The cardioprotective role of sensory nerves in adriamycin-induced experimental cardiomyopathy

PhD Thesis

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II. Boros K, Jancsó G, Dux M, Fekécs Z, Bencsik P, Oszlács O, Katona M, Ferdinandy P, Nógrádi A, Sántha P. Multiple impairments of cutaneous nociceptor function induced by cardiotoxic doses of Adriamycin in the rat. Naunyn-Schmiedeberg's Arch. Pharmacol. 2016, 389:1009-1020. Impact factor: 2.376

INTRODUCTION

Anthracycline derivatives are cytotoxic agents widely used for the treatment of various malignancies, although they have significant adverse effects. Their serious side-effects include cardiotoxicity, which may compromise the cardiac function and result in cardiomyopathy and even heart failure. Dilatative cardiomyopathy develops in a dose-dependent manner both in man and in animal models. Despite extensive clinical and experimental studies, the pathophysiology of anthracycline-induced cardiomyopathy is still unclear. Several mechanisms have been shown to contribute to the development of anthracycline-induced cardiomyopathy, including free radical production and lipid peroxidation, impairment of DNA replication and interaction with intracellular calcium homeostasis. More recently, a cardioprotective role of peptidergic sensory nerves has been revealed which significantly contributes to the mechanisms of ischemia reperfusion-induced cardiac preconditioning. It has been established that selective elimination of peptidergic sensory nerves by the specific sensory neurotoxin, capsaicin greatly reduced the cardioprotective effect of ischemic precondition in an isolated rat heart preparation. Further, it has also been demonstrated that ischemia-reperfusion-induced cardioprotection is mediated to a significant extent through calcitonin gene-related peptide released from chemosensitive sensory nerves which express the capsaicin/transient receptor potential vanilloid type 1 (TRPV1) receptor. Although the sensory innervations of the heart are well established, a possible pathophysiological role of cardiac sensory nerves in cardiac pathologies has not been considered. Therefore, we have initiated experimental studies on a well established animal model of adriamycin-induced dilatative cardiomyopathy to explore the role of sensory nerves in the development of pathological changes associated with this condition. Cardiac function was assessed with serial echocardiographic examinations to monitor the development of cardiomyopathy following the administration of adriamycin to intact rats and to rats treated with capsaicin to eliminate cardiac peptidergic afferent nerves. Our findings indicated that cardiac sensory nerves significantly contribute to the development of cardiac functional impairments induced by adriamycin. Since neurotoxicity is a well known side effect of anthracycline-type compounds, these findings raised the possibility that a toxic effect of adriamycin exerted on sensory nerves may contribute to the development of cardiac pathologies. Therefore, further studies were initiated to explore the effects of adriamycin on the functions of chemosensitive afferent nerves. Hence, the effects of adriamycin were studied on the classical sensory functions of afferent nerves, i.e. the mediation of nociceptive information, and on the sensory efferent/local regulatory, vascular functions of afferent nerves brought about

through the neural release of vasoactive and inflammatory neuropeptides. In these experiments the effects of adriamycin were examined on cutaneous sensory nerves which comprise the best characterized population of chemosensitive afferent nerves and share many morphological, functional and neurochemical traits with visceral afferents.

The aims of the present Thesis were as follows:

1) The exploration of the possible cardioprotective effects of cardiac chemosensitive peptidergic nerves in an established experimental model of adriamycin-induced cardiomyopathy by comparing the severity and dynamics of progression of cardiac failure/damage in control and in capsaicin-pretreated chemodenervated rats.

2) The evaluation of the neurotoxic effects of adriamycin on the local regulatory, sensory efferent functions of cutaneous chemosensitive afferent nerves by studying the dose- and time-dependent effects of adriamycin on cutaneous neurogenic sensory vasodilatation, neurogenic plasma extravasation, inflammatory hyperalgesia and the axonal release of CGRP.

3) The quantitative morphological and immunohistochemical analysis of the effects of adriamycin on cutaneous innervation and skin structure.

MATERIALS AND METHODS

This study conformed fully to the "Principles of laboratory animal care" and was approved in advance by the Animal Research Ethics Committee of the University of Szeged.

Adriamycin treatment

Adult male Wistar rats weighing 250 ± 30 g at the start of the experiments were used in the study. The animals received a cumulative dose of 15 mg/kg of adriamycin by systemic injection of 2.5 mg/kg of the drug three times a week for 2 weeks (introduced by Tong in 1991). The control rats received equivalent amounts of the vehicle (saline). Tests other than echocardiography were made 2-7 days after the cessation of the adriamycin treatment.

Selective sensory chemodenervation induced by systemic capsaicin treatment

A group of rats (*n*=23) was pretreated with capsaicin or its vehicle (n=6) under ether anesthesia 2 weeks prior to the induction of adriamycin treatment. This capsaicin treatment paradigm has been shown to result in a practically complete elimination of CGRP-containing cardiac sensory nerves.

Echocardiac assessment of cardiac function

Cardiac function was assessed by echocardiographic examination before and at regular intervals after capsaicin and/or adriamycin treatment as described by Schwarz et al in 1998. The left ventricle (LV) was examined in the parasternal long-axis view at the level of the mitral valve, or in the parasternal short-axis view at the level of the papillary muscles. The LV diameters were measured by means of M-mode echocardiography between the endocardial borders. The LV end-diastolic diameter (LVDD) was measured at the longest diameter of the LV. The LV end-systolic diameter (LVSD) was measured at the shortest diameter of the LV. The fractional shortening (FS) was calculated by using the LV diameters (LVDD–LVSD)/LVDD, and was expressed as a percentage. The left atrial diameter (LAD) and aortic diameter (AOD) were measured by M-mode echocardiography at the level of the longest LAD. The ratio LAD/AOD was calculated. Values are given as means ± SEM. Pericardial and pleural effusion was detected by the examination of fluid accumulation between the layers of the epicardium and the pericardium and between the layers of the visceral and parietal pleurae, respectively, with two-dimensional and M-mode echocardiography. Ascites was visualized from the subcostal four-chamber or short-axis view below the diaphragm by means of two-dimensional ultrasonography.

Immunohistochemical examination of the heart

For cardiac immunohistochemical studies additional groups of rats treated with adriamycin (*n*=3) and capsaicin plus adriamycin (*n*=3) were perfused via the left heart ventricle with Zamboni s fixative 2 weeks after the completion of the administration of adriamycin. The hearts were removed and after a post-fixation period of 3 h they were placed in a buffer solution and stored at 4°C until sectioning. Transverse sections through the ventricles were cut at a thickness of 20 μm and were processed for immunohistochemical staining with the indirect immunofluorescence technique by using rabbit polyclonal antisera raised against protein gene product 9.5 (1:1000) and CGRP (1:500). Goat anti-rabbit IgG labeled with Cy3 (1:500) was

used as a secondary antibody. The specimens were viewed under a Leitz DMLB fluorescence microscope.

Measurement of chemically induced neurogenic cutaneous vasodilatation

Animals were anesthetized with chloral hydrate. Body temperature was maintained at 37.2 ± 0.5 ^{o}C with a heating pad. Cutaneous blood flow (CuBF) was monitored with a Laser Doppler Blood Flow (LDF) Monitor. After a stable LDF signal was attained, 20 µl of mustard oil (0.2% in ethanol) was applied onto the skin through a polyethylene cannula built into the wall of the chamber and the CuBF was recorded for 15-20 min. This procedure was repeated 45 min later with a higher concentration of mustard oil (1%). We also studied the vasodilatory effect of capsaicin (1%) in a similar set of experiments. Relative increases in CuBF were calculated by comparing the 3-min pre-drug mean CuBF with the mean of consecutive 1-min LDF signal values recorded after the application of test agents. The effects of pretreatments with the specific TRPA1 and TRPV1 antagonists, HC-030031 and capsazepine, respectively, were also tested.

Measurement of chemically induced neurogenic cutaneous plasma protein extravasation

Briefly, animals were anesthetized with chloral hydrate and injected intravenously with Evans blue dye (50 mg/kg, 1% in saline). Ten min later, the dorsal skin of the hind paws was painted with a solution of 5% mustard oil, 1% capsaicin or their solvent (liquid paraffin and ethanol, respectively). The effects of pretreatments with the specific TRPA1 and TRPV1 antagonists, HC-030031 and capsazepine, respectively, were also tested. After 20 min, sacrificed by decapitation, and samples of the dorsal paw skin were removed, weighed and placed into formamide to extract the dye for quantitative photometric determination. Tissue Evans blue contents were expressed in µg dye/g tissue as mean ± S.D. Plasma extravasation induced by histamine, bradykinin and substance P was also measured with the Evans blue technique following the intracutaneous injections of these vasoactive agents into the abdominal region. Twenty minutes after the injections, rats were sacrificed by decapitation and abdominal skin samples of standardized size were removed and their dye contents were measured.

Measurement of CGRP release in vitro

The animals were anesthetized with chloral hydrate, sacrificed by decapitation, and the sciatic nerves were removed. The epineurium was carefully removed from the nerves under

microscopic control and the samples were washed with synthetic interstitial fluid (SIF), gassed with 95% O_2 and 5% CO_2 to a pH of 7.4 at room temperature for 30 min. Thereafter, the SIF was replaced with 400 µl of the release buffer consisting of SIF supplemented with 0.1% bovine serum albumin and 16 µM thiorphan. After an incubation period of 10 min to measure the basal CGRP release, the buffer was replaced either with a release buffer containing 10 µM capsaicin or with a modified release buffer containing 60 mM KCl. The tissue samples were then further incubated for 10 min to determine the capsaicin- and the depolarization-induced CGRP release. The effect of capsazepine, a specific TRPV1 antagonist, on the capsaicin-induced CGRP release was also studied. Samples were collected at the end of each incubation period into silicone-coated Eppendorf cups, frozen and stored at -70 °C until the determination of their CGRP content. The CGRP concentrations of the samples were determined with an enzyme-linked immunoassay kit. The absorbances of the samples were measured photometrically with a microplate reader, and the CGRP contents were calculated and corrected for the tissue weights. The results (mean ± S.D.) are expressed as percentages of the basal CGRP release.

Carrageenan-induced thermal and mechanical hyperalgesia

The nociceptive paw withdrawal response to radiant heat stimulation was studied by the Hargreaves method. For the assessment of carrageenan-induced thermal hyperalgesia, rats received an intraplantar injection of carrageenan (3 mg in 0.1 ml saline) into the right hind paw. The paw withdrawal latencies were measured 3 h after the carrageenan injection, and the data were expressed as percentage changes (mean ± S.D.) from the control values.

Paw withdrawal thresholds for mechanical stimulation were measured in both hind paws with an automated apparatus suitable for the application of reproducible mechanical stimuli. Mechanical hyperalgesia was assessed after an intraplantar injection of carrageenan as described above. The latency of withdrawal responses were measured 3 h after carrageenan. The data were expressed as percentage changes (mean ± S.D.) from the control.

Kinematic analysis of hindlimb locomotor function

For the kinematic analysis of the locomotion of intact and treated animals a transparent plexiglass runway with a tilted mirror fixed under the floor plate was used. Another mirror system was fixed behind the runway in order to observe the movements of the contralateral limb, too. A square grid pattern with 1 cm intervals was scratched into the front panel for calibration purposes. The rats were trained prior to the measurements to walk from one end of

the runway to the other reaching a shelter. The hair of the animals was shaved off from the hindlimbs and the skin was marked with permanent black ink above the major joints. The locomotor pattern of the rats was recorded with high resolution and high speed digital cameras.

Locomotor data analysis

We recorded 3 runs of each animal in every session and examined 5 steps complying two criteria: one similar step was required before and after the measured step and the head of the animal had to point to the direction of walk. The appropriate video sequences were divided into single frames using the VirtualDub software and the selected frames where the animals were in a defined phase of movement were analysed by using the ImageJ software. The following parameters were determined and used in the analysis of locomotion in this study. Lateral view parameters were defined as follows. Step length: the length of the step cycle measured between the first moments of the consequent stance phases expressed in cm; tarsus off angle: the angle enclosed by the tarsus and the floor plate at the last moment of the stance phase expressed in degrees; ankle flexion: the angle enclosed by the tarsus and the tibia at the first moment of the stance phase expressed in degrees; knee flexion: the angle enclosed by the tibia and the femur at the first moment of the stance phase expressed in degrees; lateral placing: the angle enclosed by the tarsus and the longitudinal axis of the animal. We measured this parameter at the first moment of the stance phase expressed in degrees. Ankle lifting: the highest point reached by the ankle joint during the swing phase compared to its lowest position on the ground (in mm); knee lifting: the highest point reached by the knee joint during the swing phase compared to its lowest position on the ground (in mm). Rear-view parameters were defined as follows. Metatarsus-surface angle: the angle enclosed by the metatarsus and the surface (expressed in degrees) at the first moment of the swing phase when the foot has just left the ground; spreading of toes: the angle opening between the 2nd and the 4th toes is determined in degrees at the first moment of the swing phase when the foot has just left the ground.

Demonstration of cutaneous innervation by immunohistochemistry and quantitative morphometry

For immunohistochemical studies, adriamycin- or vehicle-treated rats were perfused via the left heart ventricle with 4% buffered formaldehyde solution 2-7 days after the completion of treatment. The plantar skin of the hind paws was removed and, after a post-fixation period of 3 h, was placed into a buffer solution and stored at 4 °C until sectioning. Skin samples were

taken from the mid-plantar region of the paw and transverse sections were cut at a thickness of 25 μm and processed for immunohistochemical staining with the indirect immunofluorescence technique by using antisera raised against the TRPV1 receptor (rabbit polyclonal, 1:1000-1:1500) and β-tubulin (mouse monoclonal, 1:1000). Donkey anti-rabbit and anti-mouse IgG labeled with Cy3, DL-488 (1:500) were used as secondary antibodies. Control procedures for immunolabeling were performed by replacing the primary antisera by normal donkey serum. To test the specificity of the TRPV1 antibody we have also performed a preadsorption test by applying a blocking peptide (supplied by the manufacturer of the TRPV1 antibody) representing the immunogenic fragment of TRPV1 against which the antibody was generated. No staining was observed in either case. Slides were covered with ProLong Gold Antifade Mountant with or without DAPI.

The specimens were viewed under a Zeiss confocal fluorescence microscope. Z-stack image series were collected from sections of plantar skin samples obtained from control and adriamycin-treated rats. The density of epidermal axons was estimated with the vertical projection method. This design-based stereological approach allows an unbiased estimation of the length density of linear structures in a given volume (Gokhale, 1990). The epidermal thickness was also measured along a line perpendicular to the skin surface with the aid of Image-J software in sections prepared for immunohistochemistry and covered with a mounting medium containing DAPI to visualize cell nuclei. Epidermal thickness was calculated as the average of 6 measurements on each sample from control and adriamycin-treated rats.

Statistics

Statistical evaluation of the experimental cardiomyopathic data was performed with ANOVA, followed by the Bonferroni test. A probability level $p<0.05$ was regarded as a statistically significant difference between groups. At the start of the experiments the numbers of animals were 15 and 17 in the adriamycin and the capsaicin plus adriamycin-treated groups, respectively. For the statistical comparisons of the mustard oil-induced vasodilatory responses two-way ANOVA was performed with pairwise comparisons based on Estimated Marginal Means using the Bonferroni post-hoc analysis, whereas one-way ANOVA was used for comparisons of the capsaicin-induced blood flow changes. One-way ANOVA was used to compare Evans blue contents of skin samples. The Student t-test (unpaired) was applied for the statistical analyses of the data concerning the paw withdrawal responses, the *in vitro* CGRP release and the immunohistochemical experiments. In all groups normality was proved by the

Shapiro-Wilk test and homogeneity of variances was confirmed by Levene's test in advance of performing two-way ANOVA. One-way ANOVA was applied to analyze the results of the hindlimb locomotor parameters. In all comparisons, a p value of <0.05 was accepted as a significant difference.

RESULTS

Anthracycline cardiotoxicity assessed by echocardiography, effect of systemic capsaicin pretreatment

Fractional shortening at the beginning of the study was 25.3±1.5% and did not exhibit any significant change throughout the entire study period. Examination of the rats treated with capsaicin revealed that there was no significant change in FS as compared with the initial baseline value or that for the control group. During the period of the study, the follow-up examinations indicated that there was no change in FS of rats treated only with capsaicin. In rats treated only with adriamycin, echocardiographic measurements of the LV dimensions 3 and 4 weeks after the completion of drug administration demonstrated significant increases in the LVSD. LVDD did not change significantly in these animals. The ratio LAD/AOD was already significantly increased 1 week after the completion of the treatment. In the rats treated with capsaicin plus adriamycin, LVSD was increased significantly already by the end of the 1st week after the completion of adriamycin administration. By the end of the 3rd week after the completion of adriamycin administration, both LVDD and LVSD were increased significantly. The ratio LAD/AOD was already significantly increased 1 week after the completion of adriamycin treatment. In the rats treated with adriamycin, the echocardiographic examinations revealed a progressive reduction in FS, indicating a decrease in cardiac contractility, from the 2nd week onwards after the completion of the administration of the drug, but this became statistically significant only in the 4th week post-treatment. In contrast, a marked and significant reduction in FS was already observed 1 week after the completion of adriamycin treatment in the rats pretreated with capsaicin. Statistical analysis of the data showed that, with the exception of the 4th week post-treatment, the FS in the capsaicin plus adriamycin-treated rats was markedly lower than that in the rats treated only with Adriamycin. Echocardiography and ultrasonographic examination of the chest and abdomen revealed the accumulation of fluid in the pericardial, pleural, and abdominal cavities in adriamycin-treated animals. Three weeks

after the cessation of adriamycin treatment macroscopic signs of cardiotoxicity became evident in sacrificed animal. The ventricles were dilated and ventricular walls, and the septum became visible thinner than that of the control animal.

Adriamycin- and capsaicin-induced changes in cardiac innervation: immunohistochemistry

Immunohistochemical studies were performed to reveal possible changes in the population(s) of cardiac nerves which, in turn, may contribute to the aggravation of adriamycin-induced cardiomyopathy in capsaicin-pretreated rats. In control rats, immunohistochemical demonstration of cardiac nerves using an antiserum against protein gene product (PGP) 9.5, a panneuronal marker, revealed a dense innervation of both the left and right ventricles. CGRP-containing nerves innervating the ventricles were far less numerous. Examination of the distribution of PGP 9.5-positive nerve fibers of the heart ventricles of rats treated with adriamycin or capsaicin plus adriamycin 2 weeks after completion of adriamycin treatment disclosed an innervation pattern similar to that of the controls. In contrast, in rats treated with capsaicin plus adriamycin, but not in rats treated only with adriamycin, CGRP-containing nerves could not be detected 2 weeks after cessation of adriamycin treatment.

Alterations of chemically-induced cutaneous vascular reactions

In control rats, applications of mustard oil at concentrations of 0.2% and 1%, and 1% capsaicin produced significant increases in the CuBF. Subcutaneous (s.c.) injection of HC-030031 (100 μM, 50 μl) 20 min before the epicutaneous application of mustard oil significantly inhibited the vasodilatory response. The increase in CuBF evoked by mustard oil (1%) amounted to 121.80 ± 24.19%, whereas the increase in CuBF after the prior administration of the TRPA1 antagonist, HC030031 amounted to 14.47 ± 10.44% ($p<0.01$, n=6/group). The epicutaneous application of capsaicin (1%) resulted in a marked elevation in CuBF, which amounted to 167.23 ± 53.36%. Administration of the specific TRPV1 antagonist, capsazepine (10 μM, 50 μl, s.c.) 20 min before the epicutaneous application of capsaicin resulted in a significant inhibition of the vasodilatory effect which amounted to 27.94 ± 8.95% ($p<0.01$, n=6/group). In the adriamycin-treated animals, vasodilatory responses were measured 2 days after cessation of the treatment. After the administration of a cumulative dose of 7.5 mg/kg adriamycin, the CuBF increases elicited with 1% mustard oil and 1% capsaicin were reduced by 51% ($p<0.01$, n=10) and 64% ($p<0.01$, n=6), respectively, whereas the vasodilatory effect of 0.2% mustard oil was not affected significantly (n.s., n=10). However, in animals treated

with a cumulative dose of 15 mg/kg adriamycin, the increase in CuBF was markedly and significantly reduced in response to both 0.2% and 1% mustard oil (Fig. 1c), and to 1% capsaicin, amounting to 43%, 29%, and 29%, respectively, of the control values ($p<0.01$, n=6-10/group).

Neurogenic plasma protein extravasation was studied with the quantitative Evans blue technique following the epicutaneous application of mustard oil or capsaicin. In control rats, both mustard oil (5%) and capsaicin (1%) produced marked plasma protein extravasation, with In control rats, epicutaneous application of mustard oil at a concentration of 5%, skin Evans blue dye content amounted to 180 ± 68 μg/g, which was markedly and significantly reduced to 32.67 ± 17.98 μg/g by the prior s.c. administration of the specific TRPA1 antagonist HC030031 ($p<0.01$, n=6/group). Similarly, the specific TRPV1 antagonist capsazepine almost completely abolished the vascular permeability increasing effect of 1% capsaicin; the Evans blue dye content of the skin amounted to 205.50 ± 60.90 μg/g after the application of 1% capsaicin, which was reduced to 32.73 ± 19.75 μg/g by the prior administration of capsazepine ($p<0.01$, n=6/group). Following the administration of a cumulative dose of 7.5 mg/kg adriamycin, the mustard oil-induced increase in tissue Evans blue content was markedly reduced (72.85 ± 20.84 μg/g, $p<0.01$, n=8), and after a cumulative dose of 15 mg/kg, the low Evans blue dye content of the skin (24.95 ± 10.84 μg/g) indicated an almost complete abolition of the neurogenic inflammatory response ($p<0.01$, n=8). Similarly, after the administration of adriamycin at cumulative doses of 7.5 and 15 mg/kg, tissue Evans blue contents were markedly and significantly reduced to 109.93 ± 38.16, and 51.08 ± 17.43 μg/g after the application of capsaicin (for both comparisons $p<0.01$, n=8), while there was no difference in the effect of histamine-, bradykinin- and substance P– induced plasma extravasation between control and adriamycin-treated rats.

Effects of adriamycin treatment on the in vitro neural release of CGRP

Neurogenic sensory vasodilatation is mediated by CGRP released from chemosensitive afferent nerves in response to stimulation of the TRPV1 and TRPA1 receptors. Study of the capsaicin-induced release of CGRP is an established experimental approach through which to characterize the sensory efferent function of peptidergic nociceptors expressing the TRPV1 ion channel. Capsaicin and high potassium-induced release of CGRP was therefore measured with ELISA in *ex vivo* preparations of sciatic nerves from control and adriamycin-treated rats. The release of CGRP, elicited by capsaicin at concentrations of 0.1, 1.0 and 10.0 μM, was

significantly inhibited by 69.54%, 54.02 % and 23.26%, respectively, in the presence of the specific TRPV1 antagonist, capsazepine (10.0 μM; $p<0.01$, n=6/group). In the control sciatic nerve preparations, both capsaicin (10 μM) and high potassium (60 mM) elicited a marked release of CGRP (325 ± 110%, and 327 ± 77% of the basal release, respectively, n=6-8/group). In preparations obtained from adriamycin-treated rats, the high potassium-induced peptide release was similar to that in the controls (303 ± 55% of the basal release, n=8, n.s.). In contrast, the capsaicin-induced release of CGRP was significantly reduced in the adriamycin-treated animals (164 ± 71% of the basal release, $p<0.01$, n=6).

Effects of adriamycin on carrageenan-induced thermal and mechanical hyperalgesia

The findings on the effects of adriamycin treatment on the neurogenic sensory vascular responses were indicative of a marked functional impairment of the chemosensitive afferent nerves which express the TRPV1 and TRPA1 receptors. Since these nociceptive ion channels play a crucial role in the mechanism of inflammatory hyperalgesia, the effects of adriamycin treatment were studied in the carrageenan model of paw inflammation. In the control rats that received carrageenan, the paw withdrawal latencies to radiant heat stimulation decreased by 63.2 ± 2.0% ($p<0.05$, n=5). In contrast, in the adriamycin-treated rats that received carrageenan, the heat withdrawal latencies were barely different from the control values (16.5 ± 11.6%, n=5, n.s.). Similarly, the mechanical withdrawal thresholds were significantly reduced in the controls (by 61.6 ± 10.5%, $p<0.05$, n=5), but not in the adriamycin-treated animals (by 19.4 ± 7.0%, n.s., n=5).

Effects of adriamycin on hindlimb locomotor parameters

The treated animals walked in a similar manner as their intact controls. No marked difference in the locomotor pattern of intact and treated animals was observed before the detailed analysis. In adriamycin treated rats, thorough examination of the high resolution images failed to reveal any significant impairment in step length, ankle and knee lifting, lateral placing, ankle flexion and spreading of toes (n.s., n=5). The knee flexion, tarsus off angle and metatarsus-surface angle parameters showed moderate but significant differences for both treatment groups as compared with the controls ($p<0.05$, n=5).

Effects of adriamycin treatment on cutaneous sensory nerves

In control skin samples tubulin immunostaining revealed subepithelial bundles of nerve fibers, giving rise to individual epidermal axons approaching the surface. Double immunofluorescence staining revealed that almost all the tubulin-immunoreactive epidermal axons are TRPV1-positive. The densities of tubulin- and TRPV1-immunoreactive epidermal axons were 1075 ± 120 mm/mm^3 and 1065 ± 116 mm/mm^3, respectively. The density of epidermal axons decreased by roughly half following adriamycin treatment; the densities of tubulin- and TRPV1-positive axons were 503 ± 76 ($p<0.01$, n=5) and 417 ± 80 mm/mm^3 ($p<0.01$, n=5), respectively. Interestingly, although the number of epidermal axons was greatly reduced, bundles of nerve fibers were seen subepidermally. It appeared that the epidermal axons were truncated at the epidermal-subepidermal border. Adriamycin treatment also resulted in a significant reduction in epidermal thickness (control: 70.41 ± 7.52 μm; adriamycin-treated: 47.47 ± 6.75 μm; $p<0.01$, n=5).

DISCUSSION

The experiments reported in this thesis were devoted to the study of the mechanisms of the cardiotoxic and neurotoxic actions of adriamycin, a widely used anthracycline-type anticancer agent. In agreement with previous findings, administration of adriamycin over a period of two weeks resulted in the development of congestive cardiomyopathy in adult rats. The detection of early signs of cardiac dysfunction and the progression of cardiomyopathy were assessed by repeated echocardiographic examination of adriamycin-treated rats following systemic capsaicin desensitization or administration of the vehicle for capsaicin. The most important observation of the present study was the demonstration of a marked aggravation and, in particular, an acceleration of the development of the symptoms of adriamycin-induced congestive heart failure in capsaicin-pretreated rats. Hence, capsaicin pretreatment resulted in a marked deterioration of the cardiac function even only one week after the cessation of adriamycin administration, as indicated by a significant reduction in FS and a significant increase in the ratio LAD/AOD. In particular, one and two weeks after the completion of adriamycin treatment FS was significantly reduced and the ratio LAD/AOD was significantly increased in the capsaicin-pretreated rats as compared not only with the baseline values, but also with the values obtained in the rats treated only with adriamycin. We suggest that the loss

of chemosensitive CGRP-containing cardiac afferent nerves may be the most likely explanation for the marked acceleration and aggravation of the adriamycin-induced cardiac pathology in the capsaicin-pretreated rats. This assumption is supported by previous findings showing a pivotal role of chemosensitive CGRP-containing peptidergic afferent nerves in the mechanisms of the ischemia/reperfusion-induced preconditioning phenomenon. Hence, we concluded that chemosensitive CGRP-containing cardiac sensory nerves play an important protective role against adriamycin-induced cardiomyopathy. Further, the results suggest that pharmacological perturbation of chemosensitive afferent nerves and/or myocardial CGRP metabolism, e.g. by agents interfering with the function of capsaicin/TRPV1 receptors localized on cardiac sensory nerves, or with peptide metabolism, may open up new perspectives in the prevention and/or alleviation of pathological changes associated with adriamycin treatment.

These observations raised the possibility that the neurotoxic effect of adriamycin, through damaging sensory nerves, may contribute to the deterioration of cardiac function induced by adriamycin. Therefore, in further experiments we studied the effect of adriamycin on the function of cutaneous chemosensitive afferent nerves, which form the best characterized population of chemosensitive primary sensory neurons. We demonstrated that adriamycin treatment resulted in a marked reduction of both components of the cutaneous neurogenic inflammatory response, e.g. vasodilatation and plasma extravasation elicited by chemical irritants. Mustard oil and capsaicin applied onto the skin act by directly activating TRPA1 and TRPV1 receptors, respectively. Activation of epidermal chemosensitive afferents leads to the release of vasoactive sensory peptides, such as SP and CGRP. SP mediates the neurogenic plasma extravasation response by binding to NK1 receptors situated on endothelial cells of postcapillary venules, while CGRP mediates the vasodilatory response mostly by directly acting on vascular smooth muscle cells. They are stored in the sensory nerve endings and can be released via activation of TRPV1 or TRPA1 receptors. Neurogenic sensory vasodilatation is a sensitive measure of sensory nerve function since activation of even one single nerve fiber can cause detectable changes in CuBF. Indeed, evaluation of the cutaneous flare response, the human equivalent of neurogenic sensory vasodilatation was proposed as a diagnostic tool to assess the functionality of C-fiber (cutaneous) afferents. Our findings indicate that adriamycin-induced inhibition of cutaneous vascular reactions may be accounted for by an altered activation of TRPV1, and possibly TRPA1 receptors, since the capsaicin-, but not the high potassium-induced release of CGRP was significantly inhibited after adriamycin treatment. Adriamycin treatment resulted also in a significant reduction of inflammatory hyperalgesia, which is largely mediated by chemosensitive afferent nerves expressing the TRPV1 receptor. Analysis of

locomotor parameters indicated that the increase in the thermal and mechanical thresholds of the nociceptive withdrawal reflex measured in adriamycin-treated animals under inflammatory conditions may be attributed to a toxic damage of the antitumor agent on nociceptive afferent nerves, rather than an impairment of motor function/performance. Quantitative morphological and immunohistochemical findings also demonstrated a substantial and selective loss of epidermal sensory axons which are in part peptidergic and largely chemosensitive.

The mechanisms of the impairments in both the classical afferent, nociceptive and sensory-efferent, local vascular functions of chemosensitive afferent nerves expressing the TRPV1 and TRPA1 receptors cannot be fully explained on the basis of the present findings. However, the results do indicate that degenerative changes of cutaneous, and in particular epidermal sensory axons may be responsible, at least partially, for these functional disturbances. Examination of cutaneous innervation by means of immunohistochemistry and a quantitative stereological approach revealed a marked loss of epidermal axons in adriamycin-treated animals. In contrast, the density and distribution of subepidermal nerve fibers appeared normal, suggesting a selective loss of epidermal nerve endings. This might explain both the marked reduction of the sensory neurogenic vascular reactions and the inflammatory hyperalgesia in adriamycin-treated rats, since activation of the epidermal chemosensitive nerves is essential in the initiation of these responses. It is noteworthy that similar structural changes, i.e. a selective loss of terminal, epidermal, but not preterminal nerve fibers was also observed in the toxic neuropathies elicited by paclitaxel and vincristine and, interestingly, a human bacterial disease, leprosy.

In conclusion, the findings presented in this thesis have demonstrated the protective role of TRPV1 expressing chemosensitive afferent nerves in a clinically important pathological entity, anthracycline-induced dilatative cardiomyopathy. Further, these observations support and extend previous reports on cardioprotective neurogenic mechanisms involving a specific class of nociceptive peptidergic CGRP-containing sensory nerves which express the nociceptive ion channels TRPV1 and TRPA1. We also provided evidence for a neurotoxic lesion by adriamycin of nociceptive primary sensory neurons resulting in profound impairments in nociceptive functions of fundamental physiological and pathophysiological significance, such as pain sensation and neurogenic sensory vascular reactions. The findings also suggest that the neurotoxic propensity of anthracycline-type antitumor agents may contribute to the deterioration of cardiac function through the elimination of an important cardioprotective mechanism conferred by sensory nerves. Importantly, the adriamycin-induced functional impairments of the nociceptive afferent neurons observed in the present study precede the

commencement of cardiomyopathic changes. Hence, the findings raise the possibility of using specific quantitative sensory testing, such as the detection and quantification of the axon reflex flare response, the human equivalent of neurogenic sensory vasodilatation, to predict the risk of adriamycin-induced cardiac injury in clinical practice.

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