NEUROGENIC INFLAMMATION AND REFLEX SYMPATHETIC DYSTROPHY (*IN VIVO* AND *IN VITRO* ASSESSMENT IN AN EXPERIMENTAL MODEL)

M. DAEMEN[1], H. KURVERS[1], P. BULLENS[1], G. BARENDSE[2], M. VAN KLEEF[2], F. VAN DEN WILDENBERG[1]

In the chronic constriction injury (CCI) model, signs and symptoms similar to those observed in reflex sympathetic dystrophy (RSD) can be induced by loosely ligating a rat sciatic nerve. Skin microcirculatory (inflammation-like) disorders may result from release of vasoactive neuropeptides at peripheral endings of antidromically acting nociceptive nerve fibers. These antidromic mechanisms may account for vasodilation and polymorphonuclear leukocyte (PMN) accumulation in the ligated hindpaw. We assessed skin blood flow (SBF) on the ligated side, by means of laser Doppler flowmetry, before as well as at day 4 after ligation. Postligation SBF measurements were performed before and after selective (capsaicin) conduction blockade of the ligated sciatic nerve. The extent of PMN accumulation was determined by measuring myeloperoxidase (MPO) activity in muscle biopsies obtained from the ligated and contralateral nonligated side. As compared to preligation SBF values, we observed an increase at day 4. SBF returned to preligation values consequent to capsaicin application. MPO activity, when compared to the nonligated side, was higher in biopsies obtained from the ligated side. These findings indicate that in the CCI-model, antidromically acting C-nociceptor nerve fibres increase SBF at 4 days after ligation. In addition, these antidromic mechanisms may induce an inflammatory response in the ipsilateral hindpaw, mediated by release of neuropeptides from the peripheral endings of antidromically acting C-nociceptor nerve fibers. This inflammatory response may account for various signs and symptoms as observed in the CCI model and may mirror pathophysiological mechanisms of RSD.

Keywords : reflex sympathetic dystrophy ; algodystrophy ; neurogenic inflammation ; skin blood flow.

Mots-clés : dystrophie réflexe sympathique ; algodystrophie ; inflammation neurogène ; débit sanguin cutané.

Provocation of reflex sympathetic dystrophy (RSD) has been purported to involve injury to either central or peripheral neural tissue, including peripheral nerve twigs (33). Experimental animal models, such as the chronic constriction injury (CCI) model introduced in 1988 by Bennett and Xie, have been proven to be of importance to the investigation of pathophysiological mechanisms underlying RSD (1, 2, 26, 41). It has been demonstrated that mechanical (34) and electrical (11, 19) excitation of C-nociceptor nerve fibers at the midaxon level may provoke release of neuropeptides at peripheral endings of these fibers (4, 5, 19). Substance P (SP), one of these released neuropeptides, has been demonstrated to have inflammatory as well as immune modulating actions. Among others, it decreases vascular tone (19) and increases extravasation of leukocytes (13, 38). These inflammatory processes resulting from release of neuropeptides have been termed neurogenic inflammation (6, 40). Excitation of C-noci-

[1] Department of General Surgery.

[2] Department of Anesthesiology of the University Hospital Maastricht, Maastricht, The Netherlands.

Correspondence and reprints : M. Daemen, Department of General Surgery, University of Maastricht, P.O. Box 616, 6200 MD Maastricht, The Netherlands.

ceptor nerve fibers can be provoked by ligation of the common sciatic nerve in the rat (35). Hence, release of neuropeptides at the peripheral endings of nociceptor nerve fibers is likely to be present. If the latter is true, one would expect vasodilation and polymorphonuclear leukocyte (PMN) accumulation in the ligated hind paw. To investigate the actual involvement of the mentioned processes, we investigated skin blood flow before and after (at day 4) loose ligation of the sciatic nerve. In addition, we studied the possible influence of sensory nerve-fiber blockade (by means of perineural application of capsaicin) on the vasodilator response induced by sciatic nerve ligation. In a second group of rats we investigated PMN accumulation by means of measuring tissue myeloperoxidase (MPO) activity.

MATERIALS AND METHODS

Surgery

Adult male Lewis rats (n = 10), weighing 250-350 grams at the start of the experimental protocol, were used. Neuropathy was induced according to a procedure described by Bennet and Xie (1). Briefly, rats were anesthetized with 100 mg/kg ketamine hydrochloride I.P. (Nimatek, AUV, Cuijk, The Netherlands) and 0.5 mg diazepam S.C. (Centrafarm BV, Etten-Leur, The Netherlands). The administration of ketamine hydrochloride I.P. was repeated if necessary. Under aseptic conditions, the right sciatic nerve was exposed from high-thigh to mid-thigh level, just proximal to the sciatic trifurcation into the tibial, sural and peroneal nerves. Under guidance of a dissecting microscope (25 × magnification), 4 chromic catgut ligatures (4-0, Ethicon, Norderstedt, Germany) with about 1 mm spacing were tied loosely around the common sciatic nerve. The wound was closed with mersilene muscle sutures (5-0, Ethicon) and mersilene skin sutures (2-0, Ethicon). All procedures were carried out under a protocol approved by the Institutional Animal Care Committee of the University of Limburg, The Netherlands.

Laser Doppler flowmetry

Laser Doppler flowmetry (LDF) is a noninvasive technique which allows continuous evaluation of skin microcirculatory blood flow (24). The system evaluates the Doppler shift of laser light, backscattered by moving blood cells. Measurements were performed with a commercially available system (Perimed PF3 with standard angled probe (90°), Perimed, Linköping, Sweden). The sample volume is about 1 mm^3. The following settings were used : time constant of 2 seconds, to avoid heart beat oscillations in the signals, low pass band filter "on" in order to reduce movement artifacts ; frequency limited to 0.07-12 kHz band. An output of 1 volt was calibrated against 100 Perfusion Units (PU).

The animals were anesthetized as described above. Body temperature was kept at 37°C with an infrared heating lamp controlled by a thermo-analyzer system (Hugo-Sachs Elektronik, March-Hugstedden, Germany) connected to a rectal probe. The ambient temperature was maintained at 21-23°C. Each animal was placed in the prone position on a plastic floor adjustable in height. The plantar surface of the right hindpaw was secured in the desired position with adhesive tape. Subsequently, laser Doppler flowmetry was employed to assess skin blood flow (fig. 1). LDF measurements were performed within a standardized skin area, consisting of a prominence located at the lateral side of the plantar surface of the right hindpaw. Within this specific area, the probe was moved about in order to determine maximal skin blood flow. The skin area under investigation is innervated by the tibial nerve, which originates from the sciatic nerve (39). A micromanipulator (Leitz 115001, Leica BV, Rijswijk, The Netherlands) was used to position the probe close to the surface of the site of measurement. After signal

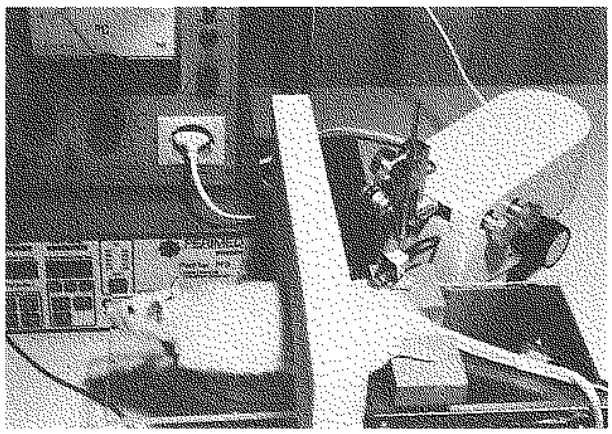

Fig. 1. — Photomicrograph showing the experimental setup for laser Doppler flowmetry. Animals were placed in the prone position on a plastic floor adjustable in height. The plantar surface of the right hindpaw was secured and laser Doppler flowmetry was employed to assess skin blood flow.

stabilization for at least three minutes, skin blood flow was assessed for three minutes. The analogue output of the laser Doppler system was digitized by an analogue-to-digital converter and saved on hard disk. Off-line analysis was performed with a custom-made software program.

LDF was employed to measure skin blood flow (SBF) in the first group of rats (n = 10) before as well as at day 4 after ligation. Measurements were performed before and after exposure of the loosely ligated sciatic nerve as well as after blockade of conduction of sensory (C-nociceptor) function by means of perineural capsaicin application distal to the site of ligation. To this end, a thin layer of plastic foil was placed underneath a segment of the exposed sciatic nerve after which cotton containing capsaicin 1% solution was applied to the nerve (20, 43). Capsaicin was used because it selectively blocks conduction of C-nociceptive nerve fibers (11). Moreover, axoplasmatic flow of vasodilator neuropeptides to the periphery is blocked (29), whereas the axoplasmatic flow of acetylcholinesterase and noradrenaline is not interrupted, indicating that efferent motor and sympathetic nerve fibers are not affected (10). The cotton containing capsaicin solution was removed after 15 min., after which excess capsaicin solution was absorbed with a dry piece of cotton. The effectiveness and selectiveness of a 15 minutes perineural application of this neurotoxin has been demonstrated previously (29). SBF was assessed for 3 min. at 10 and 60 min. following the 15 min. capsaicin application.

Myeloperoxidase (MPO) assay

Tissue MPO content can be used as a reliable index of PMN accumulation (3, 21, 31). MPO is an enzyme that is essential for the oxygen-dependent bactericidal system of PMNs (14) and 5% of the PMN weight consists of MPO (32). The extent of PMN sequestration was assessed by measuring tissue activity of the granulocyte-specific enzyme MPO. Muscle biopsies were taken from the medial gastrocnemius and anterior tibial muscle (innervated as well by the tibial nerve). A total amount of 1 g of biopsies from medial gastrocnemius and anterior tibial muscle was homogenized for 60 sec. in potassium phosphate buffer (pH 7.4) using a tissue homogenizer (Omni International 2000). Homogenates were centrifuged at 12,000 *g* for 5 min. in 1 ml potassium phosphate buffer (pH 7.4) containing 1 µl 0.4% triton X-100 and the supernates were collected. MPO activity was assayed by measuring the H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine (TMB). The reaction mixture for analysis consisted 50 µl sample and supernatant in a 1:50 dilution of potassium phosphate buffer (pH 7.4), 50 µl TMB-reagent and 50 µl H_2O_2-reagent (both were obtained from Kirkegaard and Perry, Gaithersburg, MD). The reaction was evaluated in a 96-well microtiter plate. The mixture was performed for 10 min. at 37°C and stopped with 50 µl 1M H_2SO_4, after which optic density (OD) was measured in a microprocessor-operated micro-ELISA autoreader (Murex Biotech, Dartford, UK). MPO activity was defined as the OD measured from reaction mixtures at 450 nm. MPO activity was determined, 4 days after loose ligation of the right sciatic nerve, in muscle biopsies obtained from both hindpaws (n = 10). Subsequently, the data from the ligated side were compared with those from the nonligated side.

Statistics

Results obtained with LDF are expressed normalized relative to control values (value of interest divided by the corresponding preligation value) and compared with ipsilateral preligation values. The Wilcoxon-signed ranks test for paired data and the Mann-Whitney U test for unpaired data were used to test for significant differences. All data are presented as medians with their interquartile ranges. Overall, significance was defined as $p < 0.05$.

RESULTS

Laser Doppler flowmetry

Figure 2 shows that, when compared to preligation values (normalized value = 1, interquartile ranges (absolute) 22-64 PU), SBF was increased at day 4 (1.94). Nerve exposure did not influence SBF (1.81). Subsequent capsaicin application overcame the increase in SBF induced by ipsilateral loose sciatic nerve ligation. As a result, SBF after capsaicin application did not differ from preligation values (0.83 at 10 min. and 0.80 at 60 min. after application).

MPO activity

MPO activity (fig. 3) was higher in muscle biopsies obtained from the ligated side when compared with those obtained from the nonligated side (0.91 vs 0.55 ; $p < 0.05$).

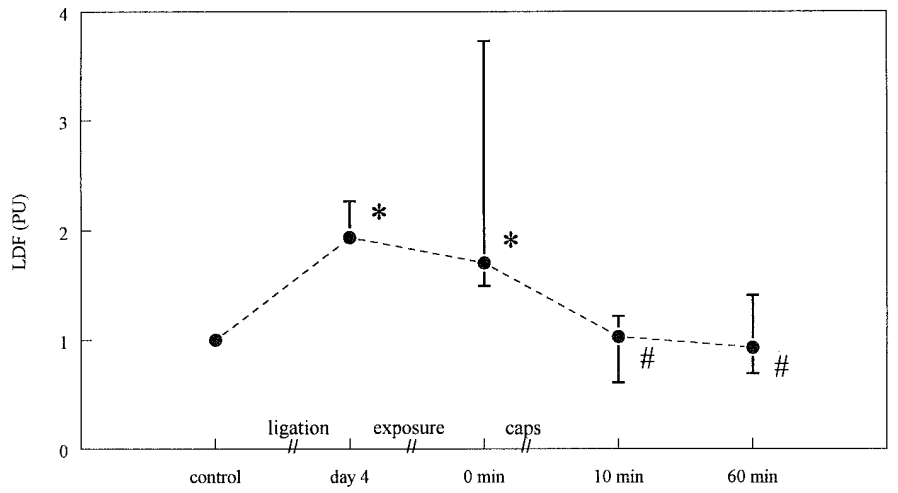

Fig. 2. — Representation of skin blood flow in the plantar footpad of the rat before and 4 days after ligation. At 4 days after ligation the ligated sciatic nerve was exposed and perineural capsaicin was applied. Skin blood flow was re-determined at 10 and 60 minutes after capsaicin application. Asterisks indicate the level of statistical significance as compared to preligation values ($p < 0.05$). Crosses indicate the level of statistical significance as compared to skin blood flow values before capsaïcin application ($p < 0.05$). All data are shown as medians with interquartile ranges.

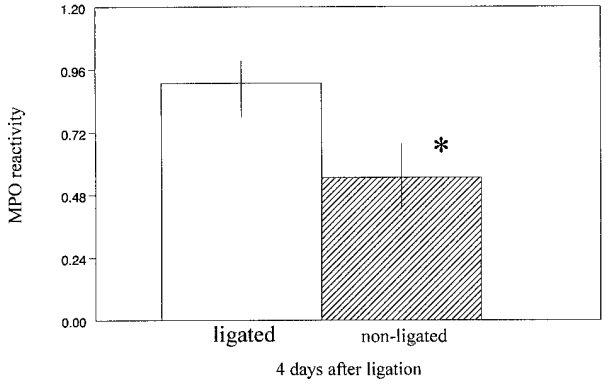

Fig. 3. — MPO reactivity in skeletal muscles obtained from the ligated and contralateral non-ligated side at 4 days after ipsilateral sciatic nerve ligation. The asterisk indicates the level of statistical significance as compared to the ligated side ($p < 0.05$). All data are shown as medians with interquartile ranges.

DISCUSSION

These results show that loose ligation of a sciatic nerve in rats induces increased microvascular skin perfusion in the ligated hindpaw, which is overcome by blockade of sensory (C-nociceptor) nerve fibers by perineural application of the neurotoxin capsaicin. Additionally, the constrictive procedure induces accumulation of polymorphonuclear leukocytes (PMN's) in muscle tissue obtained from the lower leg. These results suggest the presence of neurogenic inflammation in this animal model of RSD and attest to the involvement of antidromically acting sensory nerve fibers in this inflammatory process.

Four days after ligation, skin blood flow was increased as compared to preligation values, which agrees with our previous observations (16). Our finding that perineural application of capsaicin reduced this vasodilator response argues in favor of the involvement of antidromically acting sensory nerve fibers in the CCI-model. This vasodilator response involves only C-nociceptor nerve fibers, since C-nociceptor but not Aδ nerve fibers mediate the release of vasodilator neuropeptides (6).

In the CCI-model we observed an increase of myeloperoxidase (MPO) activity in skeletal muscle obtained from the ligated side when compared with the contralateral (nonligated) side. This finding indicates increased levels of tissue PMN's (3, 21, 31). It has been reported that neuropeptides may increase the level of tissue PMN's. In these studies, it was shown that substance P, one of the most important neuropeptides (6, 9), induces synthesis and release of monocyte-derived cytokines, stimulates T-lymphocyte proliferation and

promotes PMN chemotaxis (13, 25, 27, 28). Besides, this neuropeptide activates mast cells (8), which produce mediators (such as Eosinophil Chemotactic Factor (ECF) and Neutrophil Chemotactic Factor (NCF)) that can attract PMN's (15, 37). These observations, in conclusion, suggest that in the CCI-model the observed increase in tissue content of PMN's may also be related to the reported release of neuropeptides from the peripheral endings of C-nociceptor nerve fibers.

This study is the first to demonstrate that excitation of sensory nerve fibers through loose ligation of a peripheral nerve provokes a vasodilator response as well as increased levels of PMN's. The presence of these phenomena consequent to release of neuropeptides has been referred to as neurogenic inflammation (6, 20). Excitation of sensory nerve fibers in the CCI-model (12) may result from the constrictive procedure itself or from formation of granulation tissue.

Patients suffering from reflex sympathetic dystrophy (RSD) demonstrate clinical signs and symptoms indicative of inflammation (42). The similarities between the CCI-model and the clinical characteristics of RSD attest to the usefulness of the CCI-model in the investigation of pathophysiological mechanisms of RSD. Moreover, it is tempting to speculate that release of neuropeptides consequent to excitation of sensory nerve fibers also occurs in patients suffering from this neuropathic syndrome. The concept of involvement of neurogenic inflammation in RSD is supported by observations of increased skin blood flow (17, 18) in RSD patients. As mentioned before, release of neuropeptides from the terminal endings of peripheral nerves may induce remote inflammation. In addition, these neuropeptides have been reported to increase the excitability of sensory nerve fibers (7). Hence, in the CCI-model, release of neuropeptides may also contribute to sensory abnormalities. In line with the concept of neurogenic inflammation, pharmacologic interference with axoplasmatic flow and / or release of neuropeptides may prevent sensory disturbances and skin blood flow abnormalities.

The latter hypothesis is supported by a study of Meller *et al.* (23), demonstrating that administration of neonatal capsaicin (which induces degeneration of C-nociceptive nerve fibers that normally release neuropeptides) relieves Aδ-mediated allodynic responses in the CCI-model.

In conclusion, the findings of the present study suggest that loose ligation of a sciatic nerve induces an inflammatory response in the ipsilateral hindpaw, mediated by release of neuropeptides from the peripheral endings of antidromically acting C-nociceptor nerve fibers. This inflammatory response accounts for various signs and symptoms as observed in the CCI-model and may account for aspects of the clinical presentation of RSD.

Acknowledgements

We wish to thank Mrs. Trudi Jeunhomme for excellent technical assistance.

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SAMENVATTING

M. DAEMEN, H. KURVERS, P. BULLENS, G. BARENDSE, M. VAN KLEEF, F. VAN DEN WILDENBERG. Neurogene ontsteking en RSD. In vivo en in vitro bevindingen in een experimenteel model.

Het chronic constriction injury (CCI) model, is een diermodel waarin de symptomen van reflex sympathische dystrofie (RSD), een neuropathisch pijnsyndroom dat onder andere wordt gekemerkt door op ontsteking gelijkende symptomen, worden geïnduceerd via het aanbrengen van vier losse ligaturen rond de n. ischiadicus. Wij onderzochten de hypothese dat in dit diermodel vasoactieve neuropeptiden vrijgekomen uit perifere zenuweinden van antidromaal gestimuleerde nociceptieve c-vezels een toename induceren in zowel microcirculatiore huiddoorbloeding als accumulatie van polymorfonucleaire leukocyten (PMN). Huid doorbloeding (SBF) werd gemeten aan geligeerde zijde met laser Doppler flowmetrie zowel vóór als 4 dagen na ligatie. SBF-metingen na ligeren vonden plaats vóór en na selectieve c-vezel blokkade van de geligeerde n. ischiadicus met capsaïcine. PMN accumulatie werd bepaald door middel van het meten van myeloperoxidase (MPO) activiteit in spierbiopten verkregen uit de geligeerde en niet-geligeerde achterpoot. SBF was toegenomen op dag 4 in vergelijking met metingen verricht vóór ligatie en keerde terug naar deze normaalwaarden na applicatie van capsaïcine. MPO activiteit was hoger aan geligeerde zijde dan aan niet-geligeerde zijde. Deze bevindingen wijzen op betrokkenheid van antidromaal gestimuleerde c-vezels bij de toename in SBF na ligatie. Deze antidromale mechanismen dragen, via uiteinden van antidromaal gestimuleerde c-vezels vrijgekomen neuropeptiden, bij aan de ontstekingsreactie waargenomen in de geligeerde achterpoot. Deze ontstekingsreactie is mogelijk bepalend voor pathofysiologie en symptomatologie van zowel het CCI model alsook RSD.

RÉSUMÉ

M. DAEMEN, H. KURVERS, P. BULLENS, G. BARENDSE, M. VAN KLEEF, F. VAN DEN WILDENBERG. Inflammation neurogénique et dystrophie réflexe sympathique. Étude in vivo et in vitro à l'aide d'un modèle expérimental.

Dans le modèle de constriction chronique, une symptomatologie similaire à celle présentée par les patients souffrant de dystrophie réflexe sympathique peut être induite chez le rat par ligature lâche du nerf sciatique. Dès modifications vasomotrices cutanées caractéristiques de l'inflammation peuvent résulter de la libération de neuropeptides vasomoteurs au niveau de l'extrémité périphérique de fibres nerveuses nociceptives, agissant de manière antidromique. Ces mécanismes antidromiques peuvent rendre compte de la vasodilatation et de l'accumulation de polynucléaires au niveau du membre ayant subi la ligature. Les auteurs ont mesuré le débit sanguin cutané du côté de la ligature, par débitmétrie laser Doppler avant et au 4ème jour après la ligature. Ces dernières mesures ont été réalisées avant et après blocage sélectif de la conduction du nerf sciatique par capsaïcine. L'importance de l'accumulation des leucocytes polynucléaires a été déterminée par mesure de l'activité en myéloperoxydase sur les biopsies musculaires obtenues des côtés ligaturé et non ligaturé. Par rapport à la situation avant ligature, les auteurs ont observé une augmentation du débit sanguin cutané au 4ème jour. Le débit revenait à des valeurs normales après application de capsaïcine. L'activité en myéloperoxydase, par comparaison au côté sain, était plus élevée sur les biopsies obtenues du côté ligaturé. Ces résultats confirment l'augmentation du débit sanguin cutané, 4 jours après la ligature, sous l'action des fibres nociceptives agissant de manière antidromique. De plus, ces mécanismes antidromiques peuvent induire une réponse inflammatoire au niveau du membre ipsilateral, suite à la libération de neuropeptides par les fibres nerveuses nociceptives. Ces réactions inflammatoires pourraient expliquer la symptomatologie observée dans le modèle de constriction chronique, et expliquer la physiopathologie de la dystrophie réflexe sympathique.