Sensitized peripheral nociception in experimental diabetes of the rat

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ARTICLE INFO

ABSTRACT

Painful neuropathy is a common complication of diabetes. Particularly in the early stage of diabetic neuropathy, patients are characterized by burning feet, hyperalgesia to heat, and mechanical stimuli, as if residual nociceptors were sensitized. Such symptoms are barely explained by common pathophysiological concepts of diabetic neuropathy. Diabetes was induced in Wistar rats by streptozotocin (STZ). After 4 weeks behavioral testing (Plantar test, Randall–Selitto) was conducted. Basal and stimulated release of calcitonin gene-related peptide (CGRP), Substance P (SP) and prostaglandin E_2 (PGE_2) from isolated skin and sciatic nerve were assessed by enzyme immunoassays. Electrophysiological properties of identified nociceptors under hyperglycemic, hypoxic, and acidotic conditions were investigated using the skin-nerve preparation. The diabetic rats showed hyperalgesia to heat and pressure stimulation. The basal CGRP/SP release was reduced, but chemical stimulation with bradykinin induced greater release of SP, CGRP and PGE_2 than in control animals. In contrast, capsaicin-stimulated CGRP release was reduced in sciatic nerves. Hypoxia per se lowered von Frey thresholds of most C-nociceptors to half. Hyperglycemic hypoxia induced ongoing discharge in all diabetic but not control C-fibers which was further enhanced under acidosis. Sensory and neurosecretory nociceptor functions are sensitized in diabetes. Diabetic C-fibers show exaggerated sensitivity to hyperglycemic hypoxia with and without additional acidosis, conditions that are thought to mimic ischemic episodes in diabetic nerves. Ongoing C-fiber discharge is known to induce spinal sensitization. Together with altered receptor and ion channel expressions this may contribute to painful episodes in diabetic neuropathy.

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1. Introduction

In diabetic neuropathy dying-back of nerve fibers and, thus, negative symptoms are comparatively well explained by results from decades of clinical and experimental research. However, "positive" sensory symptoms such as paresthesias, spontaneous burning pain and allodynia/hyperalgesia have largely remained enigmatic, although they indicate a dysfunction of peripheral nociception [8]. Increased responsiveness to noxious stimulation of nociceptive neurons is reported [64,12] and it has been proposed that altered expression and functional properties of diverse ion channels could be responsible, but a complexity of up- and down-regulations of e.g. sodium channels or transient receptor potential channels led to confusion [43,31].

Clinical and experimental reports emphasize the role of micro-angiopathy in the development of painful diabetic neuropathy leading to endoneurial ischemia and hypoxia [59]. However, it has been demonstrated that the combination of hyperglycemia, hypoxia and reduced buffering capacity can rapidly increase the (electrical) excitability of healthy rat peripheral nerve axons by enhancement of depolarizing afterpotentials and suppression of inwardly rectifying K^+ currents in myelinated axons [28,57]. The high-glucose-hypoxia condition caused intracellular acidification which was found responsible for the increased excitability [55]. In this study sensory fibers were more sensitive than motor axons and recovered badly from hyperglycemic hypoxia [28]. In addition, sensory axons were depolarized and their membrane resistance increased under hyperglycemic hypoxia, which could either decrease excitability by increasing steady-state inactivation of voltage-gated sodium channels or increase the excitability of those C-fibers that are well equipped with the inactivation-resistant $Na_V1.8$ sodium channel [68]. In any case, even with prolonged hypoxic challenge no spontaneous action potential discharge was observed in this axonal model. On the other hand, nociceptive C-fibers from stretozotocin (STZ-) diabetic rats do show spontaneous activity and increased mechanical responsiveness in the isolated skin-nerve preparation [64]. This suggests quantitative biophysical differences between the peripheral nerve axons and the potentially more susceptible unmyelinated terminals, which led us to test the high-glucose-hypoxia model in the isolated skin-nerve preparation taken from diabetic and control rats.

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Another way to study small fiber functions is to assess their neurosecretory capacity measuring basal and stimulated neuropeptide release. We used the rat hindpaw hairy skin and the isolated desheathed sciatic nerve to compare the release of CGRP and SP of STZ-diabetic and control animals. Neuropeptide release provides a lump signal reflecting the degree of nociceptor activation, as well as it presents a pathogenic factor in its own right. Sensory nerve fibers release neuropeptides into surrounding tissues and cause "neurogenic inflammation" and on a longer time scale promote wound healing and regulate tissue nutrition [30,62].

Reduction of cutaneous sensory fibers and diminished release of neuropeptides contribute to trophic disturbances of diabetic skin [38,39,68]. On the other hand increased CGRP content of the unstimulated trachea and enhanced capsaicin-stimulated release of neuropeptides from rat paw skin 8 weeks after diabetes induction have been described [9,20].

The comparison of both the electrophysiological and neurochemical data with the behavioral changes in STZ rats 4 weeks after induction of severe diabetes reveals new and underlines established mechanisms that may contribute to pain and hyperalgesia in diabetic neuropathy.

2. Methods

2.1. Animals and diabetes induction

Experiments were carried out on STZ-treated Wistar rats and age-matched control rats. The experiments were approved by the animal ethics committee of the district (Regierung von Mittelfranken, Ansbach, Germany). Diabetes was induced in adult male rats (weight 250–350 g) by one injection of streptozotocin (STZ, 70 mg/kg in 2% citrate buffer i.p.) after fasting for one night. The water consumption and body weight of the animals were controlled and repeatedly measured. Animals that did not react with a dramatic increase in water consumption within 1 week after STZ treatment were excluded from further experiments (10 out of 51 rats). Four weeks after successful STZ treatment behavioral, electrophysiological and release experiments were conducted.

2.2. Behavioral studies

2.2.1. Plantar test

Thermal nociceptive sensitivity to radiant heat was quantified using the paw withdrawal test [29]. The rats were placed in a Perspex enclosure with a metal mesh floor. A movable radiant heat source was placed directly under the plantar surface of the hind paw (Ugo Basile, Comerio VA, Italy). The paw withdrawal latency (in s) was defined as the time from onset of irradiation (lamp 50 W, intensity 7, distance lamp to paw 40 mm, 24 °C room temperature) to the withdrawal of the hind paw. Testing was alternated between the left and the right hindpaw at 10 min intervals for 2 h. The rats we trained on the experimental situation for 1 h on 3 days before the real measurements were conducted. The rats were not restrained within the chamber.

Overall we tested eight diabetic rats and five healthy control rats. Data are shown as means ± SEM of the determined latencies. Differences between animal groups were evaluated using an ANOVA design for repeated measures. Effects were assessed between diabetic and healthy animals ("between" subject effects) and within the 12 consecutive measures over time ("within" subject effects).

2.2.2. Paw pressure test

Mechanical nociceptive tolerance (Randall–Selitto) was assessed using a custom-made electromechanical algesimeter and measuring withdrawal thresholds upon increasing pressure ap-

plied to the dorsal surface of the hindpaw. During testing the rats were lightly restrained in a cotton towel (individual towel for each rat) and held by hand. The rats were trained to this testing procedure on 3 days. The tested paw was placed on a wooden ball and the stimulus probe (diameter 2.5 mm) perpendicularly lowered onto the dorsal surface of the hindpaw. The feed-back force controlled stimulation had a maximal duration of 32 s, and the force increased linearly from 150 to 5000 mN. The withdrawal reaction of the rat was taken as the nociceptive threshold (in mN) and the stimulus was stopped immediately. No vocalization of the rats was ever observed.

Overall, we tested eight diabetic and five healthy control rats. Four consecutive measurements of the mechanical withdrawal thresholds were assessed at 30 min intervals. Measurement of the right and left paw alternated. Data are shown as means ± SEM. Differences of withdrawal thresholds were evaluated using ANOVA for repeated measures. The between subjects effects (diabetic and healthy animals) and within subjects effects (four measuring repeats) were assessed.

2.3. CGRP, SP and PGE$_2$ release from isolated skin and sciatic nerve

2.3.1. Preparation, sampling and stimulation procedures

Fours weeks after induction of diabetes, skin flaps of the lower leg and hindpaws of the rats [3] and their sciatic nerves [52] were harvested [5]. After sacrificing the animals in pure CO_2 atmosphere, the hairy skin of both hindpaws was subcutaneously excised sparing the bigger vessels and the saphenous and peroneal nerve stems. The preparation started at knee level and included the toes; after dissection, the flaps were wrapped around arylic glass rods (diameter 6 mm) with the corium side exposed and fixed with surgical silk.

Both sciatic nerves were excised from the origin to the trifurcation into tibial, sural and peroneal nerves and desheathed, as described previously [52]. The nerves were blotted and thereafter the desheathed nerves were wrapped around acrylic rods and fixed with surgical silk.

The skin or nerve preparations were washed for 30 min in "synthetic interstitial fluid" (SIF) [6] gassed with carbogen at 32 °C. The samples were then subjected to four or six consecutive incubation steps (S1–S4/6), each in a glass tube mounted in a shaking bath at 32 °C and filled with 1 or 0.5 ml SIF, respectively, sufficient to cover the sample. Each step lasted for 5 min with only time enough between steps to transfer the samples. After 5 min of incubation, the preparations were forwarded to the next tube, and the remaining elutions were subdivided into three aliquots. Contents of calcitonin gene-related peptide (CGRP), substance P (SP) and prostaglandin E$_2$ (PGE$_2$) were measured using enzyme immuno assays (EIA). The third incubation step (S3) contained the stimulating solution; we used bradykinin 10^{-5} M or capsaicin 10^{-6} M (SIGMA, Taufkirchen, Germany) freshly dissolved from stock solutions (10^{-3} M) in SIF.

2.3.2. Enzyme immunoassays

Details of the analytical methods are published in detail [4,53]. Briefly, CGRP and SP contents were measured using commercial EIA kits (SPIbio, Montigny, France). The PGE$_2$ assay was performed off-line after keeping the elutions at −28 °C as previously published, and the antibody was a generous gift of K. Brune (Dept. Pharmacol., University of Erlangen, Germany). The EIA plates were determined photometrically using a microplate reader (Dynatech, Channel Islands, UK).

The total released amounts of CGRP, SP and PGE$_2$ are given referring to 1 g of fresh tissue weight. Values are given as means ± SEM in Tables 1 and 2. The figures display normalized values;

Table 1
Basal (S1, S2) and bradykinin-stimulated (S3) release of neuropeptides (CGRP and SP) and prostaglandin E$_2$ from isolated hairy skin of STZ-diabetic and healthy control rats, three consecutive samples collected over 5 min incubation time (means ± SEM).

Measured (pg/g tissue)	STZ rats				Control rats			
	n	S1	S2	S3 bradykinin 10 μM	n	S1	S2	S3 bradykinin 10 μM
SP	16	50 ± 4	53 ± 5	105 ± 13	13	84 ± 12	87 ± 10	112 ± 12
CGRP	7	47 ± 7	39.5 ± 9	84 ± 19	13	63 ± 7	58.7 ± 7	85 ± 11
PGE$_2$	16	1650 ± 346	1554 ± 536	6786 ± 1307	13	1636 ± 408	1332 ± 276	2897 ± 502

Table 2
Basal (S1, S2) and capsaicin-stimulated (S3) CGRP release from isolated and desheathed rat sciatic nerves taken from STZ-diabetic and healthy control rats (means ± SEM over 5 min).

Measured (pg/g tissue)	STZ rats $n = 6$			Control rats $n = 9$		
	S1	S2	S3 capsaicin 1 μM	S1	S2	S3 capsaicin 1 μM
CGRP	734 ± 46	759 ± 52	7324 ± 549	1092 ± 153	997 ± 173	11,246 ± 1456

the actual value of CGRP, SP or PGE$_2$ measured was divided by the baseline value taken from the second sample, the last one before stimulation. For comparison of experimental groups (diabetes vs. normal rats) we used the Mann–Whitney U-test, comparisons within one group were performed with the Wilcoxon match-pairs test.

2.4. Single-fiber electrophysiology

Single-fiber recordings of identified primary afferents from skin of control and diabetic rats were carried out using the skin-nerve preparation *in vitro* which has previously been described [49]. Briefly, 27 healthy control and 34 STZ-diabetic rats were sacrificed by exposure to pure CO$_2$ atmosphere. The saphenous nerve was dissected free from the upper thigh and removed together with the complete hairy skin of the dorsal hind paw and lower leg. The skin was fixed by means of little metal hooks to the silicon bottom of an organ chamber, the corium side facing upward. The cut nerve was pulled through a hole leading into a separate recording chamber and laid on a small mirror. Under binocular control fine filaments were teased from the desheathed nerve until single-fiber activity could be recorded monopolarly through a gold wire electrode which was isolated with paraffin oil overlying the aqueous fluid in the recording chamber. The skin preparation was continuously superfused at a flow rate of 16 ml/min with a carbogen (95% O$_2$, 5% CO$_2$) saturated synthetic interstitial fluid (SIF, [6]) of pH 7.38 at 32 °C. Normal SIF consisted of (in mM) 108 NaCl, 26.2 NaHCO$_3$, 9.64 sodium gluconate, 5.55 glucose, 7.6 sucrose, 3.48 KCl, 1.67 NaH$_2$PO$_4$, 1.53 CaCl$_2$ and 0.69 MgSO$_4$.

Receptive fields of single-fibers were searched by probing the corium side of the skin with a blunt glass rod. For determination of the conduction velocity (cv), the fibers were electrically stimulated in their receptive field with a Teflon coated steel microelectrode (impedance ≈1 MΩ). Mechanical (v. Frey) thresholds were determined using a set of nylon filaments with uniform blunt tips (0.9 mm diameter) which were calibrated in the form of a geometrical series ($x_i = x_{i-1} \sqrt{2}$ mN) from 1 to 256 mN. To determine the heat responsiveness of the fibers, the receptive fields were isolated from the surroundings by a metal cylinder (inner diameter 0.8 mm) placed on the corium side of the skin; this chamber was evacuated from SIF for thermal stimulation in order to prevent convection. A focused halogen bulb was positioned so as to irradiate the receptive field through the translucent bottom of the organ chamber producing a ramp-shaped temperature increase from 32 to 46 °C within 20 s. The temperature was feed-back controlled by a miniature thermocouple placed at the corium side. For cold stimulation the isolated receptive fields were superfused with cooled SIF at about 4 °C and the temperature was monitored.

2.5. Simulation of diabetic conditions

To simulate the tissue conditions that are assumed to result from diabetic microangiopathy and diabetic hyperglycemia, three parameters were altered in the extracellular fluid (SIF) that was used to rinse the receptive fields of the characterized nociceptors:

1. Hyperglycemia was mimicked by increasing the glucose concentration from 2.5 to 25 mM in SIF.
2. Ischemia was mimicked by reducing the concentration of the physiological buffer to half (13.1 mM NaHCO$_3$).
3. Hypoxia was induced by gassing SIF with 97.5% N$_2$ and 2.5% CO$_2$ instead of carbogen at pH 7.4.

Following a neutral pH-hypoxia period of 30 min tissue acidosis was finally induced by bubbling the hypoxic superfusion fluid with pure CO$_2$ which generated a pH value of 5.7.

2.6. Electrophysiological protocols and data processing

After isolation of the single-fibers we determined conduction velocity (cv), mechanical (v. Frey) threshold, the rate of spontaneous discharge activity as well as heat and cold responsiveness. Thereafter different test phases followed:

First, spontaneous activity was recorded for 5 min. Thirty minutes superfusion of hypoxic SIF followed and the induced activity was recorded. After 10 and 30 min hypoxia period we re-determined the v. Frey thresholds. Thereafter a 10 min lasting superfusion with acidic SIF (pH 5.7) followed. The induced discharge activity was recorded, and at the end the v. Frey thresholds were re-tested.

Skin taken from diabetic rats was continuously superfused during the whole experiment with SIF containing 25 mM glucose in order to mimic hyperglycemia. As a control, normal rat skin was also superfused with 25 mM glucose, and as a further control normal skin was superfused with normal SIF (5 mM glucose). The hypoxia and low pH periods were the same in all three groups of experiments (see Fig. 4).

Unitary action potentials were passed through a custom-made low noise AC – coupled amplifier and monitored on an oscilloscope and through loudspeakers. For further spike analysis the recordings were digitized (12-bit A–D converter, 4 kHz sampling rate) and stored in a PC using the DAP 1200 interface package (Microstar, Richmond, WA, USA). The Spike/Spidi software package which employs a template–matching procedure was used to spike discrimination in for off-line analysis [23]. The statistical tests are specified in Section 3 and the STATISTICA package (Statsoft, Tulsa, USA) was used.

3. Results

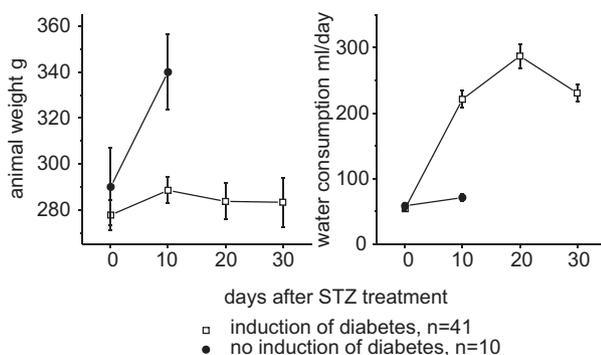## 3.1. Induction of diabetes by STZ treatment of the rats

Fifty-one rats with a mean body weight of 280 ± 6 g and 55 ± 3 ml water consumption per day were treated with STZ i.p. Every 10th day body weight (Fig. 1A) and water consumption per day (Fig. 1B) of the animals were measured. Fig. 1 shows the time courses of body weight changes and water consumption of successfully treated diabetic rats ($n = 41$) and of the rats that did not develop diabetes after STZ treatment ($n = 10$). Successfully STZ-treated diabetic rats did not gain any weight after treatment and their water consumption increased dramatically within 10 days. Ten of the initial 51 rats did not develop diabetes indicated by a further increase of their body weight and no increase of water consumption. These animals were excluded from the following behavioral, neurochemical and electrophysiological experiments, that started earliest after 30 days following STZ treatment.

## 3.2. Paw withdrawal latency upon noxious heat (Plantar test)

At the beginning of the test session with repeated measurements control rats showed a mean heat withdrawal latency of 14.2 ± 1 s ($n = 5$). During the first hour of repeated testing at 10 min interval the values dropped by about 40% to a mean level of 8.6 ± 0.5 s, in the subsequent test period of 1 h the withdrawal latencies increased again to 10.4 ± 1 s (Fig. 2A). Four weeks after STZ treatment the heat withdrawal latencies were significantly lower than in control animals (ANOVA repeated measures, $p < 0.005$, LSD post hoc test see Fig. 2A). The diabetic in contrast to healthy animals ($n = 8$) exhibited variations of the withdrawal during the observation period. The initial latency was 9.2 ± 1 s and dropped during half an hour to 6.8 ± 0.3 s, subsequently varying between these values. In both groups no significant difference could be observed between left and right paw, so data from left and right paws were pooled.

3.3. Mechanical withdrawal thresholds (Randall–Selitto test)

Control rats showed a mean mechanical withdrawal threshold of 3666 ± 268 mN ($n = 5$, mean of right and left paw, reflecting a weight force of about 370 g) that did not vary much on repeated testing (Fig. 2B). Four weeks after STZ injection diabetic rats exhibited significantly lowered mechanical thresholds of 2833 ± 193 mN reflecting a decrease by about 22% ($n = 7$, ANOVA repeated measures, $p < 0.005$, LSD post hoc test see Fig. 2B).

Fig. 1. Development of animal weight and water consumption after treatment with streptozotocin (70 mg/kg in 2% citrate buffer i.p.): in case of successful induction of diabetes the animals do not gain weight and greatly increase their daily water consumption.
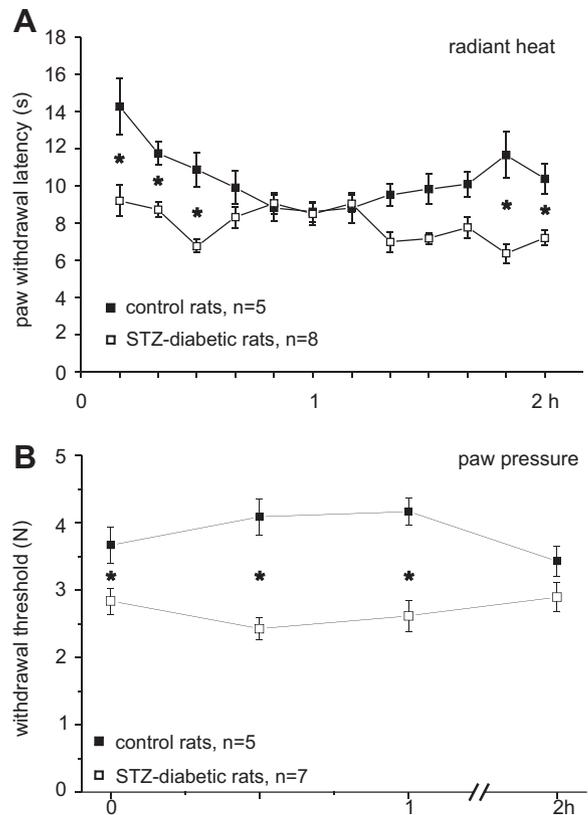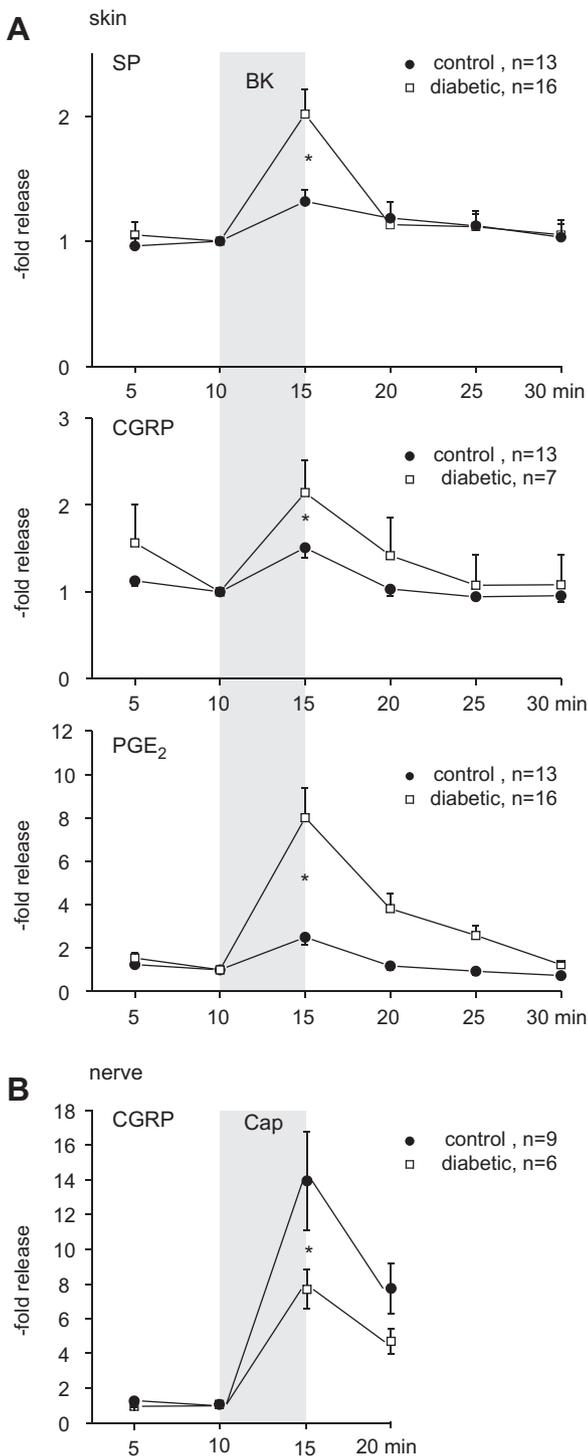

Fig. 2. Heat and mechanical hyperalgesia in diabetic rats 4 weeks after induction of diabetes by STZ. Radiant heat (A) and pressure-induced hindpaw withdrawal (B) of STZ-diabetic rats was sensitized compared to healthy controls. Both behavioral responses were repeatedly measured over a 2 h observation period; asterisks refer to $p < 0.05$ (ANOVA/LSD).

3.4. Basal neuropeptide and prostaglandin E_2 release from the isolated skin and sciatic nerve

In skin isolated from STZ-diabetic rats we found reduced basal levels (S1 and S2) of SP release (U-test, $p < 0.01$), basal levels of CGRP release were only marginally lowered as compared to healthy controls (Table 1). This result may reflect reduced neuropeptide content of nociceptors innervating the skin. Small fiber neuropathy causes degeneration of nerve fibers which normally contain neuropeptides [21,38], and a disturbance of the axonal transport of neuropeptides gives further reason for the reduced basal neuropeptide release [9,15,18,67,69]. Also, signs of neurogenic inflammation that is mediated by release of neuropeptides are diminished in diabetic neuropathy [19,38]. PGE_2 contents of the incubation fluids from the first steps were not different between diabetic and control skin. Basal CGRP release from isolated desheathed sciatic nerves was insignificantly lower in diabetic animals (Table 2) which would be in accord with the literature where a reduction of neuropeptide content in nerves is reported [16,50]. The strongly attenuated capsaicin response of the diabetic nerve (see below) may be the effigy of a reduced releasable pool of CGRP-containing vesicles [42].

3.5. Bradykinin-induced release from skin

Bradykinin (BK, 10^{-5} M) was used to stimulate skin flaps from diabetic and control animals. In both animal groups SP, CGRP and PGE_2 release was significantly increased by BK (Fig. 3A). In skin from STZ rats, BK augmented the SP levels by about 100% (Wilcoxon test, $p < 0.001$, $n = 16$) which was significantly more than in control skin (U-test, $p < 0.01$) showing only an increase by 30%

A

$p = 0.06$), in which BK caused only a 50% increase (Wilcoxon test, $p = 0.01$, $n = 13$). BK induced an enormous increase of PGE_2 in diabetic skin by about 700% (Wilcoxon test, $p = 0.006$, $n = 13$), whereas in skin from normal rats BK only doubled the PGE_2 release (Wilcoxon test, $p = 0.001$, $n = 13$). This difference again was significant (U-test, $p < 0.001$). Thus, BK-induced neuropeptide release was increased in diabetic skin relative to the lowered baseline, although the stimulated peak release was about the same as in healthy animals. In contrast, the PGE_2 peak release was more than double the amount in diabetic than normal rats, although the baseline was about the same.

3.6. Capsaicin-induced release from the isolated desheathed sciatic nerve

Capsaicin-stimulated CGRP release was significantly reduced in nerve preparations from diabetic animals (U-test, $p = 0.03$). In control preparations capsaicin 1 μM augmented the CGRP release 13-fold (Wilcoxon test, $n = 9$, $p < 0.01$), in nerve preparations from diabetic rats only about 7-fold (Wilcoxon test, $n = 6$, $p = 0.02$), and the peak release was lower by about 35%.

3.7. Single-fiber electrophysiology in the rat skin-nerve preparation

3.7.1. Sensory properties
The fibers were classified according to conduction velocity, mechanical (v. Frey testing) and thermal responsiveness. Altogether we recorded from 40 nociceptive C-fibers, 16 from STZ-diabetic rats and 24 from healthy control rats.

Table 3 summarizes the classification characteristics: fiber types, conduction velocities (m/s), median v. Frey threshold and mean heat threshold of the heat sensitive fibers, divided into the three different experimental groups: (I) healthy control rats using normal glucose concentration (5 mM) in SIF, (II) healthy control rats using elevated glucose concentration (25 mM) in SIF and (III) STZ-diabetic rats using high-glucose concentration (25 mM) in SIF. No differences between these groups were obvious with respect to sensory properties and conduction velocity.

After classification of the fibers, we recorded ongoing activity in the absence of any stimulation over 5 min. Nociceptors recorded from healthy control rats showed no spontaneous activity irrespective of superfusion with normal ($n = 8$) or glucose-enriched SIF ($n = 16$). Low-frequent ongoing activity was rarely observed in fibers recorded from diabetic rats (2/16 fibers: about 1 spike/min).

3.7.2. Hypoxia
After baseline recordings at rest the fibers were superfused with hypoxic SIF for 30 min to assess potentially-induced ongoing activity (Fig. 4). Fibers recorded from healthy control animals developed marginal spontaneous activity during 30 min under hypoxic superfusion (average: 0.1 spikes/min in five out of eight fibers), and about the same applied to hypoxic high-glucose superfusion (0.1 spikes/min in 11 of 16 fibers). However, in diabetic animals 16 out of 16 fibers developed low-frequent ongoing activity during hypoxia (2.3 ± 0.9 spikes/min over 30 min). In the first 10 min of hypoxia the ongoing activity rate was the highest (41.3 ± 18 spikes/10 min) and declined over the following 20 min. The overall response in 30 min to hypoxia was significantly increased in fibers recorded from diabetic animals (68.3 ± 27.3 spikes/30 min, $n = 16$) compared to control rats, irrespective whether the receptive fields were superfused with control SIF (2.6 ± 1.2 spikes/30 min, $n = 8$) or high-glucose SIF (5 ± 1.7 spikes/30 min, $n = 16$; $p = 0.03$ and $p = 0.01$, respectively, one-way ANOVA, with LSD post hoc comparison). Differences between the fiber subtypes in each experimental group were not observed.

B

Fig. 3. Neuropeptide (calcitonin gene-related peptide, CGRP, and substance P, SP) and prostaglandin E_2 (PGE_2) release from isolated rat skin (A) and desheathed sciatic nerves (B) was measured using EIA techniques, stimulation with bradykinin (BK 10 μM) or capsaicin (Cap 1 μM) 4 weeks after STZ treatment. Data are normalized to the second baseline value (10 min). Relative release of SP ($p < 0.01$, U-test), CGRP ($p = 0.05$) and PGE_2 ($p < 0.004$) was significantly augmented in skin from STZ-diabetic rats (A). In contrast, capsaicin-induced release from sciatic nerves (B) was significantly reduced in diabetic rats ($p < 0.01$). All stimulated increases over baseline were significant ($p \leqslant 0.01$, Wilcoxon).

(Wilcoxon test, $p < 0.01$, $n = 13$). The CGRP release in diabetic skin was increased by BK by about 110% (Wilcoxon test, $p = 0.02$, $n = 7$) which was marginally more than in control skin (U-test,
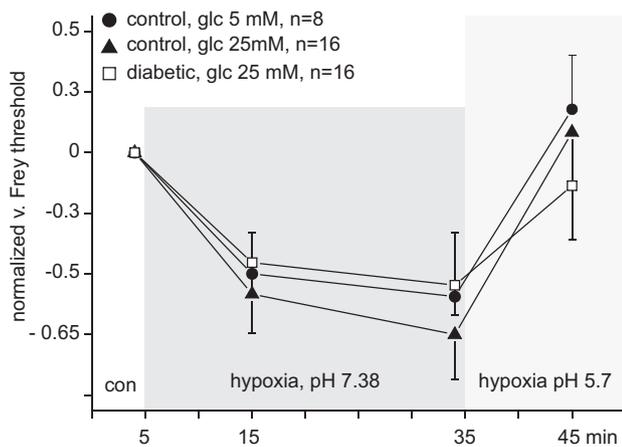
Table 3
Table of fiber populations and initial sensory properties recorded in the three experimental groups.

Protocol	Fiber type	n	Mean cv (m/s)	Median v. Frey (mN)	Mean heat threshold (°C)	Hypoxia drop of v. Frey in n/n fibers	Hypoxia mean heat threshold (°C)	Acidosis rise of v. Frey in n/n fibers
Control skin 5 mM glucose SIF	C-htm	2	0.52	64		1/2		1/2
	C-mc	3	0.6	45.3		2/3		3/3
	C-mh	3	0.48	64	42.7 ± 0.2	3/3	39.3 ± 2	2/3
Control skin 25 mM glucose SIF	C-htm	3	0.58	64		3/3		3/3
	C-mc	6	0.54	2		4/6		4/6
	C-mh	6	0.5	23.65	40.8 ± 1.7	3/6	40.3 ± 2	5/6
	C-mhc	1	0.61	45.3		1/1		1/1
STZ-diabetic skin 25 mM glucose SIF	C-htm	2	0.58	64		0/2		0/2
	C-mc	6	0.56	2		4/6		4/6
	C-mh	7	0.41	26.65	42.5 ± 0.7	6/7	42.6 ± 1.1	4/7
	C-mhc	1	0.56	45.3		1/1		0/1
	Total number of fibers	40						

C-htm, high threshold-mechano-sensitive; C-mc, mechano-cold-sensitive; C-mh, mechano-heat-sensitive; C-mhc, mechano, heat, cooling-sensitive.

During the hypoxic superfusion of the receptive fields, we determined the v. Frey thresholds after 10 and 20 min to compare with those determined before hypoxia (Fig. 4). The initial v. Frey thresholds were statistically not different between groups (group I: median 64 mN, 54.65 mN lower and 64 mN upper quartile, $n = 8$; group II: median 26.65 mN, 2 mN lower and 64 mN upper quartile, $n = 16$; group III: median 32 mN, 24 mN lower and 64 mN upper quartile, $n = 16$). In all three experimental groups hypoxia caused a drop of the v. Frey threshold by about two steps (~50%) on the geometric scale (Fig. 5): nine out of 16 fibers recorded from diabetic rats with 25 mM-Glucose SIF showed a drop of their v. Frey threshold after 20 min by two steps ($p = 0.02$, Wilcoxon test). Likewise, in control animals treated with 25 mM-Glucose SIF 11/16 fibers lowered their mechanical threshold by about 2.5 v. Frey steps ($p = 0.002$, Wilcoxon test). Finally, in the control group with normal SIF the threshold dropped during hypoxia in 6/8 fibers, but this did not reach significance due to the small n ($p = 0.1$, Wilcoxon test). The heat thresholds of the C-MH fibers measured after hypoxic superfusion are listed in Table 3, no significant changes could be detected.

3.7.3. Acidotic hypoxia

After the neutral hypoxia period the receptive fields were rinsed with acidified SIF (pH 5.7 at half buffer concentration, 100% CO_2 gassing) for 10 min. In this phase of the protocol, proton-induced discharge activity of the fibers and further changes of the mechanical v. Frey thresholds were assessed. In the diabetic group with 25 mM glucose SIF the recorded fibers responded to the acidic condition with a mean total number of 55.5 ± 22 spikes/10 min ($n = 16$). In contrast, the activity was poor and significantly lower in the experimental groups using healthy control animals. In the low-glucose group the fibers responded with a mean discharge of 7.75 ± 3 spikes/10 min ($p = 0.05$, one-way ANOVA, with LSD post hoc comparison). In the high-glucose group the fibers responded with a mean discharge of 8.75 ± 3 spikes/10 min ($p = 0.02$, one-way ANOVA, with LSD post hoc comparison). The induced ongoing activity did not show any adaptation or desensitization during the 10 min of hypoxic low pH superfusion. We found no differences between the fiber subtypes in each experimental group, so the data were averaged.

At the end of the superfusion with hypoxic SIF at low pH a "recovery" or desensitization of the v. Frey thresholds was observed in all three experimental groups. In the diabetic animals with high-glucose SIF, six out of 16 fibers showed a reversal of their mechanical thresholds (mean increase by 1.5 v. Frey steps, $p = 0.02$, Wilcoxon test) compared to the thresholds just before acidosis. The median threshold was no longer different from the very initial threshold of the fibers in this group. Notably, five out of 16 diabetic fibers completely lost their mechanical sensitivity over the acidosis period and could only be excited by electrical stimulation; the remaining four fibers maintained their lowered thresholds. In the control group with high-glucose SIF 13 out of 16 fibers showed a more than complete restitution of their mechanical sensitivity (increase by 3.9 v. Frey steps, $p < 0.01$, Wilcoxon test). The median threshold after hypoxic acidosis was 32 mN, similar to the original median value of 26.65 mN before hypoxia. The v. Frey thresholds of two fibers remained unchanged and one lost mechanical sensitivity over the acidotic period. In the control group with normal SIF, six out eight fibers restituted their median threshold by 2.6 v. Frey threshold steps upward to 128 mN compared to before acidosis ($p = 0.03$, Wilcoxon test), one fiber did not change threshold and one fiber lost its sensitivity. In conclusion, the nociceptive C-fibers became more sensitive to punctuate mechanical stimulation during hypoxia but were desensitized (to normal) upon continued acidotic hypoxia, irrespective of the experimental group. However, the diabetic nerve fibers appeared more susceptible to losing their mechanosensitivity over the additional acidotic challenge.
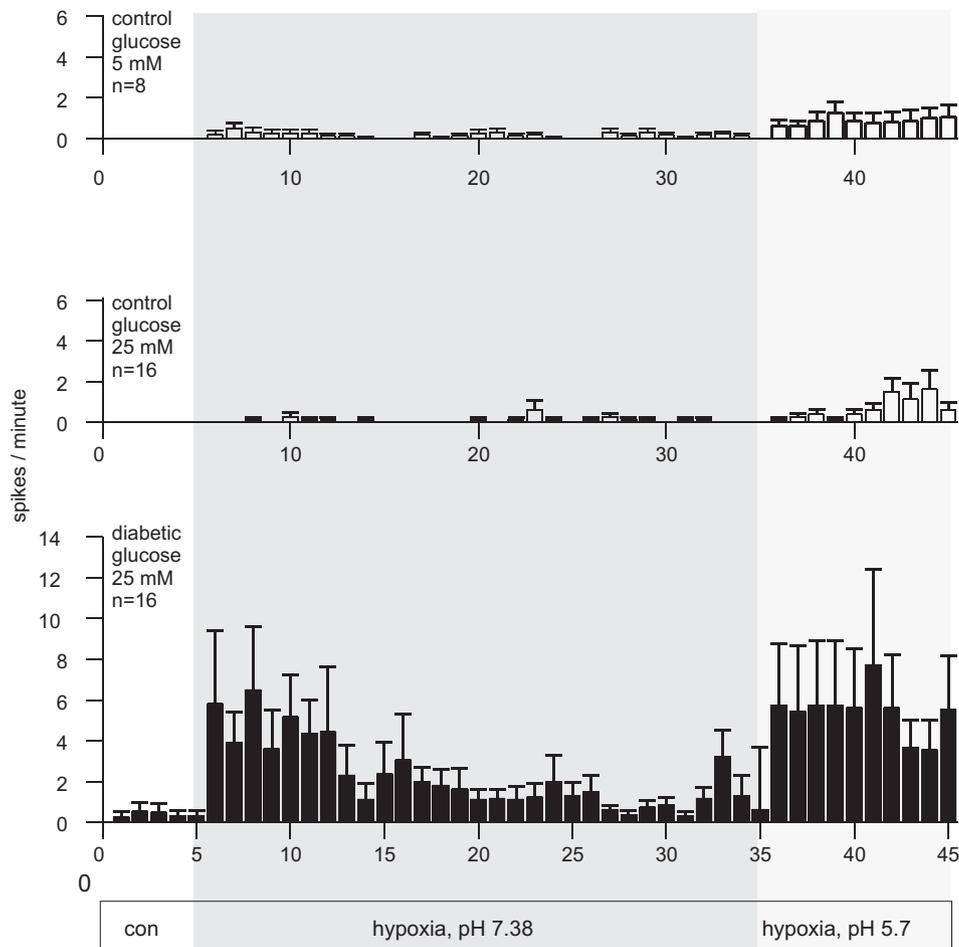

Fig. 4. Relative changes of the mechanical v. Frey thresholds of C-fibers of diabetic and healthy control rats during hypoxia and acidotic hypoxia of the receptive fields. During hypoxia mechanical thresholds decreased significantly and increased under acidic conditions to baseline values. There were no significant differences between diabetic and normal rats.

Fig. 5. Single-fiber discharge activity of cutaneous nociceptors recorded from the saphenous nerve, *in vitro*. Averaged histograms of responses to hypoxic (dark gray box) and subsequent hypoxic/acidotic superfusion (light gray box) of the receptive fields. Skin-nerve preparations, *ex vivo*, from diabetic animals were recorded 4 weeks after STZ-diabetes induction. C-fibers from diabetic animals exhibited greatly increased ongoing activity under both hypoxic and acidotic conditions providing evidence for increased excitability and acid sensitivity (for statistics see Section 3).

4. Discussion

We have used behavioral, neurochemical and electrophysiological techniques to characterize nociceptor functions in diabetic rats. The behavioral studies exhibited reduced mechanical and heat thresholds in 4 weeks-diabetic animals. Basal neuropeptide release from the skin was reduced, whereas the stimulated release was augmented relative to controls. Acutely mimicking diabetic tissue conditions by inducing hyperglycemia, hypoxia, and reducing buffer capacity, nociceptors from diabetic animals showed an increased ongoing activity compared to healthy controls under normal as well as hyperglycemic conditions. Increased nociceptor activity and sensitivity during hyperglycemic hypoxia could be a mechanism of e.g. burning pain attacks in painful diabetic neuropathy.

4.1. Hyperalgesia to heat and pressure

Four weeks after STZ induction of severe diabetes the rats exhibited hyperalgesia to heat and strong mechanical stimulation.

The results are in good agreement with the literature which frequently reports on hypersensitivity to mechanical stimulation [1,2,12,13,17,40]. A decrease of the pressure withdrawal threshold by 30–40% after 3 weeks of STZ-diabetes is reported by Romanovsky et al. and comparable to our results [51]. Hyperglycemia and the activation of aldose reductase and polyol pathway are made responsible for this development of mechanical hypersensitivity [17,51].

Reports on altered thermal sensitivity are much more variable; hyperalgesia is reported as well as hypoalgesia or unchanged thresholds [13,24,33,40]. Fox et al. observed an early thermal hypoalgesia 3 days after STZ treatment which normalized within the first 4 weeks after STZ treatment; heat hyperalgesia was not found [24]. Conversely Pabbidi et al. reported an early phase of heat hyperalgesia in mice 1–3 weeks post-STZ treatment turning into hypoalgesia after 6 weeks [45]. The discrepancies may relate to the different durations of the diabetic condition and to the relatively poor state of health of the animals 4 weeks after STZ treatment, as observed by us (Fig. 1) and reported by others [13,24].

4.2. Neuropeptide and prostaglandin E_2 release

Absolute peak values of bradykinin-stimulated release of SP and CGRP were not changed in diabetic skin (see Table 1), but the effectiveness of bradykinin to stimulate neuropeptide and PGE_2 release was significantly increased (see Fig. 3) and may be due to a reported up-regulation of bradykinin B1 receptors [66]. Different lines of functional evidence point to an involvement of the B1 receptors in diabetic hypersensitivity of nociceptors [26,10,14]. B1 receptor activation led to increased paw edema formation in STZ-treated rats [10], and diabetic B1 knockout mice did not show signs of hyperalgesia [26].

The Gq/11 protein-coupled B1 receptor exerts its sensitizing effect finally through an activation of phospholipase A2, mobilizing

arachidonic acid for prostaglandin formation, and through protein-kinase C (PKC) which increases heat responsiveness of sensory neurons [11]. A corresponding up-regulation of COX2 in peripheral nerves and DRG neurons has also been shown in diabetes [25,35]. Up-regulated COX2 should lead to increased stimulated prostaglandin formation which was in fact the case in response to BK stimulation of the diabetic skin. Secondary PGE$_2$ formation contributes essentially to nociceptor sensitization to both heat as well as acid stimulation [46,71].

Capsaicin-stimulated neuropeptide release from the desheathed sciatic nerve [52], was reduced in the diabetic rats, most likely due to the impaired axonal transport of neuropeptides [15,16,18,22]. A reduced function of the capsaicin receptor-channel TRPV1 is not likely, since the thermal hyperalgesia of diabetic mice has been reported to depend on TRPV1 [34,45]. However, in the hearts of diabetic mice lowered levels of CGRP and TRPV1 protein were found [60]. Also in diabetic rat DRG neurons, the overall TRPV1 protein was decreased, but its plasma membrane fraction, the homotetrameric form of TRPV1, and its degree of phosphorylation were increased which resulted in larger capsaicin and proton-activated currents [31].

4.3. Single-fiber electrophysiology

Consequences of hyperglycemia such as oxidative stress, activation of polyol pathways, production of Advanced Glycation End Products (AGE) lead to impairment of nerve blood flow both with endoneurial hypoxia and resulting acidosis which are considered to be important factors for the development of painful diabetic neuropathy [8,59]. By accumulating lactic acid hyperglycemic hypoxia exhausts the HCO$_3^-$ stores as it has been shown in the brain [37]. Thus, low buffering capacity of diabetic nerves is another factor that finally contributes to the hyperexcitability of sensory neurons [55]. In our electrophysiological experiments, we mimicked these pathological conditions. The most obvious result was the low-frequent ongoing activity of C-fibers of diabetic animals induced by hyperglycemic hypoxia and this was in clear contrast to healthy animals. The second potentially important result was that nerves from diabetic rats were significantly more sensitive to hypoxic acidosis. These results confirm the working hypothesis that the major pathological changes in diabetic nerves act in concert to provoke ongoing activity of primary afferents and not just an increase in excitability (see below). In human microneurography on elderly diabetic patients, some spontaneous C-fiber activity at rest was found in the subject with as well as without pain, however no provocation was tried [44]. The "submaximal effort tourniquet technique" might be able to differentiate between these patient subpopulations [32].

4.3.1. Hyperglycemic hypoxia

Hyperglycemic hypoxia is assumed to boost the metabolic rate in glycolysis leading to an accumulation of lactic acid. It is very likely that chronically diabetic nerve fibers with induced glycolytic enzymes are more efficient than healthy ones in gaining energy from anaerobic glycolysis and, thus, inducing lactacidosis [56]. Sensory nerves exhibit a particular susceptibility to hyperglycemic hypoxia [55,63] that provokes an increase in neuronal input resistance accompanied by a depolarization as measured by recording the DC potential from isolated dorsal roots [28]. The endoneurial acidification is suggested to be the reason for both increased membrane resistance and depolarization that are caused by a proton block of K$^+$ channels [28,57]. In the same experimental setting the potassium channel blockers 4-aminopyridine (4-AP) and tetraethylammonium (TEA) were only able to induce hyperexcitability as found under hyperglycemic hypoxia [57]. In cutaneous nociceptors, however, application of 4-AP and/or TEA to the recep-

tive field regularly induced ongoing activity in the absence of any stimulation [36]. Excitability testing in human diabetic nerves also revealed decreased nodal potassium conductance in myelinated fibers of hyperglycemic patients [41].

In diabetes the Na$^+$/K$^+$ pump is functionally and structurally modified [48,58]. Chronic impairment of the Na$^+$/K$^+$ pump would enhance the membrane depolarization caused by hypoxic hyperglycemia, but this would rather decrease neuronal excitability, because voltage-gated sodium channels (VGSC) would be driven into steady-state inactivation. Only nociceptive nerve terminals (rather than the axons [47]) could gain in excitability, because they are well equipped with the relatively inactivation-resistant TTX-sensitive Na$_v$1.8-subtype of VGSC that operates as a default action potential generator [7,70].

4.4. Hypoxic acidosis

In isolated peroneal nerves from healthy rats, a 30 min period of hyperglycemic hypoxia doubles the endoneurial proton concentration (pH drop by 0.3) [27,63]. In diabetic neuropathy with induced anaerobic glycolysis, the hypoxia may lead to even lower pH values in the nerve, sufficient to induce ongoing discharge. However, the pH threshold (pH 6.9–6.1) may not be reached in acid-sensing cutaneous nociceptors [61]. Therefore, we challenged the nerve endings with pH 5.7. In spite of the continued hypoxia (further 10 min), the vast majority of the C-fibers responded with an increased rate of ongoing discharge, in particular the diabetic nerve fibers. This reflects the established paradox that diabetic nerves, in spite of their precarious condition, show supernormal resistance to hypoxia with respect to nerve conduction velocity [56]. As acid sensor we consider the capsaicin receptor-channel TRPV1 that is overexpressed in the plasma membranes of DRG neurons and dermal nerve fibers of diabetic rodents, and capsaicin- and proton-induced whole-cell currents are increased [31,45]. Finally, the proton-induced CGRP release from isolated mouse skin is almost completely abrogated in TRPV1 knockout mice (S. Sauer and P. Reeh, unpublished observation).

4.5. Heat and mechanical sensitivity

In agreement with previous work on the skin-nerve preparation, no obvious differences in basal sensory properties of the C-fibers were found between diabetic and healthy animals [64]. Apparently, no correlate to the thermal and mechanical hyperalgesia of the diabetic rats could be established. However, Suzuki et al. could enhance the mechanical responsiveness of polymodal nociceptors in diabetic skin by superfusing the receptive fields with a high-glucose solution [65]. Likewise, mechanical hyperalgesia was not only induced by STZ-diabetes, but could also be elicited in healthy rats by chronic exposure of the L5-DRG or sciatic nerve to a 30 mM glucose solution [17]. While these findings point to an exaggerated glucose metabolism as a neuropathological mechanism, our experiments with mere hypoxia show an acute sensitizing effect to punctuate mechanical but not heat stimulation in healthy animals. The mechanism of this mechanical sensitization is unclear, but it may relate to the "pins and needles" sensation associated with tourniquet ischemia of a limb. The strong subsequent acidosis (pH 5.7), added to the hypoxia in our experiments, "normalized" the mechanical sensitivity, perhaps by a proton block of the nerve fibers like the one that affects voltage-gated sodium channels [54].

5. Conclusion

Four weeks after induction of diabetes in rats an increased responsiveness of the peripheral nociceptors is demonstrated on three levels of evidence. The findings present peripheral nervous

changes that may correlate to "positive" symptoms of painful diabetic neuropathy, and they support and extend the concept [27] that reduced blood flow in diabetic skin and nerve together with elevated glucose generates local lactacidosis rendering small nerve fibers hyperexcitable and eventually spontaneously active.

Conflict of interest statement

Acknowledgements

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