Diabetologia (2015) 58:1621–1625
DOI 10.1007/s00125-015-3609-5

SHORT COMMUNICATION

Overexpression of cutaneous mitochondrial superoxide dismutase in recent-onset type 2 diabetes

Dan Ziegler[1,2,3] · Alexander Strom[1] · Jutta Brüggemann[1] · Iris Ziegler[1] · Bernd Ringel[1] · Sonja Püttgen[1] · Michael Roden[1,2,3] · for the GDS Group

Received: 4 February 2015 / Accepted: 13 April 2015 / Published online: 2 May 2015

Abstract
Aims/hypothesis Oxidative stress and microvascular damage have been implicated in the pathogenesis of diabetic neuropathy, with manganese superoxide dismutase 2 (SOD2) responsible for superoxide detoxification in mitochondria. We hypothesised that patients with recently diagnosed type 2 diabetes would show an altered cutaneous expression of SOD2 and endothelial cell area.
Methods In this cross-sectional study, we assessed skin biopsies using immunohistochemistry, peripheral nerve function and heart rate variability in 69 participants of the German Diabetes Study with recently diagnosed type 2 diabetes and 51 control individuals.
Results Subepidermal SOD2 area in the distal leg was increased by ~60% in the diabetic group vs the controls (0.24 ±0.02% vs 0.15±0.02%; $p = 0.0005$) and was correlated with an increasing duration of diabetes ($r = 0.271$; $p = 0.024$) and with the low frequency/high frequency ratio ($\beta = 0.381$; $p = 0.002$) as an indicator of sympathovagal balance. The area of the subepidermal endothelial cells (measured by CD31 staining) did not differ between the groups.
Conclusions/interpretation Cutaneous antioxidative defence is enhanced in relation to the duration of diabetes and is linked to a cardiac sympathovagal imbalance towards a sympathetic predominance in individuals with recently diagnosed type 2 diabetes without evidence of endothelial cell damage. Whether cutaneous SOD2 levels can predict the development of diabetic neuropathy remains to be determined in prospective studies.

Keywords Heart rate variability · Oxidative stress · Peripheral nerve function · Superoxide dismutase · Sympathovagal balance · Type 2 diabetes

Abbreviations

GDS	German Diabetes Study
HF	High frequency
HRV	Heart rate variability
IENFD	Intraepidermal nerve fibre density
LF	Low frequency
MNCV	Motor nerve conduction velocity
NDS	Neuropathy Disability Score
NSS	Neuropathy Symptom Score
RMSSD	Root mean square of successive differences
ROS	Reactive oxygen species
SDNN	Standard deviation of R-R intervals
SNCV	Sensory nerve conduction velocity
SOD2	Superoxide dismutase 2
TDT	Thermal detection threshold
VPT	Vibration perception threshold

The GDS Group consists of M. Roden (speaker), H. Al-Hasani, A. E. Buyken, J. Eckel, G. Geerling, C. Herder, A. Icks, J. Kotzka, O. Kuß, E. Lammert, J. Lundbom, K. Müssig, P. Nowotny, W. Rathmann, J. Szendroedi, D. Ziegler and their co-workers who are responsible for the design and conduct of the GDS.

✉ Dan Ziegler
dan.ziegler@ddz.uni-duesseldorf.de

[1] Institute for Clinical Diabetology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich Heine University, Auf'm Hennekamp 65, 40225 Düsseldorf, Germany

[2] Department of Endocrinology and Diabetology, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany

[3] German Center for Diabetes Research (DZD e.V.), Partner Düsseldorf, Düsseldorf, Germany

Introduction

A single hyperglycaemia-induced process of overproduction of superoxide by the mitochondrial electron transport chain has been implicated in the pathogenesis of diabetic microvascular complications including experimental diabetic neuropathy [1]. Manganese superoxide dismutase (SOD2) is responsible for superoxide detoxification in mitochondria [2]. Its expression in skin has not been previously studied in diabetic patients. Furthermore, it is not known whether microvascular pathology is already present shortly after the onset of diabetes.

We sought to determine whether the expression of cutaneous mitochondrial SOD2 and endothelial cell area would be altered in volunteers with recently diagnosed type 2 diabetes compared with control participants.

Methods

Volunteers This cross-sectional study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of Heinrich Heine University, Düsseldorf. All participants provided written informed consent. Individuals with recent-onset type 2 diabetes (n=69) and healthy controls (n=51) matched for age, sex and height were studied. The participants with diabetes were recruited consecutively from the prospective German Diabetes Study (GDS) evaluating the long-term course of diabetes and its sequelae (ClinicalTrials.gov Identifier NCT01055093) [3]. The exclusion criteria were secondary diabetes, pregnancy, severe disease (e.g. cancer), psychiatric disorders, immunosuppressive therapy, a limited ability to cooperate and neuropathy from causes other than diabetes.

Peripheral nerve function Motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV), malleolar vibration perception threshold (VPT) and thermal detection thresholds (TDTs) on the dorsum of the foot were measured as previously described [3]. Neurological examination was performed using the Neuropathy Disability Score (NDS) and Neuropathy Symptom Score (NSS). Polyneuropathy was defined and staged according to the Toronto Consensus [4].

Heart rate variability Heart rate variability (HRV) was measured during normal breathing in the resting supine position over 5 min using a VariaCardio TF5 System (AMD, Buckinghamshire, UK) as previously described [3]. Time domain indices included the root mean square of successive differences (RMSSD) and the SD of R-R intervals (SDNN). Frequency domain indices included the low-frequency (LF) band (0.04–0.15 Hz), the high-frequency (HF) band (0.15–0.4 Hz) and the LF/HF ratio.

Skin biopsy and tissue fixation Skin biopsies were processed as previously reported [3]. In brief, 3 mm punch biopsies from the left lateral calf were fixed with 2% periodate-lysine-paraformaldehyde. After cryoprotection, the tissues were stored at −80°C.

Immunohistochemistry Intraepidermal nerve fibre density (IENFD) was measured following the free-floating method as previously described [3]. Endothelial cells were stained using a mouse anti-CD31 (clone 9G11) monoclonal antibody (R&D Systems, Abingdon, UK) followed by a biotinylated anti-mouse IgG antibody (Vector Labs, Burlingame, CA, USA). Antibody binding was visualised by incubation with Vector ABC and Vector SG substrate (Vector Labs). SOD2 was detected by immunofluorescence using a sheep anti-SOD (Mn enzyme) antibody (Merck Millipore, Schwalbach, Germany), followed by an Alexa488 conjugated donkey anti-sheep antibody (Life Technologies, Darmstadt, Germany) and Hoechst 33342 (Sigma-Aldrich, Munich, Germany).

Morphometric analysis Individual intraepidermal nerve fibres from four cross-sections were visually counted along the length of the epidermis using a Leica DMRBE inverted microscope (Leica, Wetzlar, Germany) equipped with an Olympus DP73 digital colour camera and cellSens imaging software v1.7 (Olympus, Hamburg, Germany). The areas of subepidermal CD31 (at 300 μm depth) and SOD2 (over the complete dermal area) were quantified using two 10 μm sections per participant. The light (CD31) and fluorescence (SOD2) images were acquired using a Leica DMRBE inverted microscope equipped with an Olympus DP73 digital colour camera (×10 and×20 magnification). CellSens imaging software was used to calculate the percentage marker area.

Statistical analysis Continuous data were expressed as mean ±SEM. Categorical data were given as percentages with 95% CIs. The non-parametric Mann–Whitney U test and Spearman rank correlation were applied to determine the differences between groups or the correlations between variables. Multiple linear regression analyses were performed using adjustments for sex, age and BMI. The level of significance was set at α=0.05.

Results

In the group with diabetes, HbA_{1c} was 6.5±0.1% (47.7± 1.2 mmol/mol) and the known duration of diabetes before skin biopsy was 11.5±1.0 months. The percentages of patients receiving treatment for diabetes and comorbidities were: diet only, 23.2%; insulin, 5.8%; metformin, 65.2%; sulfonylurea, 11.6%; dipeptidyl peptidase-4 (DPP4) inhibitors, 8.7%; statins, 14.5%; antihypertensive drugs, 53.6% (diuretics,

17.4%; ACE inhibitors, 23.2%; angiotensin II receptor blockers (ARBs), 21.7%; beta-blockers, 24.6%; and calcium channel antagonists, 13.0%); and analgesics, 4.3%. The percentages of patients with subclinical, confirmed asymptomatic and confirmed symptomatic polyneuropathy were 23.2%, 5.8% and 7.2%, respectively. Microalbuminuria was observed in 14.5% of patients, and none of the patients demonstrated any retinopathy.

The demographic, clinical, functional and morphological data for the two groups studied are shown in Table 1. Compared with the control participants, the individuals with diabetes had lower peroneal MNCV, cold TDT, RMSSD and IENFD values but higher values for BMI, malleolar VPT, NSS and SOD2 area (all $p<0.05$). After adjustment for age, sex and BMI, the differences between the groups for IENFD ($\beta=-0.270$; $p=0.011$) and SOD2 area ($\beta=0.218$; $p=0.046$) remained significant. No significant differences between the groups were noted for the remaining variables.

Immunofluorescence staining images representative of normal and increased SOD2 expression are shown in Fig. 1a, b. In the diabetes group, the SOD2 area correlated positively with the duration of diabetes ($r=0.271$; $p=0.024$), while the CD31 area showed an inverse association ($r=-0.317$; $p=0.025$). After adjustment for sex, age, BMI and smoking status, the association between SOD2 and duration of diabetes remained significant ($\beta=0.281$; $p=0.023$), whereas the correlation between CD31 area and duration of diabetes lost its statistical significance ($\beta=-0.257$; $p=0.086$). Furthermore, the area of SOD2 correlated positively with the LF/HF ratio ($r=0.283$; $p=0.021$) and inversely with the RMSSD ($r=-0.262$; $p=0.035$) as an index of parasympathetic activity (Fig. 1c, d). After adjustment for sex, age, BMI and smoking, these associations remained significant for both LF/HF ratio ($\beta=0.381$; $p=0.002$) and RMSSD ($\beta=-0.324$; $p=0.012$). No other significant associations were noted.

Table 1 Anthropometric, demographic, clinical, functional and morphological data for the volunteers studied

Variable	Control ($n=51$)	Diabetes ($n=69$)	p value
Age (years)	55.1±1.2	54.3±0.9	NS
Sex (% male)	54.9 (46.3, 70.5)	68.1 (57.7, 77.3)	NS
BMI (kg/m^2)	25.4±0.5	32.3±0.7	<0.0001
Height (cm)	174±1.5	173±1.1	NS
Heart rate (bpm)	66.4±1.5	70.7±1.1	0.028
Systolic BP (mmHg)	129±2.8	128±1.76	NS
Diastolic BP (mmHg)	72.9±1.4	73.0±1.2	NS
Smokers (%)	23.5 (14.2, 35.3)	34.8 (25.3, 45.3)	NS
Peroneal MNCV (m/s)[a]	47.1±0.5	43.7±0.7	0.002
Sural SNCV (m/s)[a]	46.3±0.7	44.3±0.8	NS
Malleolar VPT (μm)[a]	1.07±0.13	2.57±0.43	0.024
Warm TDT (°C)[a]	39.7±0.5	40.9±0.5	NS
Cold TDT (°C)[a]	28.9±0.3	26.6±0.6	0.021
NSS[a]	0	0.68±0.24	0.008
NDS[a]	0.81±0.20	1.39±0.21	NS
RMSSD (ms)[a]	1,110±202	548±63	0.011
SDNN (ms)[a]	59.0±2.9	51.5±2.5	NS
LF (ms^2)[a]	413±56	320±38	NS
HF (ms^2)[a]	332±63	254±33	NS
LF/HF ratio[a]	2.06±0.24	2.12±0.26	NS
IENFD (fibres/mm)	9.10±0.41	6.90±0.30	<0.0001
SOD2 area (%)	0.15±0.02	0.24±0.02	0.0005
CD31 area (%)	2.05±0.16	2.05±0.15	NS

Data represent mean±SEM, except for sex and smoking status (percentages and 95% CI)

[a] Group comparisons were adjusted for age, sex, BMI and smoking status

Discussion

This study demonstrates that at approximately 1 year after the diagnosis of type 2 diabetes, subepidermal SOD2 expression in the lower limbs was augmented by ~60% and correlated with increasing duration of diabetes, cardiac sympathetic predominance and diminished vagal activity, while the subepidermal endothelial cell area was not altered. The overexpression of SOD2 points to an early enhanced, presumably compensatory, cutaneous antioxidative defence in type 2 diabetes.

The results of this study are in line with experimental data suggesting that an overexpression of SOD2 in primary dorsal root ganglion neurons protects against the formation of mitochondrial reactive oxygen species (ROS), while a reduced expression of SOD2 in mouse models of diabetes can promote the development of diabetic neuropathy [5], and treatments aimed at reducing oxidative stress in the nervous system may ameliorate neuropathic symptoms and impairments [6].

Other novel findings are the correlation between an increasing area of cutaneous SOD2 and a rising LF/HF ratio, indicating enhanced sympathetic activity, and the association between an increasing area of SOD2 and diminishing RMSSD, indicating reduced parasympathetic activity. A spectral analysis of HRV indicates that efferent vagal activity is a major contributor to the HF component, while the LF component has been considered to be a marker of sympathetic modulation or as being under both sympathetic and vagal influence [7]. Emerging evidence suggests that ROS including nitric oxide, superoxide and peroxynitrite may contribute to a shift of cardiac autonomic modulation toward sympathetic predominance in the brainstem, peripheral neurons and cardiomyocytes [7]. Sympathetic overdrive and diminished vagal activity are a consequence of many primary cardiovascular diseases and can trigger arrhythmias [7]. Administration of the antioxidant vitamin E over 4 months to individuals with type 2 diabetes lowered their LF/HF ratio [8], suggesting that

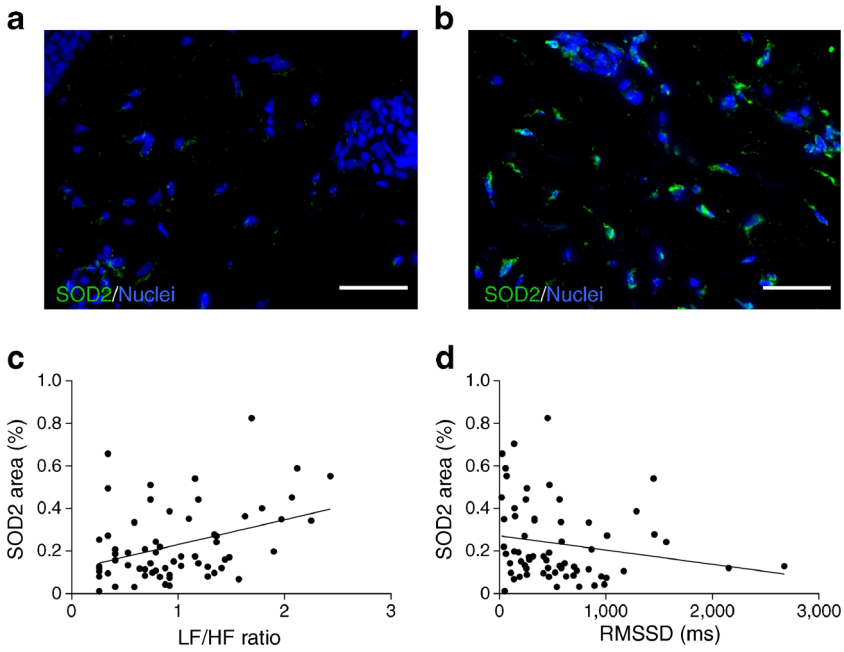

Fig. 1 Immunofluorescence staining images representative of normal SOD2 expression in a healthy volunteer (**a**) and increased SOD2 expression in an individual with recently diagnosed type 2 diabetes (**b**); scale bars, 50 μm. Correlation between subepidermal SOD2 area and LF/HF ratio (r=0.283; p=0.021) (**c**) and RMSSD (r=−0.262; p=0.035) (**d**)

antioxidant treatment may improve the sympathovagal balance by reducing oxidative stress.

In addition to diabetes, another relevant experimental setting is neurogenic hypertension, the pathogenesis of which involves central sympathetic overactivation and oxidative stress in the rostral ventrolateral medulla, a vasomotor centre in the brainstem [9]. An overexpression of SOD2 in the rostral ventrolateral medulla reduces sympathetic overactivity and hypertension in spontaneously hypertensive rats [9], suggesting a protective effect against oxidative stress. In fact, 54% of the patients in the current study were undergoing treatment for hypertension. Treated hypertension, blood pressure and heart rate were not, however, related to the LF/HF ratio. As we did not directly measure oxidative stress, we can only speculate that the increased SOD2 expression in relation to the augmented LF/HF ratio serves as a surrogate for enhanced local oxidative stress.

We found no evidence of dermal endothelial cell damage using CD31 staining. This differs from the study by Quattrini et al [10] showing that the density of dermal microvessels (number/mm^2) was increased in patients with diabetes who had moderate polyneuropathy, but reduced in those without polyneuropathy or with only mild polyneuropathy. There may be two reasons for this disparity. First, we did not quantify microvessel density but endothelial cell area, as the latter provides an estimate of the total microvascular supply. Second, we studied a population with recent-onset diabetes, while the duration of diabetes in the groups studied by Quattrini et al [10] ranged between 15 and 28 years. It is thus possible that alterations in the dermal microvasculature develop only at a later stage of type 2 diabetes.

The limitations of the current study include its cross-sectional rather than prospective design and its assessment of antioxidative defence rather than oxidative stress. The strengths of the present work are the relatively homogeneous diabetic group and the precise quantification of dermal changes at the local site that are relevant to the development of neuropathy.

In conclusion, we demonstrated that cutaneous antioxidative defence is enhanced and linked to sympathovagal imbalance towards a sympathetic predominance in individuals with recently diagnosed type 2 diabetes. Long-term prospective studies are needed to establish whether dermal SOD2 is an early marker for predicting the development of diabetic neuropathies.

Acknowledgements The authors wish to thank the staff of the GDS at the German Diabetes Center, Düsseldorf, Germany, especially M. Behler and M. Schroers-Teuber, for their excellent technical support.

Funding The GDS was initiated and financed by the German Diabetes Center, which is funded by the German Federal Ministry of Health (Berlin, Germany), the Ministry of Innovation, Science, Research and Technology of the state North Rhine-Westphalia (Düsseldorf, Germany) and grants from the German Federal Ministry of Education and Research (BMBF) to the German Center for Diabetes Research (DZD e.V.). This work was partially funded by an EFSD award supported by Novartis.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement DZ designed the study and wrote the manuscript. DZ, AS, JB, IZ, BR, SP and MR researched the data and revised the manuscript. All authors contributed substantially to the following: the conception or design of the work or the acquisition, analysis or

interpretation of the data; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. DZ is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis.

References

1. Nishikawa T, Edelstein D, Du XL et al (2000) Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404:787–790
2. Zelko IN, Mariani TJ, Folz RJ (2002) Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med 33:337–349
3. Ziegler D, Papanas N, Zhivov A et al (2014) Early detection of nerve fiber loss by corneal confocal microscopy and skin biopsy in recently diagnosed type 2 diabetes. Diabetes 63:2454–2463
4. Dyck PJ, Albers JW, Andersen H et al (2011) Diabetic polyneuropathies: update on research definition, diagnostic criteria and estimation of severity. Diabetes Metab Res Rev 27:620–628
5. Vincent AM, Russell JW, Sullivan KA et al (2007) SOD2 protects neurons from injury in cell culture and animal models of diabetic neuropathy. Exp Neurol 208:216–227
6. Papanas N, Ziegler D (2014) Efficacy of α-lipoic acid in diabetic neuropathy. Expert Opin Pharmacother 15:2721–2731
7. Danson EJ, Paterson DJ (2006) Reactive oxygen species and autonomic regulation of cardiac excitability. J Cardiovasc Electrophysiol 17(Suppl 1):S104–S112
8. Manzella D, Barbieri M, Ragno E, Paolisso G (2001) Chronic administration of pharmacologic doses of vitamin E improves the cardiac autonomic nervous system in patients with type 2 diabetes. Am J Clin Nutr 73:1052–1057
9. Chan SH, Chan JY (2012) Brain stem oxidative stress and its associated signaling in the regulation of sympathetic vasomotor tone. J Appl Physiol 113:1921–1928
10. Quattrini C, Jeziorska M, Boulton AJ, Malik RA (2008) Reduced vascular endothelial growth factor expression and intra-epidermal nerve fiber loss in human diabetic neuropathy. Diabetes Care 31: 140–145