ROLE OF INFLAMMATORY CYTOKINES AND CHEMOATTRACTANTS IN THE RAT MODEL OF STREPTOZOTOCIN-INDUCED DIABETIC HEART FAILURE

JAN DRIMAL, VLADIMIR KNEZL, JANA NAVAROVA, JANA NEDELCEVOVA, EMA PAULOVICOVA[1], RUZENA SOTNIKOVA, VLADIMIR SNIRC, DANIEL DRIMAL[2]

Institute of Experimental Pharmacology, Slovak Academy of Sciences and [1]Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia; [2]First Welding Co. Bratislava, Slovakia

Objective. It is not yet clear how oxidative stress, free radicals, inflammatory cytokines and chemoattractants produced in the heart induce chronic heart failure. The myocardial damage caused by chronic diabetes results either from the persistence of inflammatory signaling directly in the heart or from the dysregulation of anti-inflammatory signaling systems. In the rat model of streptozotocin-induced diabetes (STZD) we investigated 1/ the concentration of free radicals (FR), 2/ reduced glutathione (GSH), 3/ lysozomal enzymes, 4/ inflammatory cytokines (tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)), and monocyte chemoattractant protein-1 (mcp-1) in the myocardium.

Methods. Diabetes was induced in 12 male Wistar rats by injection of streptozotocin (STZ). The free radical scavenger and cardiac protectant SMe1EC2 (10 mg/kg/d.) was given orally for 5 days and 5 weeks and these animals were compared with the diabetic and non-diabetic controls.

Results. We found reduced heart rate and rate dependent functions of the rat heart, early release of free radicals triggering the release of cytotoxic inflammatory cytokines (like TNF-α and IL-6) and chemoattractants (mcp-1) as an example of this type of pathogens, resulting in the initiation and progression of cardiac pathology. The reduced myocardial contractility after STZD was accompanied with the increased reactive responsiveness of isolated aorta and mesenteric artery to phenylephrine, with increased production of chemoattractive proteins directly in the myocardium, with increased activity of peripheral ß-N-acetyl-glucosaminidase (NAGA), as representative of lysosomal activation processes. The pretreatment of SME1EC2 reduced increase in vascular reactivity, reduced myocardial depression and protected against myocardial toxicity.

Conclusion. The newly identified and specific cardiac protectant SMe1EC2 could serve as a prospective target in the treatment of increased myocardial cytokine and chemoattractive proteins in diabetic cardiomyopathy.

Key words: Streptozotocin diabetes – Heart – Cytokines – Monocyte chemoattractant protein-1 – Cardiac protectant SMe1EC2

Important role of diabetes in congestive heart failure was originally established in the Framingham study (TROST and LEWINTER 2001).Since then diabetes mellitus has been documented as increasing the risk of heart failure (BOUDIN et al 2007). Moreover, at present many authors believe that independently of underlying two diseases, the hypertension and/or coronary artery disease, diabetes leads directly to cardiomyopathy (WANG et al. 2006, AMOUR et al. 2007). In 1956 KISCH described a special myocardial cell with dense inclusions, JAMIE-

Corresponding author: Jan Drimal, MD, DSc, Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dubravska 9, 84104 Bratislava, Slovak Republic; Phone: +421 2 59410660, Fax: +421 2 54775928, e-mail: jan.drimal@savba.sk

SON and PALADE (1964) and later FORSSMANN (1986) characterized cardiac endocrine hormones. Consequently on the basis of these results FISMAN et al. (2003) proposed a novel interleukin classification in the "cardiovascular diabetes". For more than 50 years, investigators have unsuccessfully tried to recreate in experimental animals the cardiovascular complications of diabetes seen in humans. In particular, accelerated atherosclerosis and dilated cardiomyopathy, the major causes of mortality in patients with diabetes, have been absent in the majority of animal models of the disease. The NIH-Consortium on Animal Models of Diabetic Complications has worked to address this issue focusing on the development of mouse models (KANNEL et al. 1974). Since the recent identification of immune/inflammatory mechanisms in "human diabetic cardiomyopathy", the important involvement of oxidative stress, impairment of cardiac-specific transcription factors, production of inflammatory cytokines and chemokines has become increasingly evident (ARAGNO et al. 2005; POORNIMA et al. 2006). In the present study a rodent model of streptozotocin-induced diabetes (STZD) was used to describe the early release of free radicals (FR), lysosomal enzymes ß-N-acetyl glucosaminidase (NAGA), inflammatory cytokines (tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)), and chemoattractant monocyte-chemoattractive protein-1(mcp-1), resulting in the initiation and progression of heart failure. The most important fact is, and our results show it clearly, that inflammatory molecules were induced under stress conditions directly in the rat myocardium. Another important fact is that this type of adaptive response in animal cardiac tissues involved the release of growth factors and proteins, and activation of apoptotic or necrotic pathways, leading to further progression of myocardial pathology. In this view, we describe the adaptive immune response triggering the release of cytotoxic factors, inflammatory cytokines and chemoattractants, resulting in the initiation and progression of heart failure, as well as the cardiac protection after chronic SMe1EC2 therapy.

Materials and Methods

Experiments with animals and procedures were approved by the Animal Care and Use Committee at the Institute of Experimental Pharmacology and are conform to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, publication no. 85-23, revised 1996 and State Veterinary and Food Product Inspection (SR).

Animals and induction of diabetes. Male Wistar rats (n = 24) weighing 230 to 250 g were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg). Experimental diabetes was induced by intravenous administration of a single dose of streptozotocin (STZ, 3 x 20 mg/kg i.v., every second day). From five to ten days after STZ administration, plasma glucose levels were determined and animals with glucose >20 mmol/l were considered diabetic. Experimental groups of animals were created by a random choice from diabetic and control healthy Wistar rats. In accordance with the given protocol, drug dosage started. In regular time intervals (5 days and 5 weeks) the following parameters were monitored: mortality rate, body weight, food and water consumption, arterial blood pressure, blood biochemistry, cholesterol, creatinine. For in vitro functional measurements the heart, aorta and first order mesenteric artery were isolated from control and STZ-induced anesthetized rats, anticoagulated with heparin (1000 IU/kg). The heart was perfused with Krebs bicarbonate buffer (in mmol/l): 120 NaCl; 4.2 KCl; 1.75 $CaCl_2$; 1.25 $MgSO_4$.$4H_2O$; 12.5 glucose; $NaHCO_3$ 25.0) and temperature 37°C. The coronary flow, left ventricular pressure, ECG and heart rate (Cardiovascular Analyzer) were recorded on a six-channel NEK-6T Physiograph. Rings of the mesenteric artery (approximately 2 mm) were mounted in tissue chambers containing Krebs solution (122 NaCl, 5.9 KCl, 15 $NaHCO_3$, 11 glucose, 1.25 $MgCl_2$ and 1.25 $CaCl_2$, gassed by mixture of O_2 and CO_2), maintained at 37°C. After stabilization, the rings were precontracted with phenylephrine and relaxant responses to acetylcholine were tested at the plateau of isometric contraction.

Experiments in animals and procedures were approved by the Animal Care and Use Committee at the Institute of Experimental Pharmacology and are conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, publication no. 85-23, revised 1996 and State Veterinary and Food Product Inspection (SR).

Estimations of inflammatory cytokines. Plasmatic concentrations of tumor necrosis factor-α.(TNF-α), interleukin-6(IL-6) and the chemotactic protein monocyte-chemoattractant protein-1 (mcp-1) were measured by RIA and ELISA on using commercially available kits (Amersham Biosciences, UK) and Microplate Multiscan Reader (Finnland). Ventricular tissue was homogenized in 10 volumes of phosphate-buffered saline (PBS) along with 1 % Triton-100 and with a protease inhibitor cocktail. The homogenate was cen-

trifuged at 4.500 x g for 20 min at 4°C. The supernatant was collected and the myocardial concentrations of proteins were measured using sandwich ELISA kits. Assays were performed according to the manufacturer instruction. Absorbance of standards and samples was determined at 450 and 550 nm with the aid of Multiscan Reader (Labsystem).

Enzyme assays. The quantification of free radical concentration (FR) in plasma and in the myocardium was accomplished with Chlorophyllin-Kit (Votruba et al. 1999). The total glutathione (GSx) in myocardium was determined by enzymatic method (Baker et al. 1990). The concentration of c-AMP was assessed in myocardial muscle. Microsamples of control (45° Linea anterior-obliqua, LAO) and ischemic zone of myocardium (135 °LO posterior wall (PW) of the left ventricle) were obtained with the use of Wollenberger Clamps and cooled in liquid nitrogen. cAMP content was determined by (^{125}I) RIA (IRAPRA Prague).

Chemicals. Aprotinin, BSA ,leupeptin, pepstatin A, PMSF, p-nitrobluetetrazolium (Sigma), SMe1EC2(4a,9b)-cis-2-ethoxycarbonyl-8-methoxy-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido-[4,3b]indolin-chloride (Stolc et al. 2003), 1/ Sep-Pack C(18), (Amprep), (h)tumor necrosis factor-α, (Bachem).

Statistical evaluation. All data are expressed as mean ± SEM, where n = number of animals or experiments. Data were compared statistically by ANOVA A probability value of less than 0.05 was considered significant.

Results

In chronic streptozotocin-induced diabetic rats (STZD) blood glucose levels showed a significant increase (Fig.1). The heart rate, the rate dependent cardiac functions and left ventricular systolic pressure were reduced (-18 ± 3 %, p<0.05). The 12-lead ECG recording was routinely used to support the diagnosis. ST-segment elevation with Q-waves in standard leads or in anterior precordial leads was frequently present (Fig. 2). The QTc duration increased significantly (+47%). Chronic therapy with SMe1EC2 significantly reduced ST-segment elevation with unchanged or increased QTc. In vitro, on isolated heart, STZD was accompanied with reduced cardiac function (-13.8 %) and increased left ventricular enddiastolic pressure. The vascular reactivity of isolated aorta and isolated mesenteric artery of STZD rats showed significantly increased response to micromolar concentrations of prenylephrine. The in vivo enzyme activities showed significant alterations both in plasma and in cardiac tissue homogenate. Aortic and myocardial concentrations of GSH and NAGA showed significant decline. The quantification of free radical concentrations in plasma and in myocardium early (5 days) and late (5 weeks after induction of STZD), (Fig.3), showed a significant in-

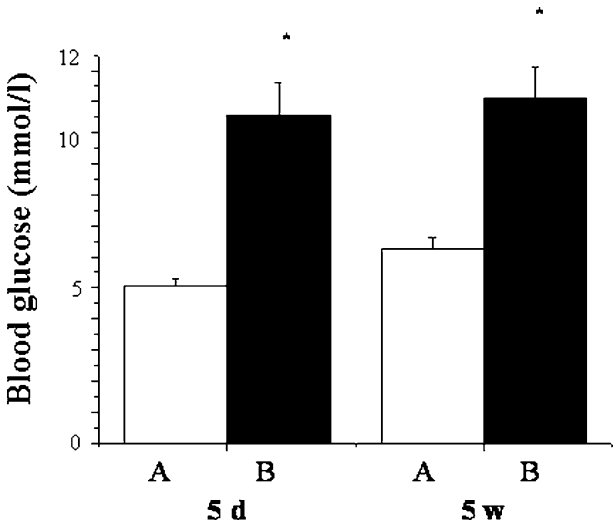

Fig. 1 Blood glucose in control (A) and (B) in streptozotocin (STZD) pretreated diabetic Wistar rats . Significant change (p < 0.05), (n = 36). Results are mean ± SEM.

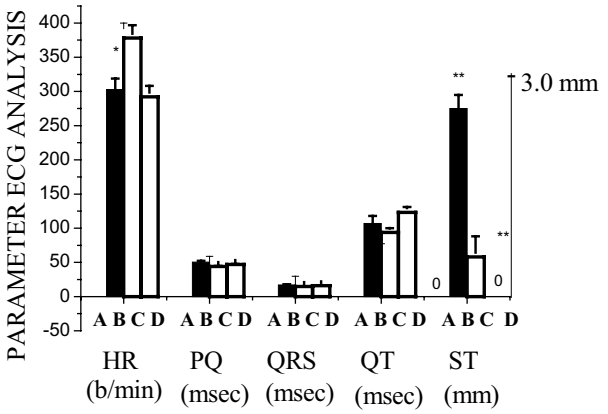

Fig. 2 ECG Diagnosis in four groups of Wistar rats (A) in Control (placebo-controls), (B) in streptozotocine (STZD) pretreated diabetes (D), in STZD + SMe 1EC pretreated animals (5 weeks of therapy). Parameters (diagonal) : Heart rate (HR), ECG Analysis : PQ, QRS and QT interval (in ms), and ST segment elevation (in mm). Values are mean ±SEM.

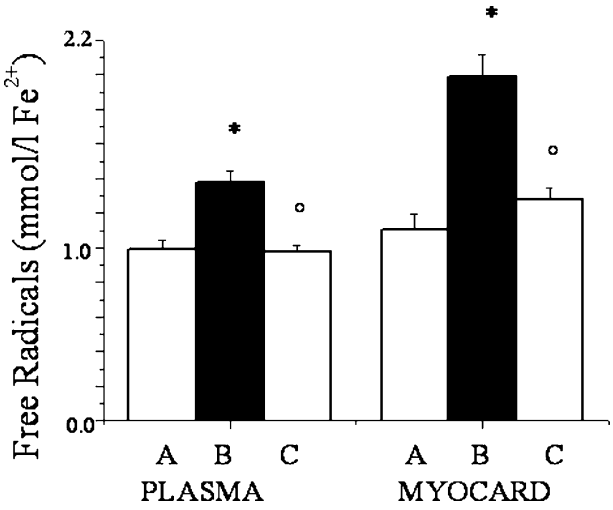

Fig. 3 Quantitative determination of free radical (FR) concentration with chlorophylline method in venous plasma and in the left ventricular myocardium. (A)-Control, (B)-STZD, (C)-STZD after Sme1EC pretreatment. Chlorophylline method uses the advantage of acceptance of electrons by chlorophylline (n = 20). Values are mean ±SEM.* Significant increase in FR concentration in STZD and ° significant reduction of FR concentration SMe1EC2 therapy (10 mg /kg/every second day for 5 weeks).

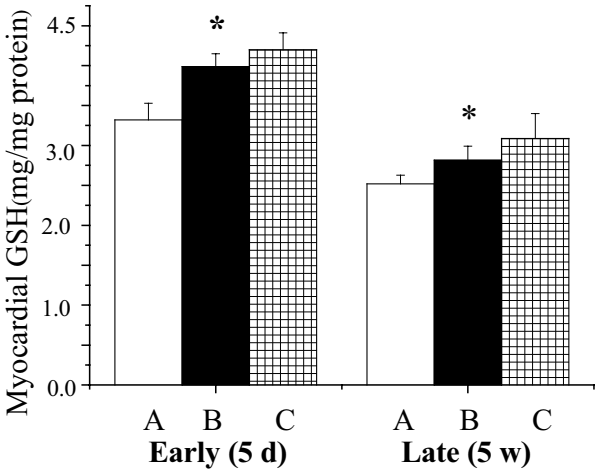

Fig. 4 Myocardial GSH in controls (A) columns), (B) early (5 days) and late (5 weeks) after induction of STZD (filled columns), and after chronic therapy with SMe1EC2 (C) columns. Note significant increase in GSH after STZD.

crease both in plasma (+39 ± 5 %) and in the left ventricular myocardium (+ 84 ± 19 %, p< 0.05). Significant reduction in free radical concentration was seen after SMe1EC2 therapy. When compared to control, the myocardium after STZD was associated with a state of increased immunomodulation. The total glutathione system in the heart (GSx) increased, and so did GSSG/GSH equivalents in STZD rats, and a further rise in the SMe1EC2 group indicated increased cell glutathione. The increase was more intensive early after STZD. With the presumably more severe mitochondrial damage in the left ventricular myocardium 5 weeks after STZD, the myocardial GSx concentration showed a significant reduction in cellular glutathione content. The glutathione concentrations were significantly increased early after induction of STZD (n = 11), (Fig.4). In all experiments specific activities of GSx were significantly reduced 5 weeks after STZD and further reduction was seen after SMe1EC2 therapy. Fig. 5 shows biochemical findings in lysosomal enzymes in total tissue homogenate early (5 days) and late (5 weeks) after induction of STZD, and after SMe1EC2 therapy. In vitro, in the isolated retrogradely perfused heart, the left ventricular systolic function and left ventricular end-diastolic pressure were significantly impaired and coronary blood flow slightly increased. *In vitro* experiments with mesenteric arterial segments (isolated from STZD rats 5 weeks after induction of diabetes), precontracted with phenylephrine, showed significant reduction in acetylcholine relaxation thus indicating endothelial dysfunction. *In vivo,* in control Wistar rats, the basal plasmatic concentrations of cytotoxic TNFα were slightly higher than limits for estimation of TNFα. In control Wistar rats basal myocardial concentrations of TNFα were also very low. Concentrations of TNFα were increased markedly 5 days after induction of STZD, and 5 weeks after STZD myocardial concentration of TNFα was increased 88 fold (Fig.6). Chronic therapy with SMe1EC2 in STZD rats significantly reduced TNFα in myocardium by - 48 % ($p<0.05$). The myocardial concentration of IL-6 remained unchanged after induction of diabetes. Marked increase in circulating levels of IL-6 as seen in STZD rats after SMe1EC2 therapy was a remarkably consistent finding in our experiments (Fig.7). The concentration of IL-6 was significantly increased also in the myocardium. The magnitude by which myocardial concentrations of IL-6 increased after SMe1EC2 therapy (+ 43% $P < 0.05$) was related to improved myocardial function.

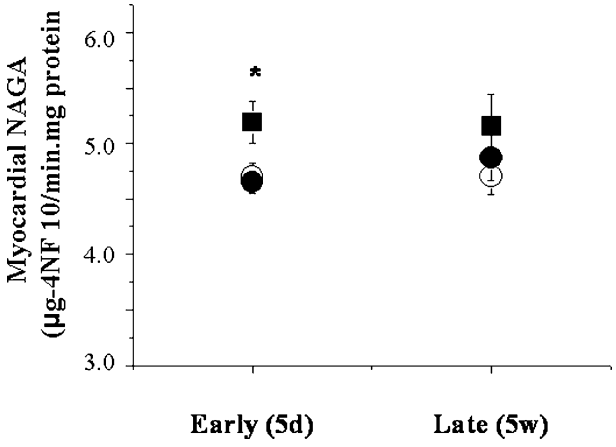

Fig. 5 N-acetylglucosaminidase (NAGA) activity in myocardial homogenates early and late after induction of STZD.Alteration in acid hydrolase activity in serum and in total cardiac tissue homogenate 5 days and 5 weeks after STZD and SMe1EC2 therapy. Control (open symbols) , STZD (squares), and therapy with SMe1EC2 (closed circles). Values are mean ± SEM, (n = 12) * Statistical significance, p <0.05.

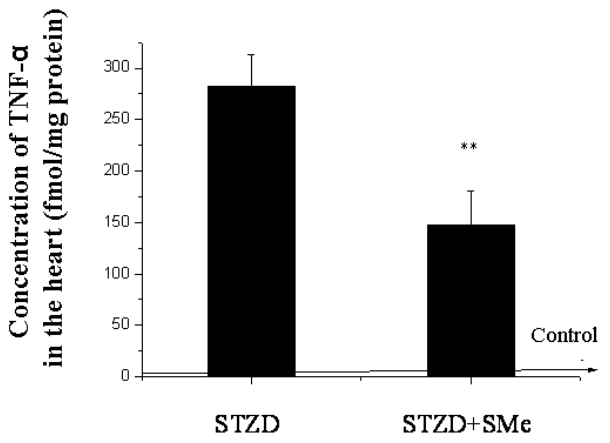

Fig. 6 Myocardial concentrations of tumor necrosis factor (TNF-?) in Wistar rats 5 weeks after streptozotocin-induction and 5 weeks after STZD+SMe1EC2 therapy (10 mg/kg/day p.o.every second day, for 5 weeks). Basal control values of cardiac concentration (Placebo-control) 3.2 ±0.5 fmol/mg of protein. Significant reduction of TNF-? after SMe1EC2.

Discussion

Principal finding of this study is the strong induction of free radicals and pro-inflammatory cytokine TNFα directly in the myocardium in the rodent animal model of streptozotocin-induced diabetes (STZD). Results of this study are consistent with the following conclusions: First, in STZD animals myocardial free radical concentrations and production of cytotoxic cytokine TNFα in myocardium were increased early after induction of diabetes. Second, elevated blood glucose, increased free radicals and inflammatory TNF-α production in myocardium were dominant features in the development of STZD diabetes and in induction of cardiac pathology. Third, the release of free radicals and pro-inflammatory TNFα dominant in myocardial tissue, contributed predominantly to the induction of immune- and inflammatory responses resulting in gradation of left ventricular dysfunction. Fourth, chronic therapy with SMe1EC2 significantly reduced myocardial free radicals and inflammatory cytokine production in STZD animals. Fifth, the early increase and late reduction in CSx concentration in myocardial tissues correlated well with the initial reduction and late expression of IL-6 after SMe1EC2 therapy. Sixth, IL-6 seems to exert also anti-inflammatory effects. IL-6, as an important signal for „healing processes“ in myocar-

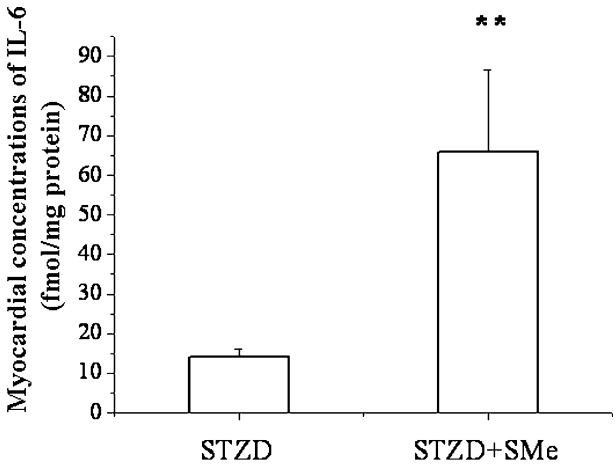

Fig. 7 Increased myocardial concentrations of interleukin-6 (IL-6) in streptozotocin diabetic rats (STZD) and after STZD + chronic treatment with SMe1EC2. Note further increase in myocardial IL-6 concentration, possibly, in response to systemically elevated IL-6.

dium, most probably reduced concentration of free radicals brought on by significant reducion in TNFα after SMe1EC2 chronic therapy. Myocardial glutathione system is considered to be a major redox system, im-

portant for the cellular redox and antioxidant defence, redox restoration, signal transduction, cell growth and apoptosis. In addition, GSH reductase in the heart regulates activities of various transport kinases and phosphatases and other processes in myocardium. However, there is very little if any transport of intact GSH from plasma to tissues. Human myocardial glutathione GSx is translocated out of cells and enters blood plasma. Only glutathione-monoesters can be effectively transported into myocardial cell and increase cell glutathione, presumably the important immune response in reducing the oxidative stress in human heart (Levy et al. 1993). Whether this mechanism is effective also in rat heart remains to be identified. Five weeks following induction of STZD in the animal model, there was probably significant GSH deficiency. TNF is ultimatively involved in myocardial inflammation. The results obtained in this study, however, suggest a new regulatory role of the TNF system linked to high plasma concentrations of TNFα and/or to a high transcription rate of TNFα late (5 weeks) after induction of heart failure in STZD Wistar rats. Thus the TNF system seems to be designated for an active adaptational response in the compromized heart. After chronic SMe1EC2 therapy, elevated IL-6 concentrations in myocardial muscle will reduce cardiac depression, brought by decreased free radical concentration. Increased GSH may further reduce TNFα and elevate IL-6 in myocardium. The strong increase in IL-6 myocardial expression (+368 ± 42 %) occurring at the end of SMe1EC2 therapy was surprising. The precise role of IL-6 in the production and release of myocardial TNFα cytotoxicity and protection, is however still an open question. Increased levels of cardiac lysosomal hydrolases (O-linked attachment of N-acetylglucosaminidase) are probably involved in the later stages of diabetic cardiomyopathy with impaired relaxation of cardiomyocytes, blunted responses to phenylephrine and vascular cell dysfunction in vascular smooth muscle. In contrast to Levy et al. (1993), others have shown that O-Glc-N-acetylglucosamine increased early in the response to acute stress and that it may be associated also with increased cell survival (Liu et al. 2006 and Fulop et al. 2007). For the majority of myocardial proteins, a gradient can be distinguished from minimal to large concentration in the diabetic heart. The present evidence suggests that the "diabetic heart" may be a source of inflammatory cytokines, lysosomal enzymes and chemoattractants both early and late after induction of diabetes.

In conclusion. Chronic treatment with the SMe1EC2 normalized cardiac cytokine levels and facilitated the repair of damaged myocardial tissue in diabetic cardiomyopathy.

Acknowledgements

This work was supported by grants from the Ministry of Education of the Slovak Republic (VEGA No 2/5007/27 and by Grant-in-Aid for Scientific Research APVV -51-017905)

References

Amour J, Loyer X, Le Guen M, Mabrouk N, David JS, Camors E, Carusio NB, Vivien B, Andriantsitohania R, Heymes C, Riou B: Altered contractile response due to increased ß3-adrenoceptor stimulation in diabetic cardiomyopathy: The role of nitric oxide synthase 1-derived nitric oxide. Anesthesiol **107**, 452-460, 2007

Aragno M, Mastrocola R, Medana C, Catalano MG, Vercellinatto I, Danni O, Boccuzzi G: Oxidative stress-dependent impairment of cardiac-specific transcription factors in experimental diabetes. Endocrinology **147**, 5967-5974, 2006

Baker MA, Cerniglia GJ, Zaman A: Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal Biochem **190**, 360-365, 1990

Boudin S, Abel ED: Diabetic cardiomyopathy revisited. Circulation **115**, 3213-3223, 2007.

Fisman EZ, Motro M, Temenlam A: Cardiovascular diabetology in the core of a novel interleukins classification: the bad, the good and the aloof. Cardiovasc Diabetol **2**, No.11, 2003

Forssmann WG: Cardiac hormones. I. Review on the morphology, biochemistry, and molecular biology of the endocrine heart. Eur J Clin Invest **16**, 439-451, 1986

FULOP N, MASON MM, DUTTA K, WANG P, DAVIDOFF AJ, MARCHASE RB, CHATHAM JC: Impact of type 2 diabetes and aging on cardiomyocyte function and O-linked N-acetylglucosamine levels in the heart. Am J Physiol Cell Physiol **292**, C1370-C1378, 2007

JAMIESON JD, PALADE GE: Specific granules in atrial muscle cells. J Cell Biol **23**, 151-172, 1964

KANNEL WB, HJORTLAND M, CASTELLI WP: Role of diabetes in congestive heart failure: the Framingham study. Am J Cardiol **34**, 29-34, 1974

KISCH B: Electron microscopy of the atrium of the heart. I. Guinea pig. Exp Med Surg **14**, 99-112, 1956

LEVY EJ, ANDERSON ME, MEISTER A: Transport of glutathione dietyl ester into human cells. Proc Natl Acad Sci USA **90**, 9171-9175, 1993

LIU J, PANG Y, CHANG T, BOUNELIS P, CHATHAM JC, MARCHASE RB: Increased hexosamine biosynthesis and protein O-GlcNAc levels associated with myocardial protection against calcium paradox and ischemia. J Mol Cell Cardiol **40**, 303-312, 2006

POORNIMA IG, PARIKH P, SHANON RP: Diabetic cardiomyopathy: the search for a unifying hypothesis. Circ Res **98**, 596-605, 2006

STOLC S, POVAZANEC F, BAUER V, MAJEKOVA M, WILCOX AL, SNIRC V, RACKOVA L, SOTNIKOVA R, STEFEK M, GASPAROVA Z, GAJDOSIKOVA A, MIHALOVA D, ALFOLDI J: Pyridoindole derivatives with antioxidant properties. Their synthesis and use in therapeutical praxis. (Slovak PP 1321, 61pages, 2003).

TROST S, LEWINTER M: Diabetic cardiomyopathy. Curr Treat Options Cardiovasc Med **6**, 481-492, 2001

VOTRUBA M, STOPKA P, HROUDOVA J, VESELY K, NEJEDLOVA L: A simple method for quantitative estimation of free radicals in serum (in Czech). Klinicka Biochemie a Metabolismus **7**, 96-101, 1999

WANG J, YOSHIDA T, NAKAKI F, HIAI H, OKAZAKI T, HONJO T: Establishment of NOD-Pdcd1-/- mice as an efficient animal model of type I diabetes. Proc Natl Acad Sci USA **102**, 11823-11828, 2005