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Effect of α-trinositol on interstitial fluid pressure, oedema generation and albumin extravasation in experimental frostbite in the rat

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1 The anti-inflammatory effect of α-trinositol (D-myo-inositol-1,2,6-trisphosphate) on oedema formation, microvascular protein leakage and interstitial fluid pressure (P_{if}) in rat skin after frostbite injury, was investigated. α-Trinositol (40 mg kg body weight^{-1}) was administered intravenously as a bolus both before and/or in the interval between freezing and thawing of the tissue.

2 P_{if} was measured in rat paw skin with micropipettes connected to a servo-controlled counterpressure system. Oedema formation was estimated by measuring the increase in total tissue water content (wet weight minus dry weight divided by dry weight). Albumin extravasation (i.e., the difference between the plasma equivalent space for ^{125}I- and ^{131}I-human serum albumin (HSA) circulating for different time intervals) was used to estimate the microvascular leakage.

3 Compared to untreated animals, α-trinositol given pre- and/or post-freeze reduced total tissue water and albumin extravasation as well as the fall in P_{if} in injured tissue significantly ($P<0.05$). α-Trinositol given only post-freeze reduced total tissue water and albumin extravasation from 4.46 ± 0.93 and 2.37 ± 1.12 to 2.51 ± 0.29 and 0.36 ± 0.18 ml g dry weight^{-1}, respectively ($P<0.05$).

4 P_{if} fell from -0.8 ± 0.2 mmHg pre-freeze to -3.4 ± 1.0 mmHg ($P<0.05$) at 20 min after tissue injury (circulatory arrest) and was attenuated by treatment with α-trinositol.

5 We conclude that α-trinositol exerts its anti-oedematous effect by acting on the extracellular matrix, attenuating the lowering of P_{if} as well as on the microvascular wall, thereby decreasing the protein extravasation.

Keywords: Inflammation; cold injury; oedema; microvascular permeability; α-trinositol; rat skin

Abbreviations: Bq, becquerel; E_{alb}, albumin extravasation rate; HSA, human serum albumin; NaCl, sodium chloride; O.d., outer diameter; P_{if}, interstitial fluid pressure; TTW, total tissue water content; DW, dry weight

Introduction

Several pharmacological strategies have been attempted to reduce the microcirculatory insufficiency and oedema induced by cold injury. Free radical scavengers (Maity *et al*., 1992), adrenocortical extracts (Serda & Wei, 1991), non-steroidal anti-inflammatory drugs (Talwar & Gulati, 1972), anti-coagulative (Webster & Bonn, 1965), fibrinolytic (Zdeblick *et al*., 1988), sympatholytic (Snider & Porter, 1975), vasodilatator (Daum *et al*., 1989) and antihistamine agents (Cummings & Lykke, 1973) have been used to improve tissue survival. Most studies on cold injuries have been performed on animals and have shown minimal improvement in tissue survival (Britt *et al*., 1991). The drugs had effect only when given immediately before freezing, with little or no effect when given during or after thawing. Provided a suitable agent is used, the use of drugs to limit oedema formation and tissue damage in cold injury should be possible because the interval between freeze and thawing offers a unique opportunity to start therapeutic intervention, either prior to or simultaneously with restoration of blood flow.

α-Trinositol (D-myo-inositol-1,2,6-trisphosphate) is an isomer of inositol trisphosphate (D-myo-inositol-1,4,5-trisphosphate) with anti-inflammatory properties. The molecular weight of α-trinositol is 529.9 D and its solubility in water at pH = 7.0 is 50 g l^{-1}. Due to its low molecular weight and hydrophilic properties, the drug should readily cross the microvascular membrane to be distributed throughout the extracellular fluid compartment.

Reduction of the inflammatory oedema after pretreatment with α-trinositol has been demonstrated in burn injury to the skin (Tarnow *et al*., 1996), smoke inhalation injury in lungs (Nakazawa *et al*., 1994), neurogenic inflammation in the trachea (Woie & Reed, 1994) and carrageenan-induced inflammation in paw skin (Claxson *et al*., 1990). In burn injury, α-trinositol reduced microvascular leakage and oedema formation even when administered 5 min after the injury (Lund & Reed, 1994). Also, α-trinositol inhibits neuropeptide Y evoked vasoconstriction by sympathetic nerve stimulation (Sun *et al*., 1991; 1992) and may have effects on vascular resistance when symphatetic activity is high (Edvinsson *et al*., 1990).

The beneficial effects of α-trinositol on inflammatory oedema formation seems related to its effect on interstitial fluid pressure (P_{if}) and/or microvascular permeability (Lund & Reed, 1994; Woie & Reed, 1994). In several acute inflammatory reactions, P_{if} decreases initially (Reed *et al*., 1997), raising the transcapillary net filtration pressure. The lowering of P_{if} seems to involve perturbation of the normal β_1-integrin function (Reed *et al*., 1992; 1997), i.e. the transmembrane heterodimeric receptors that mediate cellular attachment towards structural components of connective tissues (Hynes,

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1992). Release or loosening of the attachment between the connective tissue cells and the interstitial matrix cause the tissue to expand due to its content of hyaluronan and glycosaminoglycans, resulting in more negative P_{if} and thereby increased net filtration pressure and oedema formation. The cellular mechanism of the action of α-trinositol on P_{if} during acute inflammation is not fully elucidated but may involve the modulation and strengthening of β_1-integrin function *in vitro* and *in vivo* (Rodt *et al*., 1994) *via* the cytoskeleton (Åhlèn *et al*., 1998).

The present experiments were conducted, first, in order to investigate the acute effect of experimental frostbite on oedema formation, microvascular protein extravasation and P_{if}. Second, we evaluated the oedema-limiting effects of α-trinositol, administered both pre- and post-freeze, in the same model.

Methods

Animals

Female Wistar-Møller rats (200–250 g) from Møllegaard, Denmark were used. They were kept in transparent plastic cages and fed standard laboratory food and tap water *ad libitum*. Room temperature was kept at 21 ± 1°C in a normal 12 : 12-h light-dark cycle. The experimental protocols and procedures were approved by the Norwegian State Commission for Laboratory Animals.

Experimental cold injury

The animals were anaesthetized with pentobarbital administered intraperitoneally (50 mg kg^{-1}), prior to surgery. Supplementary anaesthetic when required and drug infusions were administered through a catheter (PE-50) placed in the right jugular vein. Frostbite was produced experimentally in the right hindpaw of the animal. The hair on the dorsal side of the hind limb was carefully cut with a pair of scissors prior to freezing. A calibrated Cu-Co thermocouple wire probe (Lab Facility, Middlesex, U.K.) covered by a polyvinyl chloride tubing (O.d.: 0.5 mm) was used to measure skin temperature to the nearest 0.1°C in the dorsal surface of the hind limb. The wireprobe was inserted through an aperture made by a syringe needle (O.d.: 0.8 mm), just proximal to the metacarpo-phalangeal joint, and the tip of the probe was advanced subcutaneously 5–6 mm proximal to the aperture. The temperature was continuously measured by a thermometer (Digitron Instrumentation Limited, Type T208, Hereford, U.K.) so that the temperature changes in the tissue could be recorded and to ensure that crystallization of the water in the tissue occurred. For freezing, a metal element perfused with a mixture of ethyl alcohol and water with an antifreeze solution added from a cooling reservoir (−30°C) was placed onto the plantar side of the hind limb 5 mm distal to the ankle joint. The dorsal side of the paw was placed against a temperature isolating plate not perfused by any liquid. There was no compression of the hind limb during this procedure. The animals were supine during the freezing procedure and rectal temperature was maintained at 38–38.5°C using a heating lamp. Both the cooling time (i.e., time of sub-zero temperature before the desired minimum temperature was reached) and the desired temperature were adjusted by regulating the flow of the antifreeze solution from the cooling reservoir through the metal element. The overall cooling time was 30 min. The minimum temperature achieved was between −15 and −18°C and was reached after 10–15 min. After freezing, the hindlimb was removed from the cooling element and permitted to rewarm under a heating lamp for 10 min. Rewarming was monitored by the thermocouple. The left paws served as non-injured controls and were neither cooled nor rewarmed.

Measurements

Interstitial fluid pressure Pressure measurements were made by sharpened glass pipettes (tip diameter 2–5 μm) filled with 0.5 M of NaCl coloured with Evans Blue and connected to a servo-controlled counterpressure device (Wiig *et al*., 1987). Using a stereo-microscope (Wild M5, Heerbrugg, Switzerland), the interstitial fluid pressure (P_{if}) in the dermal skin layer was measured by direct puncture using a micromanipulator (Leica, Heerbrugg, Switzerland) as described in detail elsewhere (Wiig *et al*., 1987).

Measurements were grouped in the following time periods after the inducement of cold injury and start of rewarming: 0–10, 11–20, 21–30, 31–45 and 46–60 min. Circulatory arrest was induced as a part of the experimental protocol by intravenous injection of potassium chloride under pentobarbital anaesthesia. Inflammation enhances microvascular filtration, thereby raising interstitial fluid volume. Due to fluid accumulation in the tissue, P_{if} will become more positive as a consequence of tissue compliance. Circulatory arrest was therefore induced in order to limit fluid filtration and oedema formation and to avoid a possible underestimation of a lowering of P_{if} (Lund *et al*., 1988).

Oedema formation and microvascular permeability Tissue samples were obtained by removing the skin on the dorsal side of the hind paws with a pair of scissors. The samples were placed in pre-weighed vials that were bottled immediately and re-weighed as soon as possible. The wet weight of the tissue samples was approximately 0.1–0.2 g. Following radioactivity measurements (see below), the samples were dried at 65°C until constant weight (usually 2–3 weeks).

Total tissue water and albumin extravasation Total tissue water in tissue samples was estimated as water content per g of dry tissue weight ((wet weight – dry weight)/(dry weight)).

Albumin extravasation was measured as the 25 min extravascular space of ^{125}I human serum albumin (^{125}I-HSA) (Institute for Energy Technology, Kjeller, Norway) during reperfusion. ^{125}I-HSA (0.05 MBq) in 0.3 ml saline was administrated at the time that the paw was removed from the cooling device. Twenty-five minutes later ^{131}I-labeled HSA (0.05 MBq) in 0.3 ml saline was injected intravenously and 5 min thereafter blood samples were obtained by cardiac puncture and the rat was killed by an intravenous injection of saturated potassium chloride. Tissue samples were obtained as described above. Radioactivity in tissue and blood samples were determined in a gamma-counting system (LBK Wallac 1285, Turku, Finland) with automatic background subtraction and spillover correction. Distribution volumes were calculated as plasma equivalent spaces (i.e., counts per min per gram dry tissue weight divided by counts per milliliter plasma). Albumin extravasation was calculated as the difference between the plasma equivalent distribution volume ^{125}I-HSA and that of ^{131}I-HSA. All calculations were made per gram dry tissue weight.

Change in paw weight The weight of the cold-injured hind paw during reperfusion was measured continuously by slightly

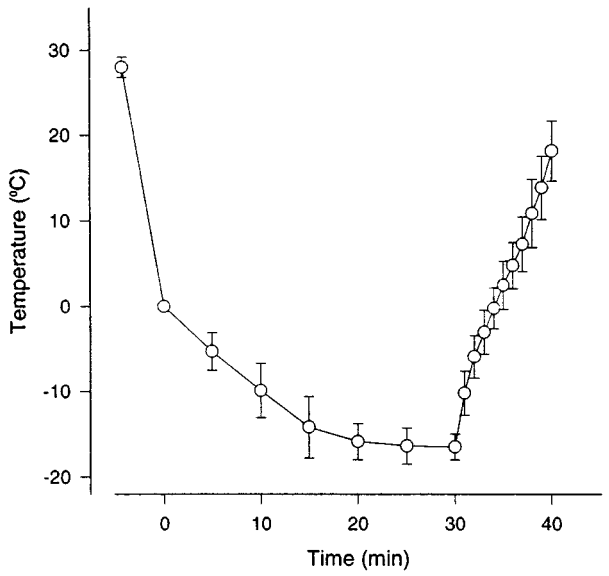

Figure 1 Temperature changes recorded in the rat paw (mean ± s.d., $n = 31$) during cooling. The temperature of the paw decreased to 0°C within 4–6 min during cooling. The tissue was kept at a low constant temperature for approximately 15 min before thawing.

elevating the hind limb and connecting the distal part of the toes of the paw to a force-displacement transducer (Grass Instrument Co, model FT.03, Quincy, Mass). The transducer was calibrated before and after each experiment. After zeroing for tare weight, a time-course for the change in paw weight (indicative of the increase in fluid content, i.e., the oedema formation) could be obtained to the nearest 10 mg.

The interstitial volume-pressure relationship

Interstitial compliance is the change in interstitial fluid volume divided by the corresponding change in interstitial fluid pressure (Guyton, 1965) and was calculated as the ratio between the change in total tissue water content and P_{if}, between control (10 min after the start of thawing) and overhydration (60 min after the start of thawing).

Drugs

The pentasodium salt of D-myo-inositol-1,2,6-trisphosphate was obtained from Perstorp Pharma (Perstorp, Sweden) in sealed vials, each containing 1 g of the freeze dried powder. The α-trinositol was reconstituted in sterile water to give a stock solution of 100 mg ml^{-1}. The reconstituted solution was frozen at −20°C in vials containing 0.5 ml. Each reconstituted vial was used the day it was opened. The dosage of α-trinositol was selected based on earlier experiments related to oedema limiting (Cassuto *et al*., 1990; Lund & Reed, 1994; Woie & Reed, 1994) and pharmacokinetic (Lennernäs *et al*., 1997) properties of the substance.

Experiments

Experiment 1: Interstitial fluid pressure Animals were studied in two separate series of experiments, each series being divided into one experimental and two control groups. One series had intact circulation throughout the experiment while in the other circulatory arrest was induced at different times following the onset of the cold injury.

Intact circulation The experimental group ($n = 5$) received 10 mg (0.2 ml) of α-trinositol 5 min prior to start of thawing and 5 mg (0.1 ml) 25 min afterwards. The first control group ($n = 7$) received 0.2 ml saline at 5 min prior to start of thawing and 0.1 ml saline 25 min afterwards. In the second control group ($n = 5$), 0.2 ml saline was given 5 min prior to thawing. Circulatory arrest was induced 60 min after start of thawing and an occluding tourniquet was placed around the lower and upper leg to prevent fluid loss from the paw. The aperture where the temperature probe was inserted was sealed with a drop of tissue glue (Histoacryl, B. Braun, Melsungen, Germany).

P_{if} was measured for more than 150 min, starting from the onset of thawing. Total tissue water in the skin of the cold injured paw was obtained at the end of each experiment (see below).

Circulatory arrest The experimental group ($n = 5$) received 10 mg of α-trinositol (0.2 ml) 5 min prior to start of thawing. Circulatory arrest was induced 15 min thereafter. P_{if} was followed for 60 min after start of thawing. Control groups received 0.2 ml of saline at 5 min prior to the start of thawing. Circulatory arrest was induced either 10 min after the start of thawing ($n = 9$, group 1) or when thawing was started ($n = 6$, group 2).

Experiment 2: Oedema formation and microvascular permeability Two groups of experiments were conducted. The first experimental group ($n = 5$) received 10 mg α-trinositol (0.2 ml) 5 min prior to start of thawing. The second experimental group ($n = 9$) received 10 mg α-trinositol (0.2 ml) and 5 mg (0.1 ml) α-trinositol at 30 and 5 min prior to start of thawing, respectively. Total tissue water at 30 min was measured after thawing and albumin extravasation was determined as the 25 min clearance of ^{125}I-HSA.

Control rats ($n = 5$) received 0.2 and 0.1 ml saline at 30 and 5 min prior to the start of thawing, respectively. Total tissue water and albumin extravasation were determined at the same time points as in the experimental groups.

When paw weight was measured continuously, from 5 to 60 min after start of thawing, two separate groups were studied: (1) The experimental rats ($n = 5$) received 10 mg (0.2 ml) α-trinositol 5 min prior to thawing and 5 mg (0.1 ml) 25 min afterwards. (2) The control group ($n = 5$) received 0.2 ml saline 5 min prior to the start of thawing and 0.1 ml saline 25 min later.

Experiment 3: The interstitial volume-pressure relationship Total tissue water and the corresponding P_{if} were measured 10 min ($n = 6$), 20 min ($n = 7$) and 60 min ($n = 6$) after start of thawing. All rats received 0.2 ml saline 30 min prior to the start of thawing.

Data analysis Two-way ANOVA with repeated measures and subsequent Bonferroni and Student's *t*-test were used to make comparison within and between the different groups. A value of $P < 0.05$ was considered statistically significant. The data are given as mean ± s.d.

Results

The standard cold injury in the anaesthetized animal produced severe frostbite. Immediately after removal from the cooling device the paw appeared white, stiff and frozen. The thawing of the superficial skin after removal from the cooling device, from

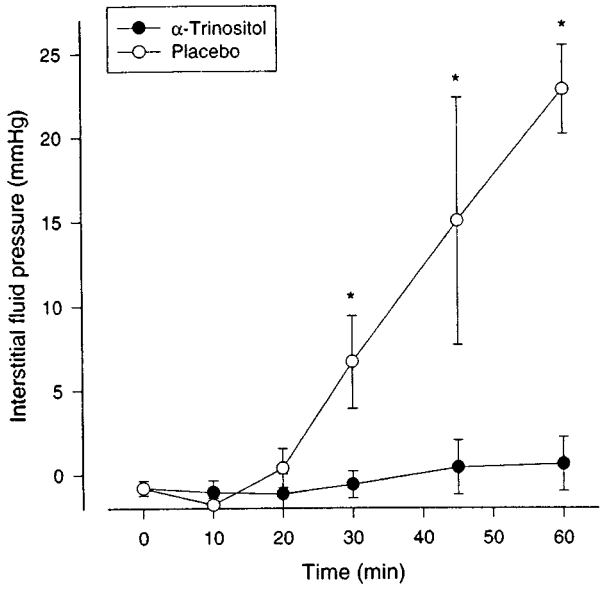

Figure 2 Effect of frostbite injury on interstitial fluid pressure in paw skin of untreated animals (placebo) with intact circulation as a function of time. Also shown is the effect of treatment with α-trinositol (intravenous bolus injection given 5 min before (10 mg) and 20 min after (5 mg) start of thawing of the tissue). Mean ± s.d. $*P<0.05$, compared to own control.

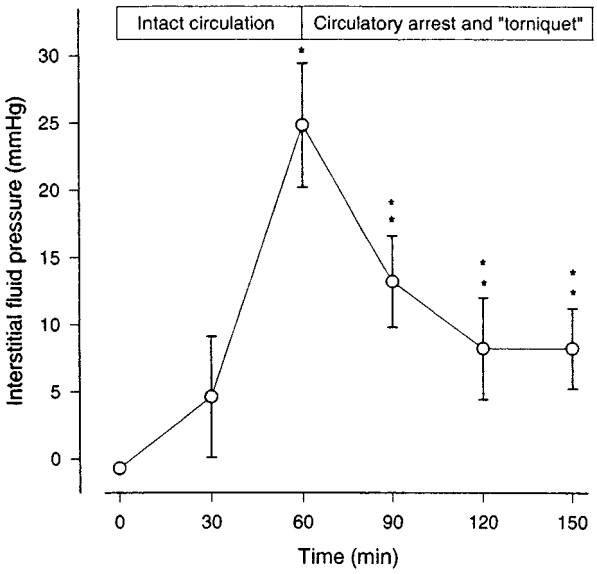

Figure 3 Effect of frostbite injury on interstitial fluid pressure (P_{if}) in paw skin of untreated animals. P_{if} is measured during intact circulation and following circulatory arrest with an occlusive tourniquet. Mean ± s.d. $*P<0.05$, compared to own control (at time zero) and $**P<0.05$, compared to values at 60 min.

the minimum temperatures to 0°C, required approximately 4–6 min (Figure 1). Thereafter the skin regained colour and became soft. Approximately 20 min post-freeze the paw was hyperaemic and swollen.

Experiment 1: Interstitial fluid pressure measurements

Intact circulation Control P_{if} before cold injury and circulatory arrest was -0.8 ± 0.2 mmHg (mean of all controls, $n=12$). During thawing P_{if} initially decreased to -1.2 ± 1.2 mmHg (Figure 2, $P>0.05$ vs control). Thereafter P_{if} rose almost linearly with time after thawing to $+21.7 \pm 3.8$ mmHg at 60 min post-freeze ($P<0.05$) (Figure 2). In animals subjected to freezing and treated with α-trinositol post-freeze P_{if} was -0.9 ± 0.4 mmHg and did not change throughout the observation period of 60 min ($P>0.05$).

In the group without α-trinositol and intact circulation, P_{if} at 60 min after start of thawing was $+24.8 \pm 4.6$ mmHg (Figure 3). Circulatory arrest was induced at 60 min after start of thawing and P_{if} decreased gradually to $+8.2 \pm 2.9$ mmHg at 30 min after circulatory arrest. Total tissue water did not change ($P>0.05$) between 90 min (3.9 ± 1.3 ml g dry weight^{-1}, $n=4$) and 60 min (3.7 ± 0.8 ml g dry weight^{-1}, $n=6$) after start of thawing in the tourniquet group indicating that fluid had not been lost from the injured tissue during this time period.

Circulatory arrest Control P_{if} before cold injury and circulatory arrest was -0.8 ± 0.3 mmHg (mean of all controls, $n=19$). When circulatory arrest was induced after the cold injury, but before the start of thawing of the tissue, P_{if} did not change from control (Figure 4) ($P>0.05$). Circulatory arrest induced 10 min post-freeze caused a lowering of P_{if} in the cold injured paw within 20 min to -3.4 ± 1.0 mmHg ($P<0.05$ vs control) (Figure 4) which was abolished when 10 mg of α-trinositol was given 5 min before starting to thaw the tissue ($P<0.05$ vs non-treated) (Figure 4).

Experiment 2: Total tissue water content, albumin extravasation and change in paw weight

Total tissue water in the non-injured paws that received α-trinositol or saline was not different ($P>0.05$) (Table 1), and was therefore combined (mean of all controls = 2.0 ± 0.15 ml g dry weight^{-1}, $n=19$). Total tissue water in the cold-injured paw receiving saline vehicle increased by 2.4 ± 0.7 ml g dry weight^{-1} compared to that in the contralateral non-injured paw ($P<0.05$) (Table 1). Treatment with α-trinositol both pre- as well as post-freeze markedly reduced the increase in total tissue water ($P<0.05$ vs saline treated control) (Table 1).

Albumin extravasation in the non-injured paws that received α-trinositol or saline were not different (Table 1), and the mean of all controls was 0.02 ± 0.017 ml g dry weight^{-1} ($n=19$). The increase in albumin extravasation in the cold injured paw receiving saline vs the contralateral non-injured control paws was 2.4 ± 1.1 ml g dry weight^{-1} ($n=9$) (Table 1). Administration of α-trinositol both pre- as well as post-freeze reduced the increase in albumin extravasation significantly (Table 1).

Administration of α-trinositol 5 min before (10 mg) and 20 min after (5 mg) the start of thawing reduced the rise in the paw weight (Figure 5) by 27% ($P<0.05$) and 36% ($P<0.05$) compared to the non-treated control group at 30 and 60 min, respectively (data not presented).

Experiment 3: The interstitial fluid volume-pressure relationship

The relationship between total tissue water content and the corresponding P_{if} is given in Figure 6. Within 40–140% increase in total tissue water (from 2.6 to 4.3 ml g dry weight^{-1}) (Figure 6) there is an almost linear relationship between volume and pressure. Compliance in this range was 0.112 ml g dry weight^{-1} mmHg^{-1}, i.e. 6% per mmHg

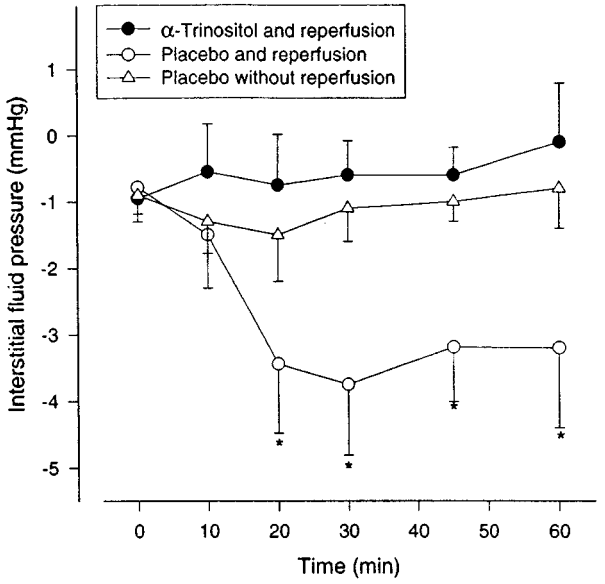

Figure 4 Effect of frostbite injury on interstitial fluid pressure in paw skin of untreated animals (placebo) in which circulatory arrest has been induced at the start (placebo without reperfusion) and 10 min after start of thawing of the tissue (placebo and reperfusion). Also shown is the effect of intravenous bolus injection of 10 mg α-trinositol given 5 min before the start of thawing and where circulatory arrest was induced 10 min after start of thawing. Mean ± s.d. $*P<0.05$, compared to own control.

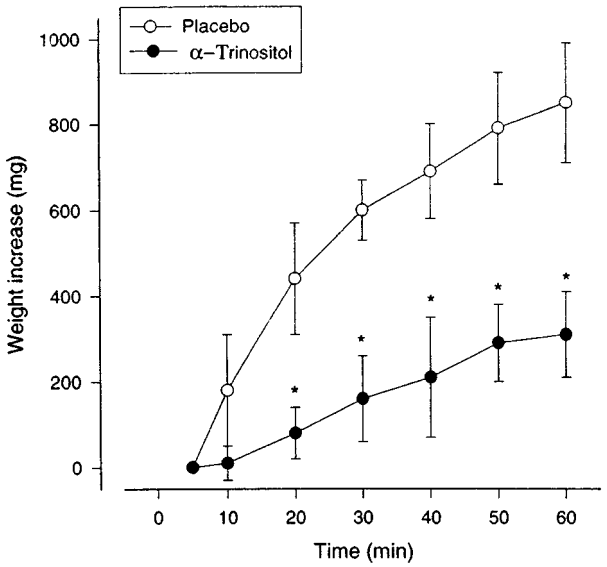

Figure 5 The time course of oedema formation expressed as gain in total paw weight evoked by cold-induced tissue injury in untreated animals (placebo) and animals treated with α-trinositol. Mean ± s.d. $*P<0.05$, compared to untreated controls in the same time group.

Table 1 Effect of α-trinositol on albumin extravasation (E_{alb}) and total tissue water content (TTW) following severe experimental frostbite injury in rat paw skin. ΔTTW and ΔE_{alb} are differences between the cold injured paw and the control paw of the same animal

		E_{alb}		*TTW*		*Δ*	
Group	n	*Injured* (ml g DW^{-1})	*Noninjured* (ml g DW^{-1})	*Injured* (ml g DW^{-1})	*Noninjured* (ml g DW^{-1})	E_{alb} (ml g DW^{-1})	*TTW* (ml g DW^{-1})
Saline control	(5)	2.37 ± 1.12	0.02 ± 0.02	4.46 ± 0.93	2.13 ± 0.16	2.3 ± 0.9	2.4 ± 0.7
α-Trinositol post-freeze	(5)	0.66 ± 0.33*	0.01 ± 0.001	2.51 ± 0.29*	2.03 ± 0.06	0.6 ± 0.3*	0.8 ± 0.2*
α-Trinositol pre- and post-freeze	(9)	0.36 ± 0.18*	0.01 ± 0.001	2.71 ± 0.35*	1.91 ± 0.11	0.3 ± 0.1*	0.5 ± 0.2*

All values are mean ± s.d $*P<0.05$ vs the corresponding value in the saline group.

when control total tissue water was 1.81 ml g dry $weight^{-1}$.

Discussion

The present study contains two novel observations on frostbite injury. First, a lowering of interstitial fluid pressure (P_{if}) will contribute to the initial and rapid oedema formation. Second, the experimental anti-inflammatory drug α-trinositol is able to abolish the oedema associated with the frostbite injury.

The mechanism of action of α-trinositol, i.e. the 1,2,6-isomer of D-*myo*-inositol trisphosphate, is not fully elucidated. Several inositol phosphates are found intracellularly in animal cells and 1,4,5-trisphosphate is one of the second messengers stimulating the release of Ca^{2+} from intracellular stores (Berridge & Irvine, 1989). The 1,2,6-isomer does not occur naturally in animal cells, but has several interesting and novel pharmacological effects (Carrington *et al.*, 1993; Edvinsson *et al.*, 1990). In skin burn injury in rats (Lund & Reed, 1994; Tarnow *et al.*, 1996) α-trinositol reduce the albumin extravasation and oedema formation when given pre- as well as post-burn (Lund & Reed, 1994). In the present model of acute frostbite injury as well as in other experimental inflammatory models (Claxson *et al.*, 1990; Woie & Reed, 1994), α-trinositol effectively inhibits oedema formation and transvascular loss of macromolecules (albumin). A possible explanation for the reduction of the oedema by α-trinositol could be a reduction of tissue perfusion and/or microvascular pressure in turn reducing microvascular fluid filtration. However, a reduction of oedema is usually associated with vasoconstriction, which reduces local blood flow (Yarwood *et al.*, 1993). In contrast, α-trinositol has vasodilatory effects causing increased peripheral blood flow (Gardiner *et al.*, 1994), and presumably increased microvascular pressure. However, α-trinositol reduces albumin extravasation in burns (Gardiner *et al.*, 1994) in spite of an increased blood flow (Cassuto *et al.*, 1990). Similarly, in smoke inhalation in sheep, reduction of lymph flow after α-trinositol treatment was associated with decreased microvascular permeability rather than increased microvascular pressure (Nakazawa *et al.*, 1994). This suggests that mechanisms other than lowering of microvascular blood pressure are important in explaining the anti-inflammatory effects of α-trinositol.

Neutrophils play a major role in the pathophysiology of frostbite injury during reperfusion of the tissue *via* generation of free oxygen radicals (Bhaumik *et al.*, 1995). The anti-inflammatory properties of α-trinositol, which is also a metal chelator, may therefore be due to the influence of free radical generation (Claxson *et al.*, 1990). The activated neutrophils

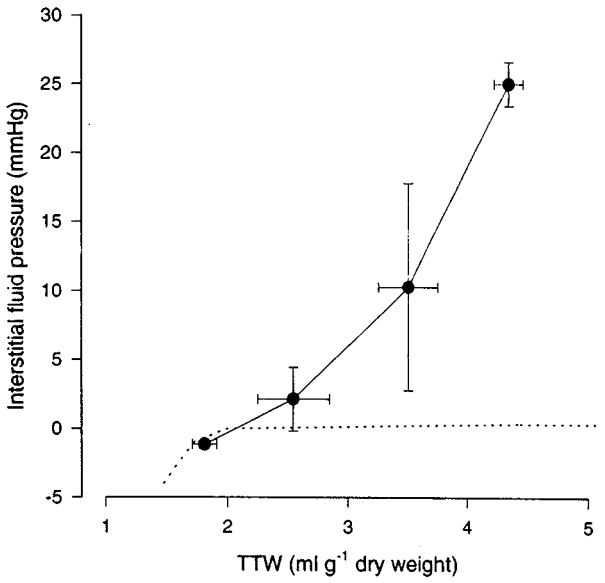

Figure 6 The relationship between interstitial fluid pressure (P_{if}) and total tissue water (TTW) (compliance) in rat paw skin during tissue overhydration due to frostbite injury. Mean ± s.d. Error bars contained within one symbol. Broken line indicates the volume-pressure curves obtained by Wiig & Reed (1981).

adhere to the microvascular endothelium, extravasate and release cytotoxic oxidants and hydrolytic enzymes to increase microvascular permeability and transvascular fluid filtration. α-Trinositol reduces the number of circulating leukocytes after burn injury (Nakazawa *et al*., 1994) but neutrophils appear not to play a significant role in the formation of acute oedema taking place in the first 4 h post-burn (Jakobsson *et al*., 1985). Finally, a possible mechanism of action of α-trinositol is modulation of the β_1-integrin function (Rodt *et al*., 1994). Anti-β_1 integrin (Rodt *et al*., 1994) and anti-$\alpha_2\beta_1$ integrin (Rodt *et al*., 1996) causes lowering of P_{if} and oedema formation when injected subdermally in rat skin and α-trinositol abolish these reactions (Rodt *et al*., 1994).

The present observation of a lowering of interstitial fluid pressure (P_{if}) accompanying frostbite injury is important since it points to an 'active' role for the loose connective tissue in generation of the oedema. Thus, the loose connective tissue rather than the microvascular wall might be targets for pharmacological therapy of this condition. The role normally assigned for P_{if} is to prevent oedema formation and maintain a constant interstitial volume. When microvascular filtration increases, interstitial volume and thereby P_{if} will increase. The increase in P_{if} will in turn act across the microvascular wall to limit further filtration. The present observation of a lowering of P_{if} from −0.8 to −3.9 mmHg implies that P_{if} following the frostbite injury will enhance and not prevent transvascular fluid transport. This observation is novel regarding frostbite injury, but has been demonstrated in several inflammatory reactions (Reed *et al*., 1997; 1998).

The oedema generated by the frostbite injury raised total tissue water from 2.1 to 4.5 ml g^{-1} dry weight in 30 min and at the same time the clearance of plasma reached a similar figure, i.e. the microvascular filtrate generating the frostbite oedema has a protein concentration similar to plasma. The oedema formation calculated from the 25 min albumin clearance, 0.01–0.02 ml g^{-1} in control, and 2.4 ml g^{-1} after injury i.e., the rate of filtration is increased by 100–200 times above control value (Table 1). Part of the rise in clearance is due to the microvascular membrane becoming less selective. Normally, the microvascular filtrate in skin has a concentration one half that of plasma (Parker *et al*., 1984), i.e. the 0.01–0.02 ml of plasma clearance represent a fluid volume of 0.02–0.04 ml. The loss of selectivity of the microvasculature is suggested by the virtually identical increase in total tissue water and albumin extravasation. Microvascular filtration will nevertheless rise by 50–100 times above control, excluding an effect of altered net filtration pressure and increased capillary filtration coefficient. The latter increases by two to three times even in major injuries like burn injury (Arturson & Mellander, 1964; Pitt *et al*., 1987) leaving a 20–50 times increase in net microvascular filtration pressure to be accounted for by the lowering of P_{if}, reduced reflection coefficient and increased microvascular hydrostatic pressure. From this reasoning it should be evident that the complete reversal of frostbite injury oedema by α-trinositol must be caused by an action of the drug on both P_{if} and an effect maintaining the integrity of the microvascular membrane since abolishment of P_{if} by −3 mmHg is not sufficient to explain the effect of α-trinositol in completely reducing the frostbite injury.

The lowering of P_{if} did not occur unless the injured tissue was reperfused, in agreement with previous observations of frostbite injury being mediated by free radicals rather than direct tissue injury (Iyengar *et al*., 1990). This could suggest that α-trinositol is a free radical scavenger, or at least is able to abolish damage induced by free radicals since it totally abolished the oedema formation when given prior to freezing or prior to start of thawing. However, the effect of α-trinositol on P_{if} is likely mediated *via* another mechanism where α-trinositol is able to modulate β_1-integrin function, i.e. the cellular receptors towards extracellular matrix components (Hynes, 1992). Through a series of studies we have demonstrated that the lowering of P_{if} is mediated *via* perturbation of the β_1-integrin system (Reed *et al*., 1992; Rodt *et al*., 1994; 1996; Åhlèn *et al*., 1998). Antibodies towards these receptors lowers P_{if} (Reed *et al*., 1992; Rodt *et al*., 1996) and α-trinositol will abolish this lowering of P_{if} (Rodt *et al*., 1994) likely mediated through an increase in cytoplasmatic free calcium in the skin fibroblasts (Åhlèn *et al*., 1998).

A rise in P_{if} is one of the 'safety factors' against oedema formation. Compliance (Guyton, 1965) gives a direct estimate of P_{if} developed from a change in interstitial fluid volume, which in turn reduces the net filtration pressure. In rat skin the P_{if} of normally hydrated tissues can maximally rise by 1–2 mmHg upon two to six times increase in interstitial fluid volume (Wiig & Reed, 1981). The compliance in rat skin has been estimated to be 14% per mmHg (Wiig & Reed, 1981) while in our study the compliance was estimated to be 6%. The present volume-pressure relationship describe P_{if} to rise almost linearly during over-hydration from total tissue water values about 2.4 ml g dry weight^{-1} (Figure 6). This is different from the pressure-volume curves obtained by Wiig & Reed (1981) although the same animal, tissue and experimental techniques were used in the experiments. However, there are several possible explanations for this discrepancy. We used paw skin while Wiig & Reed (1981) used the skin from the thigh which may be less distensible than that of the paw. Another explanation is that acute and chronic changes may follow a different volume-pressure relationship. In the present study, overhydration was induced within 15–20 min while Wiig & Reed (1981) used several hours to obtain the same degree of oedema. The two observations would fit together if the compliance increases over time, i.e. there is stress relaxation or delayed compliance (Guyton, 1965). The observation that P_{if} decreases despite unchanged volume

(Figure 3) indicates that the skin of the paw is highly distensible over time.

To summarize: α-trinositol attenuates development of the oedema following frostbite injury. The drug was effective when given both before and in the interval between freezing and thawing of the tissue. The oedema after the cold injury was reduced by α-trinositol limiting the increased microvascular permeability for macromolecules and attenuating a lowering of interstitial fluid pressure. The ability of α-trinositol to modulate P_{if} suggests that the extracellular matrix can be a target for pharmacological intervention during acute inflammatory reactions.

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