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Cardiac ischemia and reperfusion in spontaneously diabetic rats with and without application of EGb 761: II. Interstitium and microvasculature

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Summary. Besides alterations in cardiomyocytes themselves, diabetic cardiopathy is characterized by interstitial and microvascular disorders. On the assumption that a specific heart muscle disease develops due to permanently increased oxidative stress on liberation of oxygen-free radicals, adjuvant application of antioxidative therapeutics appears promising in preventing or delaying long-term diabetic complications and protecting the myocardium against acute ischemia. We have investigated the effects of Ginkgo biloba extract (EGb 761), a radical scavenger, against diabetes-induced myocardial interstitium and microvasculature damage, and against additional ischemia/reperfusion injury in spontaneously diabetic BioBreeding/Ottawa Karlsburg (BB/OK) rats modelling diabetic cardiac infarction. Morphological and morphometric parameters in the heart muscle were evaluated by light and electron microscope. We used immunohistochemistry to investigate collagen protein expression as a marker for tissue remodelling together with endothelial nitric oxide synthase (eNOS) protein expression as a marker for endothelial-dependent vasodilation. We also evaluated inflammation response caused by neuropeptide Substance P and interacting mast cells in the diabetic heart. Our results revealed that A) Diabetic myocardium appears more vulnerable to ischemia/reperfusion injury than normal myocardium with regard to myocardial interstitium and microvessel ultrastructure, as well as eNOS protein expression; B) Inflammation response increases in diabetic animals exposed to ischemia/reperfusion injury compared to controls; C) Pre-treatment of diabetic myocardium with EGb results in an improvement of impaired endothelial-dependent vasodilation in diabetes and additional ischemia/reperfusion, diminished mast cell and substance P accumulation, and better preserved myocardial ultrastructure compared to unprotected myocardium. In conclusion, EGb may act as a potent therapeutic adjuvant in diabetics with respect to ischemic myocardial injury, and may contribute to preventing late complications in diabetic cardiopathy.

Key words: Diabetes, Ischemia, EGb 761, Interstitium, Microvessels

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Introduction

Structural alterations in, and expression of antioxidant enzymes of diabetic cardiomyocytes exposed to ischemia/reperfusion (as published in part I – Schneider et al., 2008) are accompanied by interstitial and microvascular changes. The resulting mismatch of myocardial supply and demand in the diabetic heart contributes to diabetic cardiopathy (Doi et al., 2001). Increased myocardial fibrosis leads to interstitial tissue stiffness (Aragno et al., 2008), as well as capillary basal lamina thickening with endothelial dysfunction (Okruhlicova et al., 2005), and may contribute to perfusion defects, particularly involving ischemia in the absence of epicardial coronary atherosclerosis (McDonagh et al., 1997).

Bioavailability of potent vasodilator nitric oxide (NO) is impaired in diabetes and with additional ischemia/reperfusion injury (Pozo-Navas et al., 2006). Several studies (Nagareddy et al., 2005; Desjardins and Balligand, 2006) have demonstrated that endothelium-dependent vasodilation in peripheral microcirculation is

disrupted in diabetes. Metabolic disturbances may impair Ca^{2+}-dependent eNOS activity and expression (Giraldez et al., 1997).

As described in varying cases of tissue fibrosis (Fox et al., 1990; Seibold et al., 1990), perivascular mast cells (MC) may be associated with remodelling processes in diabetic myocardium in a process promoted by inflammatory cytokines. Histamine secreted by MCs plays an important role as a vasoactive mediator while stimulating collagen synthesis. Non-immunological neuropeptide substance P (SP) has been implicated in MC activation and degranulation (Patella et al., 1995; Suzuki et al., 1999). On release, SP appears to trigger a cascade of proinflammatory and pro-oxidative events (Tejero-Taldo et al., 2006). Myocardial ischemia results in local release of SP from cardiac nerve terminals (Chiao and Caldwell, 1996).

The aim of this study was to characterize (ultra)structural alterations in cardiac interstitium and microvasculature from spontaneously diabetic BioBreeding/OttawaKarlsburg rats, a rat model of human diabetes type I, additionally exposed to ischemia/reperfusion to simulate myocardial infarction in diabetes and demonstrate the protective effects of EGb ginkgo biloba extract, a known anti-inflammatory agent and radical scavenger that stabilizes cell-membranes. To gain further insight into myocardial vasodilation disturbances, we investigated eNOS protein expression. The diagnostic value of eNOS expression is restricted, but it can be improved by targeted biochemical enzyme activity. We investigated MCs and neuropetide SP to evaluate inflammatory response in diabetes, aiming to compare ischemic tolerance in diabetic myocardium against normoglycemic myocardium. Diabetes-induced alterations of BB rat myocardium with additional ischemia/reperfusion we have published in part I of this study.

Materials and methods

Animals and experimental procedure

The experiments were approved by Leipzig's regional governing board (Regierungspraesidium No. 10/00) and performed in accordance with local animal welfare statutes. Twenty-two male diabetic BioBreeding/Ottawa Karlsburg (BB/OK) rats aged eight to nine months and sixteen male non-diabetic BB/OK rats (http://www.medizin.uni-greifswald.de/labanim/available_rat. html) kept separately under semisterile conditions were divided into five experimental groups.
Group I rats – 10 non-diabetic BB/OK animals – were not subjected to any treatment.
Group II rats – 6 non-diabetic BB/OK animals – were exposed to ischemia and reperfusion using a Langendorff apparatus.

Insulin-dependent diabetes manifested after 102±31.6 days was identified by weekly measurement of blood glucose levels. When plasma glucose levels exceeded 22.1 mmol/l, the rats were supplied with a subcutaneous sustained-release insulin implant (LINDPLANT. LINSHIN Canada, INC., Scarborough, Ontario, Canada).
Group III rats – 5 diabetic BB/OK animals – were sacrificed after six months of diabetes.
Group IV rats – 12 diabetic BB/OK animals – were exposed to ischemia and reperfusion by preparing isolated hearts using a Langendorff apparatus.
Group V rats – 5 diabetic BB/OK animals after three months of diabetes – were treated daily with 100 mg/kg body weight of ginkgo biloba extract (EGb 761, IPSEN Paris, France) dissolved in a limited amount of drinking water administered overnight. After six months of diabetes and three months of EGb protection, the hearts were exposed to ischemia and reperfusion using a Langendorff apparatus.

Isolated heart perfusion (Langendorff heart)

BB/OK rats from Group II, IV and V were intraperitoneally anesthetized with pentobarbital (180 mg/kg bw). The hearts were excised after thoracotomy, the aorta cannulated, and retrograde perfusion was initiated at a pressure of 81 mm Hg using Tyrode solution saturated with 95% O_2 and 5% CO_2 gas at 37°C. In these experiments, the hearts were perfused for 30 min to allow functional stabilization, and then subjected to 35 min of 37°C global "no flow" ischemia followed by 90 min reperfusion.

Tissue processing for light microscopy

The animals were anesthetized using pentobarbital. The heart was rapidly excised after thoracotomy, and tissue samples from Groups I and III were taken from the left ventricle near the apex by scalpel and processed for histology, electron microscopy, and immunohisto-chemistry as described below. The biopsies of groups II, IV and V were taken immediately after reperfusion in the same manner.

Tissue processing for electron microscopy

Tissue samples were minced into small blocks of about 1 mm^3, fixed in cold Karnovsky's solution (buffered 2% glutardialdehyde, 2% paraformaldehyde, pH 7.4) for two hours, contrasted with OsO_4 and phosphotungstic acid, and embedded in Durcupan (FLUKA) after acetone dehydration.

Semithin sections from each block were stained with toluidine blue to select interesting areas for electron microscopy. Ultrathin sections were obtained using Ultracut E (Reichert-Jung) and contrasted with uranyl acetate and lead citrate solution. Representative electron micrographs were taken using an EM 900 (Zeiss).

Histological techniques

Deparaffinized sections were stained using methylene blue and toluidine blue according to Denk et

al. (1989).

Morphometric analysis

Measurements at histological levels were carried out using classic point-counting, intersection point-counting and SIS image analysis to determine the volume fraction of interstitium (VV_{ecm}) and cardiomyocytes (VV_{myo}), the number of MCs per mm^2 (NA_{mast}), capillary diameter in μm (D_{cap}) defined as the smallest diameter, and the number of capillaries per mm^2 (NA_{cap}). VV_{ecm}/VV_{myo} ratio, capillary surface density (SV_{cap}) and intercapillary distance (ICD) were calculated.

For ultrastructural morphometry, we analyzed twenty-five electron micrographs per animal at 20,000-fold primary magnification obtained from five tissue blocks. Damage-related parameters such as edematous (swollen) endotheliocytes, capillaries exhibiting luminal blebs, and capillaries exhibiting luminal protrusions were destined by calculating the percentage of cells or structures.

Immunohistochemical techniques

Five serial sections per animal were deparaffinized, rehydrated in a descending alcohol cascade, treated using 3% H_2O_2 solution, rinsed in distilled water, treated in TBS (Tris buffer saline) and stored in serum protein block (DAKO). Sections were stored overnight with the primary antibody at different dilutions ranging from 100 to 2,000:1 at 4°C in a moist chamber. After that, they were rinsed in TBS, stored for one hour with the diluted secondary antibody at room temperature, rinsed again in TBS, stored with PAP (peroxidase-antiperoxidase) complex (rabbit or mouse EnVision, DAKO) diluted in Tris buffer or avidin-biotinylated enzyme complex (Vectastain ABC Kit, Vector Laboratories), and rinsed three times in TBS. After reaction with the DAB set, the sections were developed for 1–5 min, rinsed in distilled water, dehydrated in an ascending alcohol cascade, and embedded in Canada balsam.

Primary antibodies: rabbit polyclonal collagen I, III and VI antibody (Chemikon); rabbit polyclonal eNOS antibody (Transduction); synthetic polyclonal SP peptide (Biotrend); rabbit polyclonal factor VIII antibody (DAKO)

Secondary antibodies: goat anti-rabbit IgG/goat anti-mouse IgG EnVision (DAKO)

Controls for immunostaining of collagen, eNOS, SP and factor VIII:

Negative controls: incubation a: without primary antibody; b: without secondary antibody.

Positive controls: incubation of known positive tissue (rat liver and kidney).

Semiquantitative evaluation of immunohistochemical staining

The extent and intensity of collagen, eNOS, and substance P protein reaction was subjectively evaluated using a semiquantitative four-level grading system. Grade 0 stands for no apparent reaction product. Focal and minimal staining intensity was graded (1), and the most prominent staining reaction covering nearly the whole area of the specimen was classified as (4). Grades (2) and (3) were intermediate levels between (1) and (4).

Statistics

Data for myocardial morphometric and immunohistochemical analyses are expressed as the means ± SD. Statistical differences between mean values were calculated using Student's t-test for unpaired values, and were considered significant at values of $p<0.05$; Wilcoxon's test was used for non-parametric variables. The SPSS+ software package was used for each statistical evaluation.

Results

Light-microscopic findings

Left myocardium of diabetic rats exposed to ischemia/reperfusion (Group IV) showed a markedly extended, irregularly distributed connective tissue matrix with pronounced fibrosis around blood vessels and areas of severely damaged cardiomyocytes compared to controls with normal appearance (Group II). As reported in our previous study, a considerable fraction of diabetic cardiomyocytes showed both hydropic degeneration with an increase in diameter and cellular disintegration or myocytolysis together with reduced cross-section number per area and volume fraction. The number of fibroblast nuclei was elevated in these interstitial fibrosis areas. Myocardial capillaries and microvessels were enshrouded in thick bundles of collagen fibers. Many irregularly arranged microvessels appeared dilated, whereas their numbers seemed to be reduced. Surprisingly, staining with toluidine blue revealed a marked accumulation of MCs in diabetic myocardium, mainly around blood vessels and within fibrotic areas. General degranulation of MCs only occurred in untreated diabetes. Morphometric analysis of several structural diabetic myocardium parameters confirmed diabetes-induced morphologic alterations (Table 1). The cardiomyocyte volume fraction (VV_{myo} [%]) was significantly decreased, while interstitium volume fraction (VV_{ecm} [%]) and interstitium/cardiomyocyte ratio (VV_{ecm}/VV_{myo}) were each significantly increased. The mean diameter of capillaries (D_{cap} [μm]) was significantly increased, while their number (NA_{cap} [mm^{-2}]) and surface density (SV_{cap} [%]) were significantly reduced. Intercapillary distance (ICD [μm]) – defined as the closest distance between endothelium and mitochondria of cardiomyocytes – was increased. The average number of MCs (NA_{mast} [mm^{-2}]) showed a marked increase in unprotected diabetic myocardium exposed to ischemia and reperfusion.

The structure of myocardium was better preserved in hearts from diabetic rats treated with EGb (Group V),

showing a weaker manifestation of fragmentation or degeneration of cardiomyocytes with more normal nuclei and homogeneous cytoplasm. Accumulation of interstitial connective tissue and frequency of fibrotic scars were both clearly diminished compared to unprotected diabetic myocardium. Furthermore, only a slight disarrangement of microvasculature and less extent of perivascular fibrosis was to be seen. Degranulation and increase in number of surrounding MCs did not occur. EGb treatment significantly improved most morphometric parameters. Diabetic myocardium protected by EGb only showed a slightly increased interstitium volume fraction, average capillary diameter and cross-sectional number of MCs, whereas cross-sectional numbers of capillaries and volume fractions of cardiomyocytes were clearly less diminished compared to unprotected diabetes.

Ischemia/reperfusion caused no significant morphometric differences at lightmicroscopic level either in control or in diabetes.

Qualitative electron microscopic findings

The ultrastructural diabetic myocardial interstitium patterns without experimental ischemia/reperfusion (Group III) confirmed pronounced fibrosis. The interstitial matrix contained bundles of small fibers in the dilated interstitium and around small vessels. The pericapillary space contained a loose web of fine fibers in varying density partly condensed to plaques in most cases. Coarse collagen fibers were closely related to capillaries. Some capillary endotheliocytes showed thickened basal lamina as well as slight to moderate swelling of mitochondria partly accompanied by disintegration of membranes, and occasionally cellular edema.

Ischemia and subsequent reperfusion of the diabetic myocardium (Group IV) slightly aggravated the ultrastructural alterations of the interstitium mentioned above, showing a moderate increase in intercellular edema, along with a fine flocculent or unstructured substance filling the entire intercellular space at some points. As typical alterations of the microvasculature, we observed alternating thickness of endothelial cells combined with luminal protrusions, pericapillary cellular debris, progressive mitochondrial damage, deposition of lipid, endothelial edema, occasional luminal blebbing, vacuoles, and fused vesicles in various shapes. In contrast, ischemia/reperfusion injury in normal myocardium (Group II) led to less severe alterations, such as slight intercellular edema, condensation or moderate swelling of a fraction of mitochondria, and less

Table 1. Histological morphometric myocardial parameters (left ventricle) in experimental groups (mean ± SD) (*p ≤ 0.05).

	Control; control + ischemia/reperfusion		Diabetes; diabetes + ischemia/reperfusion		Diabetes + EGb 761 + ischemia/reperfusion
D_{cap} (μm)	4.8 ± 0.3	*	6.1 ± 0.4	*	5.6 ± 0.4
NA_{cap} (1/mm^2)	3587.3 ± 93.4	*	2496.2 ± 92.9	*	2926.1 ± 113.3
VV_{myo} (%)	79.4 ± 1.4	*	70.6 ± 2.3	*	74.6 ± 3.0
VV_{ecm} (%)	20.6 ± 1.4	*	29.4 ± 2.3	*	25.4 ± 3.0
NA_{mast} (1/mm^2)	23.6 ± 5.5	*	49.8 ± 8.7	*	30.8 ± 4.9
SV_{cap} (mm^{-1})	54.4 ± 3.2	*	48.3 ± 2.6		50.9 ± 2.69
ICD (μm)	13.1 ± 0.3	*	15.3 ± 0.9	*	14.3 ± 0.3
VV_{ecm}/VV_{myo}	0.26 ± 0.02	*	0.42 ± 0.05	*	0.34 ± 0.05

luminal blebbing.

After protection with EGb (Group V), the ultrastructure of interstitium and microvasculature appeared less altered than in unprotected diabetic ischemic animals. Endothelial alterations were less heavily expressed, such as luminal blebs and protrusions, an increase in the number of vacuoles and fused vesicles, edema, thickening of capillary basement membrane and deposition of amorphous matrix, pericapillary debris and small fiber bundles in the interstitium and around small vessels (Fig. 1).

Quantitative electron microscopic findings

Quantitative analysis of endothelial compartments revealed that ischemia and subsequent reperfusion had led to stronger deterioration in morphological conditions in diabetic myocardium than in controls. EGb treatment improved some of these ultrastructural parameters after ischemia/reperfusion damage (Table 2).

The amount of swollen (edematous) endothelial cells was markedly increased in diabetes, and slightly more increased after ischemia and reperfusion in the unprotected diabetic group. EGb treatment led to significant improvement of this parameter. Ischemia/reperfusion damaged diabetic myocardium somewhat more than non-diabetic myocardium.

The number of capillaries showing luminal blebs was significantly increased in diabetes, and slightly more increased after additional ischemia/reperfusion, but less increased after protection by EGb. In contrast, ischemia/reperfusion damaged non-diabetic myocardium somewhat more than diabetic myocardium. Moreover, those capillaries showing luminal protrusions were significantly increased in diabetic myocardium and after additional ischemia/reperfusion, whereas this increase was absent in animals treated with EGb. Ischemia/reperfusion damaged diabetic myocardium

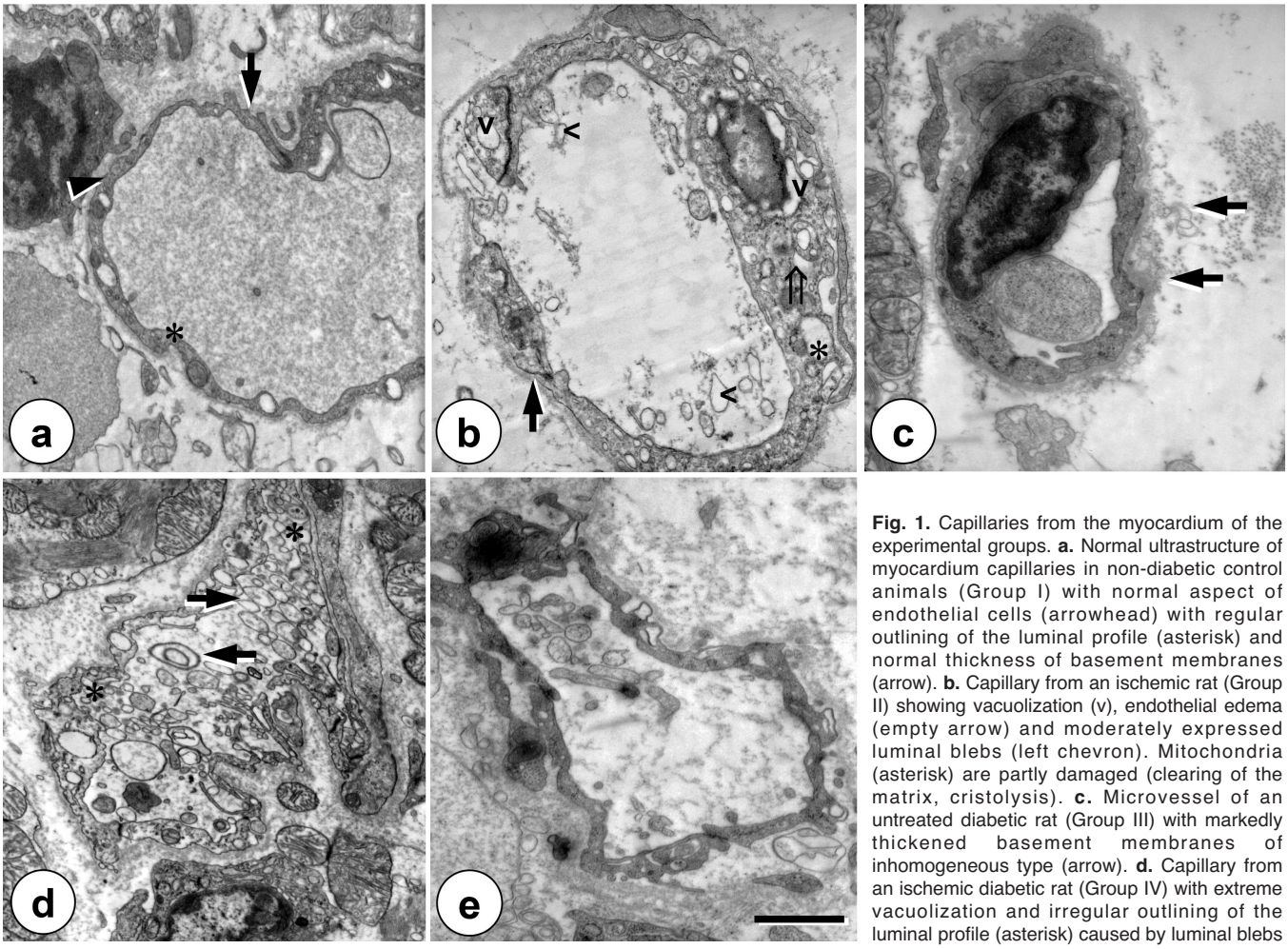

Fig. 1. Capillaries from the myocardium of the experimental groups. **a.** Normal ultrastructure of myocardium capillaries in non-diabetic control animals (Group I) with normal aspect of endothelial cells (arrowhead) with regular outlining of the luminal profile (asterisk) and normal thickness of basement membranes (arrow). **b.** Capillary from an ischemic rat (Group II) showing vacuolization (v), endothelial edema (empty arrow) and moderately expressed luminal blebs (left chevron). Mitochondria (asterisk) are partly damaged (clearing of the matrix, cristolysis). **c.** Microvessel of an untreated diabetic rat (Group III) with markedly thickened basement membranes of inhomogeneous type (arrow). **d.** Capillary from an ischemic diabetic rat (Group IV) with extreme vacuolization and irregular outlining of the luminal profile (asterisk) caused by luminal blebs (arrow). **e.** EGb-protected myocardial microvessel from an ischemic diabetic rat (Group V) exhibiting nearly normal ultrastructure. Scale bar: 2.0 μm.

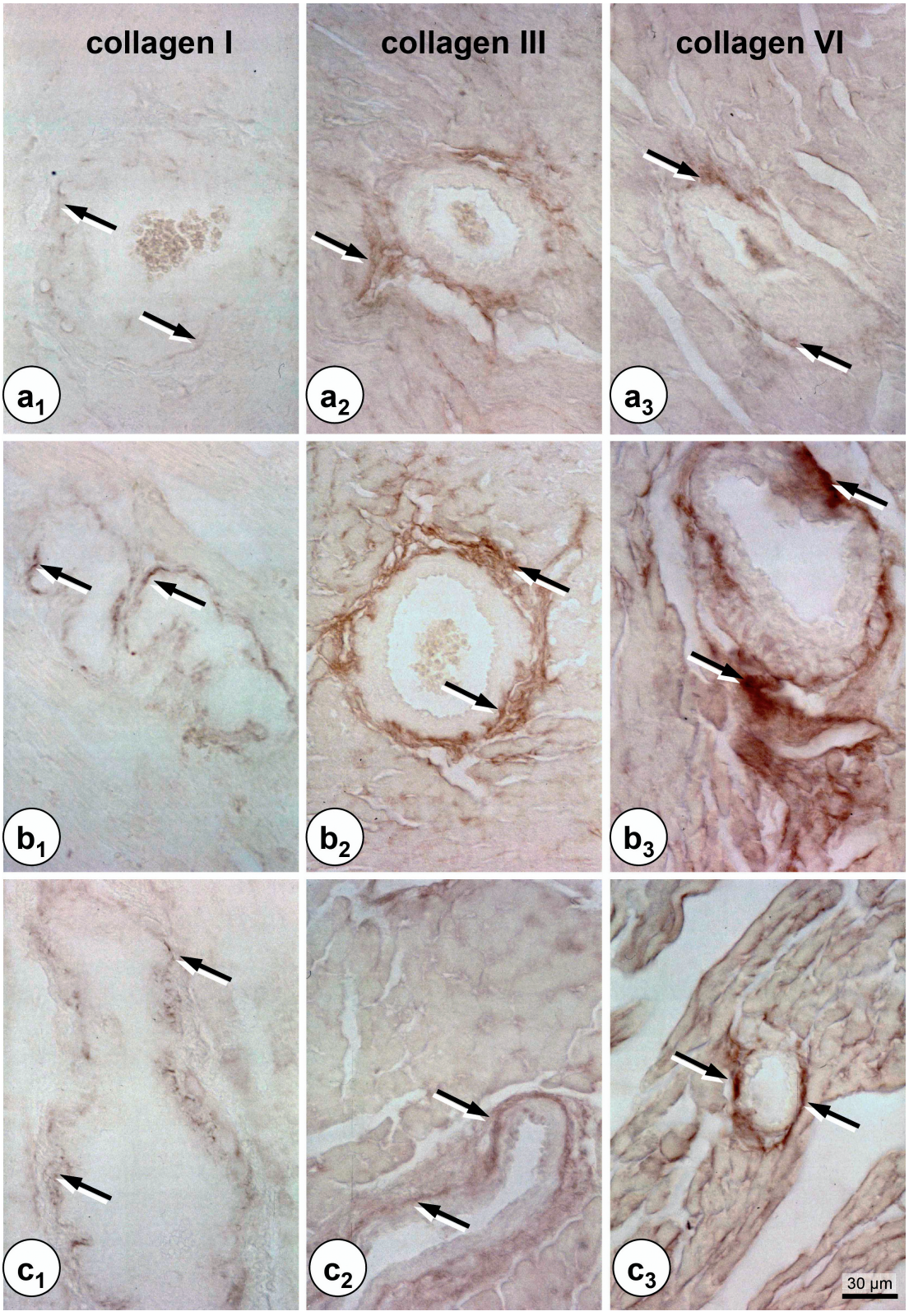

Fig. 2. Immunohistochemical demonstration of fibril-forming collagen types I and III, and microfibrillar collagen type VI in myocardium from the experimental groups exposed to ischemia/reperfusion, with an inhomogeneous pattern showing areas of stronger and no staining reaction. **a.** Control rats (Group II), normal appearance of collagen type I, III and VI (arrow). **b.** Diabetic rats (Group IV), increased amounts of type I, III and VI collagen. **c.** EGb-protected diabetic rats (Group V), smaller deposits of collagen types (arrow) than in untreated diabetes.

more than non-diabetic myocardium.

Immunohistochemical demonstration of collagen expression

Immunostaining for different collagen proteins in myocardium from all experimental groups showed an inhomogeneous, spotted pattern with areas of stronger staining reaction surrounded by areas of weak or no immunostaining (Fig. 2); fibril-forming collagen type I was mainly observed at pericapillary and interstitial locations; fibril-forming collagen type III, as well as microfibrillar collagen type VI were concentrated on basal membranes of capillaries and cardiomyocytes, as well as around larger vessels. Immunostaining of collagen types I, III and VI was weak in myocardium of control animals with and without ischemia/reperfusion, but considerably stronger in diabetes and diabetes with ischemia/reperfusion. EGb treatment reduced the number and size of deposits of collagen I, III and VI to nearly the level of the control groups (statistical analyses in table 3).

Immunohistochemical demonstration of substance P expression

Immunostaining for SP protein showed an inhomogeneous distribution of the reaction product in

Table 2. Ultrastructural morphometric parameters of cardiomyocyte components (left ventricle) in experimental groups (mean ± SD) (*p≤ 0.05).

	Control	Control + ischemia/ reperfusion	Diabetes	Diabetes + ischemia/ reperfusion	Diabetes + EGb 761 + ischemia/reperfusion
Edematous (swollen) endotheliocytes (% of all endotheliocytes)	7.2 ± 5	15.3 ± 5 *	11.3 ± 5 *	16.4 ± 5 *	6.9 ± 4
Capillaries exhibiting luminal blebs (% of all capillaries)	4.9 ± 2	8.1 ± 2 *	7.2 ± 3	7.9 ± 2	6.8 ± 2
Capillaries exhibiting luminal protrusions (% of all capillaries)	9.1 ± 2	14.4 ± 3 *	14.1 ± 2 *	17.8 ± 2 *	15.7 ± 2

Table 3. Semiquantitative evaluation of immunohistochemical stained collagen types I, III and VI, and substance P (left ventricle) in experimental groups (mean ± SD) (*p≤ 0.05).

	Control; control + ischemia/reperfusion	Diabetes; diabetes + ischemia/reperfusion	Diabetes + EGb 761 + ischemia/reperfusion
Collagen I	1.0 ± 0.1 *	2.4 ± 0.4 *	1.4 ± 0.2
Collagen III	1.6 ± 0.1 *	2.8 ± 0 *	1.7 ± 0.2
Collagen VI	1.2 ± 0.2 *	2.1 ± 0.2 *	1.8 ± 0
Substance P	1.4 ± 0.1 *	2.7 ± 0.2 *	1.6 ± 0.2

Table 4. Semiquantitative evaluation of immunohistochemical stained eNOS (left ventricle) in experimental groups (mean ± SD) (*p≤ 0.05).

	Control	Control + ischemia/ reperfusion	Diabetes	Diabetes + ischemia/ reperfusion	Diabetes + EGb 761 + ischemia/reperfusion
eNOS	3.4 ± 0.2 *	2.1 ± 0.1	2.1 ± 0.3	1.7 ± 0.3	2.1 ± 0.1

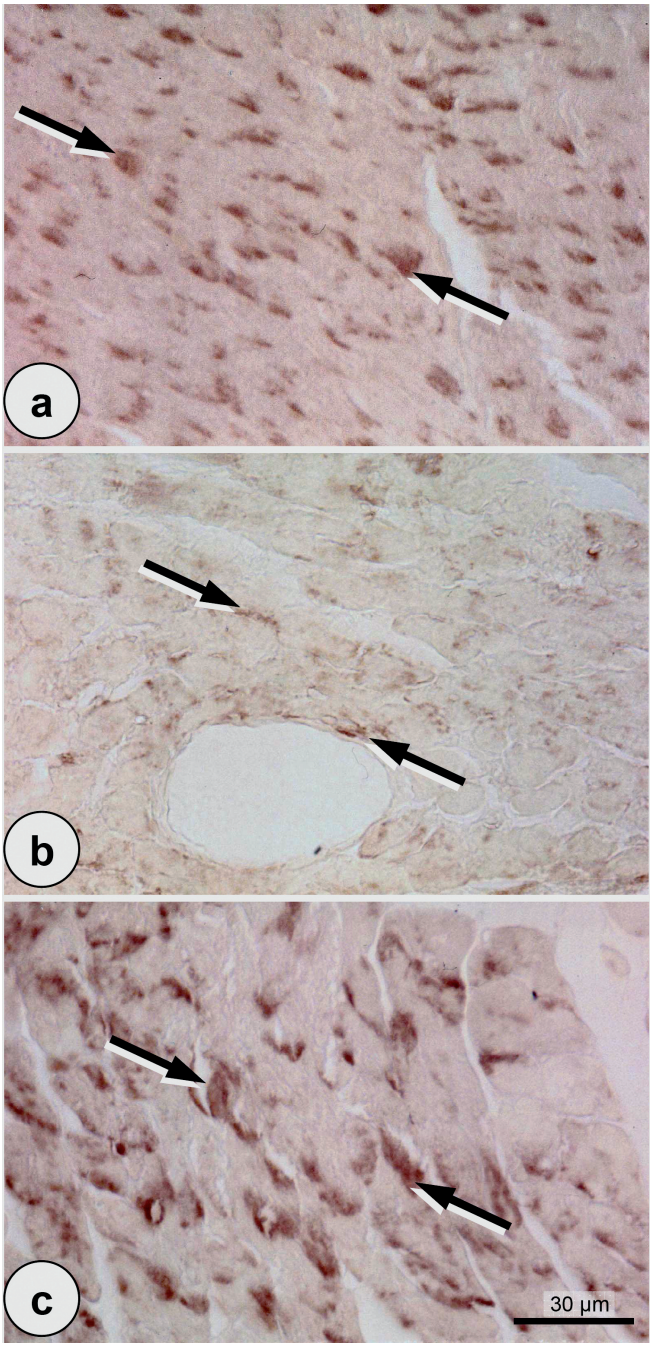

Fig. 3. Immunohistochemical demonstration of eNOS protein expression in myocardium from experimental groups after ischemia/reperfusion showing an inhomogeneous striated eNOS immunostaining pattern in cardiomyocytes from all experimental groups. Most cardiomyocytes are unstained. **a.** Control rats (Group II): strong eNOS immunostaining (arrow). **b.** Diabetic rats (Group IV): weaker eNOS immunostaining (arrow). **c.** EGb-protected diabetic rats (Group V): stronger eNOS immunostaining (arrow).

myocardial interstitium, either as fiber-associated chain-like varicosities closely related to the longitudinal axis of cardiomyocytes or as isolated perivascular structures in all experimental groups. Staining intensity was strong in diabetic myocardium with and without ischemia/reperfusion, but weak in non-ischemic and ischemic controls. EGb treatment reduced the intensity of SP protein expression to values near control levels in the ischemic-diabetic group (statistical analyses in table 3).

Immunohistochemical demonstration of eNOS protein

In general, a similar spot-like pattern of immunostained cardiomyocytes was evident in myocardium from all experimental groups with only slight differences in semiquantitative evaluation, except in non-ischemic controls (Fig. 3). Most cardiomyocytes were unstained, while stained cells showed an artificial dislocation of the reaction product to one side of some cells.

Staining intensity of eNOS protein was strong in cardiomyocytes from control rats, but reduced in diabetes, and more reduced in diabetes after experimental ischemia/reperfusion. EGb treatment in the ischemic diabetic group led to a more intense eNOS protein expression near to control levels (statistical analyses in table 4).

Discussion

As we have shown in part I of our study on cardiomyocytes (Schneider et al., 2008), spontaneously diabetic BB/OK-rats developed typical diabetic alterations (Okruhlicova et al., 2005; Aragno et al., 2008). Obviously, compensative as well as degenerative processes in both interstitium and microvasculature play an important role in the development of specific diabetes cardiopathy. Qualitative light-microscopic analysis on diabetic myocardium showed pronounced interstitial fibrosis, which may be understood as a reparative mechanism of cell loss morphometrically expressed as an increased interstitium fraction and interstitium/cardiomyocyte ratio. In addition to the apparently higher proportion of ground substance components, simple edema may contribute to the greater extracellular space volume (Warley et al., 1995). Pronounced perivascular fibrosis in unsystematically arranged myocardial capillaries and microvessels, morphometrically diminished number of microvessels per unit area and surface density at cross sections, but increased mean diameter and intercapillary distance indicated diminished vascular density, as described by Gross et al. (2004). The disproportional rise observed in collagen type I, III and VI protein expression confirmed high-grade perivascular, interstitial and replacement fibrosis in unprotected diabetic myocardium, as described in the literature (Rösen et al., 1995; Yu et al., 1997; Welt et al., 1999). Protein kinase C stimulation by hyperglycemia and OFRs (oxygen free radicals) may promote proto-

oncogene expression, leading to increased generation of inflammatory cytokines and synthesis of matrix proteins (Doi et al., 2001). Collagen digestibility seems to be impaired by an imbalance between collagenase and its inhibitors (Cleutjens et al., 1995; Bollano et al., 2007) and altered matrix-related signal transduction (Brownlee, 1995). Fibrosis has been discussed as being more a result of impaired degradation rather than enhanced synthesis (Shehadeh and Regan, 1995) as supported by a specific cascade triggered by advanced glycosylation end products (AGEs). This accumulation of connective tissue matrix, as well as compensative hypertrophy of remaining cardiomyocytes, leads to architectural changes in myocardial geometry, which Swynghedauw described as cardiac remodelling (1999).

Mast cells may be also involved in the remodelling process in diabetic myocardium as they are in other fibrotic tissues (Fox et al., 1990; Seibold et al., 1990). Direct non-immunological stimuli such as substance P may activate and degranulate MCs (Patella et al., 1995; Suzuki et al., 1999), leading to impaired vasodilatation and alteration in the OFR-nitric oxide balance via histamine, as well as stimulation of collagen synthesis and generation of chemotactic gradients necessary for recruitment of inflammatory cells (Marisa et al., 2005). MCs and SP are considered as key components in ischemia/hypoxia-induced microvascular inflammation leading to cardiomyopathy (Steiner et al., 2003). We found predominantly degranulated MC accumulations with a simultaneous increase in SP-positive structures mainly around blood vessels and within fibrotic areas in untreated diabetic myocardium, which is in agreement with Coleman et al. (1986). A close microanatomical relationship or synapse-like structure between MCs and nerve fibers positive for SP has been suggested in the literature (Crivellato et al., 1991).

To diminish late complications of chronic diabetes, it appears logical to apply adjuvant therapies to scavenge OFRs that occur during diabetic metabolism and lead to generation of AGEs. In our case, the combined activities of the constituents of EGb (24% flavonoids, 6% terpenoids, 8% ginkgolides) are responsible for the therapeutic benefit observed. The lipophilic property of the main components explains the high affinity to biological membranes. The protective effects of EGb on the myocardial structure of BB rats may be attributed to the inhibition of protein kinase C and subsequent cascade leading to production of extracellular matrix (Kantengwa and Polla, 1991; Bäcklund et al., 2004), as well as the direct antioxidative and free radical-quenching potential of EGb (Pietri et al., 1997), which results in reduced formation of AGEs. Evaluation of collagen protein expression showed a clear beneficial effect of EGb with regard to type I, III and VI collagen, as described by Welt et al. (1999) for STZ-diabetes.

The anti-inflammatory mechanism of EGb is not completely understood (Varga et al., 1999). Immunomodulating and neuromodulating properties via protein kinase C may suppress both inflammatory cytokine secretion and MC migration in diabetic myocardium, as described for neuropeptide Y in STZ-diabetes by Zhang et al. (2005). The significantly diminished SP staining, as well as decreased accumulation and degranulation of MCs in EGb-treated diabetic myocardium, result in reduced liberation of vasoactive histamine, which may support this assumption.

The underlying general mechanism of cell injury in ischemia and reperfusion conditions by OFRs is well-established in literature (Burton et al., 1990; Shattock, 1997). From our ultrastructural results, we conclude that diabetes does not improve ischemia/reperfusion tolerance in the myocardium in contrast to DaTorre et al. (1991), who postulated that diabetes desensitizes the heart against ischemia/reperfusion injury. We found increased vulnerability in diabetic myocardium, together with considerable ultrastructural and enzymatic alterations. As observed by others (Rösen et al., 1991; Pieper, 1999), the capillaries were dilated, which may have been induced by increased myocardial nutrient demand. Adenosine secretion, SP, MCs, histamine, lactate and passive dilation resulting from local perfusion disturbances have also been discussed. The possible involvement of prostaglandin endoperoxides or other cyclooxygenase products was excluded by Rösen et al. (1995). The role of microaneurysms in capillaries is unclear (Factor et al., 1980). Additionally, we found an increase in the edematous endothelial cell fraction, formation of luminal blebs and protrusions, and progressive mitochondrial damage in endothelium as typical features after ischemia/reperfusion. Impaired vascularization and increased oxygen diffusion distance from capillary lumen to myocyte mitochondria reduce oxygen supply to myocytes, resulting in a diminished ATP concentration, partial loss of function, and destructive processes in diabetic myocardium (McDonagh et al., 1997; Welt et al., 2001). Sexton et al. (1994) reported reduced microvascular diffusion capacity in small molecules, whereas the permeability of larger macromolecules was increased, leading to protein extravasation followed by interstitial edema (Zatz and Brenner, 1986), and, in our opinion, flocculent or unstructured interstitial matter deposits.

Endothelium-dependent vasodilatation impairment has been controversially discussed as a possible consequence of constant generation of free radicals interfering by quenching NO, rather than changes in expression of eNOS or sensitivity of vascular smooth muscle cells for NO (Rösen et al., 1995; McDonagh et al., 1997; Consentino and Luscher, 1998; Desjardins and Balligand, 2006). Immunostaining for eNOS protein in our study showed a significant lower protein expression in untreated diabetes, which has been proposed as a result of increased rate of mRNA degradation by AGEs and reduced enzyme activity or cofactors (Zweier et al., 2001; Goldin et al., 2006). In contrast to Zhao et al. (1999), who found an increase in mRNA, the prolonged diabetic state may lead to eNOS downregulation with

concurrent upregulation of iNOS, indicating differential regulation of NOS isoforms (Nagareddy et al., 2005; Ma et al., 2006). Given the fact that endothelial function is impaired in diabetes and that NO generation from endothelium is reduced, NO in such settings may originate from a paradoxical increase in NO production from iNOS (Schneider et al., 2008). Ischemia/reperfusion in our experiment led to somewhat lower eNOS expression in diabetic cardiomyocytes than in healthy controls. This may be due to ischemia/reperfusion-induced intracellular acidification and cellular protease activation, resulting in protein degradation (Giraldez et al., 1997), or specific fragmentation and inactivation by excess radical formation during reperfusion (Taniguchi et al., 1989).

The concentration of endothelial cells with edema, endothelial blebbing and protrusions was slight to moderate, and in some cases even significantly reduced by EGb, which may gradually improve the tolerance against ischemia/reperfusion injury. EGb attenuates further structural alterations of myocardial microvasculature, such as rarefication in the capillary network, capillary basal lamina thickening and vasodilation of diabetic BB/OK rats. The terpenoid constituents and flavonoid metabolites from EGb probably act in a complementary manner to protect against myocardial ischemia/reperfusion injury (Liebgott et al., 2000). The direct membrane-stabilizing and anti-edematous potential of EGb (Huang et al., 1981) may lead to endothelial permeability regulation, resulting in decreased protein extravasation and interstitial edema. Janssens et al. (1995) showed direct effects of endotheliocytes. Bilobalides stabilized mitochondrial function and ATP synthesis and improved the metabolic, ionic (Ca^{2+}-homeostases) and osmotic milieu. Improvement in hemodynamic features and transport of vasoactive substances promote endothelial function. The interstitial and microvascular changes observed in spontaneously diabetic rat myocardium are characteristic for uncontrolled diabetic conditions, and are probably responsible for the development of secondary complications (Kakkar et al., 1995). A connection between mitochondrial damage and generation of reactive oxygen species has been suggested (Godin et al., 1988). EGb (Ginkgolide A) evidently improves the antioxidative capacity of cardiomyocytes under high oxidative stress conditions, involving the inhibition of free-radical formation (Pietri et al., 1997), and scavenging during ischemia/reperfusion (Tosaki et al., 1994; Punkt et al., 1997). The unexpectedly stronger immunostaining for eNOS protein in protected diabetic cardiomyocytes after ischemia/reperfusion may be regarded as a protective effect against myocardial ischemia/reperfusion injury by both the terpenoid and the flavonoid constituents in the extract. In this respect, direct enzyme activation, scavenging of free radicals or general membrane stabilization of antioxidative EGb may lead to increased antiradical capacity as well as stabilization of eNOS enzyme in treated myocardium (Marcocci et al., 1994; Maitra et al., 1995).

Conclusion: Myocardium from spontaneously diabetic BB/OK rats develops the typical morphological alterations of diabetic cardiopathy under low-dose insulin therapy after six months of diabetes. After exposure to additional ischemia/reperfusion, unprotected diabetic myocardium reveals reduced structural tolerance towards O_2 deficiency. Ultrastructural integrity of myocardial interstitium and microvasculature is also damaged, along with the deterioration of eNOS protein expression.

EGb pretreatment may slightly to moderately shift these parameters towards normal values. Ultrastructural morphometric data could be interpreted as an improvement of ischemia/reperfusion tolerance. The protective effect of EGb substitution seems to consist in scavenging of radicals, the stabilizing of membranes and enzymes, as well as immunomodulating properties. EGb does not completely prevent diabetic cardiomyopathy and ischemia/reperfusion injury. However, it appears to be a promising candidate for reducing late diabetic complications and improves acute ischemic injury in diabetes.

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