Treatment with Actovegin® Improves Sensory Nerve Function and Pathology in Streptozotocin-Diabetic Rats via Mechanisms Involving Inhibition of PARP Activation

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Key words
- Actovegin®
- streptozotocin
- diabetes
- neuropathy
- neuroprotection
- PARP

Abstract

Background: Diabetic neuropathy is one of the most severe complications of diabetes, affecting approximately one-third of diabetic patients. We investigated the potential neuroprotective effect of Actovegin®, a deproteinized hemoderivative of calf blood, in an animal model of diabetic neuropathy.

Methods: A single intravenous injection of streptozotocin (STZ, 55 mg/kg) was used to induce experimental diabetes in male Sprague-Dawley rats. Actovegin® (200 or 600 mg/kg) was administered intraperitoneally from day 11 to day 40 post-STZ exposure. N-acetylcysteine (NAC) was used as a positive control and was added to drinking water (0.2 g/l) from day 2 until day 40. Measurements to assess efficacy included sensory nerve conduction velocity (SNCV), intraepidermal nerve fiber density (IENFD), and poly(ADP-ribose) content.

Results: A decrease (35%) in sensory nerve conduction velocity (SNCV) was seen in STZ-induced diabetic rats from day 10 post-STZ administration and persisted at days 25 and 39. At study completion (day 41), a decrease (32%) in intraepidermal nerve fiber density (IENFD) was found in hind-paw skin biopsies from STZ-rats. Reduced SNCV and IENFD were significantly ameliorated by both doses of Actovegin®. Moreover, 600 mg/kg Actovegin® markedly decreased poly(ADP-ribose) polymerase (PARP) activity in sciatic nerves from STZ-diabetic rats as assessed by poly(ADP-ribose) content.

Conclusion: Actovegin® improved several parameters of experimental diabetic neuropathy via mechanisms involving suppression of PARP activation, providing a rationale for treatment of this disease in humans.

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Introduction

Chronic diabetic sensorimotor distal symmetric polyneuropathy (DPN) as the most common form of diabetic neuropathy is associated with considerable morbidity, increased mortality and reduced quality of life (Boulton et al., 2005; Tesfaye et al., 2010). There is evidence that the complex interactions between metabolic and vascular factors, in particular nerve ischemia and hypoxia, are major players in the pathogenesis of DPN (Cameron et al., 2001). Several therapeutic approaches have been developed based on these mechanisms. They target, however, symptomatic treatment of neuropathic pain (Chong and Hester, 2007) rather than slowing down the disease progression. Moreover, they are associated with central nervous system side effects.

In a randomized, double-blind, placebo-controlled clinical trial, Actovegin® was shown to have beneficial effects on the clinical symptoms of neuropathy in patients with type 2 diabetes (Ziegler et al., 2009), indicating that Actovegin® may promote neuroprotection. Actovegin® is a protein-free, metabolically active hemoderivative extracted from calf blood by ultrafiltration. It contains amino acids, oligopeptides, nucleic acid derivatives, vitamins, acylcarnitines and intermediate products of carbohydrate and fatty acid metabolism. Actovegin® is a drug with favourable effects and is being used since decades for the treatment of peripheral circulatory disorders and trophic disturbances in the brain (i.e., ischemic insult and cranio-cerebral injury) and others (Buchmayer et al., 2011). On the cellular level Actovegin® is known to stimulate oxygen uptake and consumption, and to promote metabolism, shifting the redox balance of the cells towards oxidized substrates and increasing the availability of energy-rich phosphates, such as ATP and creatine phosphate (Kuninaka et al., 1991).

So far, the actions of Actovegin® have not been studied in animal models of DPN. Thus, we investigated whether Actovegin® has protective effects on neuronal parameters in the STZ-rat, a widely used animal model of diabetic neuropathy. Thereby, we attempted to get deeper insight into the potential molecular mechanisms underlying the neuroprotective effects of Actovegin®.

Materials and Methods

Drug preparation

Actovegin® (Nycomed, Linz, Austria) was supplied as a stock solution of 200 mg/ml. It was used at working concentrations of 67 and 200 mg/ml, prepared in saline solution (0.9 % NaCl); 3 ml/kg of either working concentration were administered intraperitoneally (i. p.), resulting in doses of 200 and 600 mg/kg, respectively. N-acetylcysteine (NAC; Sigma, L'Isle d'Abeau Chesnes, France) was added to drinking water at a final concentration of 0.2 g/l; the NAC solution was prepared freshly each day. STZ (Sigma, L'Isle d'Abeau Chesnes, France) was prepared in 0.1 mol/l citrate buffer pH 4.5 at a concentration of 55 mg/ml.

Experimental design

Fifty 6-week-old male Sprague-Dawley rats (Janvier, Le Genest St Isle, France) were used for the study. All animal handling was performed in accordance with French law (Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, Accreditation No. E-67-218-23, Permission no. 67-279, 67-259 and B67-173). Rats were maintained under standard conditions as described (Andriambeloson et al., 2006). Diabetes was induced by intravenous injection of STZ solution into the tail vein of the rats at a dose of 55 mg/kg and monitored as described (Andriambeloson et al., 2006; Bordet et al., 2008). Diabetic STZ-rats were randomly distributed in 4 different treatment groups (10 rats per group) and treated with one of the following: saline solution, Actovegin® 200 mg/kg, Actovegin® 600 mg/kg or NAC. Actovegin® or vehicle were administered i. p. on a daily basis from day 11 (following confirmation of neuropathic symptoms manifestation as assessed by SNCV measurement) until day 40. NAC was added to drinking water from day 2 until day 40 as described previously (Sagara et al., 1996). Body weight was recorded every day and glycemia was monitored at days 2, 7, 26, 35 and 40 after STZ administration.

Sensory nerve conduction velocity (SNCV) analysis

Electrophysiological recordings were performed on day-3 (baseline) and on days 10, 25 and 39 post-STZ administration, using the method previously described (Andriambeloson et al., 2006; Bordet et al., 2008). Velocity was expressed as m/s.

IENFD analysis

On day 41, rats were anesthetized by i. p. injection of 100 mg/kg ketamine, and a 5–10 mm diameter area of skin was taken from the hind paw. Skin samples were immediately fixed in 4 % paraformaldehyde at 4 °C, incubated overnight in 30 % sucrose (in 0.1 M phosphate-buffered saline [PBS]) for cryoprotection, embedded in Tissue-Tek® OCT™ Compound and frozen at − 80 °C. Cryosections (50 µm) were then cut vertically to the skin surface. Free-floating sections were incubated for 7 days in a bath of Rabbit Anti-Human Protein Gene Product 9.5 (1:10 000; Ultraclone, Isle of Man, UK) at 4 °C. Sections were then processed to reveal immunoreactivity using the avidin-biotin complex peroxidase method. Briefly, sections were incubated for 1 h with a biotinylated goat anti-rabbit antibody (sc-2018, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then for 30 min in the avidin/biotinylated-HRP complex at room temperature. Peroxidase activity was visualized using the diaminobenzidine system. Sections were then counterstained with eosin or hematoxylin, dehydrated, cleaned in Bioclear tissue clearing agent (EMS, Hatfield, PA, USA) and mounted with Eukitt® mounting medium (EMS, Hatfield, PA, USA). IENFD was evaluated throughout the whole length of the skin sample in a blinded fashion, and was expressed as number of IENF/mm^2 skin.

Poly(ADP-ribose) immunoreactivity analysis in sciatic nerves

On day 41 right sciatic nerves were collected from 3 rats per group, fixed in 4 % paraformaldehyde and subsequently prepared for cryosectioning (30 % sucrose/PBS at 4 °C for 24 h, Tissue-Tek® OCT™ Compound at room temperature for 24 h). Longitudinal sections (10 µm) were cut with a Leica CM3050 S cryostat and mounted on Superfrost Ultra Plus® glass slides (Menzel, Braunschweig, Germany). Sections were washed with 0.1 % Tween-20/PBS and subjected to trypsin antigen retrieval (0.05 % trypsin in 7 mM $CaCl_2$ for 20 min at 37 °C). Slides were blocked with 5 % normal goat serum and 1 % bovine serum albumin in PBS for 1 h before mouse monoclonal anti-poly(ADP-ribose) antibody (1:500 in 1 % BSA/PBS; Millipore, Schwalbach/Ts., Germany) was applied overnight at 4 °C. Samples were washed and incubated with Cy3-labelled goat anti-mouse antibody (1:300 in 1 % BSA/PBS; Dianova, Hamburg, Germany). Cell nuclei of samples were stained with Hoechst 33 258 (Polysciences, Eppelheim, Germany) prior to being cover-slipped with Dako fluorescence mounting medium (Dako, Hamburg, Germany). Image acquisition was carried out with a Zeiss Axiovert 200 microscope coupled to Zeiss Axiovision 4.7 software (Carl Zeiss, Oberkochen, Germany). For quantification of poly(ADP-ribose), numbers of poly(ADP-ribose)-immunoreactive signals were normalized to numbers of nuclei detected in the corresponding microscopic images. Quantifications were performed with ImageJ software (NIH, Bethesda, MD, USA). At least 60 microscopic images were analyzed per experimental group.

Statistical analysis

Analyses of variance (ANOVA) were performed. Fisher's protected least significant difference (PLSD) was used for pair-wise comparisons; p-values ≤ 0.05 were considered significant. Results are presented as means ± standard error of the mean (SEM). Per cent drug-induced reversions of SNCV deficit or IENF loss were calculated by setting the respective response of the Control/Saline group (non-diabetic rats) as 100 %.

Results

Characterization of diabetic rats

Following STZ administration, body weight and glycemic status were monitored throughout the study. As shown in **Fig. 1**, a marked growth arrest was observed in STZ-diabetic rats compared with normal growth in non-diabetic (Control/Saline) rats.

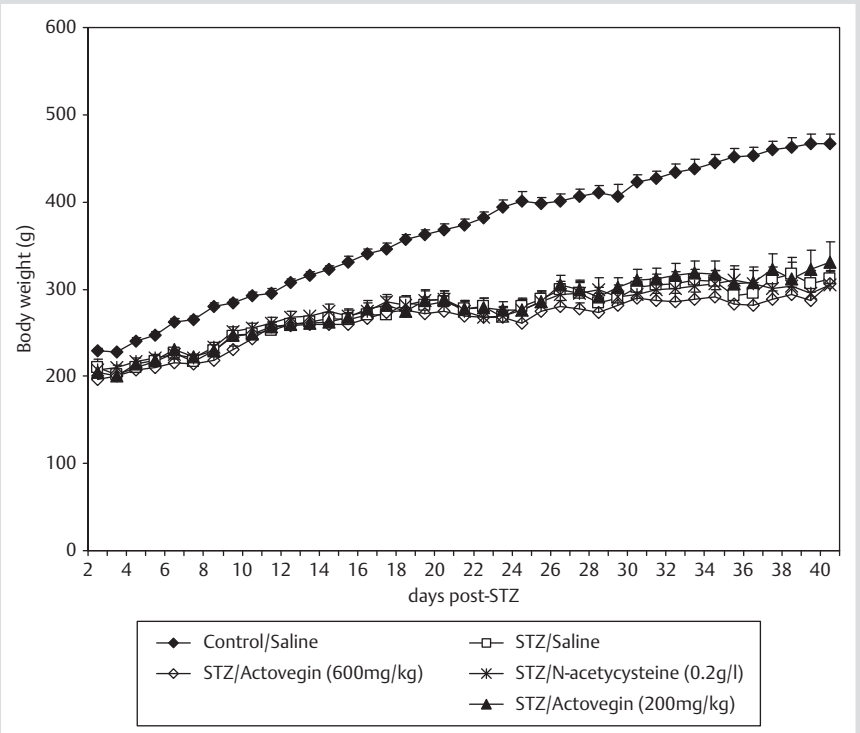

Fig. 1 Changes in body weight in the different experimental groups throughout the study. Body weight was recorded every day; results are presented as means ± SEM (n = 10 for Control/Saline, STZ/Saline, STZ/Actovegin® 200 mg/kg and STZ/NAC groups; n = 8 for the STZ/Actovegin® 600 mg/kg group).

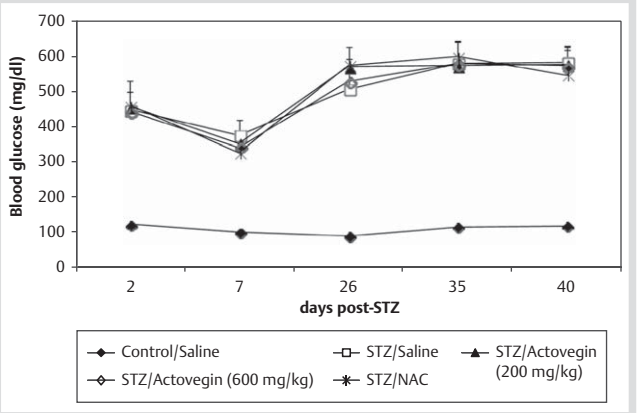

Fig. 2 Glycemia levels (mg/dl) in the different experimental groups throughout the study. Glycemia levels were monitored at days 2, 7, 26, 35 and 40 after STZ administration. Results are presented as means ± SEM (n = 10 for Control/Saline, STZ/Saline, STZ/Actovegin® 200 mg/kg and STZ/NAC groups; n = 8 for the STZ/Actovegin® 600 mg/kg group).

Table 1 Changes (%) in SNCV and IENFD in each experimental group. The Control/Saline group was set as 100% SNCV and 100% IENFD, respectively (n = 10 for Control/Saline, STZ/Saline, STZ/Actovegin® 200 mg/kg and STZ/NAC groups; n = 8 for the STZ/Actovegin® 600 mg/kg group).

Study group	SNCV		IENFD	
	Day 10	Day 25	Day 39	Day 41
Control/Saline	100%	100%	100%	100%
STZ/Saline	65%	77%	67%	68%
STZ/Actovegin® 200 mg/kg	63%	85%	85%	94%
STZ/Actovegin® 600 mg/kg	68%	89%	91%	95%
STZ/N-acetylcysteine 0.2 g/l	65%	82%	73%	88%

Treatment with Actovegin® or NAC did not affect the body weight of STZ-diabetic rats. Rats became rapidly hyperglycemic (> 450 mg/dl) within 2 days after STZ administration (**Fig. 2**); hyperglycemia persisted during the whole length of the study, and treatment with the test compounds did not induce major changes. The reason for the marked decline in blood glucose levels at day 7 in all treatment groups is unclear but could potentially be due to a compensatory enhanced insulin release of the dying β-cells.

SNCV

As shown in **Fig. 3** and in **Table 1**, SNCV values in all STZ-treated rats at day 10 were reduced by ~35% compared with non-diabetic control animals. In STZ/Saline-treated rats, SNCV values remained at reduced levels relative to control rats throughout the study. However, in STZ-diabetic rats treatment with the two doses of Actovegin® from day 11 onwards significantly ($p < 0.005$) reversed the decline in SNCV. After 14 days of Actovegin® treatment (day 25 after STZ administration), SNCV values reached 85% (200 mg/kg Actovegin®) and 89% (600 mg/kg Actovegin®), relative to control rats. A similar dose-dependent improvement in SNCV was also evident at day 39. Prophylactic treatment with the positive control NAC produced a significant ($p < 0.05$) but slightly less pronounced reversion of SNCV decline at day 25, reaching 82% relative to control animals. At day 39, however, the SNCV of NAC-treated rats was not significantly different from the STZ/Saline group.

IENFD

STZ/Saline-treated rats showed a marked decrease (32%) in IENFD compared with non-diabetic rats at the end of the study (day 41; **Fig. 4** and **Table 1**). Treatment with both doses of Actovegin® significantly ($p < 0.005$) prevented the decrease in IENFD in STZ-diabetic rats. IENFD was 6% (Actovegin® 200 mg/kg)

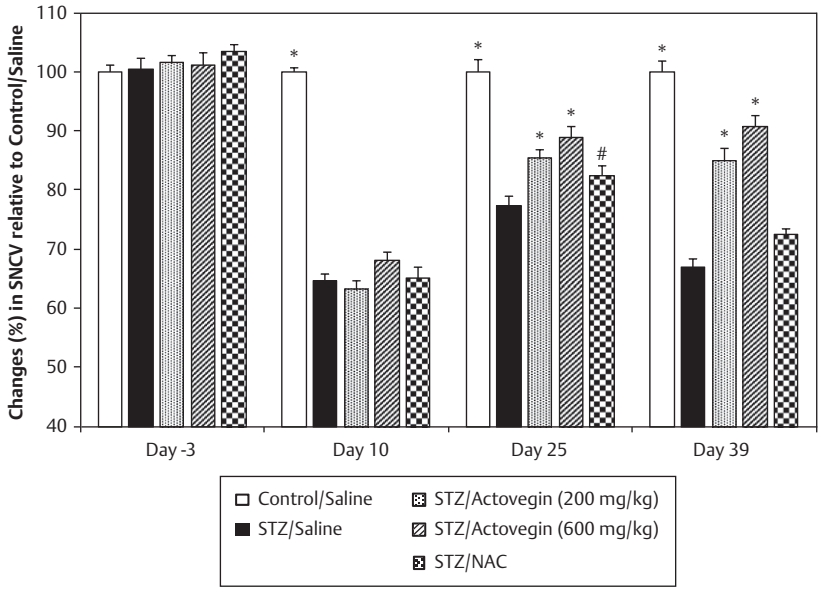

Fig. 3 SNCV performance in the different experimental groups at baseline (Day-3) and at days 10, 25 and 39 after STZ administration. Changes in SNCV of the treatment groups are depicted as a percentage of the control (Control/Saline) at the corresponding time point. Results are presented as means ± SEM (n = 10 for Control/Saline, STZ/Saline, STZ/Actovegin® 200 mg/kg and STZ/NAC groups; n = 8 for the STZ/Actovegin® 600 mg/kg group; *p < 0.005, #p < 0.05 relative to the STZ/Saline group at the corresponding time point).

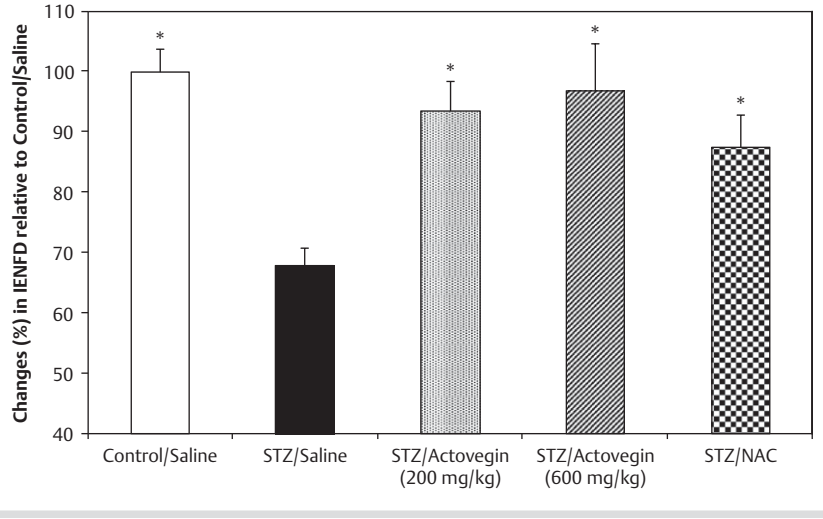

Fig. 4 IENFD in the different experimental groups at day 41 after STZ administration. Results are presented as means ± SEM (n = 10 for Control/Saline, STZ/Saline, STZ/Actovegin® 200 mg/kg and STZ/NAC groups; n = 8 for the STZ/Actovegin® 600 mg/kg group; *p < 0.005 relative to the STZ/Saline group).

and 5% (Actovegin® 600 mg/kg) lower than in healthy control rats. Treatment with NAC also demonstrated a significant ($p < 0.005$) but slightly less pronounced improvement in IENFD, which was 12% lower than in healthy control rats. Representative micrographs of IENF-stained skin tissues from control and treated animals are shown in **Fig. 5**.

Poly(ADP-ribose) analysis

Sciatic nerves of STZ/Saline-treated rats showed a ~2-fold increase in poly(ADP-ribose) content compared with non-diabetic rats. Treatment with 600 mg/kg Actovegin® brought poly(ADP-ribose) content back to levels comparable with non-diabetic animals (**Fig. 6**). Conversely, treatment of STZ-diabetic rats with Actovegin® 200 mg/kg or NAC resulted in no significant differences in poly(ADP-ribose) content compared with STZ/Saline-treated animals. Representative photomicrographs of poly(ADP-ribose)-stained sciatic nerve tissue from Control/Saline-, STZ/Saline- and STZ/Actovegin® 600 mg/kg-treated and STZ/NAC-treated animals are shown in **Fig. 7**.

Discussion

The present study was performed to elucidate the possible cellular effects standing behind the beneficial effects of Actovegin® recently observed in a clinical trial with patients having chronic diabetic sensorimotor distal symmetric polyneuropathy (DPN; Ziegler et al. 2009). Investigations were conducted on streptozotocin (STZ)-induced diabetic related neuropathy in immature rats. The rats in the present study rapidly developed massive hyperglycemia, weight loss and polydipsia after STZ-treatment. These changes were followed by symptomatic neuropathy after 10 days, which was confirmed by measurement of impaired sensory nerve conduction velocity (SCNV), as well as by reduction of the density of intra-epidermal nerve fibres (IENF) in the rat skin. Two doses of Actovegin® were administered intra-peritoneally with the lower dose being equivalent to the dose used to treat DPN patients. The treatment approach chosen in the present study was understood as curative to address the neuropathic symptoms of diabetic rats. We found that Actovegin® did

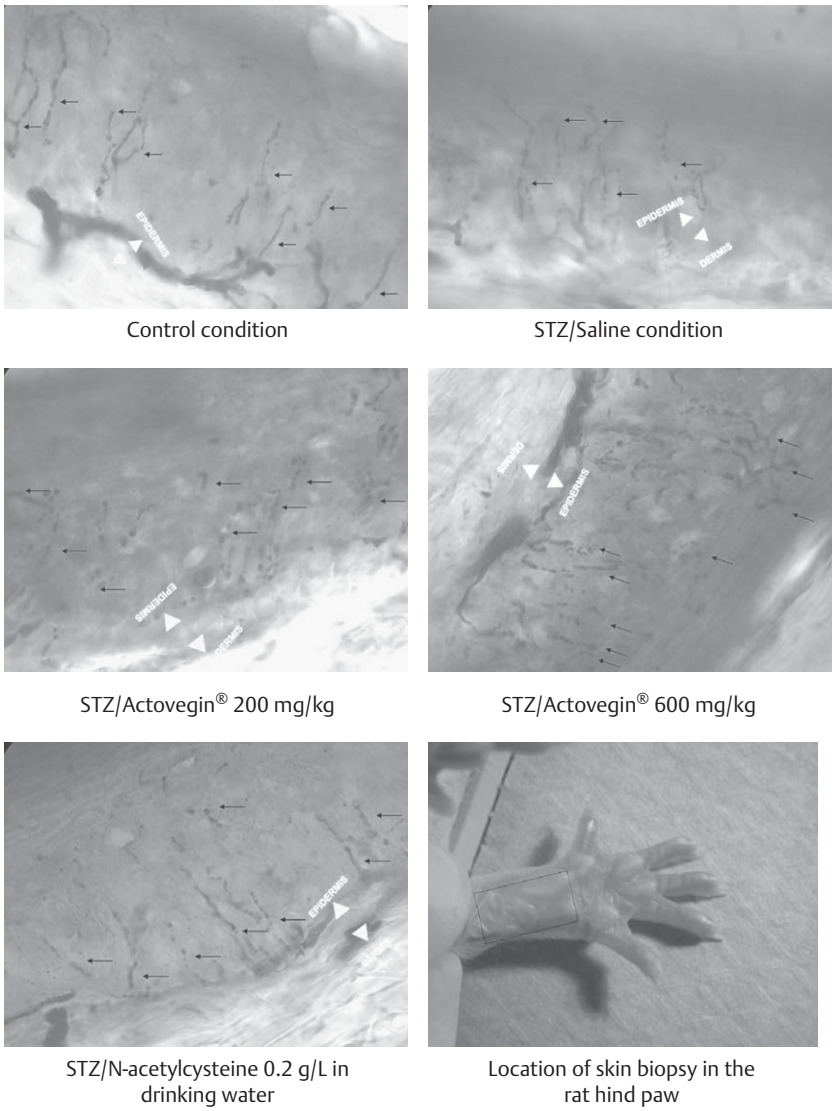

Fig. 5 Vertical sections of skin biopsies illustrating the density of IENF in the different experimental groups (100x magnification). IENF (black arrows) were immunostained with PGP 9.5 antibody. Epidermal nerve fibers that penetrate into the epidermis from the nerve bundles at the basal layer of the dermal-epidermal junction (white arrows) were counted. Secondary branching within the epidermis was excluded from the quantification. The location of the skin biopsy in the rat hind paw is depicted.

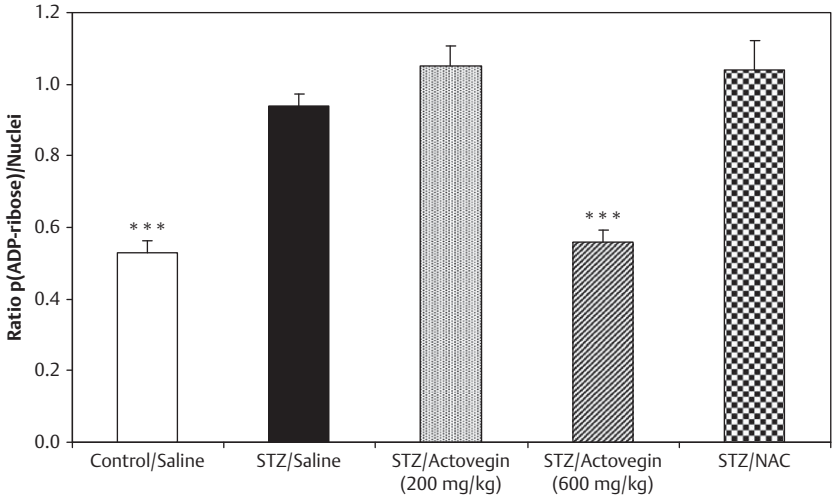

Fig. 6 Quantification of poly(ADP-ribose) content in the different experimental groups; data are presented as means ± SEM (n = 90 for Control/Saline, STZ/Saline, STZ/Actovegin® 200 mg/kg and STZ/NAC groups; n = 60 for the STZ/Actovegin® 600 mg/kg group; *** $p < 0.001$ relative to the STZ/Saline group).

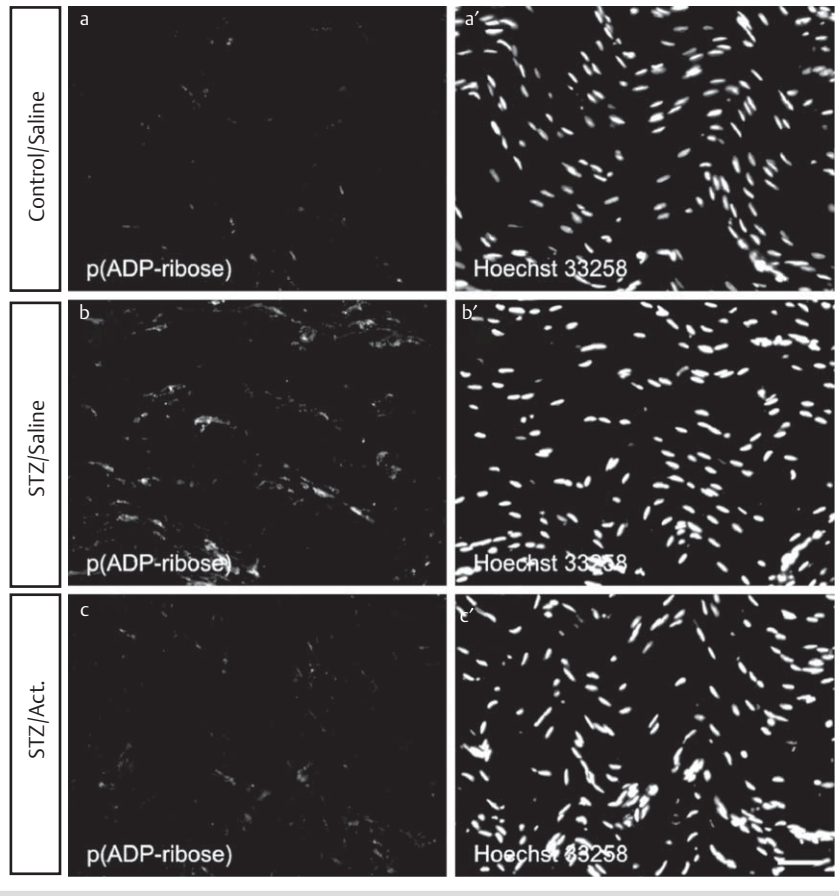

Fig. 7 Representative microphotographs of immunofluorescent staining of poly(ADP-ribose) and nuclei (Hoechst 33258) in sciatic nerves of Control/Saline-treated rats **a–a′**, STZ/Saline-treated rats **b–b′** and STZ/Actovegin® (600 mg/kg)-treated rats **c–c′**. Scale bar: 50 µm.

not decrease hyperglycemia nor did it affect animal weight, but yielded a remarkable amelioration of the SNCV after 14 days, and of the IENF density by the end of the treatment period. Notably, the known loss of IENF density observed in STZ-induced diabetic rats (Lauria et al., 2005) was essentially completely restored after chronic administration of Actovegin®. IENF densities are a quantitative measurement of cutaneous innervations and an accepted clinical endpoint in diabetic neuropathy patients. Moreover, IENF densities appear to measure disease progression, and may have a role in demonstrating improvement with therapy (Holland et al., 1997; Tesfaye et al., 2010). Thus, the morphological improvement seen here can be considered as clinically relevant and can be interpreted as a disease-modifying action of Actovegin® which could hitherto be demonstrated only for a few other drugs with this animal model (Drel et al., 2007; Francis et al., 2009; Kellogg et al., 2007; Obrosova et al., 2008; Toth et al., 2006; Vareniuk et al., 2007).

The next question was, by which cellular mechanism(s) Actovegin® may exert its beneficial effects? A potential hint came from an *in vitro* study conducted in rat hippocampal primary neurons which demonstrated that Actovegin® enhances neuronal cell maintenance and synaptic connectivity, while protecting against apoptosis and oxidative stress (Elmlinger et al., unpublished observations). Oxidative- and nitrosative stress produced by free radicals and oxidants are known to contribute to a variety of pathological effects characteristic for DPN (reviewed in Obrosova et al., 2005). One of the important effectors of oxidative-nitrosative injury and associated DNA single-strand breakage is activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP). PARP activation is involved in early diabetes-induced nerve blood flow reduction, nerve conduction deficits, reduced IENFD and neuropathic pain (Drel et al., 2010; Garcia et al., 2001; Ilnytska et al., 2006; Obrosova et al., 2008). A key role for PARP in the development of experimental diabetic neuropathy is further underlined by the earlier finding, that STZ-diabetic rats treated with a PARP inhibitor and diabetic PARP-deficient mice are protected from IENF loss and SNCV decrease (Ilnytska et al., 2006; Obrosova et al., 2008).

On these grounds, we evaluated the effect of Actovegin® on PARP activation in sciatic nerves by assessment of poly(ADP-ribose) content. Remarkably, Actovegin® 600 mg/kg potently suppressed PARP activation in sciatic nerves of STZ-treated animals down to levels of non-diabetic animals. It is thus conceivable that Actovegin® might improve diabetic neuropathy, amongst others, via mechanisms preventing PARP activation. It may be due to the limited sensitivity of the immunohistochemical detection method, that no effects could be detected with 200 mg/kg Actovegin® and NAC, respectively. The potential of Actovegin® to reduce oxidative stress levels in neuronal cells *in vitro* as demonstrated by Elmlinger and colleagues could potentially explain how Actovegin® prevented PARP activation in the present study. However, further studies are needed to explore this notion in detail.

Taken together, the present results provide the first mechanistic data to explain that the clinical benefits of Actovegin® in DPN patients could be assigned to mechanisms associated with neuroprotection, indicating that Actovegin® is a potential treatment option for DPN.

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Conflict of Interest: This study was sponsored by Nycomed GmbH, Konstanz, Germany. Andreas Dieckmann and Martin Elmlinger are employees of Nycomed. Dan Ziegler has received honoraria for speaking and consulting activities from Nycomed. Emile Andriambeloson and Martin Kriebel have no conflicts of interest to declare.

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