Microvascular inflammatory response in the skin

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To my Father

"He who does not want to solve a problem
is looking for a justification.
He who wants to solve a problem
is looking for the means"

Table of Contents

List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Evilevitch V, Wu T T, Lindgren L, Greiff L, Norrgren K and Wollmer P.** Time course of the inflammatory response to histamine and allergen skin prick test in guinea pigs. *Acta Physiol Scand* 1999, **165**, 409-413

II. **Evilevitch V, Norrgren K, Greiff L and Wollmer P.** Microvascular response in guinea pig skin at histamine challenge with and without application of skin window. *Clin Physiol Func Imaging* 2004, **24**, 5, 266-269

III. **Evilevitch V, Wu T T, Norrgren K, Greiff L and Wollmer P.** Modulation of the inflammatory response to histamine by terbutaline and NO in guinea pig skin. *Submitted*.

IV. **Movahed P, Evilevitch V, Andersson T, Jönsson B, Wollmer P, Zygmunt P and Hogestatt E.** Local hemodynamic effects of anandamide and N-acylvanillylamines in the human forearm. *Submitted*.

Introduction

The cutaneous microcirculation

The morphological structure of the skin varies depending on the place of the body. The most common pattern with epidermis, dermis and subcutis is shown in Figure 1. The epidermis lacks blood vessels and is built up by a 50-200 µm thick keratin layer, the stratum corneum. The dermis consists of a microvascular network with arterioles, capillaries, and venules - smaller than 300 µm in diameter [1]. This network has its origin in subcutaneous tissue and extends into the dermal papillae forming so-called capillary loops. The length of a capillary loop is 0.2-0.4 mm and each supplies an average of 0.04-0.27 mm^2 skin surface. The cutaneous microcirculation amounts to only about 5% of the total skin blood flow but is essential for vital functions as thermoregulation, tissue nutrition and metabolism.

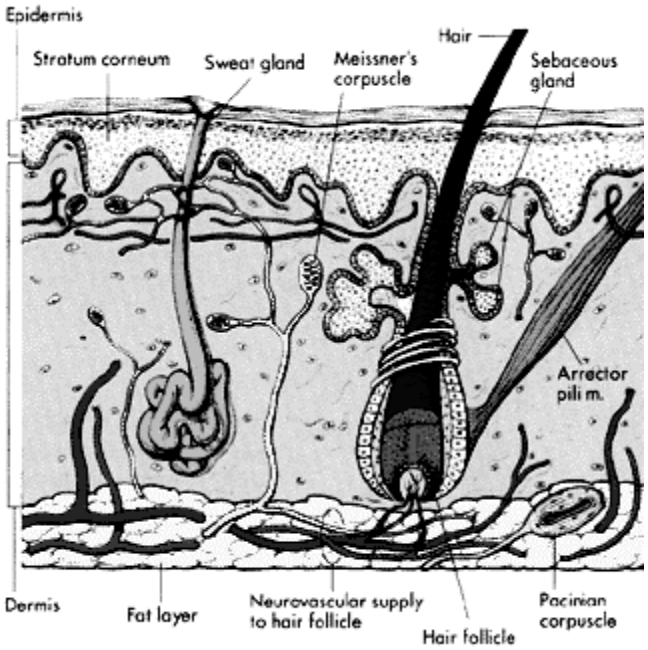

Figure 1. Schematic presentation of the skin.

The arterioles and venules of the cutaneous microcirculation form two important plexuses in the dermis: an upper horizontal network in the papillary dermis and a lower horizontal plexus at the dermal-subcutaneous interface [2-5]. Most of the microvasculature is contained in the papillary dermis 1-2 mm below the epidermal surface. In areas of skin where the dermal papillae are not well developed, arterioles connect with capillaries that run close to the dermal-epidermal junction before extending deeper into the dermis to join the postcapillary venules in the upper horizontal plexus. The microcirculatory blood flow is regulated by smooth muscle cells which are mainly located in the walls of the small arteries and

arterioles and to a lesser extent in venules and small veins. The true capillaries are free of smooth muscle cells, but the pericyte cells located in the capillary vessel walls may to some extent induce capillary contractions. In histological studies all microvascular structures can be identified by there specific characteristic: arterioles by the presence of an internal elastic lamina; capillaries by a thin vascular wall containing pericytes; and venules by thicker walls without elastic fibers [2, 5].

The inflammatory response

The inflammatory response is a defence mechanism that evolved in higher organisms to protect them from infection and injury. Its purpose is to localize and eliminate the injurious agent and to remove damaged tissue components so that the tissue can begin to heal. The four cardinal signs of inflammation—redness (Latin rubor), heat (calor), swelling (tumor), and pain (dolor)—were described in the 1st century AD by the Roman medical writer Aulus Cornelius Celsus. The inflammatory response can be subdivided into two components: vascular and cellular. The vascular component comprises changes in blood flow, and an increase in permeability of blood vessels, causing plasma exudation. This results in accumulation of fluid outside the blood vessels and oedema formation. The cellular component involves migration of white blood cells from the circulation to the site of tissue damage, platelet aggregation, mast cell degranulation and release of various mediators.

In the 19th century German pathologist Rudolf Virchow described the loss of function of the inflamed area due to pain and/or swelling that prevents movement in the area. This thesis focuses on the microvascular component of the acute inflammatory response.

Cutaneous Neurogenic Inflammation

A large number of stimuli can illicit an inflammatory reaction and activation mechanisms are also diverse. A pathway that has been given a lot of attention during the last century is neurogenic inflammation. A medline search gives us more than 970 articles only for the last 30 years.

The first observations concerning neurogenic inflammation were made more than a century ago by Bayliss who noted that activation of dorsal root ganglia neurons results in vasodilation [6]. Since then, abundant evidence has accumulated supporting the notion that activation of peripheral terminals of sensory neurons by local depolarization, axonal reflexes, or dorsal root reflexes releases bioactive substances. These substances, in turn, act on target cells in the periphery such as mast cells,

immune cells, and vascular smooth muscle producing inflammation, which is characterized by redness and warmth (secondary to vasodilation), swelling (secondary to plasma extravasation), and hyperresponsiveness (secondary to alterations in the excitability of certain sensory neurons). These inflammatory symptoms result from the release of substances from primary sensory nerve terminals.

The skin is innervated by afferent somatic nerves originating from dorsal root ganglia. These nerves contain two fibre types: fine unmyelinated (C-) or myelinated (Aδ-) fibers. Both types of fibres can be activated and respond to chemical and mechanical stimuli (heat, cold, nociception, mechanical distension and trauma). Moreover, biological agents and plant extracts (Capsaisin, papain) are capable of stimulating cutaneous nerves in the skin. After stimulation, the nerves rapidly release active neuropeptides such as neuropeptide Y, calcitonin gene-related peptide (CGRP), atrial natriuretic peptide, vasoactive intestinal peptide (VIP) and substance P (SP). From previous studies [7, 8] it is known that these substances are involved in regulation of, for example, sweat gland function and vasomotion. Besides these autonomic functions they play an important role in the first phase of an acute inflammatory reaction. This phenomenon is called "neurogenic inflammation", that is, inflammatory symptoms resulting from the release of substances from primary sensory nerve terminals.

A number of substances can be released from capsaicin-sensitive sensory neurons. Most of the available evidence indicates that substance P (SP) and CGRP are the major initiators of neurogenic inflammation [9]. These putative neurotransmitters are located in a subset of small dorsal root ganglion cells, which give rise to the lightly myelinated Aδ- and unmyelinated C fibres [10]. Stimulation of C fibres releases SP and CGRP peripherally and in the dorsal spinal cord [9]. C fibres in the skin seem to interact with adjacent mast cells in that SP mobilizes histamine while histamine stimulate C fibres to release SP and CGRP [11]. Substance P and CGRP therefore appear to produce symptoms of neurogenic inflammation by interacting with endothelial cells, mast cells, immune cells, and arterioles [12]. These symptoms can be mimicked by administration of SP or CGRP agonists and attenuated by administration of antibodies directed against these peptides or by antagonists at their receptors [12].

Besides SP and CGRP there are even other substances for example prostaglandins, which are synthesized and released from small diameter sensory neurons [13] and give rise to neurogenic inflammation. But the

knowledge concerning their role in inflammatory regulation is still limited.

Histamine as an inflammatory mediator

Since its discovery in 1910 [14], histamine has been regarded as one of the most important biogenic amines in medicine and biology. In addition to the three well-known pharmacological functions, i.e. the contraction of smooth muscles, the increase in vascular permeability, both of which are mediated through the histamine H1 receptor, and the stimulation of gastric acid secretion through the H2 receptor, histamine has been known to play various roles in neurotransmission, immunomodulation, proliferation regulation, etc [15]. In 1927, histamine was isolated from liver and lung tissue, followed by several other tissues, demonstrating that it is a natural component of the body, and hence the name histamine was coined after the Greek word for tissue, *histos*.

Mast cells and basophiles are the major source of granule-stored histamine. Histamine is released when these cells derganulate in response to various immunologic and nonimmunologic stimuli.

The pharmacological effects of histamine are mediated through four types of membrane histamine receptors: H1, H2, H3 and H4, all of which are heptahelical G-protein-coupled receptors [16-19].
Histamine released from mast cells is an established mediator of acute allergic reactions but may also play a role in chronic inflammation. The close connection between mast cells, microvessels and sensory C-fibres has been well demonstrated [20]; hence, histamine, in conjunction with other mast cell products like nerve growth factor, may contribute to vascular leakage as well as pain sensations [21]. So histamine can be classified as a neurotransmitter, which is involved in nociception. This is supported by recent observations of decreased sensitivity to nociceptive stimuli in H1 receptor knockout mice [22].

A multitude of studies have suggested a role for mast cells in the development and maintenance of neurogenic inflammation [23, 24]. Intranasal application of histamine in rats and guinea pigs produces CGRP and SP released from sensory C fibres, probably via the H1-receptor [25, 26]. The vasodilatation evoked by topical injection of histamine in pig skin could be decreased following systemic capsaicin pretreatment associated with CGRP and SP depletion [27]. These observations suggest that histamine may activate sensory C fibres. In turn, CGRP probably stimulates histamine release from mast cells and potentiates histamine action, though less potently compared to SP [28-31].

This possible indirect, CGRP-mediated histamine release following sensory nerve stimulation can be experimentally confirmed by attenuation of CGRP-induced vasodilatation following pretreatment with histamine blockers. Therefore, histamine released from mast cells appears to require co-action of sensory neuropeptides [32].

Petersen *et al*. [33] showed that intradermal injection of allergen, opiates and neuropeptides releases histamine at the site of injection. Histamine diffusion within the skin is restricted to 1-2 mm, and no histamine is detected in the periphery of the wheal. Capsaicin elicited intense pain but no histamine release by either intradermal injection or by prolonged topical application.

In additional studies [34], infusion of SP via microdialysis membranes in human skin induced dose-related vasodilatation and plasma exudation with secondary release of histamine.

Vanilloid receptor TRPV1

The vanilloid receptor TRPV1 belongs to the large family (currently containing 26 members) of transient receptor potential (TRP) channels that comprise a diverse group of non-voltage-gated cation channels involved in sensory signalling, ranging from thermal and mechanical nociception to vision, taste, olfaction, touch, and osmosensation [35]. TRPV1 received the vanilloid designation because it is activated by capsaicin and its ultrapotent analog, resiniferatoxin, both substances with vanillyl moiety (4-hydroxy-3-methoxybenzyl). Originally described as a receptor for capsaicin, TRPV1 is now believed to function as a molecular integrator of noxious stimuli (Figure 2) including heat, acid, pollutants with negative electric charge and endogenous pro-inflammatory substances [36].

Recent studies showed that the heat threshold for stimulation of the TRPV1 receptor is about 43°C in rat [37] and human [38]. The temperature threshold is not fixed but modulated by chemical ligands and the phosphorylation state of the channel [39]. The various activating ligands have synergistic effects, so that any specific chemical ligands concentration will result in a unique setting of the temperature sensitivity of the channel. The phosphorylation state is also important. For example, phosphorylation of the receptor by protein kinase C results in activation of the channel at normal body temperature [40].

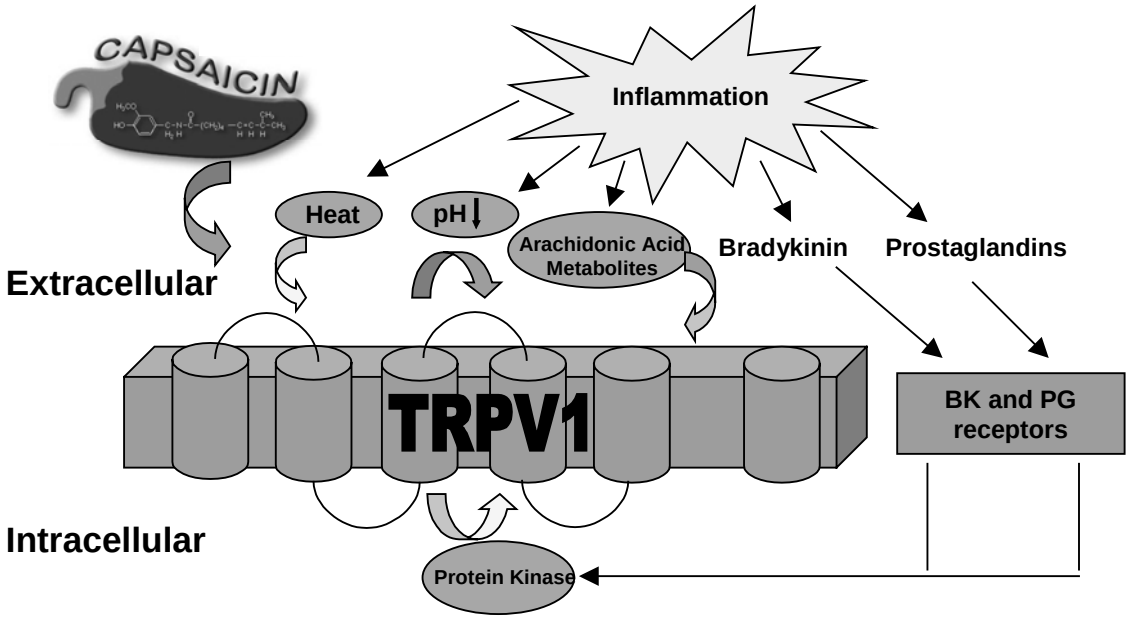

Figure 2. TRPV1 receptor activation mechanisms [41].

Rat studies showed that the density of vanilloid receptors is relatively high in dorsal root ganglia compared to other tissues and organs [42]. Vanilloid receptors are also present in the spinal cord (primarily in sensory efferent fibers), in various brain nuclei (hypothalamus, hippocampus, substantia nigra) [43], as well as in non-neuronal tissues, for example kidney [42, 44], urinary bladder epithelium [45], gastrointestinal tract [46], epidermal keratinocytes [47] and along blood vessels [48-53].

Activation of TRPV1 on unmyelinated (C-fibres) and thinly myelinated (Aδ-fibres) primary sensory neurons results in a rapid rise in intracellular Ca^{2+} and Na^{+} levels. This leads to cell depolarisation, firing of actions potential and gives sensation of pain. Activation of TRPV1 even leads to release of CGRP, substance P (SP) and neurokinin A (NKA), which are involved in the neurogenic inflammatory process. In blood vessels, these neuropeptides cause vasodilatation and increased vascular permeability, leading to plasma protein leakage and oedema formation. Furthermore, inflammatory mediators such as bradykinin, prostaglandins and serotonin can sensitize the vanilloid receptor via activation of protein kinase C or A and subsequent phosphorylation of the channel (Figure 3) [54].

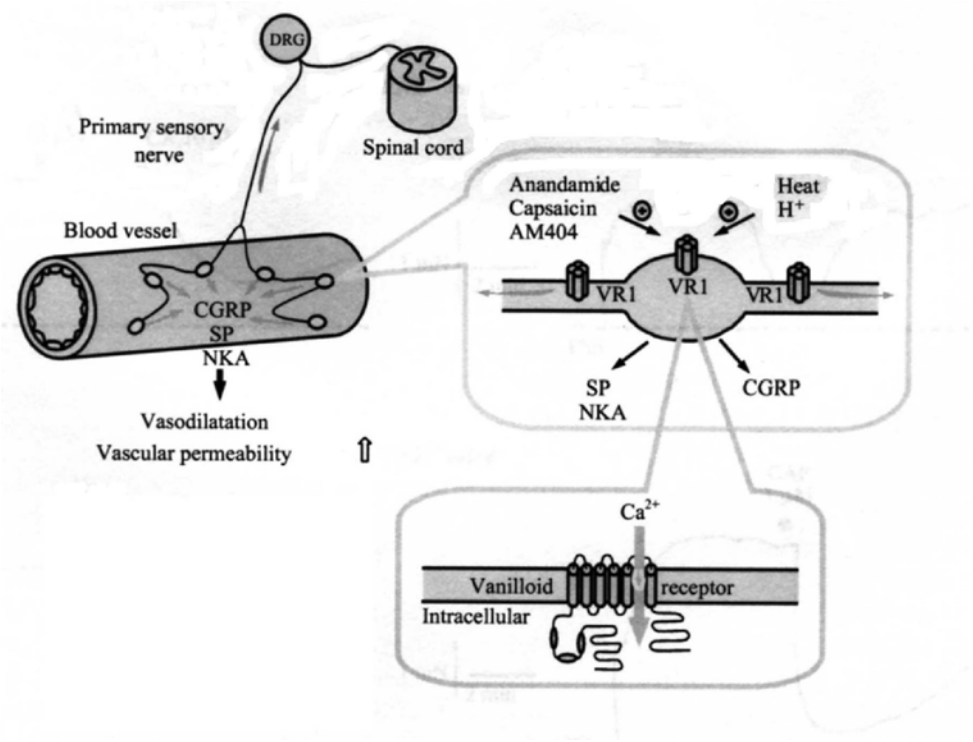

Figure 3. TRPV1 activation pathway.

These findings have prompted massive efforts on part of the pharmaceutical industry to identify active TRPV1 antagonists in the hope that a block of vanilloid receptors might be useful in treatment of chronic pain and inflammatory hyperalgesia.

Vanilloid receptor antagonists

Capsazepine (Figure 4) was reported in 1992 as the first competitive TRPV1 antagonist [55]. This compound has non-specific actions at voltage-gated Ca^{2+} channels [56]. However, in a recently published review, Szallasi [57] concluded that the use of the vanilloid antagonist capsazepine in animal models of human disease has so far been disappointing.

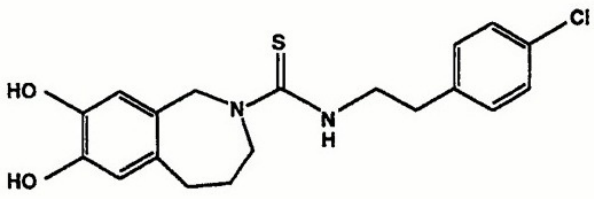

Figure 4. Capsazepine

Vanilloid receptor agonists

In the central nervous system, under normal physiological conditions, TRPV1 is unlikely to be activated by heat or low pH. Therefore it has

been suggested [58] that other endogenous ligands of this ion channel exist. Three different classes of lipids, all derived from the metabolism of arachidonic acid, that have been recently characterized, can activate TRPV1: anandamide, some lipoxygenase products of arachidonic acid and *N*-arachidonoyldopamine. All these compounds can be considered "endovanilloids", because they are formed by cells and released in an activity-dependent manner in sufficient amounts to evoke a TRPV1-mediated response by direct binding and subsequent activation of the channel. One of them, anandamide, will be described in more detail.

Endogenous: Anandamide

Anandamide (AEA, arachidonoylethanolamide) is formed in many organs and cell types including neurons, macrophages and endothelial cells [59-61]. It is likely that the accumulation site of anandamide, as a lipophilic compound, is near the cell membrane where its concentration can be estimated to 10-90 nM [62]. Anandamide is structurally related to capsaicin and olvanil, compounds that all have an amide bond and an aliphatic side chain (Figure 5). Zygmunt *et al*. showed that anandamide induces vasodilatation by activating vanilloid receptors on perivascular sensory nerves and causes release of vasodilator neuropeptides such as CGRP (Figure 3) [52]. Varga *et al*. noted in his animal study that intravenous injection of anandamide induces a complex haemodynamic response comprising an initial transient hypotension and bradycardia, followed by a vasopressor response and a final drop in blood pressure [63].

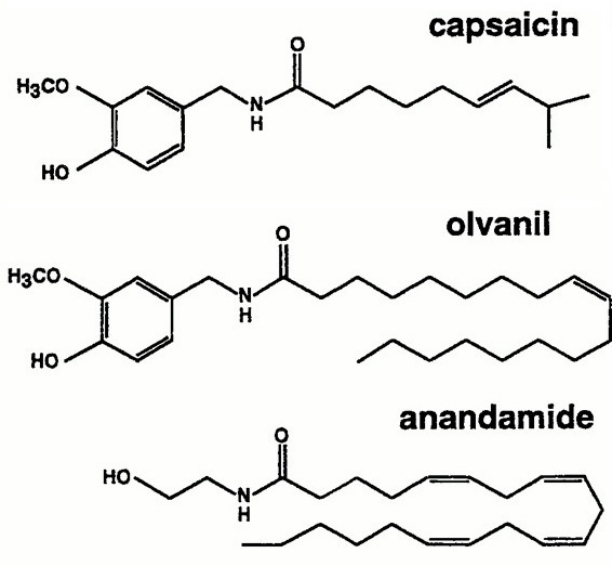

Figure 5. Structural comparison of molecules capable of activating TRPV1 receptor.

Exogenous: Olvanil, Arvanil

TRPV1 hybrid agonists were designed as possible analgesic, anti-inflammatory and antitumor agents [64]. The chemical similarity between anandamide and one of them, an oleic acid homolog of capsaicin (better known as olvanil) (Figure 5), suggests that certain substances might interact with vanilloid receptors. It is not known whether olvanil binds to the TRPV1 vanilloid receptor [37]. Di Marzo and co-workers reported that olvanil facilitates anandamide transport into cells where it blocks both the uptake and the hydrolysis of anandamide [65]. So olvanil might activate the receptor directly or indirectly by raising the levels of endogenous anandamide through inhibition of its inactivation [66].

Another chemical compound with vanilloid agonist properties is arvanil, a structural "hybrid" between the endogenous anandamide and capsaicin. It is a potent agonist for both the capsaicin receptor VR1 (vanilloid receptor type 1) and the cannabinoid receptor CB1. It also inhibits the anandamide membrane transporter (AMT). In addition, it directly activates CB1/VR1 receptors and inhibits fatty acid amide hydrolase (FAAH), thereby producing cannabimimetic effects [67].

Plant extract: Capsaicin

“Capsaicin, the purified extracted alkaloid from red chilli peppers, is a topical cream that has been found to help relieve pain from some arthritic conditions such as osteoarthritis and rheumatoid arthritis and neuralgic pain.” This text is taken from patient information about Zostrix cream produced by AFT Pharmaceuticals Ltd.

Hot chili peppers have been cultivated in South America for over 7000 years and in the rest of the world since the 16th century. Today, nearly one-fourth of the world's population consumes hot peppers or related foods on a daily basis [36]. In the mid-19th century, Thresh isolated the principal pungent component of peppers of the genus Capsicum and named it capsaicin [68]. Several decades later, Hogyes proposed that Capsicum extracts act selectively on sensory neurons to promote a sensation of pain and trigger heat loss through sweating [69]. In 1919, Nelson reported the structure of capsaicin as being an acylamide derivative of homovanillic acid, 8-methyl-N-vanillyl-6-noneamide [70]. Later in the middle of the 60’s Jancso demonstrated that this compound not only activates sensory neurons but also renders animals resistant to painful stimuli [71]. Since this discovery, capsaicin sensitivity has proven to be an extremely useful functional marker for a subset of neurons that are specialized to detect unpleasant or painful (noxious) stimuli. Studies

of capsaicin action have provided insights into the activation of primary afferent nociceptors and have revealed the ability of some nociceptors to act in an efferent capacity by stimulating inflammation, smooth muscle contraction, or secretion in target tissues. Apparently, it is not capsaicin but heat that has the capability of opening the channel pore of VR1, whereas capsaicin and protons only serve to lower the heat threshold of the receptor. Consequently, even room temperature is able to gate VR1 in the presence of mildly acidic conditions and/or capsaicin [72].
Moreover, an appreciation of the mechanisms by which capsaicin desensitizes neurons has provided a rationale for the use of capsaicin and related compounds in the treatment of painful disorders ranging from diabetic neuropathy to arthritis.

Vanilloid agonists in neurogenic inflammation

During tissue inflammation, we can see the release and accumulation of inflammatory mediators as bradykinin and histamine. These compounds are able to recruit intermediate-size neurons (240-320 μm^2), normally unresponsive to capsaicin, to respond to vanilloids [73]. Consequently, the number of nociceptors that innervate inflamed tissues increases. This mechanism may play an important role in the development of inflammatory hyperalgesia and cascade of neurogenic inflammation (Figure 6) according to Szallasi [74].

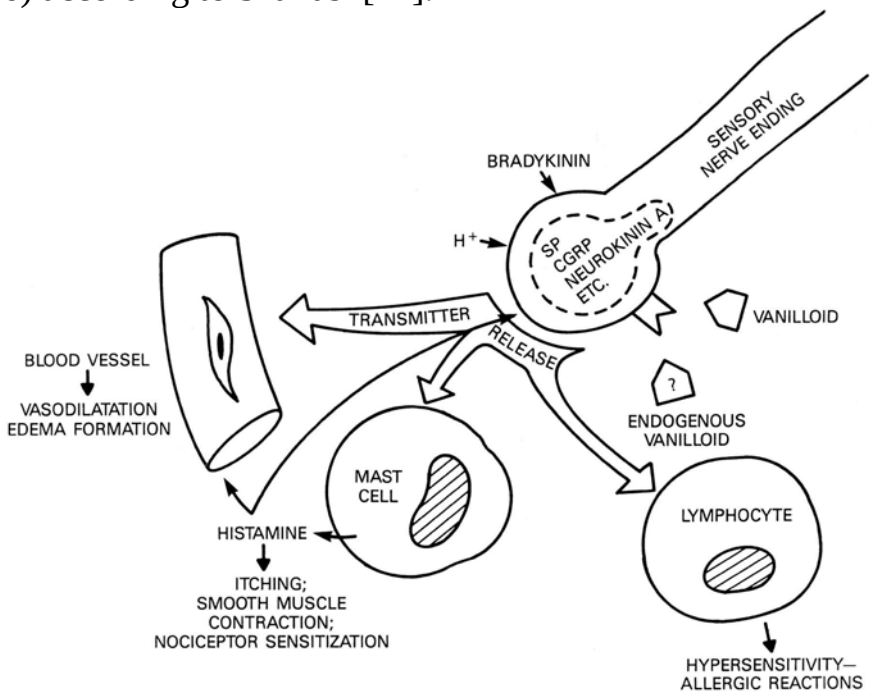

Figure 6. Schematic illustration of the role of peripheral nerve endings in neurogenic inflammation.

It has been shown that capsaicin is capable of releasing sensory neuropeptides such as SP, somatostatin, and calcitonin gene-related peptide (CGRP) from the peripheral endings of sensitive nerves in the presence of lignocaine, tetrodotoxin, ω-conotoxin, or agatoxin, suggesting a direct mechanism for peptide release not mediated by an axon reflex [75]. Vanilloid-sensitive nerves may be stimulated to release prestored proinflammatory neuropeptides by both exogenous and endogenous stimuli. Some of these agents, like bradykinin, have their own receptors; others may act on vanilloid receptors (VR). Protons are unique in that they have their own receptors (called acid-sensitive ion channels or ASICs) but they act also on VRs. Tachykinin SP released from vanilloid-sensitive nerves causes smooth muscle cells to contract (e.g., bronchospasm) and opens endothelial gaps causing plasma extravasation. Also, SP can activate various inflammatory cells. For example, SP released from vanilloid-sensitive nerves activates mast cells. Mast cells liberate histamine, which, in turn, stimulates vanilloid-sensitive nerves to release more SP and CGRP resulting in vasodilatation through action of CGRP.

Intradermal injection of capsaicin rapidly produces hypersensitivity and flare, and these symptoms can be prevented by denervation or by preexposure to capsaicin, which presumably depletes neuropeptide content in the terminals [9]. Additionally, destruction of capsaicin-sensitive fibers attenuates neurogenic inflammation produced by antidromic stimulation of sensory fibers.

Laser Doppler in skin perfusion measurements

Laser Doppler flowmetry

There are two closely related laser Doppler techniques for blood flow / perfusion studies. The first one is laser Doppler flowmetry (LDF). This technique has been used widely both clinically and experimentally as a continuous, real-time, non-invasive (in skin studies) method for tissue perfusion measurements. LDF is based on the phenomenon that laser light from a 2 mW Helium-Neon laser operating at a wavelength of 632.8 nm passing through an optical fibre illuminates the tissue i.e. the skin. The penetration depth of the light into the skin is about 0.7 mm for red light [76]. The penetration depth is dependent on factors such as laser light wavelength [77], skin pigmentation and probe type. Light is transmitted to the tissue via a fibre-optic probe. All probes use a silica fibre with a core diameter of 0.125 mm giving flexibility and a small bending radius.

Light undergoes multiple scattering and absorption in a small volume of tissue. When scattering occurs from a moving object such as an erythrocyte, the wavelength of the light is changed (Doppler effect) and the light reflected from the illuminated volume will therefore consist partly of unchanged light and partly of Doppler shifted light. Some of the illumination will be directed out of the tissue and picked up by optical fibres in the head of the probe and guided back to the instrument for signal processing.

Probes are available with fibre separations (distance between transmitting and receiving fibres) from 0.15 to 1.2 mm (standard separation is 0.25 mm). The fibre separation also influences the measuring depth. With multichannel probes, vascular beds at different depths can be measured simultaneously (Figure 7). The basic condition for successful measurement is a tight contact between probe and skin.

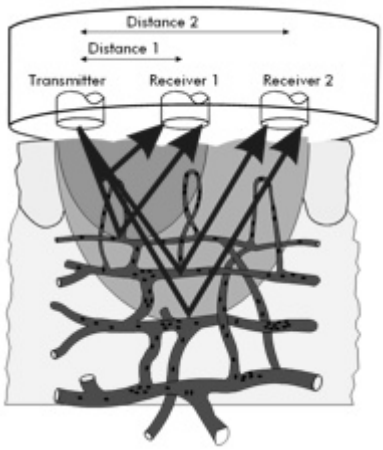

Figure 7. Principles of fibre separation in the probe.

The Doppler shifted part of the signal is isolated and converted into a voltage output signal which is linearly related to flux of blood cells [78]. A high intensity of the spectrum relates to a high flow, and vice versa. The output signal can be presented graphically by a recorder and transformed to perfusion units (PU) where 1 PU equals 1 volt at gain x 100. An example of the equipment is PeriFlux 5000 made by Perimed AB, Järfälla, Sweden [79].

LDF has been widely used in studies of vasomotion. Vasomotion can be observed by registration of tissue perfusion changes, which arise because of autonomic alteration in the calibre of blood vessels. Vasomotion is believed to be impaired with development of atherosclerosis and linked to

development of cardiovascular-related events, such as death, myocardial infarction, stroke, and unstable angina [80-82].

Laser Doppler imaging

The other laser light using equipment developed by Lisca Development AB, Linkoping, Sweden and manufactured by Perimed AB is Laser Doppler Perfusion Imager (LDPI). LDPI is also designed to be used non-invasively to produce tissue perfusion images. The instrument consists of a laser scanner with a detector and a signal processor, a computer. This laser Doppler imaging system has several advantages compared to point-monitoring LDF:

- Measurement of area rather than point perfusion with the possibility to produce two-dimensional images of microvascular perfusion.
- Non-contact measurements of skin blood perfusion and less sensitivity to movement artefacts, resulting in more stable mean value. It was suggested that direct contact of the probe with the skin surface could itself alter skin blood flow [77] or give rise to artefacts [83].
- The result of the tissue perfusion is presented as a colour image and photo of skin surface. The majority of graphic computer software allows easy and quick analysis of obtained data.
- The instrument is assisted with a statistical analysis menu that makes it possible rapidly to perform basic data analysis.
- LDPI uses more neighbouring measuring points of a two-dimensional skin area, which increases the reproducibility [84].

Disadvantages of LDPI include low temporal resolution, which e.g. limits the possibility to perform vasomotion measurements.

Measurement of the cutaneous microcirculation using laser Doppler technology is being applied to many areas of clinical medicine, such as peripheral vascular disease[85], rheumatology [86, 87], dermatology [88-90], gynecology [91, 92], wound healing of skin flaps [93], breast cancer [94-96], assessment of organ transplantation [97-101] and numerous other applications. This perfusion parameter can measure the severity of the clinical problem, monitor the progress of individual patients, and determine the value of treatment, including failure of treatment before clinical observation.

Laser Doppler Applications

The laser Doppler perfusion imager (LDPI) is a recent development in the field of laser Doppler flowmetry. It has great potential in many medical

and surgical applications for the non-invasive diagnosis of problems based on microvascular perfusion. The first biological application of the laser Doppler technique was by Riva *et al*., who investigated blood flow through retinal vessels in rabbits [102]. They demonstrated a linear relationship between the shifted frequency and the observed blood flow. LDPI has since been applied to a number of tissues. Some examples of LDPI studies of skin perfusion are presented below.

Skin allergy and irritant patch testing

A major dermatological application of LDPI is in the evaluation of irritant and allergic skin reactions, in which high sensitivity and reproducibility have been obtained (*Table 1*).

For many test substances the test time and concentration used can be lower than for visual assessment. LDPI allows user independent recordings of blood flow changes caused by the allergic reactions.

Substance / Condition	***Reference***
Nickel sulphate	[103]
Corticosteroid	[104]
Budesonide	[105]
Diesel oil	[106]
Sodium dodecyl sulphate	[107]
D vitamins	[108]
Calcipotriol	[109]
Methyl nicotinate	[110]
Gold	[111]
Atopic dermatitis	[112]

Table 1. Papers reporting skin irritant and allergy patch testing.

Skin disease

Laser Doppler Imaging technique has been tested for a variety of diagnostic uses in the field of dermatology. Seifalian *et al*. [113] used LDPI to measure the overall mean blood flow in the hands of patients with scleroderma as well as those of healthy volunteers. The readings were significantly lower for patients with scleroderma than for their

controls, although in both groups there was a considerable range of variation in flux values.

In a comparative study of patients with segmental-type and nonsegmental-type vitiligo, laser Doppler flowmetry results showed increased cutaneous blood flow in both types of lesions, but the increase in segmental-type lesions was nearly twice that of non-segmental-type lesions [114].

Segmental-type lesions alone exhibited a significant increase in cutaneous sympathetic response, which suggests that the pathogenesis of this type of vitiligo involves sympathetic nerve dysfunction in the affected skin.

Skin Tumors

Laser Doppler imaging also shows promise in the differentiation of skin tumors. In a comparison of the perfusion patterns of benign melanocytic nevi, malignant melanomas, and basal cell carcinomas, Stucker *et al*. [115] found that malignant melanomas were significantly more perfused than basal cell carcinomas and tended to be more perfused than melanocytic nevi. Furthermore, perfusion was observed to be higher in the center of the lesion than at the periphery for malignant melanomas and melanocytic nevi but was more uniform for basal cell cancers.

Skin Ulcers

Bornmyr *et al*. [116] reported the use of laser Doppler imaging to evaluate the progressive healing of a venous foot ulcer treated with pinch grafting, while simultaneously introducing the combination of skin perfusion imaging with digital photography.

Skin Burns

By using the LDPI the depth of a burn wound can be assessed. This method gives the possibility to adjust the therapy if sufficient tissue recovery is not obtained. When the skin is burned, the blood perfusion increases with severity of the burn as long as the microvascular network is intact (first- and superficial second-degree burns). When the microvascular network is destroyed, the blood perfusion is dramatically reduced and the tissue becomes necrotic (third degree burn). Measuring the blood perfusion helps to diagnose the actual severity and depth of the burn and can show if the burn will heal spontaneously or if it is necrotic. The scan flux values correlated well with both clinical assessment and histology reports, showing high flux for superficial dermal or epidermal depth wounds and low flux for deep dermal or full-thickness wounds [95].

Barachini *et al*. used LDPI for monitoring of skin blood flow changes after the burn repair process in self-repaired burn wounds and grafted skin wounds to obtain a better understanding of scar formation [117].

Skin Flap

In plastic and reconstructive surgery – flap surgery – it is of paramount importance that perfusion in the flap is adequate. Using the LDPI system, a perfusion image of the flap can be recorded at the end of the procedure to give valuable information about the microvascular condition of the flap. At this point, a simple correction of a poorly perfused flap may save it from necrosis in the post-operative phase. Measuring the perfusion in a post-operative phase can also provide information regarding possible malfunction of the blood supply to the flap. Stone *et al*. [118] demonstrated the usefullness of the method for skin free flap blood flow after plastic surgery where early diagnosis of vascular insufficiency is of paramount importance.

Other applications

In addition to the applications above, LDPI technology has been succesfully applied in a number of other fields including: diabetes neuropathy (cold provocation) [119], angiogenesis and growth factor research [120, 121], psoriasis [122], Raynaud's disease [123].

Isotope technique for monitoring of plasma exudation

Plasma exudation is a key feature of acute inflammatory reaction in the skin [124]. This exudative response may be observed as a wheal formation. The wheal response is routinely assessed in allergy investigations by skin prick testing followed by measurement of wheal diameter or area. For more accurate quantification for example in scientific work, the novel isotope technique has been developed for external detection of radiolabelled plasma proteins at the site of plasma exudation [125].

The detector used in this thesis was a photomultiplier tube (PM) with a polystyrene crystal (diameter 6 mm) with an aluminium foil window (thickness 0.014 mm) as protection for the PM from ambient light. The plastic scintillator was coupled to the PM tube by silicone grease. The detector, covered by a thin (0.006 mm) plastic film for contamination protection, was connected to a high-voltage supply (bias 900 V). The external electronics were a linear amplifier and a multichannel analyser. ^{113m}In was chosen for measurement of plasma exudation because it forms a stable complex with iron-free serum transferrin *in vivo* after intravenous

injection [126]. Transferrin is the iron-binding transport protein in plasma and has a high affinity for indium. ^{113m}In is also ideal for *in vivo* measurements because of the short half-live ($T_{1/2}$=99.5 min) and its simple decay scheme with only isomeric transition and monoenergetic γ emission at 392 keV. The conversions electrons from ^{113m}In with energy of 364 keV, which are detected with the probe, have a short range in soft tissue (1.1 mm).

In principle, several other radionuclides may be used in studies of inflammation. For example, Bergh *et al*. [127] used ^{111}In to label transferrin and red blood cells, separately, to study histamine induced inflammation in the skin. The results demonstrated dose-dependent accumulation of plasma (up to 6.5-fold increase) and blood (up to 2.0-fold increase) due to histamine provocation. Hence, about one-third of accumulation of plasma induced by histamine may be explained by vasodilatation and two-third by plasma exudation.

The advantages of this isotope detector system are a very low sensitivity to background gamma radiation and that it provides information about time course of plasma exudation. This method also directly provides quantitative information. Limitations include its rather poor energy resolution and the great geometry dependence. The count rate recorded from a point source declines sharply as the source is moved towards the periphery of the detector.

Aims of the study

The aims of this study were to further increase our understanding of the physiological process and role of microvasculature in skin in acute inflammation by investigating the following:

1. Measurement of duration of the inflammatory response in the skin after histamine provocation.

2. Comparison of histamine and allergen induced inflammatory response in skin.

3. Compare the inflammatory response in skin in two experimental models, the skin prick test and the skin window.

4. Study the modulation of the microvascular response to histamine by terbutaline and NO.

5. Study the microvascular response to vanilloid receptor (TRPV1) agonists and antagonists in human skin.

Material and Methods

Animals (Study I-III)

In all animal experiments male guinea-pigs were used, each weighing about 250-400 g. The animals were shaved on their backs (Study I, III) or bellies (Study II) 12 h before the experiment. Anaesthesia was induced by administration of a 3:2 mixture of ketamine (Ketalar, 50 mg ml^{-1}) and xylazine (Rompun, 20 mg ml^{-1}) intramuscularly in a dose of 1.0 ml kg^{-1} body weight [128]. The external jugular vein was exposed and a catheter (PE-50) was introduced through an incision and secured. The animal was then put on a height-adjustable table under the detector. The animals were kept warm by an infrared lamp. After the measurement the animal was sacrificed by intravenous administration of pentobarbital (Mebumal, 60 mg/ml) in a dose of 0.2 ml/kg body weight. The study was approved by the animal research ethics committee.

Radionuclide tracers and detector for conversions electrons (Study I-III)

In studies I - III the inflammatory response was measured by external detection of electron radiation from transferrin labelled with ^{113m}In. After intravenous injection, ^{113m}In forms a stable complex with transferrin *in vivo* [126]. ^{113m}In was injected intravenously as an InCl solution in a volume of approximately 1 ml, which is about 10% of total blood volume in guinea pigs.

The measurement technique is based on detection of the conversion electrons from ^{113m}In [125, 127]. The electrons have a short range in soft tissue (1.1 mm). Measurement of radioactivity is therefore confined to a superficial layer of the skin and background radiation from surrounding tissues is very low. The detector, a plastic scintillator, was placed on a photomultiplier tube and the signal was amplified and fed into a multichannel analyser which was preset with a 10 s dwell time.

Skin Window technique (Study II)

A skin window technique was used in study II. The method used in these studies is a modification of the technique described by MacPhee *et al*. [129]. Briefly, a perspex suction chamber with a 3 mm diameter is applied on the shaved belly skin of the animal. Using a suction pump, a negative pressure of about 0.2 kg/cm^2 is applied for about 50 min until blister was fully developed. The blister top is then removed with a knife.

Subjects (Study IV)

Forearm blood flow measurement

This part of the study was performed on six healthy male subjects, 22 to 31 years old (mean age 25 years). All subjects were non-smokers without any medication. Local anaesthesia on the forearm was provided with 1% Lignocaine. Experiments were performed in a temperature-controlled laboratory, 24-26°C.

The study protocol was approved by the Ethics Committee of Lund University and the study was performed according to the declaration of Helsinki.

Skin blood flow measurement

The study was performed on twenty-five (9 males and 16 females, mean age 26.7 years, range 20-38 years) non-smoking healthy volunteers without any medication or allergy, according to history. Informed consent was obtained from all subjects. Each subject participated 2-3 times in experiments with different mediators, which were tested separately.

All measurements were made on the forearm (both right and left). Before the measurements began, each subject was physically examined with heart- and lung auscultation, measurement of blood pressure and skin examination concerning rash or other allergic manifestations. The subject rested in the supine position for about 15 min and all measurements were made in this position. The room was darkened during measurements and the ambient temperature was 20-22°C. All conversation was avoided.

The study was approved by the Ethics Committee of Lund University, Medical Faculty in accordance with the Helsinki Declaration.

Laser Doppler Perfusion Imaging (LDPI) technique (Study IV)

The LDPI method is described in detail elsewhere [113, 130]. In brief, the LDPI comprises a 2mW helium-neon laser beam, which is directed at the forearm skin surface in a rectilinear mode by means of a mirror system, measuring up to 4096 points sequentially, and stored in matrix 50 x 64. Figure 8 shows a diagrammatic representation of the LDPI set-up. A maximum area of some 120 x 120 mm can be scanned in about 4 min. The sampling depth of the laser beam depends on the optical properties of the tissue and is approximately 300 µm for normal forearm skin [131]. According to the Doppler effect, moving blood cells reflect the beam partly. The intensity of this Doppler shifted backscatter corresponds to the

flux, which is a product of the number per volume and the velocity of the blood cells [78]. The back-scattered and Doppler-shifted light is detected by a photodetector and is processed in a personal computer. The computer also controls the mirrors directing the laser beam, stores the data, and displays a color-coded image of the spatial distribution of forearm skin perfusion on the monitor.

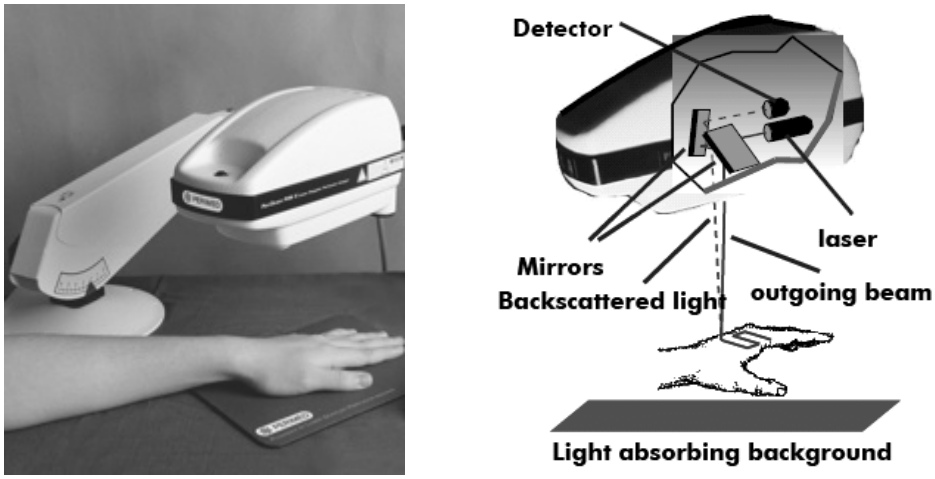

Figure 8. LDPI device (left) and its principles (right).

Colour coding of image

Images are produced on a colour scale. The colour of each pixel in the image reflects the value of the perfusion at the corresponding measurement site. Six colours are used to give an overview of the perfusion and its spatial variation in the forearm skin. Light blue and dark blue colours represent low flux and red and orange represent high flux. The colour coding procedure is performed in relative or absolute mode [132]. In relative mode the entire span of the perfusion values is divided into six intervals. The highest of the captured perfusion values is set at 100%, and the other values are scaled relative to the highest value. A pixel coded in light blue corresponds to a low perfusion value (less than 16% of the highest), while a pixel coded in red corresponds to a high perfusion value. The relative mode is preferable when the maximal dynamic range is desirable to compare adjacent areas within a single perfusion image. In absolute mode the highest and lowest perfusion values are selected before colour-coding and presentation. The absolute mode is useful when different perfusion images are to be compared in the same patient or single extreme values are to be excluded in the presentation.

Experimental protocol

Paper I

The study was designed in two parts. In the first part, the duration of the inflammatory response was examined in guinea-pig skin after prick test challenged with histamine. In the second part we studied the differences between histamine and allergen provocation in guinea pigs sensitised to ovalbumin. Sensitisation was accomplished in young animals by intraperitoneal injection of 1 µg ovalbumin and 100 mg $Al(OH)_3$ as adjuvant and experiments began 21-28 days after sensitisation when animal weight was doubled [133, 134].

The skin prick test was performed with histamine (40 mg/ml), ovalbumin (30 mg/ml) and isotonic saline by using lancet. In the first part, concerning the duration of inflammatory response, ^{113m}In was injected immediately, 5, 10, 15 and 25 min after skin prick test with histamine and immediately and 5 min after skin prick test with saline. Measurement of radioactivity was started immediately before ^{113m}In injection and lasted for 30 min.

In the groups with sensitisation animals ^{113m}In was injected immediately, 15 and 30 min after skin prick test with histamine and allergen.

Paper II

The inflammatory response in guinea pig skin after histamine prick test vs. skin window preparation and topical histamine administration was studied in this paper. All animals were divided into six groups where three groups were exposed to prick test with histamine and the rest of the animals were exposed to skin blister formation with topical histamine application. The control situation was saline solution in both prick test and skin blister groups. ^{113m}In was injected immediately or 10 min after provocation with inflammatory mediator. The measurement of radioactivity was started immediately after histamine challenge in skin prick test or blister groups. Results were presented as time-activity curves after correction for physical decay of ^{113m}In.

Paper III

Modulation of the inflammatory response to histamine in guinea pig skin was examined in this paper. All animals were subdivided into six equal groups and exposed to skin prick test according to the model described in previous study. The skin prick tests were performed with different agent depending on animal group: 1) control (isotonic saline 9 mg/ml); 2)

histamine (40 mg/ml); 3) terbutaline sulphate (0.25 mg/ml); 4) a combination of histamine (40 mg/ml) and terbutaline sulphate (0.25 mg/ml); 5) sodium nitroprusside (25 mg/ml); 6) a combination of histamine (40 mg/ml) and sodium nitroprusside (25 mg/ml). Each agent or combination of agents was used in one animal only.

Once again we used ^{113m}In *i.v.* and the detector was placed over the shaved skin. The baseline level of radioactivity was recorded for approximately 10 min followed by the skin prick test with one of the agents mentioned above, according to the procedure described previously [135]. The count rate was then recorded for another 20 min.

Paper IV

Forearm blood flow measurement

Forearm blood flow was measured in both arms simultaneously using strain gauge plethysmography. A 27 gauge needle was inserted into the left brachial artery after local anaesthesia. Basal blood flow was recorded during an initial 18 min of saline infusion. Anandamide was injected *i.v.* at a constant rate of 1 ml/min by infusion pump. Anandamide was injected in six-minutes long periods in concentration of 0.3, 1, 3, 10, 30 nmol. Forearm blood flow was measured during the last 3 min of each infusion period. Blood pressure and heart rate were measured in the right arm at the end of each 6 min period.

Skin blood flow measurement

All measurements were made in a maximally darkened room at a constant room temperature of 20-22°C. Three to six different substances were tested by skin prick test on ventral side of the forearm. Each substance was administrated as a droplet of 50 µl, immediately followed by prick test by lancet. After approximately 30 sec the droplets were dried off and laser scanning began.

Data processing and statistical methods

Paper I – II

All measurements were corrected for physical decay of ^{113m}In. The time-activity curve obtained from the detector consists of two phases (Figure 9). The first phase is a rapid rise in count rate corresponding to distribution of the tracer in the body and its arrival in to the sampling volume of the detector. The second phase is a slow increase in count rate which gradually levels off into a plateau. The second phase of the curve corresponds to the tracer accumulation during the inflammatory response, i.e. vasodilatation and plasma exudation [125, 127]. The second phase of the time-activity curve was analyzed by fitting the equation:

$$\mathbf{C_t = C_1 + C_2(1\text{-}e^{-kt})} \qquad (1)$$

to the experimental data [125], where C_t is the count rate at time t, C_1 is the count rate at the end of phase I of the time-activity curve, C_2 is the asymptote approached during the inflammatory response and *k* a constant. The magnitude of the inflammatory response was expressed as C_2/C_1.

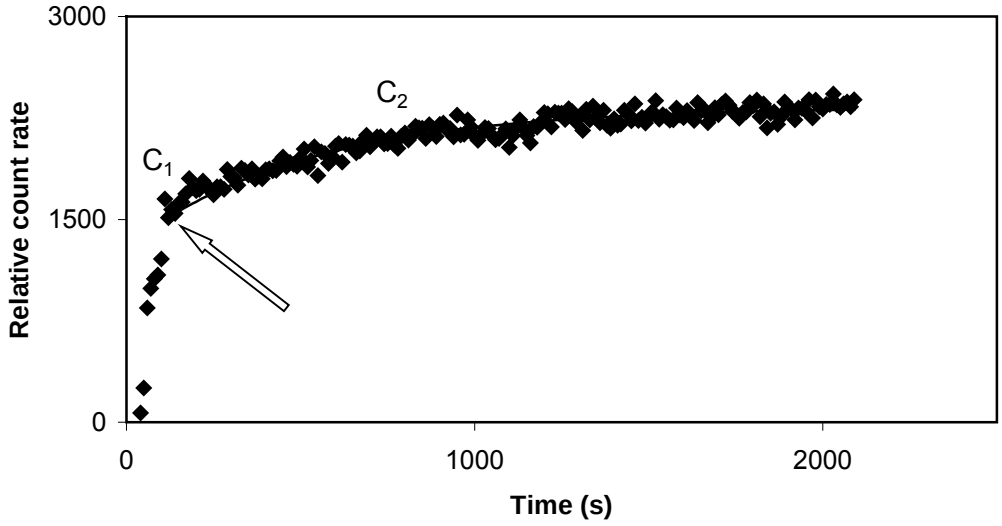

Figure 9. Time-activity curve obtained from an animal experiment with histamine skin prick test. Arrow indicates the phase transition point C_1.

Statistical significance of changes between groups of animals was assessed by one-way analysis of variance (ANOVA) followed by LSD test (t-test for independent samples) in study I and Duncan's multiple

range test in study II. P<0.05 were considered significant. The F test (variance ratio test) was performed for variance comparison of variables in control situation with skin prick test and skin window.

Paper III

The data processing in this study resembled those in the previous studies. The data analysis was performed by using equation (1) after physical decay correction for ^{113m}In. The curve consist of baseline recording made before provocation and the later part after skin prick test. The C_1 was obtained as a value at the end of baseline recording and C_2 was calculated as an asymptote to the maximum response point (Figure 10).

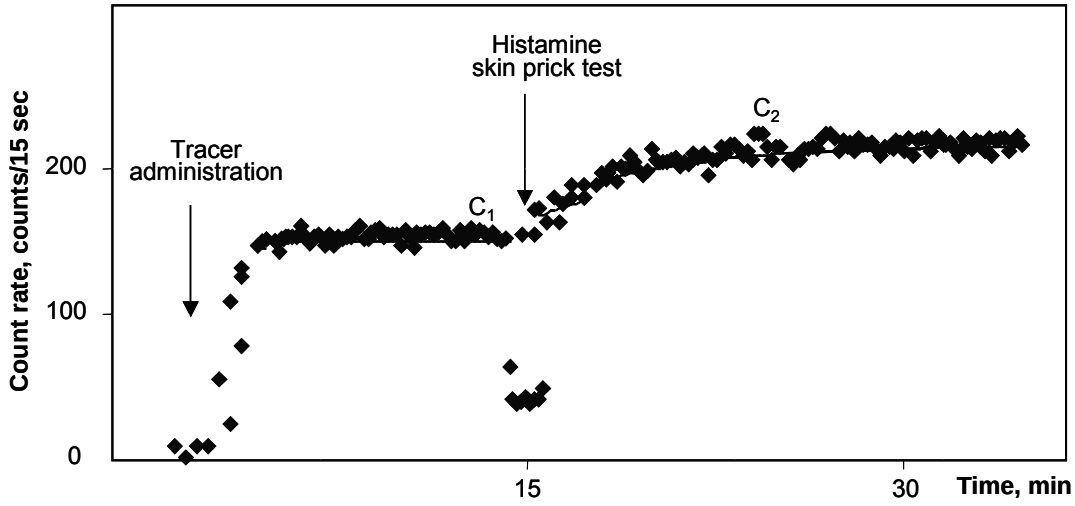

Figure 10. Time-activity curve for histamine guinea pig skin prick test.

Statistical significance of changes between groups of animals was assessed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. P<0.05 were considered significant. F test was performed for control groups.

Paper IV

In forearm blood flow experiments the blood flow was measured in ml per minute per 100 ml of forearm volume. The results were presented as a ratio between blood flow in infused arm to blood flow in control arm. Results were presented as a mean ± SEM in curves and columns. Statistical significance of changes between groups was assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test for multiple comparisons and Student's t-test for two groups comparison.

For the skin perfusion measurements, ten successive measurements were made over the area of skin prick tests. Regions of interest were centered over each provocation site, and the mean value recorded. Results are presented both as changes in perfusion over the ten minutes and as the mean value of the ten measurements.

Results

Paper I

The microvascular response was measured at increasing time after histamine provocation. When the tracer was injected immediately after histamine provocation, the response was 40% compare with 12% after provocation with saline. We found that the microvascular response declined gradually with increasing delay of tracer injection and finally was absent 25 min after histamine provocation (Figure 11).

Regarding the comparison between the inflammatory response to histamine and allergen in sensitized animal we found no significant differences. The response was slightly higher after allergen provocation than after histamine provocation only after delayed tracer injection.

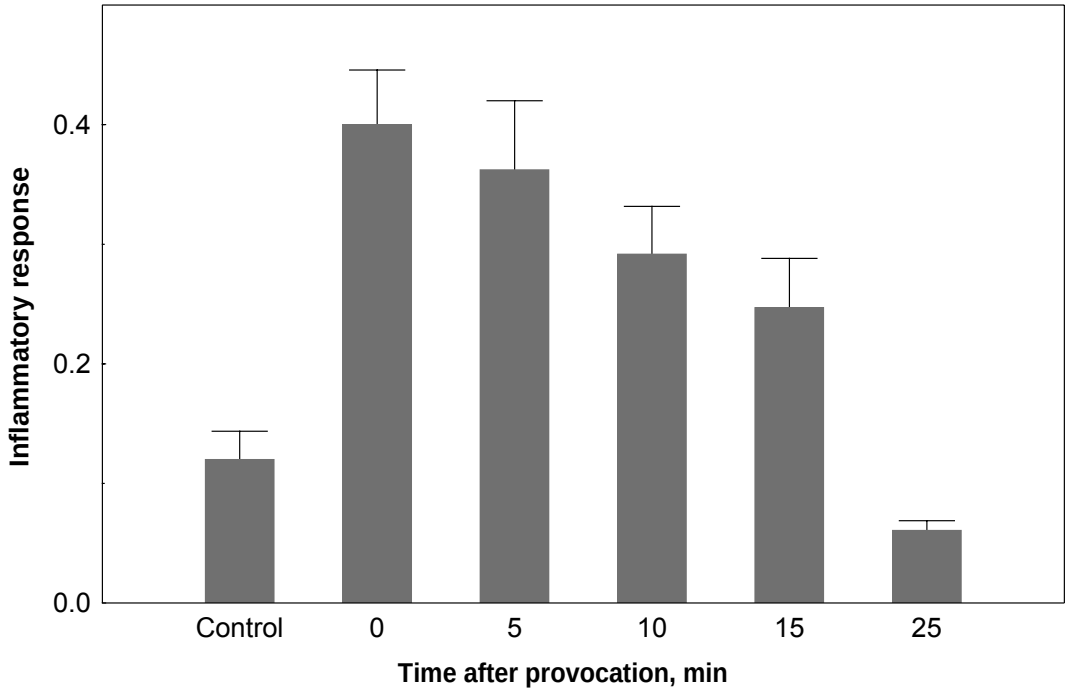

Figure 11. Mean ± SEM of the microvascular inflammatory response in the control group and groups with histamine skin prick test.

Paper II

The inflammatory response in the different experimental groups are shown in Figure 12.

Histamine prick test groups and skin window groups are presented in pairs according to the time of ^{113m}In injection. The smallest respons was seen in groups with saline provocation. There was no significant difference between skin window and skin prick test, but a considerably

larger scatter in the skin blister preparations. F-test showed the difference in variance between skin window and skin prick test to be significant in the control situation ($p<0.01$).

Similar response pattern was seen in skin window and skin prick test groups with ^{113m}In injection immediately and 10 min after histamine provocation. When ^{113m}In was injected immediately after histamine provocation, there were significant differences compared to control for both groups. After delayed injection, the difference from control was significant for skin prick test, but not for skin window.

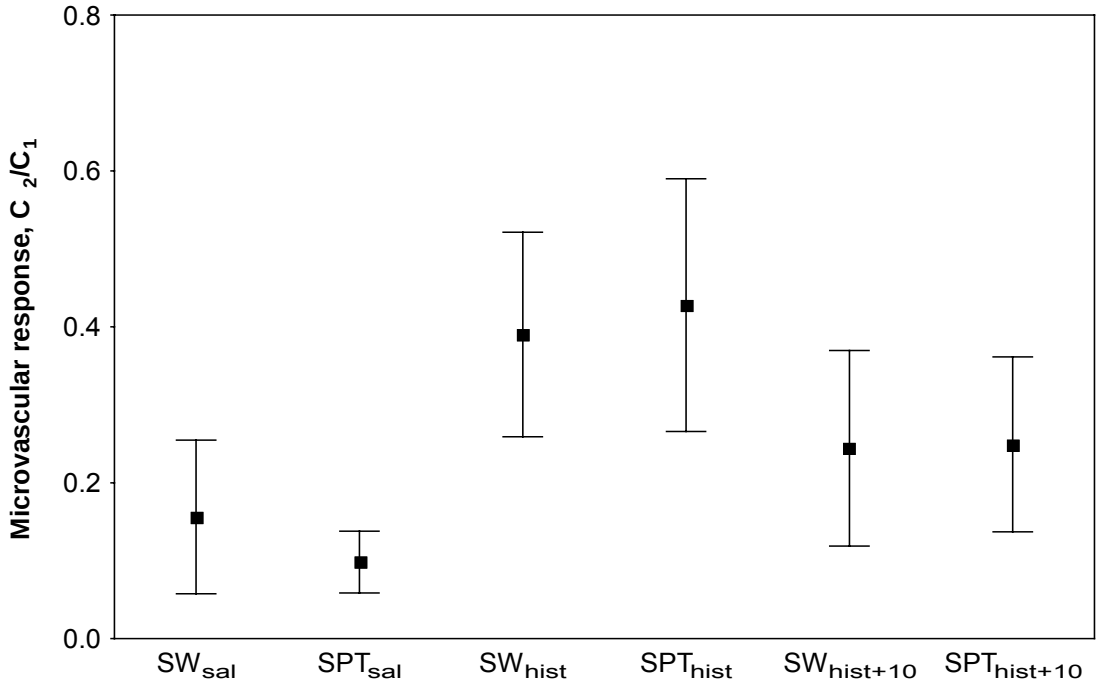

Figure 12. Microvascular response to histamine or NaCl in different experimental groups.

Paper III

Microvascular response after skin prick test with histamine, terbutaline, sodium nitroprusside and their mixture is shown in Figure 13.

The inflammatory responses to the different tested compounds were highly significant ($p<0.001$) compared to control group with saline challenge.

A significantly smaller response, compare to histamine, was seen in the group subjected to skin prick test with terbutaline. The provocation with combination of histamine and terbutaline showed somewhat greater

response than that to terbutaline alone (p=0.09) but lower than histamine alone (p=0.01).

Provocation with sodium nitroprusside resulted in a slightly lower response than with histamine (p=0.3). Provocation with the combination of sodium nitroprusside and histamine resulted in a slightly and not significantly higher response than with histamine alone (p=0.35).

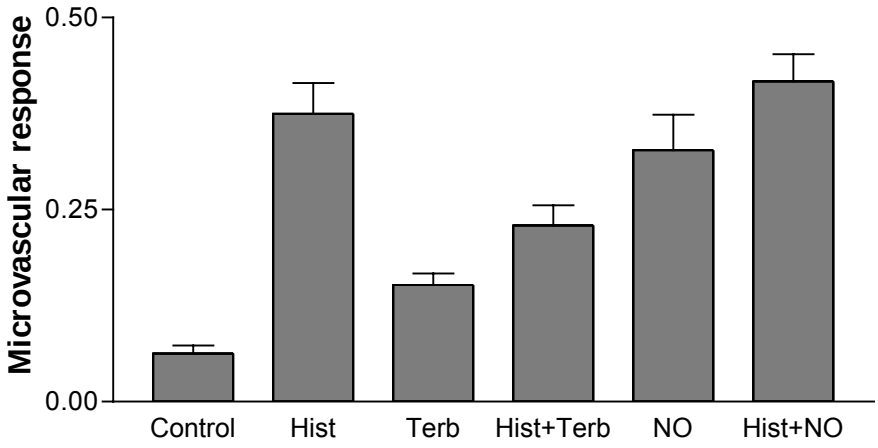

Figure 13. Microvascular response (C_2/C_1) in different experimental groups.

Paper IV

The intra-arterial administration of anandamide had no appreciable effect on forearm blood flow in man. This response can be compared with ethanol vehicle response where neither heart rate nor blood pressure changes were obtained. However, we obtained significant increase in forearm superficial skin flood circulation after anandamide administration in skin prick test (Figure 14).

This long lasting and strongly dose dependent response has reached its maximum after approximately 17 min, which is about 8 times slower, compare to histamine prick test.

Skin prick test with other TRPV1 agonists, such as olvanil, arvanil and capsaicin, showed similar microvascular response in the human skin. But olvanil and arvanil, which are exogenous compounds, caused greater blood flow increase than capsaicin in equimolar concentrations.

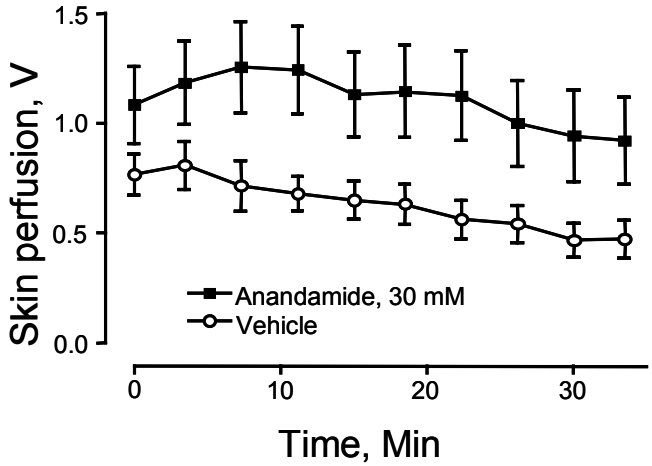

Figure 14. Microcirculation changes after skin prick test with anandamide.

Capsazepine administration in skin prick test together with the agonists mentioned above, caused significant inhibition of the effect. One example of microvascular response after arvanil skin prick test with or without presence of capsazepine is shown in Figure 15. This observation supports our assumption about TRPV1 role in microvascular regulation mechanism.

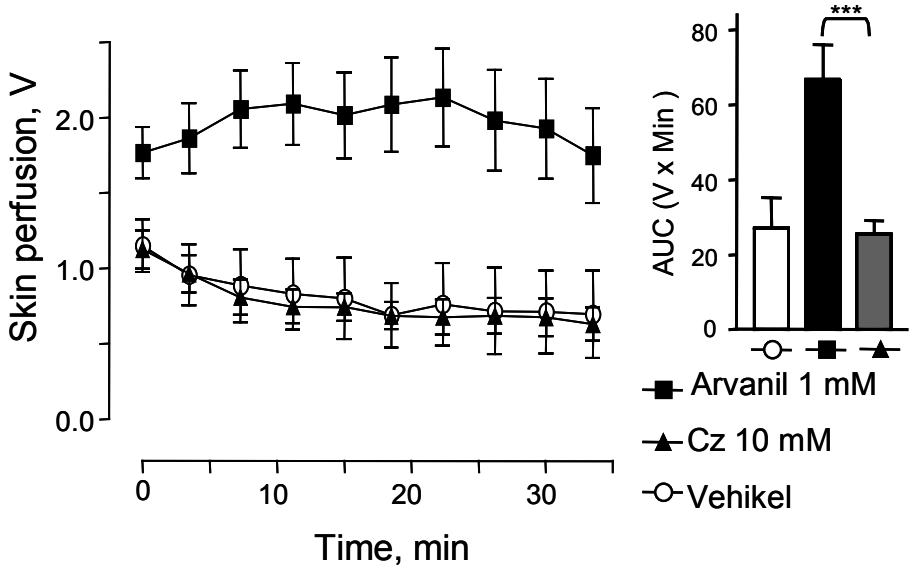

Figure 15. Microvascular response in human skin after prick test with arvanil and its inhibition with capsazepine.

General discussion

The microvascular inflammatory response comprises vasodilatation and plasma exudation. Vasodilatation results in increased blood flow and volume. Plasma exudation due to increased microvascular permeability leads to extra vascular accumulation of plasma proteins in the interstitium. The proteins will eventually be cleared via the lymphatics.

Plasma exudation as a key feature of acute inflammation has been studied in this project. Plasma exudation can be observed and studied in most tissues but the target organ in our investigations was the skin. This covering apparatus is about 1.85 m^2 in adults, "keeps us inside" and is the largest organ of the body. Inflammatory reactions are very common in the skin and there are many different experimental models to study plasma exudation. The most frequently used is skin prick test with visual assessment. A considerably more elaborated technique allowing more accurate quantification is the Evans blue dye technique, by which the dye or other tracers are measured in biopsy samples. This technique is invasive, demands histological skills and is a time consuming procedure. Another important disadvantage of this technique is that it does not allow distinction between intra- and extravascular accumulation of dye. A common limitation of all these methods is that they cannot be used to assess the time course of plasma exudation. To obtain this measurement we used a recently developed technique with *i.v.* Indium isotope administration and further ß-detection of conversion electrons in the skin. The measurements with transferrin labeling by ^{111}In and ^{113m}In and the red blood cells labeling by ^{111}In demonstrated that histamine induced microvascular response produce dose-dependent accumulation of plasma and blood in the skin where about one-third of accumulation may be explained by vasodilatation and two-thirds by plasma exudation [127].

This method allowed us to omit all the handling of skin biopsies, and its destructive tissue effects. Furthermore this allowed us to study microvascular response *in vivo*, with intact circulation in the skin. An additional advantage of this technique is the possibility to measure the inflammatory response in a superficial portion of the skin at a well-defined depth. This avoids influence from deeper tissue structures, which results in greater sensitivity to detect plasma exudation responses.

Another frequently used technique for examination of microvascular inflammatory response is laser Doppler flowmetry / perfusion imaging. The main difference from the previous technique is that the laser Doppler output signal is related to the number of moving cells and the mean velocity of these cells within the measured tissue volume. This signal

reflects the dynamic changes in tissue perfusion per unit time compare to external detection of conversions electrons where we register static amount of plasma protein locally accumulated in the place of inflammation.

To study the microvascular inflammatory response in the skin we used skin prick testing and external detection of conversion electrons. The detection of these mono-energetic electrons allowed us to measure the inflammatory response in the well-defined depth of the superficial layer of the skin without influence of the deeper structures. By using this technique we examined the duration of the microvascular response after histamine and allergen challenge. We found that the inflammatory response attains its maximum value after a couple of minutes, gradually declines thereafter and is absent after 25 min. We could not obtain any significant difference between histamine and allergen microvascular response. The opposite result was provided in laser Doppler flowmetry experiments by Olsson *et al*. [136]. They showed the long lasting inflammatory response after allergen provocation. A more complex inflammatory process, with activation of different inflammatory pathways e.g. neurogenic inflammation, might be expected after allergen provocation [137]. This would, however, probably result in a greater microvascular response as measured by the ^{113m}In technique.

Instead, we suggest that the difference between our results and those of Olsson *et al*. [136] reflect differences between measurement techniques, whereas the ^{113m}In is sensitive to increased blood volume in the superficial skin, the laser Doppler technique measures flux. A high flux may be maintained for a long period of steady vasodilatation and a constant blood volume.

The skin prick test used in our study caused a minor mechanical trauma, which can be detected for about 5 min after saline provocation. This fact indicates a rapid restitution of the microcirculation after minimal injury. Uncertainty, however, may be held regarding the trauma imposed by “skin window”, another technique widely used in skin inflammatory studying. In skin window models, the perfusion chamber is put over the base of a suction induced skin blister. The plasma exudation response may be monitored by analyzing the perfusion fluid levels of plasma proteins or labeled plasma tracers including the Evans blue dye.

Because the skin window technique is widely used in measurements of exudative effects of inflammatory stimuli, we wanted to compare this model to provocation by skin prick test using the ^{113m}In-technique. Roquet

et al. [138] showed that the concentration of plasma proteins or plasma tracers in perfusion fluids obtained shortly after the formation of the blister tends to be higher than baseline level recorded later. This suggests some degree of plasma exudative effects by the skin blister induction. The skin window technique allows the inflammatory process to be studied *in situ* with maintained tissue response. Moreover, it is less invasive than the Evans blue dye biopsy method. However, suction and warming of the skin during the blister induction may cause some damage that in itself can give rise to a microvascular response. This also appears to be reflected in our study. As we previously found, the skin prick test caused a minimal microvascular response, indicating that the mechanical trauma is minor. In contrast, the response recorded after skin window induction even without inflammatory mediator tended to be greater with considerably larger scatter. This factor might be diminished by increasing the time between skin window induction and the start of measurements. But in case of animal studies this time delay may still led to false positive results because of animal's itching and scratching which could give a higher grade of inflammation.

We compared the effects of histamine in the skin prick test and the skin window. We were able to measure increased microvascular response immediately after histamine provocation in the skin window, but after 10 min this response was not significantly different from the control. Our suggestion was that a lower degree of inflammatory response might be difficult to detect with the skin window technique because of the somewhat higher and more variable microvascular response at baseline. To test this assumption further we performed a measurement of the inflammatory response in human skin after histamine prick test and histamine challenged skin window. Skin perfusion was measured with the LDPI technique. We have observed a long lasting (over 40 min) response after skin window challenge with histamine.

For further investigation of histamine induced inflammatory microvascular response we designed the study with pharmacological modulation of this response by using terbutaline and the NO donor sodium nitroprusside (SNP) alone or in combination with histamine. We used the intermediate histamine concentration that allowed us to measure inhibitory as well as potentiating effects of terbutaline and SNP. The response was measured by external detection of β-radiation from ^{113m}In labeled transferrin.

Terbutaline, which activates β_2-adrenoceptor, is one of the most studied compounds in inflammatory context. It has been shown that beta

adrenergic agonists, depending on the experimental model, can inhibit mediator release from mast cells [139-142] and reduce microvascular permeability [143, 144], effects that may contribute to observed anti-allergic properties of these compounds [145, 146]. Another well-known influence of terbutaline on blood vessels is the relaxing effect on smooth muscle cells, leading to attenuation of the plasma exudation.

By using terbutaline with or without histamine as a mediator in skin inflammatory study we could confirm that terbutaline significantly reduce the microvascular response to histamine in skin.

Besides inflammatory properties of histamine, it may also stimulate endothelian production of NO [147]. This process requires Ca^{2+} entry from the extracellular space. The Ca^{2+} concentration is directly proportional to histamine concentration. This Ca^{2+} influx occurs via opening of TRPV1, leading to the excitation of sensory neurons. We know that TRPV1 receptor is strictly involved in neurogenic inflammatory process and that its activation by for example anandamide led to release of CGRP and SP. These mediators, in turn, cause mast cell degranulation [148].

On the other side, NO has been shown to inhibit mast cell degranulation [149, 150]. These opposing effects show the complexity of the regulation mechanisms behind the microvascular inflammatory response.

NO is a powerful vasodilator, acting directly on vascular smooth muscle. Concerning the vasodilatory effect of histamine during the inflammatory response in skin, this process can involve several different mechanisms of regulation. One of them can be a model, described by Wilkins *et al*. [151], where he suggested a possible increase of NO concentration by activation of histamine receptor H1.

Our results show that microvascular response to combination of histamine and SNP was similar to that of either substance alone. There was no sign of an additive effect of histamine and SNP on the microvasculature, as might have been expected. If we assume that SNP response most likely represents vasodilatation, and that histamine result in full vasodilatation as well, the additional administration of SNP would not give higher response. If the vasodilatation is not maximal, the lack of additive effect might indicate that SNP actually modifies the response to histamine by some way.

Another possible vasodilatory mechanism involves TRPV1 receptor and starts with its activation. Endogenous activation of this receptor by anandamide result in hypotention and bradycardia in rats [63]. These receptor agonists can also bind to cannabinoid receptor CB1, which gives rise to increase of endothelium produced NO in e.g. renal vasculature [59]. This process results in vasodilatation. On the other side the activation of TRPV1 receptor on sensory nerve terminals led to firing of action potentials that results in release of CGRP and substance P which gives vasodilatation [52].

The final part of this thesis concerns the study of TRPV1 receptor agonists and antagonists. For a more detailed study of this receptor triggering and the effect on microvascular system, we were using both exogenous (arvanil, olvanil) and endogenous compound (anandamide) as well as a plant extract, capsaicin.

We found that all these compounds cause dose-dependent activation of the receptor, which means increased blood flow by vasodilatation. From previous studies we know that anandamide is an activator of cloned human TRPV1 [152] and has ability to activate the receptor on primary sensory nerve in many vascular preparations [51-53, 153]. The salient feature of this study was its human application and the demonstration that anandamide activates the human TRPV1 receptor. This causes the release of neurotransmitters and start the neurogenic inflammation cascade with key features as vasodilatation and plasma exudation. By using laser Doppler technique we could, for the first time, show *in situ* effect of anandamide in human skin. However, we did not find any anandamide intravascular vasodilator activity. Possible explanations could be the high affinity of anandamide to plasma albumin [154] and therefore insufficient concentration of free anandamide in plasma, or that the TRPV1 receptor is not accessible via the intravascular route.

From the previous studies we know that anandamide could be produced in vascular wall and tissues around as a response to inflammatory process or tissue ischemia [155-160]. In such conditions anandamide will be produced by endothelian cells and macrophages [59, 161]. From the animal studies we also know that plasma circulating monocytes and macrophages can provide high concentration of anandamide locally i.e. at the site of inflammation, which give rise to vasodilatation which result in hypotension and bradycardia [63].

To summarize, we could provide that anandamide is able to cause vasodilatation in human skin during the local extra-vassal administration

but we have no evidence that anandamide is a circulating vasoactive hormone in man.

Conclusions

1. We found that the time course of the microvascular response is similar after histamine and allergen provocation. This inflammatory response has a rapid onset and is completed within approximately 20 min and that lymphatic clearance is negligible during the phase of plasma exudation.

2. The microvascular response to histamine is similar after provocation with the skin prick technique as well as the skin window technique. The skin window technique may have a lower sensitivity than the skin prick testing owing to a higher scatter in the control situation. Skin window trauma is somewhat greater than after prick test.

3. We conclude that terbutaline has an anti-inflammatory effect that can be measured by external detection of beta radiation from transferrin labeled with ^{113m}In. We found no indication of a pro-inflammatory effect of sodium nitroprusside when combined with histamine. Rather, the lack of additive effect may suggest an anti-inflammatory effect of SNP on the response to histamine.

4. We have proved that anandamide has a biological activity in man by activation of TRPV1 on perivascular sensory nerves. This activity can be inhibited by TRPV1 antagonists. We could not provide the evidence of anandamide as a circulating vasoactive hormone in man. We demonstrated advantages of skin prick technique in combination with LDPI to assess the activity of drugs on native TRPV1 in man.

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Svensk sammanfattning

I denna avhandling studeras den akuta inflammatoriska reaktionen i huden. Akut inflammation beskrevs redan under romersk tid av Aulus Cornelius Celsus. Han karakteriserade denna reaktion med rodnad, svullnad, värme och smärta. Fysiologiskt består akut inflammation av vidgning av små blodkärl (vasodilatation) som ger upphov till rodnad samt av läckage av plasmaproteiner (plasmaexsudation) ut ur blodbana via små porer som bildas i kärlväggen och ger upphov till svullnad. Det sammansatta svaret kallade vi för mikrovaskulär reaktion i huden vid inflammation.

Den mikrovaskulära reaktionen kan påvisas i huden med flera olika metoder vid akut inflammation. I vår studie har vi använt oss av ett radioaktivt ämne som fungerar som märkning av plasmaproteinet transferrin. Det radioaktivt märkta proteinet finns initialt bara i blodbanan. Vid inflammation sker ansamling dels i de vidgade kärlen och dels utanför kärlen vid plasmaexsudationen. Metoden mäter således både vasodilatation och plasmaexsudation.

I det första arbetet har vi framkallat en akut inflammatorisk reaktion hos marsvin genom att utföra ett pricktest i huden med histamin och ett protein som djuren gjorts allergiska mot (allergen). Pricktest används både i forskningssammanhang och kliniskt, t ex vid allergiutredningar. Vid pricktest med histamin och allergen kunde vi observera rodnad lokalt i huden samt svullnad som resultat av kärldilatation och plasmaexsudation. Vi kunde exakt mäta tidsförloppet på den inflammatoriska reaktionen som varar ca 20 min vilket är betydligt kortare än vad som tidigare beskrivits från mätningar av blodflödet vid inflammation.

En annan ofta använd metod för inflammatoriska studier i hud är så kallad fönstermetod. Man använder en liten kammare som placeras på huden. Genom att applicera både negativt tryck och värme i kammaren kan man erhålla en vätskefylld blåsa i huden. Därefter plockar man försiktigt bort taket av blåsan och får därmed ett fönster där inflammatoriska processer kan studeras. I vårt andra arbete har vi utfört en jämförelse mellan pricktest och fönstermetoden i huden. Vi misstänkte att det trauma som man få efter uppkomsten av hudblåsan kan ha betydelse för inflammatoriska processer. Vi visade att det traumat är betydligt större än det vi såg vid pricktest. Detta kan innebära att det är svårare att påvisa lindrig inflammation med fönstermetoden än med pricktest.

Syftet med vårt tredje arbete var att studera lokala hudeffekter hos marsvin vid prick test med terbutalin och natriumnitroprussid som är en kväveoxiddonator. Terbutalin är en känd substans som resulterar i vidgning av både luftväggar och blodkärl. Kväveoxid är en kroppsegen substans som vidgar blodkärlen. För oss var det intressant att studera deras effekt på blodkärl då de verkade ensamma eller i kombination med histamin. Terbutalin i kombination med histamin orsakar mindre hudreaktion jämfört med den reaktionen man ser efter enbart histamin. Detta bekräftar terbutalins anti-inflammatoriska egenskaper. Däremot gav kombinationen av natriumnitroprussid och histamin liknande reaktion som enbart histamin eller enbart natriumnitroprussid. Dessa resultat kan antingen betyda att kväveoxid orsakar lika stark kärldilatation som histamin eller att kväveoxid på något sätt förändrar svaret på histamin.

Förutom ovan nämnda metoder som används för att påvisa och studera inflammatorisk reaktion i huden finns laserdopplermetoden. Principen för mätningar med laserdoppler bygger på att en laserstråle som skickas genom yttersta hudlagret stöter på rörliga blodkroppar i kärlen och ändrar därmed sin frekvens. En del av det frekvensförändrade ljuset reflekteras och kan registreras med en mottagare. Genom att analysera det frekvensförskjutna ljuset kan man framställa en bild av blodgenomblödningen i huden både hos djur och människa. Vi använde denna teknik i vårt fjärde arbete när vi ville studera hur aktivering av en särskild receptor påverkar hudens blodflöde. Den receptor vi studerade kallas för vanilloidreceptor och återfinns på sensoriska nervtrådar längs små blodkärl i huden. Det är känt att aktivering av vanilloidreceptorn leder till uppkomst av en inflammatorisk reaktion. Denna receptor kan aktiveras av naturliga substanser som extrakt av chilipeppar och med syntetiskt framställda substanser som arvanil och olvanil. Ett kroppseget ämne, anandamid, kan också aktivera receptorn, vilket tidigare påvisats i djurmodeller. Vårt syfte var att studera effekter av stimulering av vanilloidreceptorn hos människan. Pricktest på underarmens hud hos friska försökspersoner utfördes med anandamid, olvanil, arvanil och capsaicin (substans som finns i chilipeppar). Med hjälp av laserdoppler kunde vi påvisa kraftig ökning av blodflödet i huden på platsen för pricktest med dessa substanser. Inflammationen kunde hämmas genom användning capsazepin, en substans som blockerar vanilloidreceptorn. Studien är den första som visar att stimulering av vanilloidreceptorn ger effekter på kärlen hos människa. Däremot kunde vi inte påvisa någon effekt av anandamid vid direkt administrering i blodbana.