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Combined analysis of biochemical parameters in serum and differential gene expression in circulating leukocytes may serve as an *ex vivo* monitoring system to estimate risk factors for complications in *Diabetes mellitus*

M. Kapalla[1], **K. Yeghiazaryan**[2], **M. Hricová**[1], **H. Moenkemann**[2], **A. Pírek**[1], **H. H. Schild**[2], and **O. Golubnitschaja**[2]

[1] Department of Clinical Biochemistry and Haematology, Alpha Medical s.r.o., Ružomberok, Slovakia
[2] Department of Radiology, Division of Molecular/Experimental Radiology, University of Bonn, Bonn, Germany

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Summary. *Objective*: Oxidative stress plays a crucial role in the development of complications in Diabetes mellitus (DM). Individual sensitivity against stress, however, varies among DM-patients and results, therefore, in differential severity of consequent complications. To allow more complex interpretation of a delicate antioxidant/free radicals balance and its effect on cellular functions in individual DM-patients, we analysed a correlation between total antioxidant status (TAS), antioxidant gap (AtxGap), level of free radicals (FR), routine clinical biochemical parameters in blood and differential gene expression in circulating leukocytes of DM-patients versus non-diabetic individuals.

Results and conclusions: Positive correlation was found between TAS and creatinine ($p = 0.05$), AtxGap and iron ($p = 0.025$), and between AtxGap and anti-streptolysin O ($p = 0.025$). Whereas no correlation was found between FR and any of the routine clinical parameters tested, a negative correlation was observed between AtxGap and glucose content ($p = 0.025$) and between AtxGap and γ-glutamyltransferase ($p = 0.05$). An increased content of FR was shown to influence significantly an expression of selected stress responsible genes in leukocytes. Transcription levels of NF-kappaB, XRCC1 and 90-kDa heat-shock protein A were increased in all DM-patients compared to non-diabetic individuals. In contrast, an expression of XIAP and cytochrome P450 reductase was up-regulated in patients with decreased levels of both FR and TAS and increased body mass index. This differential expression of the stress responsible genes might be further considered as a potential risk factor for diverse DM-complications helping also in reliable monitoring of supplemental antioxidant therapy and more complex interpretation of delicate antioxidant/free radicals balance.

Keywords: Molecular monitoring *ex vivo* – Diabetic complications – Risk factors – Free radicals/antioxidant status – Circulating leukocytes – Differential gene expression

Introduction

Increased oxidative stress and subsequent activation of red-ox sensitive transcription factors has been linked to the development of secondary DM-complications (Hofmann et al., 1999; Bierhaus et al., 2001; Collins and Cybulsky, 2001). Pathophysiologically relevant metabolites produced under hyperglycemia are advanced glycosylation end products (AGEs) which trigger enhanced production of free radicals (Baynes, 1991; Nishikawa et al., 2000). Being extremely reactive species free radicals induce the destruction of cellular membranes, structural proteins, enzymes, lipids, DNA, and other metabolically important compounds (Riley, 1994; Nohl et al., 1994; Reiter, 1995; Maxwell, 1995) followed by clinical manifestations of DM-complications (Baynes, 1991; Giugliano et al., 1996). Oxidative damage to DNA has been well documented in cells isolated from DM-patients (Frustaci et al., 2000). Individual sensitivity against the oxidative stress, however, varies among DM-patients and results, therefore, in differential severity of consequent complications. A monitoring of the expression patterns of genes which are relevant for the regulation of cell cycle, DNA-synthesis and DNA-repair particularly, have been shown to be important for an estimation of the cellular response in cardiac tissue under diabetic stress conditions (Moenkemann et al., 2002). These genes play an important role in a controlled cell loss that is supposed to be the clue to the pathogenesis of cardiomyopathy in patients with DM. However, the gene expression monitoring using cardiac tissue requires an invasive approach and can not be performed routinely.

It has been shown that an activation of circulating leukocytes contributes to a variety of DM-complications (Hofmann et al., 1999; Aljada et al., 2001). A degree of this activation has been monitored using an *ex vivo* quantification of the generation of reactive oxygen species and specific gene expression (Aljada et al., 2001). These results indicate that differential gene expression in circulating leukocytes could be a powerful instrument in the non-invasive investigation of DM-complications. A development of non-invasive molecular monitoring systems is one of the most important tasks in the understanding and prediction of individual DM-complications. The goal of this study was an analysis of a wide spectrum of biochemical parameters (glucose, free radicals, antioxidants, albumin, uric acid, etc.) in serum, combined with an *ex vivo* gene expression profiling in circulating leukocytes of non-diabetic individuals versus DM-patients selected for two groups with higher and lower content of free radicals in serum. This should provide a stable correlation between measurable metabolic markers in serum and corresponding gene expression patterns in leukocytes which may be further used for the development of specific molecular markers in order to estimate the risk factors for DM-complications. The results are discussed.

Material and methods

Subjects

24 individuals were recruited for this investigation (see Table 1). The diagnosis of DM type 2 as well as the identification of non-diabetic individuals was based on detailed history, physical and biochemical examination of the recruited persons. This was performed in accordance with the national guidelines for healthcare and person protection.

Measurement of biochemical parameters in serum

All biochemical tests were performed using fresh serum under routine conditions on analyser AU 400-Olympus with the original reagents by Olympus Diagnostica (Ireland). For all particular tests (albumin (Alb), uric acid (UA), creatinine (Cre), urea, total bilirubin (TBil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GMT), alkaline phosphatase (ALP), cholinesterase (CHE), creatine kinase (CK), lactate dehydrogenase (LD), α-hydroxybutyrate dehydrogenase (HBD), α-amylase (α-AMY), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), magnesium (Mg), iron (Fe), phosphorus (P), total protein (TP), transferrin, unsaturated iron-binding capacity (UIBC), glucose, apolipoprotein A1 (Apo A1), apolipoprotein B (Apo B), total cholesterol, HDL-cholesterol, LDL-Cholesterol, triacylglycerols (TG), antistreptolysin O (ASLO), rheumatoid factor (RF), C-reactive protein (CRP)) protocols supplied by the producer of reagents were used. All routine methods underwent both, regular internal quality assessment on the daily basis and regular international external quality assessment (SEKK, Czech Republic).

Table 1. Information about the non-diabetic individuals and DM-patients: "n" means a number of persons

Group	n	Male/Female [n]	Mean age [years]
DM-patients	13	8/5	47.9 ± 4.9
Non-diabetic individuals	11	5/6	30.1 ± 9.3
Males	13	13/0	42.0 ± 9.2
Females	11	0/11	37.0 ± 13.1
Total	24	13/11	39.7 ± 11.5

Total antioxidant status (TAS) was measured by ABTS (2,2′-azino-di-(3-etylbenztiazolin)sulfonate) method (Miller et al., 1993) (Randox, UK) on analyser AU400-Olympus according to the protocol supplied by manufacturer. Levels of free radicals (FR) were measured by a novel chlorophyllin method (Sevapharma, Czech Republic) (Votruba et al., 1999) on analyser AU 400-Olympus according to the supplied protocol. Antioxidant Gap (AtxGap) was calculated according to the formula suggested by K. MacKinnon: AtxGap [mmol/l] = TAS [mmol/l] − ((Albumin[g/l]/65) $*$ 0.69 + Uric acid [mmol/l]) (MacKinnon et al., 1999). Statistical significance was calculated by the two-sided unpaired Student's t-test and considered at the $p < 0.05$ level.

Isolation of leukocytes

Blood samples (20 ml) anti-coagulated with K_3EDTA were collected from DM-patients and non-diabetic individuals (controls). Leukocytes (including monocytes and stem cells) were separated using Ficoll-Hipaque gradients (Histopaque 1077, Sigma, USA) as described previously (Kalmar et al., 1988). Briefly, blood samples were diluted with equal volume of physiological buffer solution (PBS, Gibco™, USA). Then, 2 ml of histopaque were placed into 10 ml sterile centrifuge tubes and 5 ml of diluted blood samples were carefully layered onto each histopaque gradient. Gradients were centrifuged at 475 g and 20°C for 15 minutes. The leukocytes bands were removed from the interface between plasma and histopaque layers of each tube and collected into one 50 ml tube. The total volume was brought to 50 ml with cold Dubecco's Modified Eagle Medium (DMEM, Gibco™). The cell suspension was washed three times with DMEM and the total number of cells was determined. Cells were finally suspended in PBS and aliquoted into Eppendorf tubes at 10^7 cells/tube. After centrifugation cell pellets were dried and stored at −80°C until isolation of RNA.

Isolation of total RNA, mRNA and first-strand-cDNA synthesis

Isolation of total RNA from aliquoted leukocyte samples and the concomitant mRNA isolation was performed using RNAzol™ B (WAK-Chemie Medical GmbH, Germany) and Oligotex mRNA Mini Kit (Qiagen, Germany) respectively according to the protocols supplied by manufacturers. cDNA synthesis was performed using the "First-Strand cDNA Synthesis Kit" (Amersham Biosciences, UK). For each cDNA synthesis, 1 μg mRNA were reverse transcribed using oligo$(dT)_{18}$ primer in a final volume of 15 μl each, according to the protocol supplied by manufacturer.

Hybridisation to "Atlas Array"

Preparation of biotin-labelled cDNA probes

100 ng of cDNA was labelled using SpotLight™ Random Primer Labelling Kit (BD Biosciences Clontech, USA). Template cDNA together with 5 μl of 10× Random Primer Mix were heated to 97°C for 3 min in a final volume of 31 μl and chilled quickly on ice. After adding of reaction mix (5 μl 10× Klenow Reaction Buffer, 5 μl 10× Klenow Labelling Mix,

1 μl Klenow Enzyme and 8 μl ddH_2O) the labelling reaction was performed at 37°C during 30 min. Afterwards the reaction was stopped by adding 2 μl of 0.5 M EDTA (pH 8.0).

Purification of biotin-labelled probe

Non-incorporated biotin-labelled nucleotides and small (< 0.1 kb) cDNA fragments were removed using the NucleoSpin Extraction columns according to the protocol supplied by manufacturer (BD Biosciences Clontech, USA). A concentration of the newly synthesised biotin-labelled probes was determined by UV spectroscopy. The biotin-labelled probes were stored at −20°C until hybridisation to Atlas Array.

Hybridisation of biotin-labelled probes to Atlas Array

For the hybridisation Atlas Human 1.2 Array (Cat. #7850-1 BD Biosciences Clontech, USA) and SpotLight™ Chemiluminescent Hybridisation & Detection kit (BD Biosciences Clontech) were used. Each Atlas Array (membrane) was wetted by placing it in a dish of de-ionised H_2O, transferred to the hybridisation bottle and pre-hybridised in the hybridisation mix at 42°C for 3 hours before the hybridisation. Each hybridisation was performed overnight at 42°C with individual biotin-labelled cDNA probes. These probes were denatured in 100 mM NaOH at 68°C for 20 min and neutralised with 0.5 M NaH_2PO_4 (pH 7.0) at 68°C for 10 min before the hybridisation in Hybridiser (Techne, UK).

Stringency Washes

Hybridisation solution was discarded and the membranes were washed 4 times in 200 ml of Wash solution 1 (2× SSC and 1% SDS) for 30 min at 60°C with continuous agitation. Wash solution 1 was then replaced with Wash solution 2 (0.1× SSC and 0.5% SDS) and the membranes were washed two more times for 30 min at 48°C.

Probe detection and signal visualisation

The membranes were incubated first in 25 ml Blocking Buffer per Atlas Array at room temperature for 1 hour before the incubation with Streptavidin-HRP Conjugate (final dilution of 1:300) for 1 hour with constant gentle agitation. Afterwards the membranes were washed 4-times in 1× Wash Buffer (SpotLight™ Chemiluminescent Hybridisation & Detection kit, BD Biosciences Clontech) for 10 min with concomitant equilibration in Substrate Equilibration Buffer (kit) for 5 min at room temperature before the incubation in 8 ml Working Solution (the mix of Luminol/Enhancer Solution and Stable Peroxide Solution, supplied in the kit) per membrane for 5 min. After removing excess liquid the Atlas Arrays were processed for auto-radiography with exposure time of 1, 2, 5, 10 and 30 min. The exposed spots were further scanned and analysed with AtlasImage software (BD Biosciences Clontech, USA).

Results

Biochemical parameters in serum

All values of biochemical parameters measured which were relevant for this study are shown in Table 2. Taken all data together (independent from the presence or absence of DM) a positive correlation between TAS and creatinine ($p = 0.05$), AtxGap and iron ($p = 0.025$), and between AtxGap and antistreptolysin O ($p = 0.025$) was found. Negative correlation was observed between AtxGap and glucose content ($p = 0.025$) as well as between AtxGap and GMT ($p = 0.05$).

A relatively increased or decreased level of FR content in serum was further used as the criterion in order to make two groups from the pool of DM-patients. Correspondingly the cDNA samples from patients with either significantly lower or higher free radical content as well as samples from non-diabetic individuals were grouped and taken for their further hybridisation to the Atlas Array in groups A, B and C respectively (see Tables 3 and 4).

The level of TAS corresponded to the level of FR (lower in group A and higher in group B) and was lower in both DM-groups A and B compared to the non-diabetic group C. No significant differences were found between DM-groups A and B for the following routine biochemical

Table 2. Summarised basic statistical data (mean, median, standard deviation (SD), co-efficient of variation (CV) and 95% confidence interval (CI)) for biochemical parameters measured

Group	Age (Years)	Glucose (mmol/l)	TAS (mmol/l)	FR (% of normal)	Albumin (g/l)	Uric acid (μmol/l)	AtxGap (mmol/l)	GMT (μkat/l)	Creatinine (μmol/l)	Iron (μmol/l)	ASLO (IU/ml)
DM patients											
Mean	47.85	11.26	1.51	106.47	52.63	328.13	0.62	0.93	82.92	13.56	105.92
Median	47.00	10.67	1.49	100.71	52.70	314.50	0.63	0.51	81.00	13.40	91.00
SD	4.97	3.44	0.07	20.82	2.30	73.38	0.04	1.05	12.31	7.55	69.06
CV%	10.39	30.57	4.37	19.55	4.37	22.36	6.39	113.63	14.84	55.66	65.20
95% CI	1.99	1.38	0.03	8.33	0.92	29.36	0.02	0.42	4.92	3.02	27.63
Non-diabetic individuals											
Mean	30.09	5.36	1.56	115.40	53.26	313.84	0.68	0.40	75.36	19.17	182.82
Median	27.50	5.40	1.57	101.92	53.20	294.30	0.68	0.27	74.00	16.10	107.00
SD	9.30	0.55	0.09	27.45	2.58	105.19	0.05	0.37	10.39	9.60	149.29
CV%	30.90	10.17	5.92	23.79	4.83	33.52	6.90	91.72	13.78	50.06	81.66
95% CI	3.72	0.22	0.04	10.98	1.03	42.08	0.02	0.15	4.16	3.84	59.73

Table 3. Biochemical characteristics of the DM-groups selected

Group	Characteristics	n	M/F	Age (years)	Glucose (mmol/l)	TAS (mmol/l)	FR (% of normal)	Albumin (g/l)	Uric acid (μmol/l)	AtxGap (mmol/l)
A	Lower FR among the DM-patients	4	2/2	49.4 ± 6.7	11.41 ± 3.08	1.47 ± 0.01	90.69 ± 5.3	51.15 ± 2.8	320 ± 22	0.60 ± 0.05
B	Higher FR among the DM-patients	4	2/2	47.6 ± 3.9	10.87 ± 4.15	1.51 ± 0.09	128.9 ± 11.6	51.78 ± 1.0	307 ± 101	0.65 ± 0.01
C	Non-diabetic individuals	4	2/2	32.0 ± 7.5	4.95 ± 0.63	1.56 ± 0.06	115.3 ± 31.9	52.70 ± 1.4	317 ± 57	0.68 ± 0.01

Table 4. Statistical significance of the differences in values measured: ''NS'' means a non-significant difference, ''ND'' means non-diabetic individulas

Group	Characteristics	Glucose (mmol/l)	TAS (mmol/l)	FR (% of normal)	Albumin (g/l)	Uric acid (μmol/l)	AtxGap (mmol/l)
A	Low FR, DM	11.41	1.47	90.69	51.15	320	0.60
B	High FR, DM	10.87	1.51	128.90	51.78	307	0.65
Significance of differences		NS	NS	$p = 0.05$	NS	NS	NS
A	Low FR, DM	11.41	1.47	90.69	51.15	320	0.60
C	ND	4.95	1.56	115.30	52.70	317	0.68
Significance of differences		$p = 0.05$	$p = 0.05$	NS	NS	NS	$p = 0.05$
B	High FR, DM	10.87	1.51	128.90	51.78	307	0.65
C	ND	4.95	1.56	115.30	52.70	317	0.68
Significance of differences		$p = 0.05$	NS	NS	NS	NS	$p = 0.05$

parameters measured: albumin, uric acid, urea, creatinine, ALT, ALP, CHE, GMT, CK, LD, HBD, α-AMY, Na, K, Cl, Ca, Mg, P, iron, total protein, transferrin, UIBC, Apo A1, Apo B, total cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerols, rheumatoid factor, ASLO and C-reactive protein. In addition, a significant difference ($p = 0.05$) was found between the values of AST in groups A and B ($0.31 \pm 0.06\,\mu$kat/l and $0.40 \pm 0.04\,\mu$kat/l respectively) being, however, within the reference range for healthy population. Significant difference ($p = 0.05$) was also found between total billirubin values in group A ($8.88 \pm 1.85\,\mu$mol/l) and the control group ($14.78 \pm 4.23\,\mu$mol/l) being for both groups within the reference range for healthy population.

Body mass index

Values of body mass index were calculated in the selected DM-groups A and B and in non-diabetic individuals (group C). The results showed slightly increased obesity in group A compared to group B. Compared to the non-diabetic group C, the obesity in group A was significantly increased (see Table 5).

Table 5. The degree of an obesity of the DM-patients in groups A and B, and non-diabetic individuals (group C). The reference values of body mass indexes (BMI) for non-obese population is 15 to 24.9. ''NS'' means a non-significant difference, ''ND'' means non-diabetic individulas

Group	Characteristics	BMI
A	Low FR, DM	32.6 ± 3.9
B	High FR, DM	29.3 ± 1.7
Significance of differences		NS
A	Low FR, DM	32.6 ± 3.9
C	ND	25.4 ± 4.2
Significance of differences		$p = 0.05$
B	High FR, DM	29.3 ± 1.7
C	ND	25.4 ± 4.2
Significance of differences		NS

Expression profiling of the stress responsive genes

Among the stress responsive genes the following genes were equally expressed in both DM-groups A and B:

- NF-kappaB (GenBank accession number: X61498)
- XRCC1 (GenBank accession number: M36089)

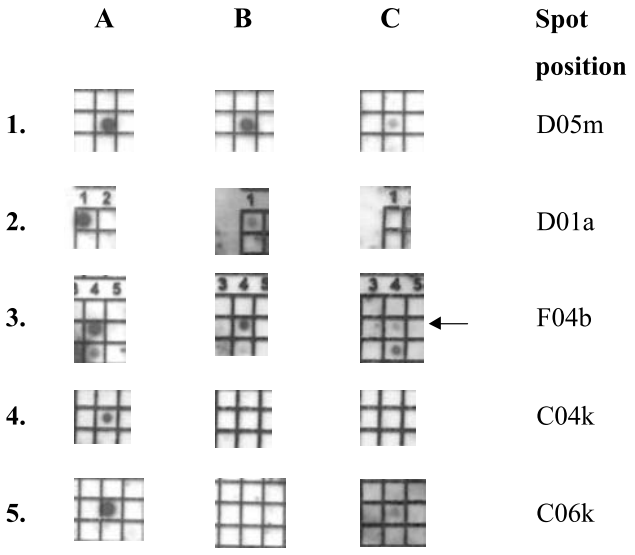

Fig. 1. Expression profiling of stress responsible genes: *1* – NF-kappaB, *2* – XRCC1, *3* – HSP90, *4* – XIAP, *5* – cytochrome P450 reductase. **A**, **B** are the groups of DM-patients with lower and higher free radical levels respectively, and **C** is the group of non-diabetic individuals

- 90-kDa heat-shock protein A (GenBank accession number: X07270) (see Fig. 1.1–1.3 respectively). Compared to the non-diabetic group C the expression of the above mentioned genes was considerably enhanced in both DM-groups.

The expression of the following two genes was up-regulated only in the DM-group A:

- XIAP (GenBank accession number: U45880)
- cytochrome P450 reductase (GenBank accession number: S90469) (see Fig. 1.4 and 1.5 respectively).

Discussion

DM-patients suffer from a variety of secondary complications supposed to be a result of vascular disease. Diabetic vascular dysfunction has an underlying inflammatory component, manifesting leukocyte recruitment and induction of their adhesive functions (Joussen et al., 2002). Increased oxidative stress is well documented in DM. Induced expression of some stress responsible genes was further observed in leukocytes of DM-patients (Hofmann et al., 1999; Collins and Cybulsky, 2001). The purpose of this investigation was to look for a possible correlation between enhanced free radical generation monitored in serum and differential gene expression in circulating leukocytes of DM-patients.

We found significant differences in the generation of free radicals among DM-patients. Thereby the intracellular reduction-oxidation state becomes altered that consequently leads to an activation of red-ox sensitive signalling pathways including transcription regulators (Collins and Cybulsky, 2001; Joussen et al., 2002). These molecular events potentially result in the red-ox status-dependent differential gene expression in circulating leukocytes. Indeed our results show the differential gene expression not only in the DM-group compared to the non-diabetic one, but also a significant difference in gene expression between two DM-groups selected for higher and lower free radical and antioxidant contents in serum. Following stress related genes were up-regulated on the transcription level in DM-patients: NF-kappaB, XRCC1, HSP90, XIAP and cytochrome P450 reductase.

NF-kappaB

Transcription rates of this gene were equally enhanced in both DM-groups compared to the non-diabetic. This is in agreement with findings of other authors demonstrated enhanced binding activity of NF-kappaB protein product in blood mononuclear cells of DM-patients (Hofmann et al., 1999; Aljada et al., 2001). The mechanism of NF-kappaB activation under stress conditions is very complex and not completely understood yet. Since the transcription of this gene is equally activated in both DM-groups, it is unlikely that NF-kappaB is directly activated by oxidation. Nevertheless, the enhanced generation of reactive oxygen species leads on the protein level to IkB degradation, permitting NF-kappaB to enter the nucleus and activate gene transcription (Ghosh et al., 1998; Baldwin, 1996). The NF-kappaB system obviously co-ordinates the activation of multiple inflammatory genes and anti-apoptotic factors. NF-kappaB activation suppresses caspases by induction of the inhibitors of apoptosis (IAP) such as XIAP. NF-kappaB is also known to suppress the release of mitochondrial cytochromes (Collins and Cybulsky, 2001).

XIAP

Expression of XIAP was increased only in the DM-group with the lower levels of free radicals and antioxidants. The role of XIAP in DM has not been elucidated yet. Increased levels of XIAP mRNA, however, were found in blastocytes under hyperglycemic conditions (Jimenez et al., 2003). Moreover, female blastocytes were shown to produce significantly higher levels of XIAP transcripts than males and this could be crucial in explaining the higher survival rates of female blastocytes observed *in vitro* under hyperglycemic conditions. XIAP is further

supposed to have an emerging role in the onset of atherosclerosis (Collins and Cybulsky, 2001).

Cytochrome P450 reductase

Induction of the cytochrome P450 system was observed and supposed to be responsible for the substrate-specific alterations in drug metabolism in DM (Past and Cook, 1982). The cytochrome P450 system is insulin sensitive (Shimeno et al., 1991). The up-regulation of the cytochrome P450 reductase expression was recently studied under the treatment of obesity using liver X receptor alpha (LXR) agonists and shown to be involved in cholesterol and lipid metabolism (Stulnig et al., 2002). This treatment is, therefore, proposed for possible beneficial effects of LXR agonists in DM.

Heat-shock-protein 90 (HSP90)

This 90-kDa protein is known to be induced in activated leukocytes when an expression of a variety of cytokines, surface molecules and nuclear proteins is up-regulated (Kaneko et al., 2002). Our results showed the enhanced expression of HSP90 in both DM-groups confirming the general activation of circulating leukocytes in DM and its association with vascular inflammation. Recent studies revealed that hyperglycemia induces loss of NO via calpain-dependent decrease in the association of HSP90 with endothelial nitric oxide synthase (Stalker et al., 2003). Inhibition of calpain activity decreases endothelial cell surface expression of the pro-inflammatory adhesion molecules ICAM-1 and VCAM-1 during hyperglycemia. HSP90 plays further a crucial role in necrotic signalling. An inhibition of HSP90 expression may alter the composition of the TNFR1 complex, favouring the caspase 8 dependent apoptotic pathway (Vanden Berghe et al., 2003). These facts open new perspectives for beneficial effects under the treatments down-regulating HSP90 expression in DM.

XRCC1

The expression of this gene was up-regulated in both DM-groups compared to the non-diabetic one. The gene product is involved in protein complexes which repair both single- and double-stranded DNA breaks and known with its recombination activity in DNA-repair mechanisms (Golubnitchaya-Labudova et al., 1997). XRCC1 expression on the level of transcription was described by other authors (Wei et al., 1995). DNA damage has been well characterised in DM (Frustaci et al., 2000). The equal activation of XRCC1 gene in both DM-groups shown in this study indicates a constant damage to DNA primarily to the disease and independently from additional alterations in the levels of free radicals and antioxidants.

On the basis of our results we conclude that similar activation of NF-kappaB, XRCC1 and HSP90 in both DM-groups indicates the involvement of these genes in general pathomechanisms conditioned by hyperglycemia which are relatively resistant to additional alterations in levels of free radicals and antioxidants. In contrast, the differential expression of XIAP and cytochrome P450 reductase among DM-patients indicates an activation of additional mechanisms which are relatively sensitive to the factors regulating the cellular red-ox status and may, therefore, lead to individual expressions and intensity of DM-complications. These red-ox sensitive mechanisms should be further studied in view of relevant factors which influence the cellular red-ox status such as nutrition, smoking, genetic pre-disposition, etc. Noteworthy, the reduction of free radical and concomitantly antioxidant levels we observed in the DM-group with significantly increased body mass index. This finding should be taken into consideration for the further monitoring of obesity as an additional risk factor in DM.

Taken together this is a potentially attractive approach for the routine prediction of DM-complications, since all markers can be tested using non-invasive *ex vivo* monitoring of the specific gene expression in leukocytes.

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Authors' address: Olga Golubnitschaja, PhD, Assoc. Prof., Department of Radiology, Division of Molecular/Experimental Radiology, University of Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany,
E-mail: Olga.Golubnitschaja@ukb.uni-bonn.de