animals (32 ± 13 vs 7 ± 3 cell/mm², p = 0.01), treatment with AV-153 almost normalised the level of expression of this protein (8 ± 5 vs 32 ± 13 cell/mm², p = 0.03, Fig. 1A).

PARP1 expression was detected in the cardiomyocytes, in some inflammation cells in the interstitium and around the blood vessels. Development of STZ-induced diabetes mellitus significantly induced PARP1 protein expression in heart tissue (42 ± 13 vs 13 ± 4 cells/mm², p = 0.008 while AV-153 administration decreased PARP1 expression (12 ± 6 vs 42 ± 13 cell/mm², p = 0.007; Fig. 1B).

Development of DM was followed by a significant increase of iNOS protein expression in the heart (26 ± 11 vs. 9 ± 4 cell/mm², p = 0.008) while AV-153 down-regulated the enzyme expression up to normal levels (8 ± 3 vs. 26 ± 11 cell/mm², p = 0.007). AV-153 increased iNOS expression per se (Fig. 1C).

Induction of DM drastically downregulated GLUT1 expression, which was detected in cardiomyocytes in perivascular areas and in endocardium, from 12 ± 2.0 cell/mm² in control group to 5.8 ± 1.2 cell/mm² in STZ group, p = 0.02. In turn AV-153 normalised expression of GLUT1 protein to 12.5 ± 3.2 cell/mm² (p = 0.03) (Fig. 1E) in diabetic rat hearts.

Similarly, GLUT4 expression also decreased from 13.9 ± 1.7 cell/mm² to 6.2 ± 1.7 cell/mm² p = 0.02 in myocardium of the rats with the experimental model of the DM, and AV-153 raised it up to the control level 11.9 ± 2.8 cell/mm² p = 0.03 (Fig. 1F).

AV-153 did not change the level of expression of GLUT1 and GLUT4 protein in any of the diabetic rat hearts, respectively 13.7 ± 3.2 cell/mm² for GLUT1 and 13.8 ± 2.2 cell/mm² for GLUT4 (Fig. 1E, F).
DISCUSSION

Cardiomyocytes derive about 10% of their energy from glucose (Chanda et al., 2016). Glucose transporter 1, which is constitutively present in the sarcolemma of cardiomyocytes, provides basal glucose uptake (Chen et al., 2015; Szablewski, 2017), while insulin-dependent GLUT4 is incorporated into the cell membrane in response to contraction and insulin (Faramounshi et al., 2016; Maria et al., 2016). The protein levels of these two glucose transporters in cardiomyocytes in our experiment decreased more than twice already on the 10th day after STZ injection (Fig. 1E, F).

This suggests that glucose uptake into cardiomyocytes is reduced and energy production is largely due to fatty acid oxidation (Isfort et al., 2014; Maack et al., 2018), which causes lipotoxicity, mitochondrial dysfunction, and increases the intensity of free radical formation, and the development of diabetic cardiomyopathy (Isfort et al., 2014; Maack et al., 2018).

It can reduce ATP synthesis, worsen cardiac contractility, alter calcium metabolism, cause inflammation and myocyte death (Ormazabal et al., 2018). In rat cardiomyocytes, a marked increase in iNOS protein was observed at day 10 of STZ-induced type 1 diabetes (Fig. 1 C). This may indirectly indicate increased nitric oxide production, which together with elevated free radicals formed under altered glucose and fatty acid metabolism in the heart, forming a toxic molecule-peroxynitrite, which is responsible for damage to large cellular molecules, including DNA (Faria and Persaud, 2016). Thus, oxidative and nitrosative stress directly affects the integrity of the DNA molecule in the myocardium in diabetic conditions and DNA breaks are formed (Ivanović-Matić et al., 2014). The DNA repair system is activated, including the PARP1 enzyme (Qin et al., 2016; Waldman et al., 2018). A marked increase in its levels was also observed in the hearts of diabetic rats (Fig. 1B). Excessive activation of the PARP1 protein may deplete its substrate the NAD⁺ intracellular reserves, thereby further slowing down glycolysis, ATP synthesis, and activating cell necrotic processes (Varga et al. 2015). Our data are coherent with previous reports on PARP1 expression in the myocardium of diabetic animals (Qin et al., 2016; Waldman et al., 2018). Evidently, AV-153 produced a benevolent effect on DNA integrity, normalising the effect of the AV-153 (Fig. 1B), which is comparable to effects of other remedies targeted to PARP1. In the hearts of diabetic rats, there was also an increase in the expression of the DNA double-stranded marker H2AX, indicating that the level of DNA damage was significant and that in diabetic animals, administration of AV-153 reduced H2AX to near healthy levels (Fig. 1A). AV-153 protects the DNA helix, thus protecting cardiomyocytes from irreversible changes and death.

It should be considered that AV-153 does not normalise the glucose level in the STZ-treated rats (Osina et al., 2016). Thus, these effects cannot be attributed to normalisation of insulin production and decrease of the blood glucose level. In our work, we observed that AV-153 normalises the expression levels of GLUT1 and GLUT4 proteins in rat cardiomyocytes in STZ-induced type 1 diabetes mellitus (Fig. 1E, F), thus normalising the altered glucose uptake levels in cardiomyocytes, thereby restoring the proportion of glucose and fatty acid oxidation that is altered due to a lack of insulin in the body, resulting in a reduction in lipotoxicity and free radical formation. AV-153 also reduces iNOS protein expression in type 1 diabetes mellitus. (Fig. 1C), which could indicate that inflammatory processes in cardiomyocytes have decreased.

Judging from the expression data of GLUT1, GLUT4, iNOS, PARP1, and H2AX proteins, we can speculate AV-153 normalises altered levels of these proteins in diabetes, possibly through normalisation of GLUT1 and GLUT4 protein expression levels, thereby normalising glucose uptake in cardiomyocytes and consequently the proportion of oxidation of glucose and fatty acids, as well as the reduction of inflammatory processes in cardiomyocytes occur, thus reducing the production of free radicals and NO and peroxynitrite, thereby reducing DNA damage and hyperstimulation of DNA repair pathways, and protecting cardiomyocytes from death.

The mechanism by which AV-153 restores protein levels of glucose transporters 1 and 4 in cardiomyocytes in diabetes remains to be clarified.

AV-153 intercalates a DNA molecule (Buraka et al., 2014; Leonova et al., 2020), and like many substances that intercalate DNA molecules, AV-153 has DNA molecule-damaging properties (Leonova et al., 2018). In this study, we observed a tendency of AV-153 to increase PARP1 levels in the hearts of intact animals (Fig. 1B). PARP1 is involved in the regulation of proinflammatory genes, including iNOS expression through NFXβ (von Lukowicz et al., 2008; Henning et al., 2018), and in this way, AV-153 might stimulate iNOS expression in the heart. Previously we observed a stimulatory effect of AV-153 on gene expression in the form of an increase in Parp1 gene expression in healthy kidneys (Osina et al., 2016). In our previous studies, we also detected differences in the expression levels of different genes and proteins in different rat organs, both in STZ-induced type 1 diabetes (Osina et al., 2017) and in response to 1,4-dihydropyridine administration (Osina et al., 2017). Regarding the question of why there are such differences in the effects of 1,4-DHP in healthy and damaged tissues, the only thing that can be said is that ITCD affects the amount and activity of proteins in cells at different levels ranging from epigenetic regulation (Wang et al., 2021) to protein utilisation processes (Osińa et al., 2016). As in diabetes, all body systems respond to the processes caused by hyperglycaemia in different ways, but all processes are interconnected (Forbes and Cooper, 2013). Analysing versatile molecules such as AV-153 that interact with both DNA molecules (Leonova et al., 2020) and proteins (Leonova et al., 2018), which have antioxidative, DNA molecular protective (Leonova et al., 2019; 2018), DNA repair enzyme activity (Ryabokon et al., 2008; 2009) and
ubiquitin-proteasome system gene transcription stimulatory properties (Dislere et al., 2021), we came to the conclusion that the mechanism of action of AV-153 is most likely a set of processes that includes all of the above-mentioned properties, which also depend on the environment (cells, organs, organisms, pathological processes) and also on the concentrations of the administered substance itself.

CONCLUSIONS

AV-153 is a promising member of the 1,4-dihydropyridine class that requires increased attention and research.

ETHICS

All experimental procedures were carried out in accordance with guidelines of the Directive 86/609/EEC “European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes” (1986) and were approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia).

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