

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

PhD Thesis

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## LIST OF ABBREVIATIONS

ABC	ATP-binding cassette protein
ACE	angiotensin converting enzyme
ANP	atrial natriuretic peptide
AOD	aortic diameter
ATP	adenosine-5'-triphosphate
CALC	calcitonin
CARP	cardiac adriamycin-responsive protein
CuBF	cutaneous blood flow
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor-like receptor
CNS	central Nervous System
cTnT	cardiac-specific isoform of troponin T
DNA	deoxyribonucleic Acid
DRG	dorsal root ganglion
EF	ejection fraction
FS	fractional shortening
GAL	galanin
GDNF	glial cell derived neurotrophic factor
IB4	isolectin B4 isolated from <i>Griffonia simplicifolia</i> (formerly <i>Bandeiraea simplicifolia</i> )
LA	left atrium
LAD	left atrial diameter
LTB	leukotriene B
LV	left ventricle
LVDD	left ventricular end diastolic diameter
LVSD	left ventricular end systolic diameter
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-associated protein
NGF	nerve growth factor
NK1	neurokinin 1
NKA	neurokinin A
NO	nitrogen monoxide
PACAP	pituitary adenylate cyclase-activating polypeptide

PAR proteinase activated receptor  
PCR polymerase chain reaction  
PGE prostaglandin E  
PGP protein gene product  
PKA protein kinase A  
PKC protein kinase C  
PLC phospholipase C  
RAMP receptor activity-modifying proteins  
SF left ventricular fractional shortening  
SIF small intensely fluorescent  
SP substance P  
TG trigeminal ganglion  
THC (tetra-hydro cannabinol)  
TMP thiamine monophosphatase  
Trk A neurotrophic tyrosine kinase receptor type 1/ high affinity nerve growth factor receptor/  
TRK<sub>1</sub>-transforming tyrosine kinase protein  
TRP transient receptor potential  
TRPA<sub>1</sub> transient receptor potential cation channel, subfamily A, previously: Transient  
receptor potential ankyrin type  
TRPM<sub>8</sub> transient receptor potential cation channel, subfamily M, member 8  
TRPV transient receptor potential vanilloid type  
VIP vasoactive intestinal polypeptide  
VMA vanillin mandelic acid  
VR vanilloid receptor

## INTRODUCTION

### *Capsaicin, chemosensitive primary sensory neurons and TRP channels*

Capsaicin is the pungent component of capsicum species (hot chili peppers) that selectively excites and, in large doses selectively desensitizes a subpopulation of primary sensory neurons. Nicholas Jancsó's classical studies have revealed that application of capsaicin onto the skin, conjunctiva, or other mucous membranes induces intense burning pain in man (Jancsó, 1960; Jancsó et al., 1967), and elicits pain-related behavior and nociceptive reflexes in animals. Jancsó has also recognized that these fibers possess dual function: they have a role not only in the signalization of pain, but also in the mechanism of local vascular responses. Hence, local application of chemical irritants, such as capsaicin and mustard oil, produces not only burning pain but local vasodilatation and flare in man, and local vasodilatation and plasma extravasation, i.e. a neurogenic inflammatory response in rats (Jancsó, 1960; Jancsó et al., 2009a). The term „capsaicin desensitization” was first introduced by N. Jancsó (Jancsó and Jancsó-Gábor, 1959; Jancsó, 1968; Holzer, 1991; Jancsó, 1994). He observed that repeated local or systemic administration of capsaicin inhibited not only the transmission of afferent nociceptive impulses, but also the efferent vascular responses mediated by chemosensitive afferent nerves, both in man and animals. Capsaicin desensitization also inhibited the pain-producing effect of other irritants of widely different chemical structure, like xylene, mustard oil or chloroacetophenone, while mechano-nociceptive reflexes remained intact (Jancsó and Jancsó-Gábor, 1959; Jancsó et al., 1968).

Capsaicin-sensitive neurons comprise a special, quantitatively and functionally important population of somatic and visceral primary sensory neurons. The knowledge on the morphological characteristics, anatomical organization and functions of these nociceptive primary afferent neurons was greatly advanced by the discovery of the selective neurotoxic action of capsaicin by G. Jancsó and his co-workers (Jancsó et al., 1977; Jancsó and Király, 1981; Jancsó and Király, 1984). Systemic administration of capsaicin to new-born rats led to a selective degeneration of about 50% of sensory ganglion cells. In peripheral cutaneous nerves a 70 % loss of unmyelinated C fibers was observed (Jancsó et al, 1977). In lumbar dorsal roots an about 95% loss of C-, and about a 10% loss of thin myelinated A-fibers was reported after neonatal capsaicin treatment (Jancsó et al., 1981; Jancsó and Király, 1981; Nagy et al., 1981). Different types of systemic and regional treatments with capsaicin or other vanilloids have become standard techniques in pain research to reveal the participation of

chemosensitive primary afferent neurons in different somatic and visceral functions under physiological and pathological conditions.

In 1997 Caterina and his co-workers successfully cloned the capsaicin receptor (Caterina et al., 1997; Caterina and Julius, 2001). The cloning of the receptor was based on an expression screening strategy exploiting the phenomenon of capsaicin-induced increase in intracellular free calcium (Jancsó et al., 1978; Joó et al., 1980; Jancsó et al., 1984). The capsaicin receptor was first named vanilloid receptor type 1 (VR1). Later, it has been recognized that this receptor highly resembles the transient receptor potential (TRP) ion channels (Caterina et al., 1997). These results were published in *Nature* by Michael Caterina 50 years after the publication of N. Jancsó's article in *Nature* in 1947, explaining the phenomenon of histamine desensitization) (the idea that finally brought him to the recognition of capsaicin desensitization (Jancsó and Jancsó-Gábor, 1947; Jancsó, 1994; Jancsó and Sántha, 2015), and 20 years after the discovery of the selective neurotoxic action of capsaicin by Gábor Jancsó published in *Nature* in 1977 (Jancsó et al., 1977). The TRP (transient receptor potential) family of ion channels comprises more than 30 ion channels, and can be divided into seven subfamilies based on their sequence homology. In the case of the TRP channels, polymodality lies in a single channel. TRP channels are multifunctional sensors of chemical, mechanical and thermal stimuli. TRPV<sub>1</sub>, TRPV<sub>2</sub>, TRPV<sub>3</sub>, TRPV<sub>4</sub>, TRPM<sub>8</sub> (transient receptor potential cation channel, subfamily M, member 8, previously melastatin type 8) and TRPA<sub>1</sub> (transient receptor potential cation channel, subfamily A, previously: ankyrin type) are known to be expressed in dorsal root ganglia (DRGs) neurons. So far TRPV<sub>1</sub> is the only known receptor that is responsive to capsaicin. The molecular site of the desensitizing effect of capsaicin is located on the sixth transmembrane domain. TRPV<sub>1</sub> is present in the cell membrane, in the endoplasmic reticulum, and in cytoplasmic vesicles (Guo et al., 1999; Morenilla-Palao et al., 2004; Nagy and Sántha, 2008). It is synthesized in the soma of the neuron, packaged and transported by fast axonal transport into the central and peripheral terminals of the neurons to fulfill its function in sensory conduction.

The fact that capsaicin applied topically onto peripheral nerves resulted in a selective loss of neurogenic plasma extravasation (Jancsó et al., 1980; Gamse et al., 1982; Dux et al., 1999) increase in heat pain threshold (Jancsó et al., 1980; Fitzgerald and Woolf, 1982), and inhibition of reactive hyperemia (Domoki et al., 2003) suggested the presence of functioning capsaicin receptors in peripheral sensory axons. Perineural application of capsaicin resulted in a blockade of axoplasmic transport of neurotransmitter peptides (Gamse et al., 1982), a selective and permanent reduction of both afferent sensory (heat- and chemo nociception),



and efferent regulatory (neurogenic inflammation) functions, and lead to a dramatic loss of epidermal fibers in the skin area which is innervated by the treated nerve. (Dux and Jancsó, 1994) The peripheral axon is not only a transporting route for the TRPV<sub>1</sub> receptors, functioning receptors are also present in the axonal membrane, although their physiological/pathophysiological role need to be clarified (Tominaga et al., 1998). TRPV<sub>1</sub> is expressed in about 30-60% of rat DRG neurons with considerable variations with respect to spinal segmental levels (Tominaga et al., 1998; Michael and Priestley, 1999; Malin et al., 2011). TRPV<sub>1</sub> is expressed almost exclusively in small- and medium-sized neurons with polymodal nociceptive function. These results are in accordance with earlier findings on sensory nerve fiber and ganglion cell loss after systemic neonatal capsaicin treatment (Jancsó et al., 1977; Nagy et al., 1983; Lawson, 1987). Small and medium-sized nociceptive DRG neurons can be divided into two main, partially overlapping populations. Peptidergic DRG neurons are characterized by the constitutive expression of several neuropeptides, especially calcitonin gene-related peptide (CGRP), substance P (SP) and somatostatin (Kruger et al., 1989; Lawson, 1995), contain neurotrophic tyrosine kinase receptor type 1 (trkA), respond to nerve growth factor (NGF) in the adult rat and comprise about 40% of DRG neurons. Non-peptidergic DRG neurons bind the *Bandeiraea simplicifolia* isolectin B4 (IB4), contain thiamine monophosphatase (TMP), respond to GDNF and make up about 30% of small DRG neurons. TRPV<sub>1</sub> is expressed in the majority of both peptidergic (60% is TRPV<sub>1</sub>-positive) and non-peptidergic (75% is TRPV<sub>1</sub>-positive) neurons. TRPV<sub>1</sub> is expressed only in few large and medium-sized DRG neurons with myelinated axons that express the 200 kD neurofilament (Michael and Priestley, 1999).

Early studies by N. Jancsó (Jancsó, 1960; Jancsó et al., 1968) demonstrated that besides capsaicin-type compounds, mustard oil (allyl-isothiocyanate) also stimulates chemosensitive afferent nerves and elicits neurogenic inflammation. The phlogogenic effect of mustard oil, similarly to capsaicin, was completely abolished by denervation, capsaicin desensitization of adult rats (Jancsó et al., 1967) neonatal treatment with capsaicin (Jancsó et al., 1977) indicating the mediation of mustard oil-induced vascular reactions by chemosensitive afferent nerves. Similarly, perineural application of capsaicin produced a permanent loss of mustard oil-induced vascular reactions in the innervation area of the capsaicin-treated nerve (Jancsó et al., 1980). Further, mustard oil- but not capsaicin-induced neurogenic inflammation could be elicited in TRPV<sub>1</sub><sup>-/-</sup> mice, indicating the existence of another TRP receptor (Caterina et al., 2000; Bánvölgyi et al., 2004). Experiments on rodents confirmed that a large population of TRPV<sub>1</sub>-positive neurons are also responsive to mustard

oil (Story et al., 2003; Jordt et al., 2004), and express the TRPA<sub>1</sub> receptors (Kobayashi et al., 2005; Nagata et al., 2005; Bautista et al., 2006). Besides mustard oil, TRPA<sub>1</sub> can be activated by cinnamaldehyde (in cinnamon), allicin (in garlic), methyl salicylate (in wintergreen oil), gingerol (in ginger), eugenol (in clove oil) and bradykinin. The data on its role in cold-nociception are controversial (Story et al., 2003; Macpherson et al., 2005; Mckemy, 2005).

Both somatic and visceral afferents involve a large chemosensitive population. They innervate, inter alia, the skin, the airways, the cardiovascular system, the gastro-intestinal tract, the urogenital system, the dura mater and have essential role in chemo-nociception and in a number of organ-specific local physiological and pathological events (Jancsó, 1960; Jancsó et al., 1968; Jancsó, 1984; Maggi et al., 1984; Jancsó et al., 1985; Rózsa et al., 1986; Jancsó et al., 1987; Holzer, 1998a; Holzer, 1998b; Dux et al., 2003). Neurogenic inflammation, the most thoroughly studied local regulatory process, is essential in the modulation of local blood flow, vascular permeability, inflammatory processes and tissue defense mechanisms in many organs (Jancsó, 1960; Jancsó et al., 1968; Rózsa et al., 1986; Maggi et al., 1987; Brain, 1997; Holzer, 1998a; Holzer and Holzer-Petsche, 2001; Jancsó, 2009; Jancsó et al., 2009a). Besides their organ- or tissue-specific characteristics, visceral and somatic chemosensitive afferents share many common traits in terms of their morphology, neurochemistry and function. The cutaneous chemosensitive afferent nerves are the best-characterized class of this particular population (Roosterman et al., 2006; Jancsó et al., 2009b).

### ***Chemosensitive innervation of the skin with special regard to neurogenic inflammation and inflammatory hyperalgesia***

The skin has a remarkably rich TRPV<sub>1</sub> positive innervation. Perineural treatment of rat peripheral nerves led to a loss of about 30% of cutaneous afferents (Jancsó and Lawson, 1990; Pini et al., 1990), and about 80% loss of epidermal nerves (Dux and Jancsó, 1994), an almost complete loss of SP-immunoreactive epidermal fibers and a less pronounced decrease of CGRP-immunoreactive axons (Dux and Jancsó, 1999). According to the results of Dux and co-workers, perineural capsaicin treatment resulted in a dramatic loss of intraepidermal nerves in skin areas innervated by the treated nerve: they found that 90% of nerves stained with the PGP 9.5 antiserum, a panneuronal marker, disappeared from the epidermis already three days after perineural capsaicin treatment (Dux et al., 1999). Repeated topical application of capsaicin onto the skin resulted in a temporary hypalgesia and loss of epidermal fibers in rats (Orosz et al., 1998) and also in man (Nolano et al., 1999).

Structural difference between peptidergic and non-peptidergic fibers was first described by Dux and coworkers (Dux et al., 1999) who showed that epidermal axons with simple morphology are peptidergic and those with a complex morphology are non-peptidergic. Zylka et al. (2005) showed that in mice peptidergic and non-peptidergic epidermal nerve endings terminate in different epidermal layers (CGRP+ fibers appeared to end in the stratum spinosum and non-peptidergic fibers seemed to penetrate into the stratum granulosum); he also realized that peptidergic and non-peptidergic fibers had different morphology, peptidergic were straight and non-peptidergic had a zig-zag structure as described earlier by Dux et al. (1999).

Cutaneous TRPV<sub>1</sub>-positive fibers primarily act as polymodal nociceptors that are responsible for heat- and chemo-nociception. Besides transmitting nociceptive impulses towards the central nervous system, these sensory neurons release peptides from their peripheral endings, and maybe along their peripheral axons, in response to noxious stimuli. Sensory neuropeptides such as CGRP, SP or neurokinin A (NKA) released from the activated nociceptors mediate local regulatory functions involving the microvasculature, inflammatory- and dermal/epidermal cells and the sensory neurons themselves. Among the local regulatory roles of sensory neurons, the vascular changes associated with sensory nerves were recognized even before the exact identification of the mediators. The triple response of the human skin evoked by noxious stimuli of different modalities was observed by Lewis (Lewis and Grant, 1924). It is composed of local vasodilatation, edema formation (wheal), and spreading vasodilatation (flare). In rats, the flare response is missing. Because the classical signs of inflammation, „rubor, tumor cum dolore et calore” were present in the reaction induced by noxious stimuli at least in humans, and because the existence of a substantial neurogenic component was evident, N. Jancsó named it „neurogenic inflammation” (Jancsó, 1960). In the human skin the sensory fibers mediating the flare response are chemosensitive (Jancsó, 1960; Jancsó et al., 1968; Jancsó et al., 1985) and mechano-insensitive C-fibers (Schmelz et al., 2000). In the rat, sensory vasodilatation is primarily mediated by capsaicin-sensitive C-fibers, but activation of A $\delta$  fibers might also cause vasodilatation (Kenins, 1981; Lynn et al., 1996). While cutaneous vasodilatation and flare responses could be elicited by relatively mild chemical, physical or electrical stimuli, the production of neurogenic plasma extravasation requires excessive stimulation. Substance P (SP) was the first peptide verified as a mediator of sensory neurogenic vascular responses (Lembeck and Holzer, 1979; Gamse et al., 1980). It is a member of the tachykinin group together with the structurally similar NKA and B. SP causes plasma extravasation by binding to the NK<sub>1</sub> receptor (a G protein

coupled receptor) situated on endothelial cells of postcapillary venules (Lembeck and Holzer, 1979; Kenins, 1981; Kenins et al., 1984; Chahl, 1988). SP also activates cutaneous mast cells (Bueb et al., 1990; Holzer, 1992) and sensitizes nociceptors (Li et al., 2012). Calcitonin gene related peptide (CGRP) is a sensory neuropeptide which is found in both small and large primary afferent neurons (Kruger et al., 1989). CGRP is a member of the calcitonin (CALC) superfamily: a group of mainly hormone peptides that derive from the CALC genes. CGRP I (or  $\alpha$ ) is a 37 amino acid long alternative mRNA splice product of the CALC I gene, together with calcitonin and pro-calcitonin. CALC II leads to CGRP II (or  $\beta$ , which differs in 3 amino acids from CGRP I or  $\alpha$ ), and CALC IV and V give rise to amylin and adrenomedullin, respectively, while CALC III is a non-translated pseudogene. Based on their structural homologies, calcitonin peptides have overlapping bioactivities, which they exert by binding to the same family of receptors. There are two subgroups of these G protein-coupled receptors with seven transmembrane domains: CALC receptors and calcitonin receptor-like receptor (CLR). Three accessory proteins, receptor activity-modifying proteins (RAMPs 1–3), bind to these receptors and alter their specific responsiveness and ligand affinity (Russell et al., 2014). The presence, the concentration, and the timing of the three RAMPs determines the specific cellular phenotype of the receptor that is biologically active on the cell surface (Christ-Crain and Müller, 2007). CGRP-immunoreactive fibers are more numerous in the human and rat skin than SP immunoreactive ones (Wallengren et al., 1987; Dux et al., 1999). In rodent as well as in human sensory nerves, CGRP is, at least in part, colocalized with substance P (Gibbins et al., 1985; Franco-Cereceda et al., 1987). It is a very potent vasodilator in the skin in many animals and in man (Brain et al., 1985). CGRP exerts its vasodilatory effect by directly acting on vascular smooth muscle cells (Ralevic et al., 1992) and also by activating endothelium-dependent NO pathways (Brain and Grant, 2004).

Both peptides are transported into the nerve endings via fast axonal transport (Morton and Chahl, 1980; Gamse et al., 1982) where they are stored and can be released via TRPV<sub>1</sub> or TRPA<sub>1</sub> mediated mechanisms (Pethő et al., 2004; Bautista et al., 2006). CGRP can also be released from isolated peripheral nerves via TRPV<sub>1</sub>-mediated mechanisms (Bernardini et al., 2004). Electrophysiological studies *in vivo* suggested a role for CGRP in regulating thermal sensitivity, sensitization and ectopic spike generation in peripheral nociceptive axons (Hoffmann et al., 2008). Some other neuropeptides expressed by cutaneous sensory neurons include pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), somatostatin (SOM) (Wallengren et al., 1987), and galanin (GAL) (Bárfai et al., 1992; Hokfelt et al., 1994; Sántha et al., 1998a; Sántha et al., 1998b; Sántha et al.,

1999). In the periphery, the main source of histamine is the mast cell. Mast cell degranulation mainly occurs when a tissue is inflamed or allergens are present (Benditt et al., 1955). Many factors cause histamine release, so does SP in the human skin (Bunker et al., 1991). There are four known types of histamine receptors, all of them are coupled to G-proteins (Shim and Oh, 2008). H<sub>1</sub>R is found on endothelial cells and on sensory nerve endings. Histamine-induced plasma extravasation (Jancsó and Jancsó-Gábor, 1947) is mediated by endothelial H<sub>1</sub>R and consequent PLC activation. Histamine also has a vasodilator effect via binding to H<sub>2</sub>Rs on vascular smooth muscle. Histamine has vasoactive and proinflammatory effects, but it does not appear to have an essential role either in the initiation or in the final vascular response of the neurogenic inflammation (Jancsó et al., 2009a). Bradykinin, the „slow” kinin is a nonapeptide, which is formed from HMW (high molecular weight) kininogen by the action of kallikrein and other substances. Bradykinin is a potent endothelium-dependent vasodilator, inflammatory mediator and pain-producing substance. There are two known bradykinin receptors: B<sub>1</sub> and B<sub>2</sub>, both of them are G-protein coupled receptors. Activation of B<sub>1</sub> receptor is responsible for neutrophil recruitment during inflammation, while endothelial B<sub>2</sub> receptor is involved in the vasodilator activity. Bradykinin causes plasma extravasation in many tissues by a B<sub>2</sub> receptor-mediated pathway (Hall, 1997). Bradykinin can be considered as an endogenous activator of the TRPV<sub>1</sub> through B<sub>2</sub> receptors (Vellani et al., 2004). Arachidonic acid derivatives, local release of proteinases and NO also increase the complexity of neurovascular reactions. Neurogenic inflammation is not only a cutaneous phenomenon; it also appears in many other organs, including the airways, the urogenital system, several parts of the gastrointestinal tract and the dura mater (Saria et al., 1987; Maggi and Meli, 1988; Abelli et al., 1991; Dux et al., 2003; Klukovits et al., 2004).

Cutaneous fibers that express the TRPV<sub>1</sub> receptor are key players of sensitization that are initiated by inflammation or tissue injury and lead to hyperalgesia and allodynia (Fischer and Reeh, 2007; Watanabe et al., 2015). Tissue damage and inflammation produce an array of chemical mediators such as ATP, bradykinin, prostanoids, protons, cytokines and peptides including substance P that can excite or sensitize nociceptors to elicit pain at the site of injury. Pain hypersensitivity can take two forms: hyperalgesia is when noxious stimuli produce an exaggerated and prolonged pain and allodynia is when thresholds are lowered so stimuli that normally don't cause pain, become painful (Costigan and Woolf, 2000). Hypersensitivity may be of peripheral or central origin. Peripheral sensitization involves increased excitability of the nociceptive nerve terminal and begin very early, even within seconds after injury. Central sensitization evolves slower and implicate long term changes in the synaptic transmission in

the central pain pathway. Heat hyperalgesia is said to be a result of peripheral sensitization and mechanical allodynia is considered to be central. As both central and peripheral mechanisms are closely related to processes that take place in the primary sensory neuron as a sensory unit, clear-cut separation of peripheral and central sensitization might not be inevitable although it serves as a helpful model. Several experimental models exist for inflammatory hyperalgesia including those induced by formalin, Freund's adjuvant and carrageenan (Hargreaves et al., 1988; Ren and Dubler, 1999; Gould, 2000). Intraplantar injection of carrageenan in experimental animals causes paw edema, heat hyperalgesia and mechanical allodynia that develops in the inflamed area (Kocher et al., 1987; Hargreaves et al., 1988) and reaches its peak within four hours. In the pathomechanism of carrageenan-induced hyperalgesia sensitization and up-regulation of the TRPV<sub>1</sub> receptors play a crucial role and the NGF signalization pathway is involved (Sammons et al., 2000; Tohda et al., 2001). Systemic neonatal capsaicin treatment significantly decreases the carrageenan-induced heat- and tactile hyperalgesia (Lee et al., 2004).

Sensory denervation of the skin in humans and in experimental animals results in the development of cutaneous lesions, impaired wound-healing (Kishimoto, 2016), and decreased hair growth (Maggi et al., 1987; Thomas et al., 1994). Toxic effects and metabolic disorders that affect cutaneous sensory neurons often lead to impaired homeostasis and/or structural lesions of the skin. Measurement of the efferent vascular functions (Jancsó and Janka, 1981) and morphological analysis of tissue samples (Beiswenger et al., 2008) are reliable methods for the detection of the structural and functional integrity of cutaneous nerves not only in experimental animals but in man, too. A quantitative assessment of the vascular reactions elicited through activation of TRPV<sub>1</sub> and/or TRPA<sub>1</sub> receptors localized on chemosensitive afferent nerves serves as a reliable indicator of the functional condition of these afferent nerves in both man and animals (Jancsó and Janka, 1981; Jancsó et al., 1985; Baron et al., 1988; Lynn et al., 1996; Dux et al., 2003; Bernardini et al., 2004; Namer et al., 2005). Further, measurements of the capsaicin- or mustard oil-induced release of the sensory neuropeptide CGRP in *ex vivo* preparations provide valuable information on the functional integrity of chemosensitive afferent nerves (Gamse et al., 1980; Saria et al., 1987; Lundberg et al., 1992; Dux et al., 2007; Fuchs et al., 2010).

***Sensory innervation of the heart and the protective role of peptidergic afferent nerves against cardiac injury***

In many cultures the heart is considered as the center of emotions, but what we really sense with our heart, is not an easy issue. As for non-painful sensations, everybody can sense palpitation, extrasystole and arrhythmia. Interestingly, many pathologies of the heart, including myocarditis or rupture of the valves do not lead to sensation of cardiac pain (Malliani et al., 1986). Ischemic pain of the heart is the most common cardiac pathology associated with the activation of cardiac nociceptors. Cardiac chemo-nociceptive nerve endings can be activated by hypoxia, acidity, and potassium ions. Importantly, cardiac sensory nerves may respond to these stimuli with the release of sensory neuropeptides such as CGRP or SP (Franco-Cereceda et al., 1987; Holzer, 1992; Sosunov et al., 1996; Nagy et al., 2004).

The existence of sensory endings in the heart was first suggested by Berkley (1894), Smirnov (1895)), and Dogiel (1898). An excellent monograph on the innervation of the heart and the blood vessels has been published by (Ábrahám, 1969). Anatomically, the heart receives dual sensory innervation. Nociceptive spinal afferent nerves, whose parent cell bodies are located in the thoracic DRGs 1-6, run together with postganglionic sympathetic fibers. Vagal afferent nerves, whose parent cell bodies are situated in the nodose ganglion, join vagal parasympathetic fibers. The relative contribution of vagal and spinal sensory nerves to the innervation of the heart is controversial. Langley (1903) stated, that most of the afferent fibers, the stimulation of which leads to pain, run with the sympathetics and are of spinal origin. Woollard (1926) suggested, that a large proportion of cardiac sensory nerves are of vagal origin. Nettleship (1936) came to a different conclusion by showing that removal of thoracic DRGs resulted in the degeneration of nerves innervating the apical ventricular endocardium and the coronary arteries. Khabarova (1963) observed that vagal and spinal endings frequently run side by side. Vagal afferents form plexi associated with cardiac ganglia, where they provide dense pericellular ending on the small intensely fluorescent (SIF) cells. In the myocardium afferents are in close contact with the cardiac muscle and conduction fibers, and in the endocardium they form end-net or flower spray terminals. Some fibers have both endocardial and myocardial intramuscular endings (Cheng et al., 1997).

Vagal left ventricular receptors are C-fiber afferents that mediate the Bezold-Jarish reflex. Bezold reported the cardiodepressive reflex evoked by veratrum alkaloids (von Bezold and Hirt, 1867) and Jarish clarified the reflex (Jarish and Henze, 1937). According to (Hainsworth, 1991), the receptors involved in the Bezold-Jarisch reflex respond to bradykinin,

prostaglandins, capsaicin, serotonin and phenylbiguanidine (PBG). Jancsó and Such (1986) demonstrated in cats that perivagal capsaicin treatment greatly reduced i.v. capsaicin-induced reflex bradycardia, hypotension and apnea but left these reflex responses evoked by intravenous administration of PBG and veratrine unaffected. These findings indicated that vagal afferent fibers mediating cardiovascular and respiratory chemo-reflexes are separated into chemo-specifically different populations.

A large population of cardiac sensory nerves is sensitive to capsaicin (Jancsó and Such, 1983; Ferdinandy et al., 1997). Several studies concluded that capsaicin induces positive chronotropic and inotropic effects in the isolated guinea pig atrium (Fukada and Fujiwara, 1969; et al., 1969; Lundberg et al., 1984). It was also shown that in guinea pig whole-heart preparations, CGRP stimulates the rate and force of atrial contractions (Franco-Cereceda and Lundberg, 1988). Franco-Cereceda and coworkers furnished indirect evidence that activation of cardiac nerves by capsaicin results in the release of CGRP, which is at least partly responsible for the capsaicin-evoked cardiac effects. In the isolated guinea-pig right atrium capsaicin ( $10^{-6}$  M) evoked an increase in contractile rate and tension with a simultaneous outflow of CGRP-LI. In the isolated whole heart, capsaicin increased the outflow of CGRP-LI and stimulated heart rate while a negative inotropic effect was observed. The negative inotropic effect of capsaicin is unrelated to the activation of sensory nerves (Franco-Cereceda et al., 1991).

Application of capsaicin ( $10\mu\text{g/ml}$ ) onto the epicardial surface of the left ventricle in anaesthetized rats resulted in cardiogenic sympathoexcitatory response (Zahner et al., 2003) similar to that induced by bradykinin (Baker et al., 1980; Veelken et al., 1996; Tjen-A-Looi et al., 1998; Li and Pan, 2000; Pan and Chen, 2002). Capsaicin and bradykinin seem to act on the same subpopulation of neurons, but on different receptors, TRPV1 and B2, respectively.

The study of the involvement of cardiac sensory nerves in the development of cardiac pathologies is a rapidly growing field of cardiovascular research. CGRP is to be found in approximately 40% of primary sensory neurons, including those innervating the heart (Gibbins et al., 1985; Mulderry et al., 1985; Franco-Cereceda et al., 1987; Franco-Cereceda and Lundberg, 1988). CGRP-containing nerves are present in all regions of the rat heart, especially in association with the coronary arteries, the papillary muscles, and the sinoatrial and atrioventricular nodes (Mulderry et al., 1985). The atria have a denser CGRP-positive innervation than the ventricles. CGRP immunopositive nerve fibers also occur in the cardiac ganglia between ganglion cells. In the guinea pig, most of the CGRP-positive fibers display SP immunopositivity, but in the rat, CGRP containing cardiovascular nerves are far more



numerous than those containing SP (Wharton et al., 1986). CGRP exerts marked coronary vasodilatory (Holman et al., 1986) and positive inotropic and chronotropic effects in several species (Franco-Cereceda and Lundberg, 1988; Franco-Cereceda and Lundberg, 1989; Yaoita et al., 1994; Gasparetti et al., 2002). CGRP is localized in cardiac primary sensory neurons which are sensitive to the potent sensory neurotoxin capsaicin (Franco-Cereceda and Lundberg, 1988; Ferdinandy et al., 1997; Gasparetti et al., 2002) and which express the TRPV1 receptor (Zahner et al., 2003). Systemic administration of capsaicin results in a depletion of CGRP from cardiac nerves (Ferdinandy et al., 1997), and a decrease in basal NO synthesis in the rat heart (Csont et al., 2003). It has been demonstrated that the depletion of CGRP by capsaicin results in a marked increase in the incidence of adverse reactions, which commence following the experimental induction of myocardial ischemia (Franco-Cereceda and Lundberg, 1989; Kallner, 1989; Kallner and Franco-Cereceda, 1989). Further, CGRP is essential for the development of myocardial protection: the depletion of CGRP from sensory nerves by prior pre-treatment with capsaicin greatly inhibits or even abolishes the protective effect of ischemic preconditioning (Ferdinandy et al., 1997; Zhou et al., 1999) or heat stress on reperfusion injury (Song et al., 1999), and attenuates the nitroglycerine-induced improvement of preservation with cardioplegic solution (Zhou et al., 2001). TRPV1 gene knockout impairs postischemic recovery (Wang and Wang, 2005) and N-oleoyldopamine, an endogenous activator of the TRPV1 receptor has been shown to protect the heart against ischemia-reperfusion injury (Zhong and Wang, 2008). CGRP seems to play an important role in both local (when conditioning stimulus is applied on the same organ), and remote (when stimulus is applied to a distant organ or tissue) ischemic conditioning (Wolfrum et al., 2005; Veighey and Macallister, 2012; Russell et al., 2014; Hausenloy and Yellon, 2016). Remote preconditioning via activation of peripheral nociceptors by non-ischemic stimuli (traumatic wound injury) were also studied (Jones et al., 2009). TRPV1 activation is involved in remote postconditioning (Gao et al., 2015) and rutacarpine, a useful herbal drug in the treatment of hypertension and angina pectoris has been shown to exert its cardioprotective effect, at least in part through an action on capsaicin-sensitive nerves (Hu et al., 2003). CGRP is also involved in human cardiac pathologies: an early increase in plasma CGRP level has been demonstrated in patients with myocardial infarction (Lechleitner et al., 1992) and administration of this peptide has been shown to delay the onset of myocardial ischemia upon physical exercise in patients with stable angina pectoris (Uren et al., 1993). In conclusion chemosensitive cardiac nerves play a crucial role in the adaptive/protective responses of the heart. The importance of cardiac peptidergic sensory nerves in cardiac adaptation to ischemic

stress is well established, but little is known about their possible protective role in drug-induced cardiotoxicity.

### ***Anthracycline-induced cardiotoxicity, and neurotoxicity***

Anthracyclines have been introduced into clinical oncology in the past century (Di Marco et al., 1969). Despite serious side effects, anthracyclines have soon become indispensable in the treatment of many malignancies (Davis and Davis, 1979). Hence, anthracycline-type cytostatic agents are still widely used in both adult and pediatric oncology. Adriamycin exerts its antimitotic effect by intercalating DNA base pairs leading to breaks in the helical strands (Pigram et al., 1972; Buja et al., 1974) and by inhibiting DNA-dependent enzymes. Adriamycin was shown to inhibit protein synthesis in cell-free systems but not in intact cells (Mompalmer et al., 1976). Studies on anthracycline distribution within tissues and cell compartments are based on its natural fluorescence (Egorin et al., 1974; Danesi et al., 1974; Hindenburg et al., 1989) or on the use of immunosilver staining techniques (Henneberry and Aherne, 1992). After intravenous administration of the drug, the access to the cells depends on the vascularization of the tissue, the strength of the endothelial barrier systems and the binding of the drug to membrane sites (Tritton and Yee, 1982). The unchanged form of the drug is transported via passive diffusion across the cell membranes (Mayer et al., 1986; Frézard and Garnier-Suillerot, 1991). Adriamycin is a weak base ( $pK=8.3$ ), and the cellular uptake is driven by the pH gradient and the concentration gradient. Binding of the drug to the DNA (Chaires et al., 1985; Capranico et al., 1990), to intracellular membranes (de Wolf et al., 1993), and its accumulation in acidic organelles (Willingham et al., 1986) can play a role in maintaining the concentration gradient. The actual intracellular concentration of adriamycin is the result of a dynamic equilibrium of influx and efflux processes. Therefore the expression pattern of efflux transporters such as ATP-binding cassette proteins (ABC) seems to be an essential factor in the cellular vulnerability for anthracyclines. Overexpression of the most studied ABC transporter, the P-glycoprotein, was shown to be associated with an acquired resistance to the cytostatic agent (Couture et al., 2006). ABC transporters, on the one hand, can protect the organism from the toxic effect of the xenobiotic but, on the other hand, their overexpression in tumor cells can limit the therapeutic effect.

The cardiotoxic effects are the most serious side effects of anthracyclines which are difficult to predict and even more difficult to manage. Congestive dilatative cardiomyopathy is the most serious manifestation of adriamycin cardiotoxicity which develops in 1.7% to

6.8% of the patients; the prevalence is highly dependent on the total dose administered (Minow and Benjamin, 1975; Lefrak et al., 1975; Praga et al., 1979; Suzuki et al., 1979). Potentially fatal cardiotoxicity has a rising probability at a cumulative dose of >550 mg/m<sup>2</sup> (Lenaz and Page, 1976). Children appear to be at greater risk at any given dose (Gilladoga et al., 1975). The outstanding clinical feature of its cardiotoxic effect is the insidious onset, followed by a rapid progressive biventricular failure and death. The lack of presently available effective treatment of established cardiomyopathy lays special emphasis on the development of possible preventive treatments. Therefore, extensive research has been and is being done to understand the pathomechanism of adriamycin cardiotoxicity. Experimental cardiomyopathy induced by adriamycin has been studied in rats (Schwarz et al., 1998a), dogs (Van Vleet et al., 1980), rabbits and mice (Lambertenghi-Delilieri et al., 1976).

The special ability to bind to the phospholipid cardiolipin and to form a nearly-irreversible complex with this mitochondrial inner membrane protein is considered as the first step of adriamycin cardiotoxicity in several studies (Rahman et al., 1985; de Wolf, 1991). Accumulation of adriamycin in the heart also depends on the rate of its transport out of the cells (Couture et al., 2006). Several members of the P-glycoprotein group of ABC transporters, named multidrug resistance-associated proteins (MRP) were found in high levels in the heart of many species including man (Flens et al., 1996; Meissner et al., 2004). Polymorphism in ABC transporter genes have been shown to modulate drug disposition, and might be responsible for interindividual variability in both the therapeutic and the toxic effect of the drug, but its exact impact on cardiac drug levels remains to be elucidated. It is probable that some drugs, for example verapamil or cyclosporine A, that are clinically proved to enhance the cardiotoxic effect of anthracyclines, act by inhibiting P-glycoprotein or other ABC transporters; paclitaxel might also have a similar effect (Couture et al., 2006). Because of the heart's unique vulnerability to oxidative stress, adriamycin-induced free radical production and subsequent lipid peroxidation (Bachur et al., 1977) has been extensively studied, and the participation of several mitochondrial enzymes (Davies and Doroshov, 1986) has been disclosed. Increased endothelial nitric oxide synthesis and apoptosis (Kalivendi et al., 2001; Bruynzeel et al., 2007) also contribute to the cardiac injury. The cardiotoxic effect of adriamycin is likewise related to impairment of DNA replication (Singal et al., 1997), disturbances of calcium homeostasis (Olson-RD and Mushlin, 1990), and altered iron homeostasis (Xu et al., 2005). Adriamycin affects cardiac-specific gene expression as well. Cardiac adriamycin-responsive protein (CARP) appears to function as a negative regulator of cardiac-specific gene expression and its overexpression in cardiomyocytes suppresses cardiac

troponin C and atrial natriuretic factor transcription (Umlauf and Horkey, 2002), but the downregulation of  $\alpha$ -actin, myosin light- and heavy chains and sarcoplasmic reticulum ATP-ase has also been suggested to play a role in adriamycin cardiotoxicity. Similarly, changes in the high energy phosphate pools, disturbances in the endothelin, cannabinoid, and neuroregulin signaling pathways, ceramide accumulation and extracellular matrix remodeling can also contribute to the pathomechanism (for a review see Octavia et al., 2012).

Besides the chronic cardiotoxic effect, acute cardiotoxicity of adriamycin also occurs, presenting mainly with arrhythmias and myocardial apoptosis (Arola et al., 2000). In humans the incidence of acute cardiotoxicity is relatively high (~11%), and occurs during and within 2-3 days of the administration of the drug, and the manifestations are usually chest pain due myo-pericarditis, sinus tachycardia, paroxysmal nonsustained supraventricular tachycardia, and premature atrial and ventricular beats. The electrocardiogram may show nonspecific ST-T changes, decreased QRS amplitude and left axis deviation. Acute left ventricular failure is rare, and the acute effects of the drug are usually reversible (reviewed by Chatterjee et al., 2010).

Anthracycline cardiotoxicity is a multifactorial process, many mechanisms of which are different from those implicated in the therapeutic effect of the drug. Jeon et al concluded that damage to cardiac adrenergic innervation might also contribute to the pathomechanism of adriamycin-induced cardiomyopathy, but little is known about the possible role of sensory nerves in the mechanism of anthracycline cardiotoxicity (Jeon et al., 2000).

Human histopathologic effects of the drug have been studied at autopsy (Suzuki et al., 1979) and in endo-myocardial biopsy specimens (Billingham et al., 1978). The lesions are disseminated but are yet focal: vacuolar degeneration due to distension and swelling of the sarcoplasmic reticulum, and later myofibrillar loss can be seen. „Adria Cell” is a term used for cardiomyocytes with myofibrillar loss, cytoplasmic vacuolization and central clumping of the nuclear chromatin seen by electron microscopy in adriamycin-treated patients. Lesions progress to death of the myocyte, at which point the mitochondria degenerate with swelling and cristolysis and finally dead myocytes are replaced by fibrotic tissue. In many animal models similar histopathologic changes were observed. Animal studies pointed to the fact that a number of morphological signs appear before the onset of congestive heart failure (for a review see (Saltier and McGuire, 1983).

Similar changes were recognized in post mortem examination of dorsal root and cardiac ganglia of adriamycin- or daunorubicin-treated patients (Smith, 1969). Adriamycin does not pass the blood brain barrier *in vivo*, but has rapid access to cells in the dorsal root

ganglia (Bigotte et al., 1982) and exerts a pronounced neurotoxic effect in experimental animals. In rats, administration of a single high dose of adriamycin lead to selective sensory neuropathy, with characteristic morphological changes and cell death in DRGs (Cho, 1977; Eddy, 1983). Cellular degeneration was also present in cultures of DRGs exposed to adriamycin (Zagoren et al., 1984). In mice, intramuscular or intraneural injection of the drug reaches the CNS via retrograde axonal transport and induces degeneration of motoneurons and other types of nerve cells (Bigotte and Olsson, 1983).

It is difficult to estimate the clinical prevalence of the neurotoxic effect of adriamycin because it is often given in combination with other cytostatic agents with known neurotoxic propensity. Vinca alkaloids are particularly neurotoxic. Other cytostatic drugs can cause peripheral neuropathies, but are less neurotoxic than vincristine (Rosenthal and Kaufman, 1974). The clinical features of vincristine neuropathy are well documented (Holland et al., 1973). Other oncolytic drugs associated with peripheral neuropathy include cisplatin, suramin, paclitaxel, chlorambucil, altretamine, carboplatin, cytarabine, docetaxel, dacarbazine, etoposide, fludarabine, tamoxifen, teniposide, and thioguanine, (for reviews see Scripture et al., 2006; Kanbayashi et al., 2010; Grisold et al., 2012). Most drug-induced neuropathies affect solely the sensory nerves or are mixed sensorimotor in nature. Usually mixed peripheral neuropathies present sensory symptoms prior to progressing to motor impairments. Autonomic neuropathy may also develop following the administration of anticancer agents.

### ***Aims of the study***

Peptidergic cardiac nerves have been demonstrated to play a significant role in cardiac function under pathophysiological and possibly physiological conditions. However, the participation of cardiac sensory nerves in the pathomechanism of anthracycline-induced toxic cardiomyopathy has not been considered, yet. With regard to the pivotal significance of chemosensitive afferent nerves expressing TRPV1 and CGRP in myocardial protective mechanisms, the present experiments were conducted in an attempt to reveal a possible role of these particular nociceptive sensory nerves in the development of adriamycin-induced experimental cardiomyopathy in the rat.

Chemosensitive afferent nerves innervating different organs and tissues share many common traits in terms of their morphology, neurochemistry and function. The cutaneous chemosensitive afferent nerves are the best-characterized class of this particular population of sensory nerves (Roosterman et al., 2006; Jancsó et al., 2009a). Through application of a

treatment paradigm previously shown to produce congestive cardiomyopathy in the rat, an additional aim of the present work was therefore an explanation of the neurotoxic effects of adriamycin on chemosensitive afferent nerves through studies of cutaneous neurogenic sensory vasodilatation, neurogenic plasma extravasation, inflammatory hyperalgesia and innervation, and measurement of CGRP release from sensory nerves.

The specific aims of this thesis were as follows:

- 1) The exploration of the possible contribution of cardiac chemosensitive peptidergic nerves to protective mechanisms against adriamycin-induced cardiotoxicity by comparing the severity and dynamics of progression of cardiac failure/damage in intact and chemodenervated rats.
  
- 2) The evaluation of the neurotoxic effects of adriamycin on the local regulatory, „sensory efferent” functions of cutaneous chemosensitive afferent nerves. The assessment of the dose- and time-dependent effects of adriamycin on cutaneous neurogenic vasodilatation, plasma extravasation, inflammatory hyperalgesia and the axonal release of CGRP.
  
- 3) The quantitative analysis of the morphological effects of adriamycin on cutaneous innervation and skin structure.

## MATERIALS AND METHODS

This study conformed fully to the “Principles of laboratory animal care” (NIH publication No. 85-23, revised 1985) and was approved in advance by the Animal Research Ethics Committee of the University of Szeged.

### *Adriamycin treatment*

Adult male Wistar rats weighing  $250 \pm 30$  g at the start of the experiments were used in the study. Adriamycin (doxorubicin) treatment was induced according to the treatment schedule introduced by Tong et al (Tong et al., 1991). The animals received a cumulative dose of 15 mg/kg of adriamycin (Pharmacia Italia, Milan, Italy) by systemic injection of 2.5 mg/kg of the drug three times a week for 2 weeks. 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 (Fluka, 10, 20 and 100 mg/kg subcutaneously injected on 3 consecutive days) or its vehicle (6% ethanol, 8% Tween 80 in saline,  $n=6$ ) 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 (Ferdinandy et al., 1997).

### *Echocardiatic assessment of cardiomyopathy*

Cardiac function was assessed by echocardiographic examination before and at regular intervals after capsaicin and/or adriamycin treatment. Echocardiography was performed essentially as described by Schwarz et al (Schwarz et al., 1998a; et al., 1998b). The rats were anesthetized with ether, the chest was shaved and the animal was placed in the supine position. Two-dimensional and M-mode echocardiographic examinations were performed in accordance with the criteria of the American Society of Echocardiography, with a Desmin F ultrasound scanner (Echo-Son, Poland), using 5 and 7.5 MHz phased-array transducers. The investigator who analyzed the echocardiograms was unaware of the modes of treatment that the animals had undergone. At all-time points, three measurements were made in each animal, and the mean values were calculated and used for statistical evaluation of the data. 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. Three cardiac cycles were measured, and the mean values were calculated and utilized for the evaluation of the data. The ratio LAD/AOD was calculated. Values are given as means  $\pm$  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 cardiac tissues***

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  $\mu$ m and were processed for immunohistochemical staining with the indirect immunofluorescence technique by using rabbit polyclonal antisera raised against protein gene product 9.5 (PGP 9.5, 1:1000; Ultraclone, Cambridge, UK) and CGRP (1:500, Sigma Chemicals, St Louis, MO, USA). Goat anti-rabbit IgG labeled with Cy3 (carboxymethylindocyanin, 1:500, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as a secondary antibody. The specimens were viewed under a Leitz DMLB fluorescence microscope equipped with an appropriate filter combination and photographed with a digital camera (Nikon Coolpix 950).

### ***Measurement of chemically induced neurogenic cutaneous vasodilatation***

Animals were anesthetized with chloral hydrate (400 mg/kg, i.p., Reanal, Budapest, Hungary). Body temperature was maintained at  $37.2 \pm 0.5$  °C with a heating pad. A small silicone chamber (5 mm in diameter) was mounted on the middle region of the dorsal skin of



the hind paw to avoid the lateral spreading of the applied solutions. Cutaneous blood flow (CuBF) was monitored with a Laser Doppler Blood Flow (LDF) Monitor (type MBF3, Moor Instruments Ltd, Axminster, Devon, UK) by positioning the probe over the surface of the skin in the center of the chamber. After a stable LDF signal was attained, 20  $\mu$ l of mustard oil (0.2%, allyl-isothiocyanate, Merck, Darmstadt, Germany) 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%, Fluka, Buchs, Switzerland) 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 TRPA<sub>1</sub> and TRPV<sub>1</sub> antagonists, HC-030031 (Tocris, Bristol, UK) and capsazepine (Sigma-Aldrich GmbH, Germany), respectively, were also tested. Capsaicin was dissolved in ethanol. Stock solutions of HC-030031 (100 mM) and capsazepine (10 mM) were prepared with dimethyl sulfoxide and ethanol, respectively. Mustard oil was diluted with ethanol.

#### ***Measurement of chemically induced cutaneous plasma protein extravasation***

Neurogenic plasma extravasation was evoked and quantified as described previously. Briefly, animals were anesthetized with chloral hydrate and injected intravenously with Evans blue dye (50 mg/kg, 1% in saline). Ten minutes 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 TRPA<sub>1</sub> and TRPV<sub>1</sub> antagonists, HC-030031 and capsazepine, respectively, were also tested. After 20 min, the animals were perfused transcardially with saline, and samples of the dorsal paw skin were removed, weighed and placed into formamide to extract the dye for quantitative photometric determination (Jancsó et al., 1977). Tissue Evans blue contents were expressed in  $\mu$ g dye/g tissue as mean  $\pm$  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. The doses of these agents were chosen to produce dye leakage responses of similar magnitude. Skin sites injected with the solvent (artificial interstitial fluid, 0.1 ml) served as controls. Twenty min after the injections, rats were sacrificed by decapitation and abdominal skin samples of standardized size were removed and their dye contents were measured as described above.

### ***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, containing in mM: 107.8 NaCl, 26.2 NaHCO<sub>3</sub>, 9.64 Na-gluconate, 7.6 sucrose, 5.55 glucose, 3.48 KCl, 1.67 NaH<sub>2</sub>PO<sub>4</sub>, 1.53 CaCl<sub>2</sub> and 0.69 MgSO<sub>4</sub>) gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> 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 (Price et al., 2005). 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 TRPV<sub>1</sub> 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 wet weights of the nerves were also measured. The CGRP concentrations of the samples were determined with an enzyme-linked immunoassay kit (ELISA, Bertin Pharma, Montigny-le-Bretonneux, France) according to the protocol provided by the manufacturer. The absorbances of the samples were measured photometrically with a microplate reader (DY-NEX MRX, Dynex Technologies, Chantilly, USA), 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 (Hargreaves et al., 1988). The animals were placed on a glass surface in a transparent plastic cage and were allowed to adapt to their environment for 15 min before testing. The heat stimulus was directed onto the plantar surface of the hind paw. The withdrawal latency was measured three times on both hind paws of each rat, the means of the measured data serving as baseline for the left and right hind paws. The apparatus was calibrated to give a withdrawal latency of about 10 s in control animals. To prevent tissue damage, a cutoff-time of 20 s was chosen. For the assessment of carrageenan-induced thermal hyperalgesia, rats received an intraplantar injection of carrageenan (3 mg in 0.1 ml saline, Sigma-Aldrich GmbH, Steinheim, Germany) 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  $\pm$  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 (Dynamic plantar aesthesiometer, Ugo Basile, Gemonio, Varese, Italy). Rats were placed on a metal mesh table and allowed to adapt to their new compartments. With the help of an adjustable angled mirror, the mechanical stimulus was directed to the plantar surface of the hind paw from below the floor. When the unit was started, a steel rod was pushed against the plantar surface of the hind paw with increasing force of 0 to 50 g over a 20-s period. As the animal withdrew its hind paw or an applied force of 50 g was reached, the stimulation was stopped and the force at which the response occurred was recorded. This procedure was repeated three times and the withdrawal responses were averaged. 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  $\pm$  S.D.) from the control.

### ***Kinematic analysis of hindlimb locomotor function***

#### ***Experimental setup***

For the kinematic analysis of the locomotion of intact and treated animals a transparent plexiglass runway (100 cm [L] x 15 cm [W]) 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. The analysis of locomotor function was performed on basis of the observations published earlier by other laboratories (Ribotta et al., 2000; Fey et al., 2010) and of our own observations on animals that had suffered from an ongoing motoneuron degeneration and/or loss (Nógrádi and Vrbová, 2001; Nógrádi et al., 2007; Pájer et al., 2014)). A methodological manuscript detailing the description of the above set up and analysis is currently being prepared for publication.

### ***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 analyzed by using the ImageJ software (NIH, USA). 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; ankleflexion: 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 TRPV<sub>1</sub> receptor (rabbit polyclonal, 1:1000-1:1500, Alomone Laboratories, Jerusalem, Israel) and β-tubulin (mouse

monoclonal, 1:1000, Sigma-Aldrich GmbH, Steinheim, Germany). Donkey anti-rabbit and anti-mouse IgGs labeled with Cy3, DL-488 (1:500, Jackson Immunoresearch Laboratories, West Grove, PA, USA) 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 TRPV<sub>1</sub> antibody we have also performed a preadsorption test by applying a blocking peptide (supplied by the manufacturer of the TRPV<sub>1</sub> antibody) representing the immunogenic fragment of TRPV<sub>1</sub> 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 (Invitrogen, ThermoFisher Scientific, Budapest, Hungary).

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). Systemic random projection images of the epidermis were obtained from 25- $\mu$ m-thick cryostat sections of the plantar skin of the hind paw. By using the filter grid cycloid arc plug-in of the Image-J image analysis software (Image Processing and Analysis in Java, National Institutes of Health, USA), a counting template containing cycloid curves was defined and superimposed onto the images. The major axis of the cycloid curves was aligned perpendicular to the skin surface. The total length of the cycloid curves and the number of counting grid points were optimized by performing pilot experiments on control skin sections. The factor  $l/p$  (length of test line per grid point) was set at 12.046  $\mu$ m. Intersections of the epidermal nerve fibers with the cycloid arcs, and the number of grid points coinciding with the area representing the cross-section of the epidermal layer were determined on each image. The length density ( $L_v$ ) of the intraepidermal nerve fibers was determined via the equation  $L_v = 2 \times li/d \times Pi \times (l/p)$ , where  $li$  is the number of cycloid intersections,  $d$  is the thickness of the section and  $Pi$  is the number of grid points hitting the cross section area of the epidermis.

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.

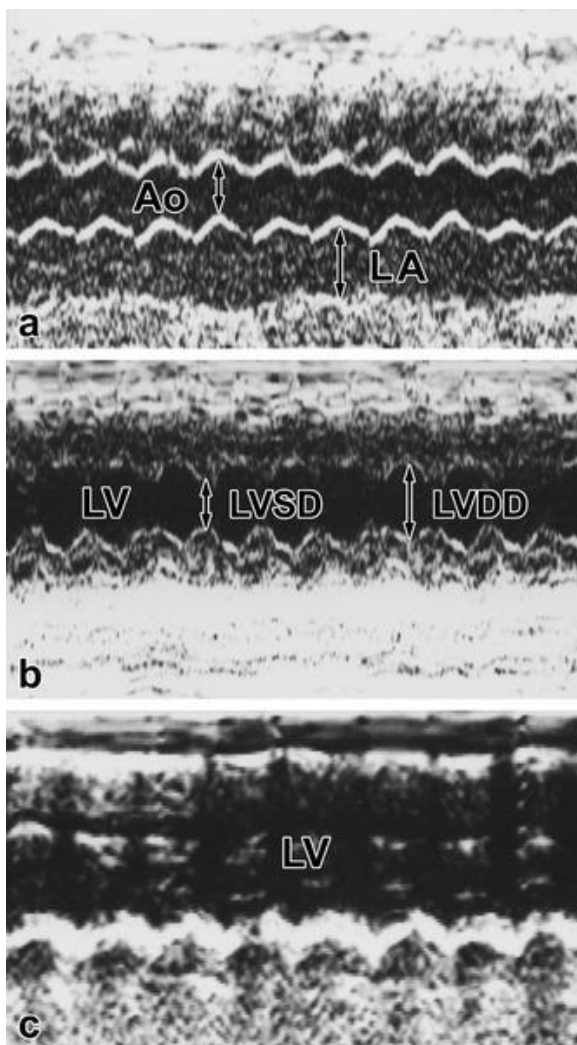
### ***Statistics***

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. The values of the survival proportions  $\pm$  SEM were calculated. To compare the survival experience of the animals of the two groups, the non-parametric log rank test was applied. Since more than one event was registered at certain times of observations, we calculated the  $X^2$  value by a formula described by (Altman, 1991). Pairwise comparisons between the survival proportions observed at the completion of adriamycin treatment and at the end of the 2nd, 3rd, and 4th weeks of the post-treatment period were performed using Fisher's exact test and the  $p$  values were corrected by Bonferroni's method. 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*

The cardiac parameters measured by echocardiographic examination in control, capsaicin-, adriamycin-, and capsaicin plus adriamycin-treated rats are presented in Table 1. The echocardiograms in Fig. 1 illustrate the techniques used to measure the echocardiographic parameters involving AOD, LAD, LVSD, and LVDD. Sequential echocardiographic examination of the control animals demonstrated little change in the cardiac parameters during the period of the study. Since there was no significant difference between the parameters of the two control groups, treated with the vehicles for capsaicin and adriamycin, respectively. For subsequent statistical evaluation, they were lumped together as a single control group. None of the rats in this control group died or developed pathologies associated with cardiac failure during the period of the study. Fractional shortening at the beginning of the study was  $25.3 \pm 1.5\%$  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.



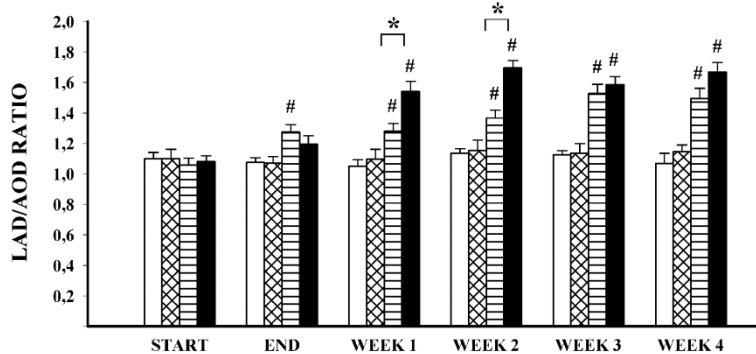
**Fig. 1** (a) M-mode echocardiogram of the aorta (*Ao*) and the left atrium (*LA*) illustrating the technique used to measure the aortic (AOD) and left atrial dimensions (LAD). A normal ratio of LAD and AOD is measured in the parasternal long axis view in a control rat. (b) M-mode echocardiogram of the left ventricle (*LV*) in the parasternal long axis view illustrating the method used to measure the ventricular dimensions in a control rat. The left ventricular end-diastolic diameter (*LVDD*) and the left ventricular end-systolic diameter (*LVSD*) measurements are done from the leading edge at the end of the diastole and at the end of the systole. Normal contractility of the left ventricle can be seen. (c) An enlarged LV can be seen by M-mode echocardiography in the parasternal long axis view in a rat treated with capsaicin plus adriamycin. The contractility of the left ventricle is markedly decreased

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 (Fig. 2a, Table I). 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 (Fig. 1, Table 1). In the rats treated with adriamycin, the echocardiographic examinations revealed a progressive reduction in FS, indicating a decrease in cardiac contractility (Fig. 1c), 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 (Fig. 2b, Table I). In contrast, a marked and significant reduction in FS was already observed 1 week after the completion of adriamycin treatment in the rats pretreated

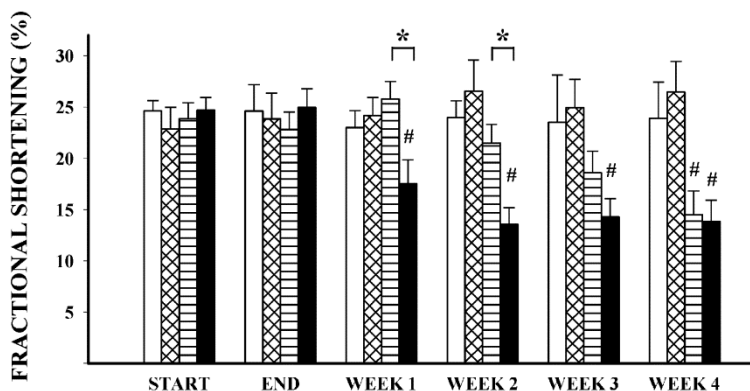


with capsaicin. Statistical analysis of the data showed that, with the exception of the 4th week post-treatment, the FS in the capsaicin plus adriamycin-treated rats was markedly lower than that in the rats treated only with adriamycin (Fig. 2b, Table I).

**A**



**B**



**Fig. 2 (a)** Ratio of LAD and AOD and (b) fractional shortening of the left ventricle in control (open bars), capsaicin-treated (cross-hatched bars), adriamycin-treated (horizontally hatched bars), and capsaicin plus adriamycin-treated (filled bars) groups of rats before (Start), and 3 days (End) and 1–4 weeks after the completion of the adriamycin treatment. The asterisk and the hash sign denote statistically significant differences ( $p < 0.05$ ) between selected groups or in comparison with the initial control value respectively. Each column depicts the mean of values obtained from at least five rats.

Treatment period	Aortic Diameter (AOD)				Left Atrial Diameter (LAD)				LAD/AOD			
	Control	Capsaicin	Adriamycin	Capsaicin + Adriamycin	Control	Capsaicin	Adriamycin	Capsaicin + Adriamycin	Control	Capsaicin	Adriamycin	Capsaicin + Adriamycin
Start	3.42±0.12	3.06±0.22	3.39±0.12	3.29±0.09	3.77±0.26	3.35±0.26	3.59±0.15	3.56±0.12	1.10±0.04	1.10±0.06	1.06±0.05	1.08±0.04
End	3.37±0.09	3.37±0.24	3.36±0.13	3.03±0.14	3.63±0.12	3.56±0.26	4.33±0.16*	3.59±0.17	1.08±0.02	1.07±0.05	1.27±0.07	1.20±0.05
1 week <sup>§</sup>	3.35±0.10	3.45±0.15	3.47±0.13	3.00±0.17	3.52±0.07	3.80±0.13	4.42±0.16* <sup>#</sup>	4.10±0.22* <sup>#</sup>	1.05±0.01	1.10±0.06	1.28±0.05* <sup>#</sup>	1.37±0.07* <sup>#</sup>
2 weeks <sup>§</sup>	3.60±0.16	3.28±0.16	3.28±0.14	3.28±0.12	4.11±0.28	3.41±0.28	4.44±0.17* <sup>#</sup>	5.54±0.25* <sup>#</sup>	1.14±0.03	1.25±0.07	1.37±0.05* <sup>#</sup>	1.69±0.05* <sup>#</sup>
3 weeks <sup>§</sup>	3.52±0.16	3.20±0.11	3.27±0.16	3.36±0.14	3.97±0.26	3.64±0.23	5.03±0.22* <sup>#</sup>	5.31±0.27* <sup>#</sup>	1.13±0.02	1.14±0.06	1.53±0.06* <sup>#</sup>	1.58±0.05* <sup>#</sup>
4 weeks <sup>§</sup>	3.62±0.12	3.48±0.19	3.15±0.17	3.24±0.16	3.87±0.14	3.79±0.36	4.69±0.22* <sup>#</sup>	5.41±0.31* <sup>#</sup>	1.07±0.02	1.09±0.05	1.49±0.07* <sup>#</sup>	1.67±0.06* <sup>#</sup>

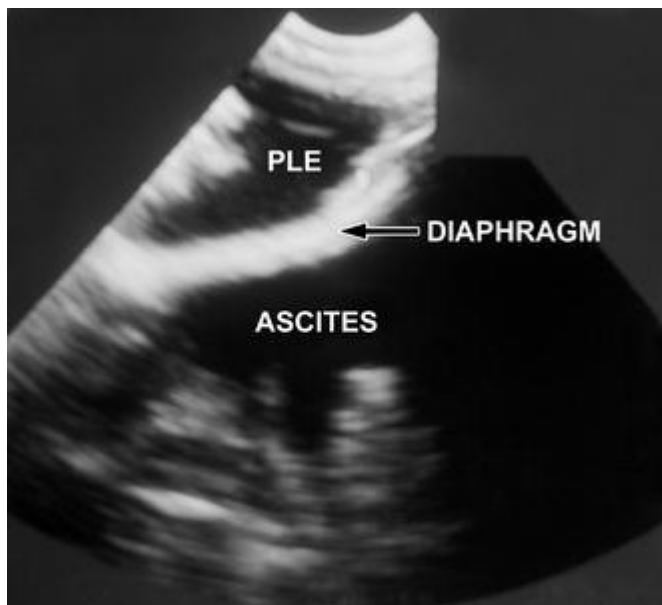
  

Treatment period	Left Ventricular End Diastolic Diameter (LVDD)				Left Ventricular End Systolic Diameter (LVSD)				Fractional Shortening (FS)			
	Control	Capsaicin	Adriamycin	Capsaicin + Adriamycin	Control	Capsaicin	Adriamycin	Capsaicin + Adriamycin	Control	Capsaicin	Adriamycin	Capsaicin + Adriamycin
Start	5.46±0.19	5.20±0.26	5.33±0.30	5.46±0.17	4.11±0.14	3.94±0.24	4.06±0.17	4.11±0.14	0.25±0.01	0.24±0.02	0.24±0.02	0.25±0.01
End	5.70±0.11	5.31±0.34	5.52±0.21	5.68±0.24	4.31±0.23	4.04±0.30	4.23±0.19	4.23±0.21	0.24±0.05	0.24±0.02	0.23±0.02	0.25±0.02
1 week <sup>§</sup>	5.65±0.14	5.53±0.18	5.40±0.17	6.01±0.30	4.33±0.13	4.19±0.17	4.00±0.16	4.95±0.27* <sup>#</sup>	0.23±0.02	0.24±0.02	0.26±0.02	0.17±0.02*
2 weeks <sup>§</sup>	5.76±0.11	5.49±0.22	5.40±0.32	5.83±0.22	4.41±0.12	4.21±0.26	4.28±0.27	5.03±0.19* <sup>#</sup>	0.23±0.02	0.23±0.03	0.22±0.02	0.14±0.02* <sup>#</sup>
3 weeks <sup>§</sup>	5.83±0.18	5.58±0.57	5.65±0.29	6.45±0.24* <sup>#</sup>	4.33±0.07	4.18±0.43	4.69±0.18*	5.52±0.21* <sup>#</sup>	0.26±0.04	0.25±0.03	0.17±0.02	0.14±0.02* <sup>#</sup>
4 weeks <sup>§</sup>	5.80±0.14	5.81±0.44	5.40±0.51	6.68±0.28* <sup>#</sup>	4.45±0.12	4.31±0.48	4.64±0.19*	5.75±0.24* <sup>#</sup>	0.23±0.03	0.26±0.03	0.14±0.02* <sup>#</sup>	0.13±0.03* <sup>#</sup>

**Table I** Cardiac parameters measured by echocardiographic examination in control, capsaicin-, adriamycin- and capsaicin plus adriamycin-treated rats.

AOD aortic diameter, LAD left atrial diameter, LVDD left ventricular end diastolic diameter, LVSD left ventricular end systolic diameter, FS fractional shortening

Values are expressed in mm as mean ± SEM obtained from at least 5 animals. § Time after completion of the treatment with adriamycin.\* Significantly different from the initial baseline value.# Significantly different from the values of the corresponding control group.



**Fig. 3** Demonstration of the large amount of pleural effusion (*PLE*) and ascites in the subcostal cross-sectional view of an adriamycin-treated rat by two-dimensional ultrasound examination

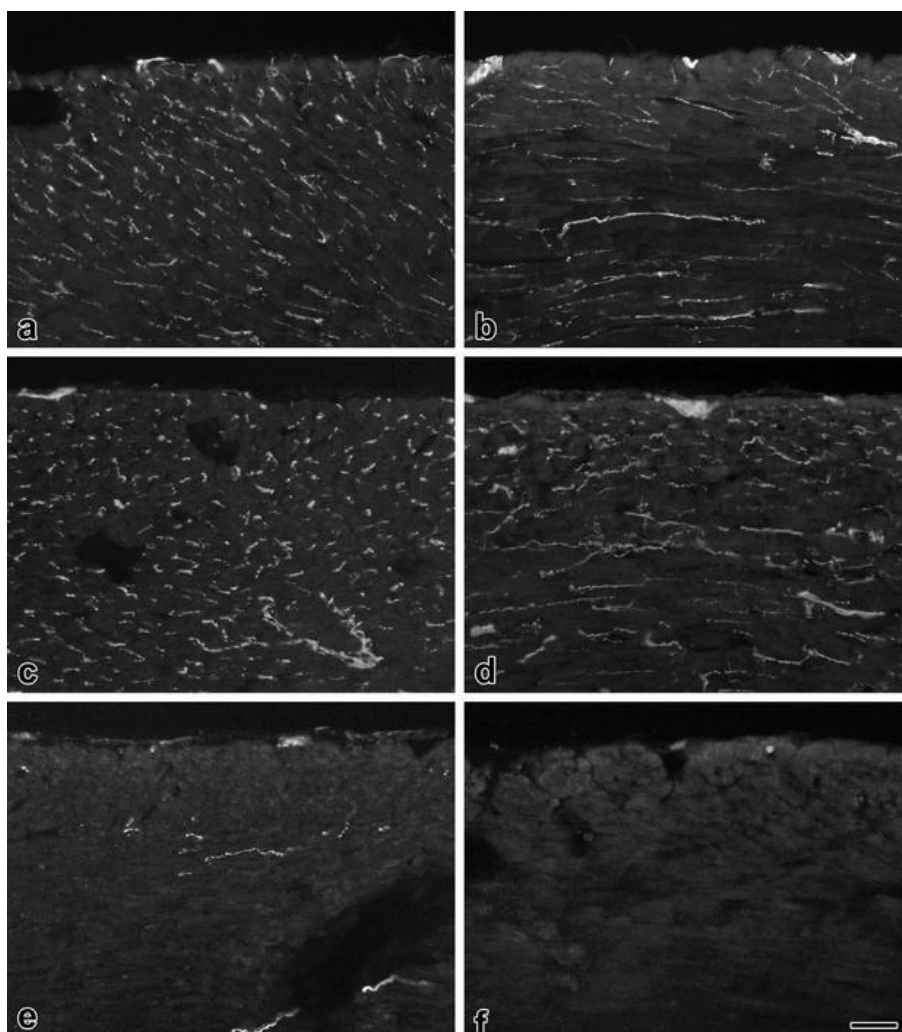


**Fig.4** Cross section of the ventricles of control (left) and adriamycin-treated (right) rats shows dilation of the both ventricles with thinning of the ventricular walls, which are characteristic signs of congestive dilatative cardiomyopathy

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 (Fig. 3). The cumulative incidences of pericardial effusion, pleural effusion, and ascites by the end of the study period were 8, 3, and 8 of the 15 adriamycin-treated rats, and 11, 3, and 8 of the 17 capsaicin plus adriamycin-treated rats, respectively. Three weeks after 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.

***Anthracycline- and/or capsaicin-induced changes in the cardiac innervation: immunohistochemistry***

Immunohistochemical studies were performed to reveal possible changes in the populations 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 (Fig.5a, b). CGRP-containing nerves innervating the ventricles were far less numerous (Fig.5e). 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 (Fig.5c, d). 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 (Fig.5f).



**Fig. 5 a–d** Protein gene product (PGP) 9.5 immunoreactive and **e, f** calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibers in tissue sections obtained from the left (**a, c, e, f**) and right (**b, d**) heart ventricles of control (**a, b**) and capsaicin plus adriamycin-treated (**c–f**) rats. The distribution pattern of PGP 9.5-positive cardiac nerves is apparently similar in control and treated rats.

CGRP-immunopositive nerves cannot be detected in the capsaicin-pretreated rat. The scale bar in **f** indicates 50  $\mu$ m and applies to all microphotographs

### *Alterations of chemically-induced vascular reactions*

#### *Effects of adriamycin on neurogenic sensory vasodilatation*

In control rats, applications of mustard oil at concentrations of 0.2% and 1%, and 1% capsaicin produced significant increases in the CuBF (Fig. 6a, c, d). Subcutaneous (s.c.) injection of HC-030031 (100  $\mu$ M, 50  $\mu$ l) 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  $121.80 \pm 24.19\%$ , whereas the increase in CuBF after the prior administration of the TRPA<sub>1</sub> antagonist, HC030031 amounted to  $14.47 \pm 10.44\%$  ( $p < 0.01$ ,  $n = 6/\text{group}$ ). The epicutaneous application of capsaicin (1%) resulted in a marked elevation in CuBF, which amounted to  $167.23 \pm 53.36\%$ . Administration of the specific TRPV<sub>1</sub> antagonist, capsazepine (10  $\mu$ M, 50  $\mu$ l, s.c.) 20 min before the epicutaneous application of capsaicin resulted in a significant inhibition of the vasodilatory effect which amounted to  $27.94 \pm 8.95\%$  ( $p < 0.01$ ,  $n = 6/\text{group}$ ). 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. 6c), and to 1% capsaicin (Fig. 6d), amounting to 43%, 29%, and 29%, respectively, of the control values ( $p < 0.01$ ,  $n = 6-10/\text{group}$ , Table II).

**Table II** *Effect of adriamycin pretreatment on chemically-evoked cutaneous sensory neurogenic vasodilatation*

	Mustard oil (0.2%)	Mustard oil (1%)	Capsaicin (1%)
Control	54.00 ± 26.30	121.80 ± 24.19	167.23 ± 53.36
Adriamycin (7.5 mg/kg)	46.55 ± 30.73	58.17 ± 22.60*	60.88 ± 16.40*
Adriamycin (15 mg/kg)	23.55 ± 19.24*	35.67 ± 12.55*	48.33 ± 27.09*

Cutaneous blood flow was measured with a laser Doppler flow probe positioned over the dorsal skin of the hindpaw. Test agents were applied as described in the text. Percentage changes in blood flow are expressed as mean ± S.D. \* Significantly different from the control ( $p < 0.05$ ,  $n = 6-10/\text{group}$ ).

### ***Effects of adriamycin on neurogenic plasma protein extravasation***

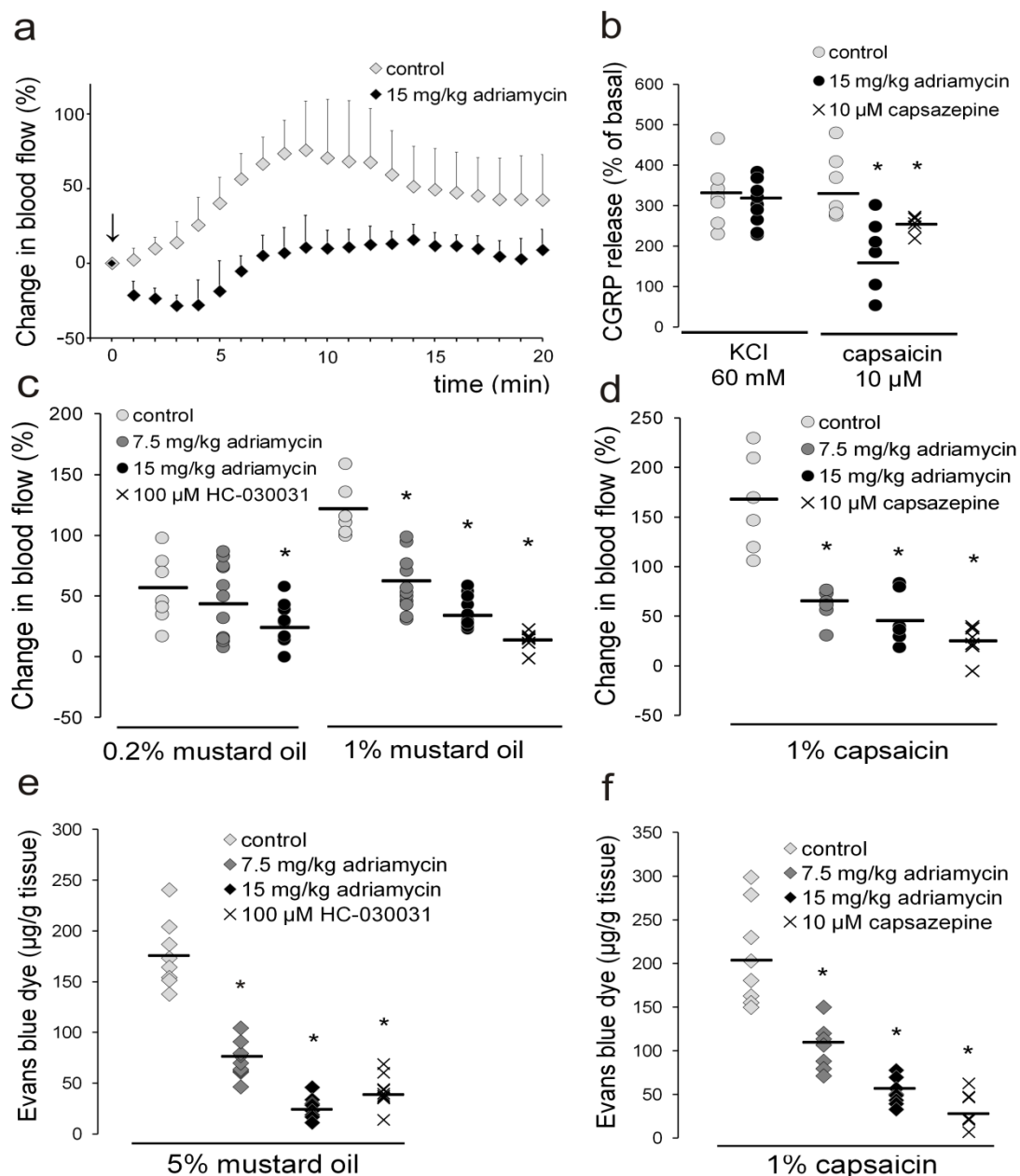
Neurogenic plasma protein extravasation was studied with the quantitative Evans blue technique following the epicutaneous application of mustard oil or capsaicin. In control rats, both mustard oil (5%) and capsaicin (1%) produced marked plasma protein extravasation, with In control rats, epicutaneous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

**Table III** *Effect of doxorubicin treatment on the plasma extravasation induced by various vasoactive compounds*

Evans blue concentration of the abdominal skin ( $\mu\text{g/g}$ tissue)	Histamine (10 $\mu\text{g}$ in 0,1 ml SIF)	Bradykinin (4 $\mu\text{g}$ in 0,1 ml SIF)	Substance P (10 $\mu\text{g}$ in 0,1 ml SIF)
control	501.0 $\pm$ 70.9	236.1 $\pm$ 70.2	115.8 $\pm$ 35.7
adriamycin (15 mg/kg i.v.)	506.3 $\pm$ 182.2	305.0 $\pm$ 126.9	125.9 $\pm$ 136.3

Data show the Evans blue dye content of standard size skin samples after the intracutaneous application of different vasoactive compounds. Note, that there is no significant difference between control and adriamycin treated rats. Data are expressed as mean  $\pm$  SD,  $n = 6-9$ /group



**Fig. 6** Effects of adriamycin treatment on chemically-evoked cutaneous sensory neurogenic vasodilatation (**a**, **c**, **d**), plasma extravasation (**e**, **f**), and axonal CGRP release (**b**). The graph (**a**) demonstrates percentage changes of blood flow of the dorsal paw skin after the application of mustard oil (0.2 %, arrow) in control and adriamycin-treated animals as assessed by laser Doppler flowmetry. Each marker indicates changes of blood flow measured in consecutive 1-min periods expressed as mean + SD of six animals. The graph (**b**) shows the effect of adriamycin treatment (cumulative dose: 15 mg/kg) on capsaicin- and high KCl-induced release of CGRP from rat sciatic nerves *in vitro*. (n = 6–8/group). The graphs (**c**), (**d**) show the effect of adriamycin treatments on mustard oil- and capsaicin-induced cutaneous vasodilatation. (n = 6–10/group). (**e**), (**f**) show mustard oil- and capsaicin-evoked plasma extravasation in control and in adriamycin-treated rats (n = 8 for each group). The effects of capsaicin and mustard oil were inhibited significantly by the specific TRPV<sub>1</sub> and TRPA<sub>1</sub> antagonists capsazepine and HC-030031, respectively. In graphs b–f, each marker indicates an individual value and the horizontal lines show the mean of the group. \* Significantly different from the control,  $p < 0.05$

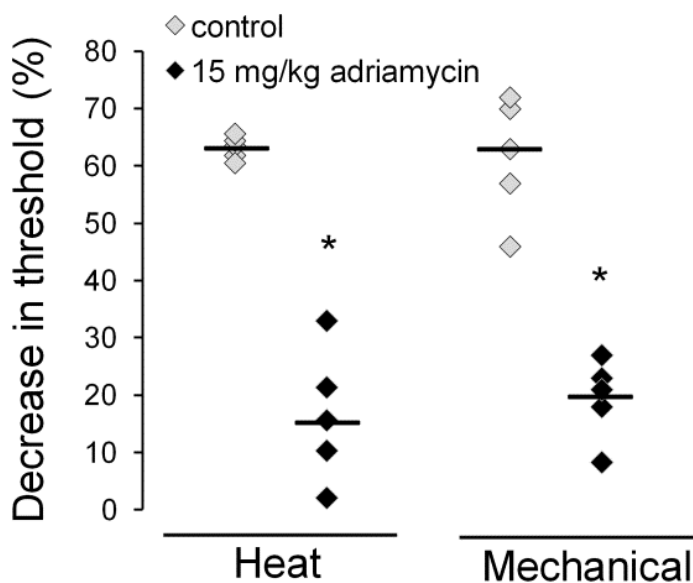


### ***Effects of adriamycin treatment on the in vitro neural release of CGRP***

Neurogenic sensory vasodilatation is mediated by CGRP released from chemosensitive afferent nerves in response to stimulation of the TRPV<sub>1</sub> and TRPA<sub>1</sub> 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 TRPV<sub>1</sub> 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  $\mu$ M, was significantly inhibited by 69.54%, 54.02 % and 23,26%, respectively, in the presence of the specific TRPV<sub>1</sub> antagonist, capsazepine (10.0  $\mu$ M;  $p < 0.01$ ,  $n = 6$ /group). In the control sciatic nerve preparations, both capsaicin (10  $\mu$ M) and high potassium (60 mM) elicited a marked release of CGRP ( $325 \pm 110\%$ , and  $327 \pm 77\%$  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 ( $303 \pm 55\%$  of the basal release,  $n = 8$ , n.s.). In contrast, the capsaicin-induced release of CGRP was significantly reduced in the adriamycin-treated animals ( $164 \pm 71\%$  of the basal release,  $p < 0.01$ ,  $n = 6$ ; Fig. 6b).

### ***Effects of adriamycin on carrageenan-induced thermal and mechanical hyperalgesia***

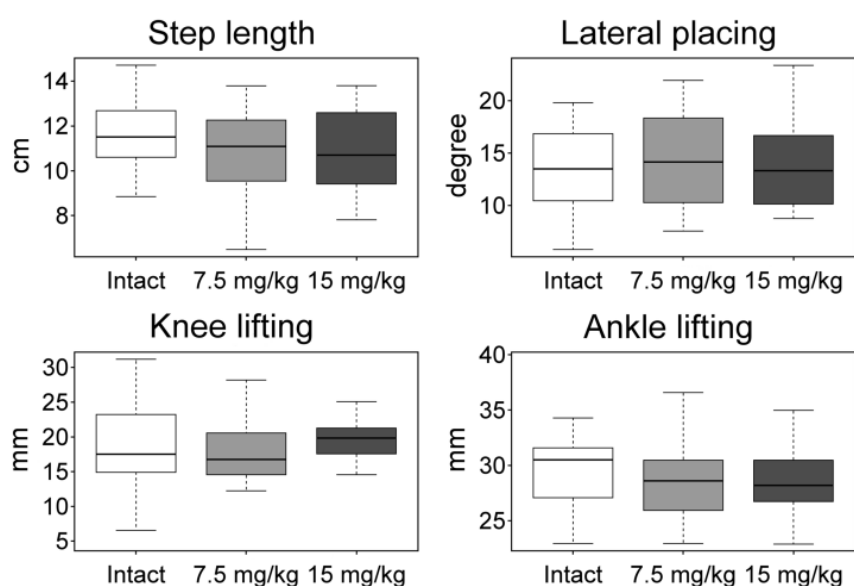
The findings on the effects of adriamycin treatment on the neurogenic sensory vascular responses were indicative of a marked functional impairment of the chemosensitive afferent nerves which express the TRPV<sub>1</sub> and TRPA<sub>1</sub> receptors. Since these nociceptive ion channels play a crucial role in the mechanism of inflammatory hyperalgesia (Caterina et al., 2000), 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  $63.2 \pm 2.0\%$  ( $p < 0.05$ ,  $n = 5$ ). In contrast, in the adriamycin-treated rats that received carrageenan, the heat withdrawal latencies were barely different from the control values ( $16.5 \pm 11.6\%$ ,  $n = 5$ , n.s.). Similarly, the mechanical withdrawal thresholds were significantly reduced in the controls (by  $61.6 \pm 10.5\%$ ,  $p < 0.05$ ,  $n = 5$ ), but not in the adriamycin-treated animals (by  $19.4 \pm 7.0\%$ , n.s.,  $n = 5$ ; Fig. 7).



**Fig. 7** Effect of adriamycin treatment (cumulative dose: 15 mg/kg) on carrageenan-induced thermal and mechanical hyperalgesia. Percentage decreases in withdrawal responses to heat and mechanical stimuli are shown. Each symbol indicates the data of an individual animal; horizontal lines show the mean values of the groups (n=5 for each group). \* Significantly different from the control,  $p < 0.05$ .

#### *Effects of adriamycin on hindlimb locomotor parameters*

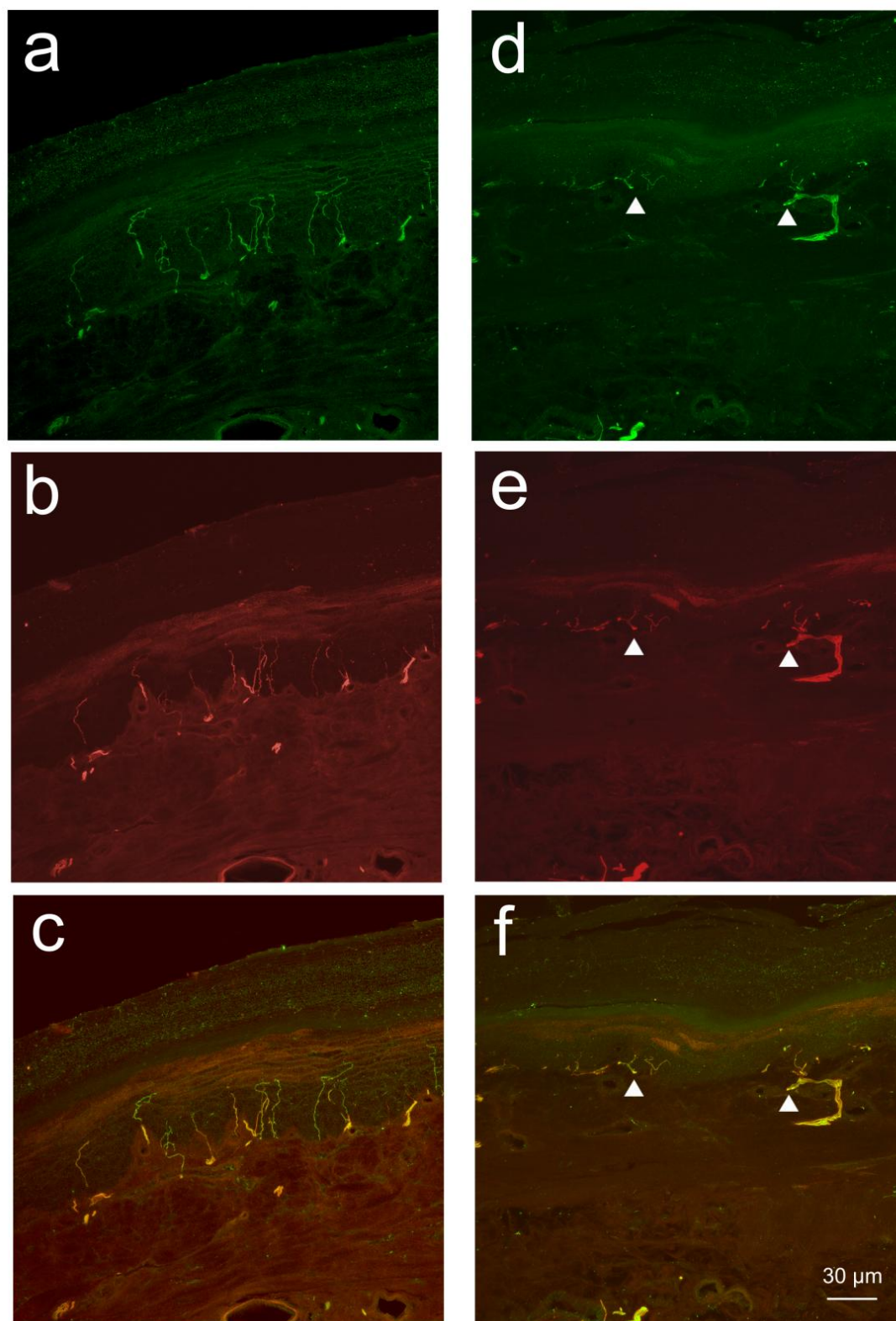
The treated animals walked in a similar manner as their intact controls. No marked difference in the locomotor pattern of intact and treated animals was observed before the detailed analysis. In adriamycin treated rats, thorough examination of the high resolution images failed to reveal any significant impairment in step length, ankle and knee lifting, lateral placing (Fig. 8), 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).



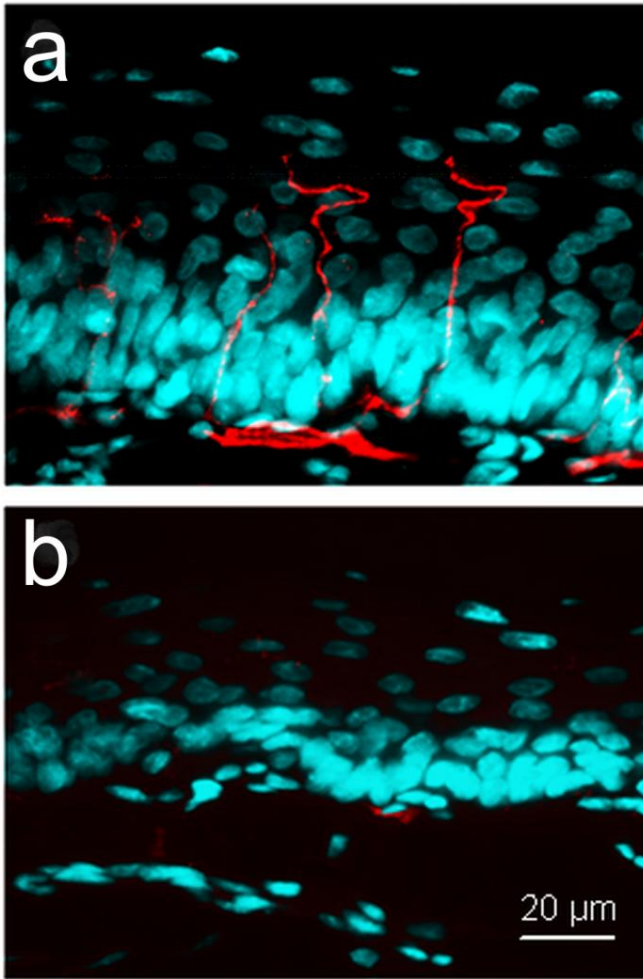
**Fig. 8** Hindlimb locomotor parameters directly dependent on the integrity of motoneuron function. Box whisker plots showing the knee lifting, ankle lifting, step length and lateral placing parameters measured in control and adriamycin-treated rats. There is no indication of motor impairment after adriamycin treatment. (n=5 for each group). \* Significantly different from the control,  $p < 0.05$

### ***Effects of adriamycin treatment on cutaneous sensory nerves and on skin structure***

The effects of adriamycin treatment on the cutaneous innervation were studied with immunohistochemistry, quantitative stereological methods being used to determine the epidermal nerve fiber density of the rat plantar paw skin. In control skin samples tubulin immunostaining revealed subepithelial bundles of nerve fibers giving rise to individual epidermal axons approaching the surface. In accord with our previous findings, two morphological types of epidermal axons were seen: axons with simple morphology, exhibiting little branching and a straight course, and axons which take a tortuous course and develop elaborate arborization. It was earlier established that the axons with simple morphology are peptidergic, whereas those with complex morphology are non-peptidergic (Dux et al., 1998; Zylka et al., 2005). Double immunofluorescence staining revealed that almost all the tubulin-immunoreactive epidermal axons are TRPV<sub>1</sub>-positive. The densities of tubulin- and TRPV<sub>1</sub>-immunoreactive epidermal axons were  $1075 \pm 120$  mm/mm<sup>3</sup> and  $1065 \pm 116$  mm/mm<sup>3</sup>, respectively. The density of epidermal axons decreased by roughly half following adriamycin treatment; the densities of tubulin- and TRPV<sub>1</sub>-positive axons were  $503 \pm 76$  (p<0.01, n=5) and  $417 \pm 80$  mm/mm<sup>3</sup> (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 (Fig. 9.). Adriamycin treatment also resulted in a significant reduction in epidermal thickness (control:  $70.41 \pm 7.52$  μm; adriamycin-treated:  $47.47 \pm 6.75$  μm; p<0.01, n=5; Fig. 10).



**Fig. 9** Effects of adriamycin treatment (cumulative dose: 15 mg/kg) on the distribution of intraepidermal axons of the rat plantar skin. Z-stack confocal microscopic images were taken from specimens double immunostained for tubulin (b,e) and TRPV<sub>1</sub> (a,d). Note the colocalization of tubulin and TRPV<sub>1</sub> (c,f) and the massive loss of intraepidermal axons after adriamycin treatment (d,e,f). Subepidermal nerves (arrowheads) can still be seen after adriamycin treatment (d,e,f). The scale bar in f holds for all microphotographs



**Fig. 10.** Z-stack confocal microscopic images of the plantar skin of control (a) and adriamycin-treated (b), cumulative dose: 15 mg/kg) rats. Epidermal nuclei are visualized with DAPI and intraepidermal axons with TRPV<sub>1</sub> immunohistochemistry. Note the marked thinning of the epidermis after adriamycin treatment. The scale bar in b holds for both microphotographs.

## DISCUSSION

### *Evaluation of adriamycin-induced cardiac impairments using echocardiography*

Several methods are used to evaluate cardiac function under physiological and pathological conditions in both humans and experimental animals. Electrocardiography has been the standard technique for the assessment of pathophysiological changes in heart function (Minow and Benjamin, 1975). It has been used to detect deterioration of cardiac function following the administration of cardiotoxic drugs, such as adriamycin. However, ST-T changes are neither specific nor characteristic of cardiomyopathy, and reduction of the QRS amplitude occurs late in the therapy (Takemura and Fujiwara, 2007). Further, these alterations often do not precede the clinical onset of cardiomyopathy and, therefore, they have little predictive value. Other techniques used to detect cardiac dysfunction include radionuclide angiocardiology, biochemical parameters, and histopathological grading of transvenous endomyocardial biopsy specimens (Billingham et al., 1978; Morandi et al., 2001; Panjra and Jain, 2006). Unfortunately, these techniques lack specificity and clinical validation and/or are invasive (Gabrielson et al., 2008; Octavia et al., 2012). The introduction of echocardiography heralded the advent of a new era in the clinical and experimental investigation of heart function. Echocardiography, a non-invasive technique, is now generally used to evaluate cardiac function by measuring pivotal cardiac morphological and functional parameters (Parisi, 1978; Lewis et al., 1978; Friedman et al., 1979; Biancaniello et al., 1980).

In our study, therefore, we used echocardiography as the most reliable and least invasive technique, which can be used for serial examinations to detect early signs of cardiac dysfunction and the progression of adriamycin-induced cardiomyopathy. Administration of adriamycin to rats over a period of two weeks resulted in a progressive deterioration of cardiac function as reported previously (Tong et al., 1991). Follow-up measurements of the cardiac parameters revealed that the changes indicative of an impaired cardiac function were most marked from the third week onwards after the cessation of adriamycin administration. The FS displayed a noteworthy reduction by three weeks after the cessation of treatment, and by the fourth week the reduction reached the level of statistical significance. Similarly as in previous reports, only minor changes in LVDD were detected in the course of the study, but LVSD exhibited significant increases from the third week onwards (Schwarz et al., 1998). The animals showed other signs of congestive heart failure, as indicated by pericardial and pleural effusions and ascites. These findings corroborate the earlier observations and furnish

further evidence that adriamycin causes a progressive deterioration of cardiac function resembling congestive cardiomyopathy, which can be reliably monitored by means of follow-up echocardiographic examinations in the rat.

### ***Cardioprotective effect of chemosensitive fibers***

The most important observation in the present study was the demonstration of a marked aggravation and, in particular, 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. Similarly, as in the rats treated only with adriamycin, pericardial and pleural effusions and ascites were observed in many animals upon ultrasonographic examination. Administration of capsaicin did not produce significant changes in cardiac parameters examined.

Capsaicin is a selective sensory neurotoxin which is known to induce depletion and/or degeneration of somatic and visceral chemosensitive C-fiber sensory nerves (Jancsó et al., 1977). Histochemical studies demonstrated peptide-containing sensory nerves in the heart of various species, including the rat (Mulderry et al., 1985; Gibbins et al., 1985). The depletion of CGRP-immunoreactive nerve fibers has also been demonstrated after systemic administration of capsaicin (Wharton et al., 1986; Ferdinandy et al., 1997). This was confirmed in the present study by showing a practically complete elimination of CGRP-immunoreactive nerves in both the atria and ventricles of adriamycin-treated rats. However, a similar reduction in cardiac nerves visualized with PGP 9.5 immunohistochemistry was not observed. CGRP plays a cardinal role in the myocardial protection provided by ischemic preconditioning, heat stress or nitroglycerine which is dependent on the integrity of the capsaicin-sensitive sensory nerves and is abolished after capsaicin treatment (Ferdinandy et al., 1997; Song et al., 1999; Zhou et al., 2001; Wang and Wang, 2005). Accordingly, we suggest that the loss of capsaicin-sensitive CGRP-containing cardiac afferent nerve fibers may be the most likely explanation for the marked acceleration and aggravation of the adriamycin-induced cardiac pathology in the capsaicin-pretreated rats. Elimination of an

important cardioprotective mechanism by the depletion of CGRP through capsaicin pretreatment may result in the earlier onset and rapid progression of the myocardial changes leading to congestive cardiomyopathy. This assumption is supported by earlier findings showing the deleterious effect of sensory denervation on experimentally-induced myocardial ischemia, and the beneficial effect of CGRP in experimental models of cardiac ischemia and arrhythmia (Kallner and Franco-Cereceda, 1989; Csont et al., 2003). Taken together, these observations strongly suggest that local release of sensory neuropeptides, in particular CGRP, from capsaicin-sensitive afferent nerves may be a significant protective mechanism which counteracts the deleterious effects of adriamycin in this experimental model of congestive cardiomyopathy. Since capsaicin acts through the activation of the TRPV1 receptor, these findings also suggest an implication of this nociceptive ion channel in the mechanisms of cardioprotection conferred by chemosensitive afferent nerves expressing the TRPV1 receptor. Importantly, the expression of TRPV1 receptor has been demonstrated in cardiac sensory nerves (Zahner et al., 2003). This assumption is supported by recent findings which demonstrated that postischemic recovery is impaired in TRPV1 gene knockout mice (Wang and Wang, 2005). On the contrary, activation of the TRPV1 receptor through N-oleoyldopamine, a novel endogenous capsaicin-like lipid, protects the heart against ischemia-reperfusion injury (Zhong and Wang, 2008).

In addition, other mechanisms may also contribute to cardioprotection afforded by capsaicin-sensitive afferent nerves. Hence, stimulation of capsaicin-sensitive afferents has been suggested to exert a protective effect by triggering cardiogenic sympathoexcitatory reflexes (Zahner et al., 2003). Further, endocrine mechanisms may also contribute to the cardioprotective effect of capsaicin-sensitive afferent nerves. Hence, B-type natriuretic peptide has been shown to limit the size of ischemia-induced infarcts in isolated rat hearts (D'Souza et al., 2004) and the stretch-induced release of atrial natriuretic peptide has been shown to be inhibited in capsaicin treated rats (Rankin and Scott, 1990).

In conclusion, the present study has demonstrated that elimination of capsaicin-sensitive afferent nerves promote the development and progression of adriamycin-induced myocardial dysfunction. The results suggest that perturbation of the function of capsaicin-sensitive afferent nerves and/or myocardial CGRP metabolism, e.g. by agents interfering with capsaicin/TRPV<sub>1</sub> receptors localized on cardiac sensory nerves, or with peptide metabolism, may open up new perspectives as concerns prevention and/or alleviation of the pathological changes that follow adriamycin treatment.



### *Effects of adriamycin on cutaneous sensory nerve functions*

Our studies indicated that the sensory nerves which express the archetypal nociceptive ion channel TRPV1 and contain the neuropeptide CGRP exert a protective effect against adriamycin-induced cardiotoxicity in a rat model of congestive cardiomyopathy. These observations suggested the possibility that the neurotoxic propensity of this anthracycline-type cytotoxic agent may play a role in the development of cardiac pathologies by virtue of their neurotoxic effect through the elimination of an important cardioprotective mechanism conferred by the sensory nerves. The effects of antitumor agents on sensory functions have been the subject of extensive investigations (Grisold et al., 2012), but studies on the effects of these compounds on nociceptor functions are scarce. In subsequent studies, therefore, we sought to further explore the effects of adriamycin on sensory nerve functions. Since somatosensory and visceral chemosensitive nerves share common functional characteristics, our studies related to the effects of adriamycin on the morphology and function of cutaneous afferent nerves, whose morphological and functional traits are best characterized (Roosterman et al., 2006; Jancsó et al., 2009a). Hence, through application of a treatment paradigm previously shown to produce congestive cardiomyopathy in the rat, we have explored the possible neurotoxic effects of adriamycin on cutaneous chemosensitive afferent nerves through studies of cutaneous neurogenic sensory vasodilatation, neurogenic plasma extravasation, inflammatory hyperalgesia, cutaneous innervation, and measurement of CGRP release from sensory nerves.

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 acts by directly activating TRPA1 and TRPV1 receptors, respectively. TRPV1 and TRPA1 are expressed mostly by the same population of chemosensitive afferents (Jordt et al., 2004; Bautista et al., 2005; Kobayashi et al., 2005; Nagata et al., 2005). Activation of epidermal chemosensitive fibers leads to the release of vasoactive sensory peptides. SP is responsible for plasma extravasation by binding to NK1 receptors situated on endothelial cells of postcapillary venules (Lembeck and Holzer, 1979; Kenins et al., 1984; Chahl, 1988), while CGRP exerts its vasodilatory effect mostly by directly acting on vascular smooth muscle cells (Ralevic et al., 1992). They are stored in the sensory nerve endings and can be released via TRPV1 or TRPA1 mediated mechanisms (Pethő et al., 2004) Irritant-induced neurogenic vasodilatation is mediated by chemosensitive afferent nerves and both non-selective and

selective (capsaicin-induced) denervation of the skin abolishes it completely (Jancsó et al., 1977). 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 (Lynn et al., 1996). Investigation 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 (Jancsó and Janka, 1981; Jancsó et al., 1983; Baron et al., 1988; Husz et al., 1991; Baron et al., 1999). In our experiments we found that epicutaneous application of chemical irritants produced a transient dose-dependent increase in regional CuBF which was significantly reduced following adriamycin treatment. Quantitative measurement of neurogenic plasma protein extravasation both in response to epicutaneous administration of capsaicin or mustard oil showed similar dramatic decreases in adriamycin-treated animals. At the same time, permeability changes induced by chemical agents acting directly on blood vessels were unaffected, which suggest that vascular responsiveness is unaffected by adriamycin. These findings indicated that the local regulatory, sensory-efferent functions of nociceptive sensory nerves are profoundly affected by adriamycin treatment. Since these efferent responses are mediated by vasoactive peptides released from activated sensory nerves, we measured the capsaicin-induced release of CGRP in peripheral nerves obtained from control and adriamycin-treated rats using ELISA. The results disclosed a significant decrease of the capsaicin-evoked release of CGRP from sciatic nerves. High potassium-induced release of CGRP was unaffected by adriamycin treatment, suggesting an impairment of mechanisms involved in the activation of the TRPV1 receptor.

Inflammatory hyperalgesia is a sensitive measure of classical nociceptor function. The examination of heat and mechanical nociceptive responses revealed a marked attenuation of carrageenan-induced inflammatory hyperalgesia in adriamycin-treated rats. This suggests an impairment of the afferent, nociceptive function of chemosensitive sensory nerves expressing the TRPV1/TRPA1 receptors, which have been shown to be critically involved in the mechanism of inflammatory hyperalgesia (Davis et al., 2000; Pogatzki-Zahn et al., 2005). The results of the quantitative locomotor analysis failed to reveal major motor impairments in adriamycin treated rats. Except three parameters all measurements yielded results similar to the controls. Locomotor parameters directly dependent on the integrity of spinal motoneuron function, such as step length, ankle and knee lifting and lateral placing remained unaffected by the treatment (Nógrádi et al., 2007; Pájer et al., 2014). The significant differences observed in parameters tarsus off angle, metatarsus-surface angle and knee flexion, suggest

disturbances in proprioception rather than a true impairment of motor function. Hence, the increased latency of the nociceptive withdrawal reflex following the administration of carrageenan in adriamycin-treated animals may be attributed to a toxic damage of the antitumor agent on nociceptive afferent nerves, rather than an impairment of motor function/performance.

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 (Maggi and Meli, 1988; Szolcsányi, 1988; Holzer, 1998b; Davis et al., 2000; Caterina et al., 2000; Jancsó et al., 2009a). 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 (Boyette-Davis et al., 2011) and vincristine (Siau et al., 2006) and, interestingly, a human bacterial disease, leprosy (Miko et al., 1993).

Epidermal thinning was pronounced after the administration of adriamycin. This may well be explained by a direct antiproliferative action of the drug on the germinative cells of the epidermis, but the contribution of neuronal mechanisms cannot be excluded. Indeed, sensory denervation-induced thinning of the plantar skin has been demonstrated in the rat (Chiang et al., 1998). Hence, the marked loss of epidermal axons observed in the present study may also play a role in this phenomenon.

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. Taking into consideration that adriamycin- cardiomyopathy once developed carries a poor prognosis and has no definitive treatment at the moment, cutaneous sensory tests as tools for the prediction of cardiac damage could open a door for the more effective preventive cardioprotective therapies.

The specific conclusions of this study were as follows:

- 1) Chemosensitive cardiac sensory nerves play a crucial protective role against the chronic cardiotoxic effect of anthracyclines in the rat model. Systemic capsaicin pretreatment intensify and accelerate the development of cardiomyopathy.
- 2) Adriamycin treatment severely impairs TRPV<sub>1</sub>/TRPA<sub>1</sub>-mediated afferent and efferent cutaneous sensory nociceptive functions, dramatically decreases epidermal nerve fiber density and causes significant epidermal thinning. Adriamycin treatment decreases capsaicin-induced CGRP release from peripheral nerves, while depolarization-induced CGRP release remains intact. Adriamycin-induced sensory changes appear prior to the manifestation of the cardiotoxic effect.
- 3) Present study raises the possibility of functional sensory testing as a predictive monitoring tool in anthracycline-induced cardiomyopathy. Our results highlight the potential usefulness of testing cutaneous neurogenic vascular reactions, such as the detection and quantification of the axon reflex flare response to predict the risk of adriamycin-induced cardiac injury in clinical practice.
- 4) The severe toxic effect of adriamycin on cutaneous chemo-nociceptive fibers suggests that sensory neural deficit could play a role in the pathomechanism of adriamycin-cardiotoxicity.

## SUMMMARY

Selye's (1936) recognition of the "general adaptation syndrome", later renamed by Selye "stress response" not only initiated a still growing wave of medical research, but also raised the idea, that diverse injury can activate the same coping systems of the body or an organ. Chemo-nociceptors, that innervate most organs including the skin and the heart, exert nociceptive afferent and local regulatory efferent functions by releasing sensory neuropeptides, and express the TRPV<sub>1</sub> and/or TRPA<sub>1</sub> receptor, can be considered as an important innate coping system for heterogeneous noxa.

In this study, we examined the possible protective role of CGRP containing chemosensitive afferent nerves in an established model of adriamycin-induced experimental cardiomyopathy in rats. Systemic treatment with capsaicin was utilized to deplete sensory neuropeptides from cardiac afferent nerves. Echocardiography was applied to assess the cardiac function in adriamycin-treated rats pretreated with capsaicin or its vehicle. In control rats, adriamycin treatment produced a reduction in the fractional shortening of the left ventricle and an increase in the ratio of the left atrial diameter and the aortic diameter, indicative of a decreased myocardial contractility and heart failure at 3–4 weeks post-treatment. In contrast, in capsaicin-pretreated rats, a deterioration of the cardiac function was already evident 1 week after the cessation of adriamycin administration, while the clinical signs associated with cardiomyopathy were more severe and displayed a significantly more rapid progression. Immunohistochemistry revealed a complete depletion of calcitonin gene-related peptide from cardiac sensory nerves after systemic capsaicin treatment.

Besides their deleterious action on cardiac muscle, anthracycline-type cytostatic agents exert significant neurotoxic effects on primary sensory neurons. Therefore, we also examined the effects of cardiotoxic doses of adriamycin on the function and morphology of epidermal nerves, that share common traits with cardiac chemosensitive nerves. Sensory neurogenic vasodilatation, plasma extravasation and the neural CGRP release evoked by TRPV<sub>1</sub> and TRPA<sub>1</sub> agonists *in vitro* were examined by using laser Doppler flowmetry, the Evans blue technique and ELISA, respectively. Carrageenan-induced hyperalgesia was assessed with the Hargreaves method. Immunohistochemistry was utilized to study cutaneous innervation. Adriamycin treatment resulted in profound reductions in the cutaneous neurogenic sensory vasodilatation and plasma extravasation evoked by the TRPV<sub>1</sub> and TRPA<sub>1</sub> agonists capsaicin and mustard oil, respectively. The *in vitro* capsaicin-, but not high potassium-evoked neural

release of the major sensory neuropeptide, CGRP, was markedly attenuated after adriamycin treatment. Carrageenan-induced inflammatory hyperalgesia was largely abolished following the administration of adriamycin. Immunohistochemistry revealed a substantial loss of epidermal TRPV<sub>1</sub>-expressing nociceptive nerves and a marked thinning of the epidermis.

The results suggest that TRPV<sub>1</sub>-expressing cardiac peptidergic nerves have a protective role against anthracycline-induced cardiac pathologies. Furthermore, these findings indicate early impairments in the functions of TRPV<sub>1</sub> and TRPA<sub>1</sub> receptors expressed on cutaneous chemosensitive nociceptive nerves and the loss of epidermal axons following the administration of cardiotoxic doses of adriamycin.

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**APPENDIX**