

# **Damage to vascular endothelium and smooth muscle contribute to the development of non-freezing cold injury in the rat tail vascular bed *in vitro***

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## **INTRODUCTION**

Non-freezing cold injury (NFCI) is a clinical condition characterised by damage to peripheral tissues, usually the extremities, as a result of prolonged exposure to cold and wet conditions usually, but not always, just above freezing point (Montgomery *et al.*, 1954). The clinical progression of NFCI was first characterised by Ungley (Ungley *et al.*, 1945); early symptoms include a period of intense vasoconstriction with ischaemia to affected tissues.

On removal from the cold environment the affected area is hyperaemic with swelling, impairment of microcirculation and slowed capillary refilling. Initial numbness is often replaced by pain which may be severe and often resistant to even the strongest analgesics. Pain is also a common symptom in later, or chronic, stages of the condition. Lasting sequelae include cold-sensitivity, hyperhidrosis (excessive sweating) and lasting nerve damage (Francis & Oakley, 1996). In extreme cases extensive tissue necrosis and onset of gangrene may necessitate amputation of the affected limb.

Despite the potentially severe consequences of NFCI, the largest number of reported cases is of a mild form, which does not exhibit the more severe symptoms. Because of difficulties in diagnosing this milder form of NFCI, individuals so afflicted may unknowingly return to a cold environment prematurely, thereby running the risk of a relapse, which will usually exacerbate the overall level of injury (Francis & Golden, 1985). Therefore, a reproducible model of mild forms of NFCI would be of substantial clinical benefit to possible future diagnosis and prevention of long-term cold injury.

Previous *in vitro* studies have identified the isolated, perfused rat tail as a suitable preparation for *in vitro* measurement of vascular responses to pharmacological agents (Francis & Oakley, 1997). Subsequent attempts to develop a suitable *in vivo* model of rat tail, in unaesthetised animals, proved unsuccessful (Golden *et al.*, 2003). The aim of the present study was to investigate the pharmacology and pathophysiology of NFCI in the isolated, perfused rat tail *in vitro*, with a view to identifying the mechanisms underlying the condition.

## **METHODS**

Male Wistar rats (250-300g) were killed by rising concentration of CO<sub>2</sub>, followed by cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. The caudal artery was exposed through an incision in the upper portion of the tail, and immediately

cannulated using a 18-gauge venflon<sup>®</sup> intravenous catheter and perfused with Krebs solution maintained at 37°C, and aerated with a 95% O<sub>2</sub> / 5% CO<sub>2</sub> mixture at a constant rate of 2.5ml min<sup>-1</sup> using a peristaltic pump. An initial equilibration period of 30 min was allowed before commencement of experiments. Drugs were injected in a volume of 10µl or 30µl into the perfusate through a rubber injection port close to the cannulation site. Alterations in perfusion pressure were recorded with a pressure transducer (Harvard<sup>®</sup>), and displayed on a Biopac MP150 acquisition system (Biopac Systems<sup>®</sup>). For experiments involving vasodilator responses the perfusion pressure of the vascular bed was increased by a 6µM phenylephrine infusion via a constant rate infusion pump (Harvard<sup>®</sup>).

*Polymerase chain reaction (PCR) analysis of eNOS and iNOS mRNA expression.* Total RNA was isolated from a 10cm length of caudal artery using the GenElute Mammalian RNA Miniprep kit (Sigma<sup>®</sup>). Concentration and purity were determined by measuring absorbance at 260 and 280 nm following treatment with DNase I (Sigma) to remove contaminating genomic DNA. cDNA was reverse transcribed from 2 µg of total RNA using Moloney murine leukaemia virus reverse transcriptase (Sigma). PCR amplifications were carried out in 50 µl reaction volumes comprising 2 µl cDNA template, 25 µl master mix (Promega), gene-specific forward and reverse primers for (1) eNOS and GAPDH or (2) iNOS and GAPDH. PCR products were subjected to agarose gel electrophoresis (2 % agarose gel/ 1X TBE/ 125 V/ 30 min). Bands were visualized by ethidium bromide fluorescence under ultraviolet light. Densitometry was then carried out using SYNGENE software, to quantitate the relative amounts of each NOS gene with the internal control, GAPDH (an invariably expressed housekeeping gene).

*Immunolocalisation of endothelial NOS (eNOS).* Frozen sections of caudal artery of 30µm thickness were mounted on glass slides, fixed with 4% formaldehyde and stained with rabbit anti-eNOS antibody (Sigma<sup>®</sup>) for 24 h at 4°C in a humidified environment. Secondary antibody, Alexa Fluor 488 goat anti-rabbit (Invitrogen<sup>®</sup> Molecular Probes) was applied for 30 min, along with a nuclear stain (propidium iodide, 3µg/ml), and slides were washed and mounted with Vectashield mounting medium (Vector<sup>®</sup>). Sections were viewed using a Zeiss<sup>®</sup> 510 Meta confocal laser scanning microscope using laser wavelengths of 488 and 543nm.

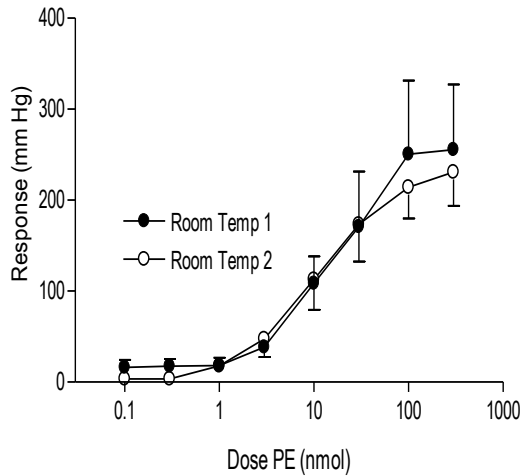
*Analysis of results.* Increases in perfusion pressure produced by constricting agents are expressed as mm Hg. Decreases in perfusion pressure produced by dilating agents are expressed as percent decrease in phenylephrine-induced tone in mm Hg, normalised to a 300nmol bolus dose of papaverine. All data shown are mean ± S.E.M. For statistical analysis, either 2-way analysis of variance (ANOVA) with repeated measures, or a paired t-test was used, followed by a Bonferroni *post-hoc* test. *P* values of less than 0.05 were considered significant.

## RESULTS

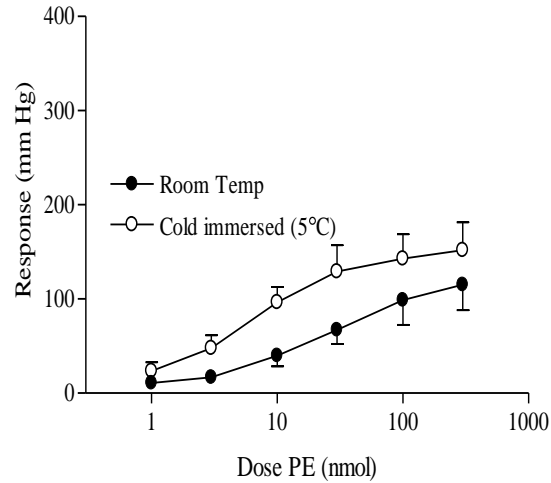
Vasoconstrictor responses to the  $\alpha_1$  agonist phenylephrine (PE) were significantly augmented following 5°C cold immersions for between 30 minutes and 2 hours relative to time-matched, dry perfused preparations (Figure 1). Vasoconstrictor responses were not affected by a 30-minute cold immersion. Specific nitric oxide synthase (NOS) inhibition with 300µM L-NAME elicited a significant leftward shift in vasoconstriction to PE, which was subsequently shown to be abolished by prior cold water immersion, suggesting endothelial damage following cold water

immersion. The leftward shift in PE responses to L-NAME was partially restored by pre-treatment with the Rho kinase inhibitor fasudil (3 $\mu$ M).

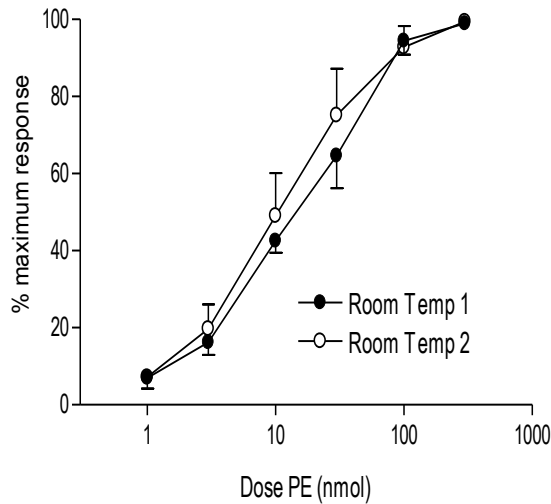
A. Time-matched control



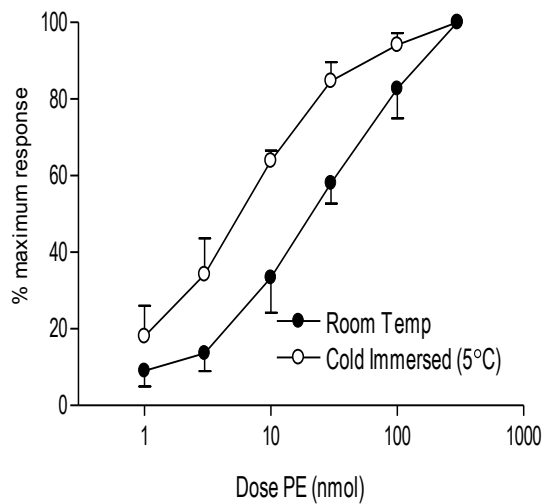
B. Response to 5°C immersion



C. Normalised time-matched control responses



D. Normalised response to 5°C immersion



**Figure 1.** Effect of a 120-minute cold (5°C) water immersion on PE-induced increases in perfusion pressure. Part A shows the time-matched control group ( $n=3$ ). The maximum values (mm Hg) did not change over time ( $259.95 \pm 75.98$  vs.  $232.22 \pm 37.55$ ;  $p>0.05$ , ns). Part B shows the response to cold immersion ( $n=4$ ). Cold did not significantly affect maximum values ( $115.25 \pm 27.18$  vs.  $153.12 \pm 30.06$ ;  $p>0.05$ , ns). Part C shows control data expressed as % maximum response ( $n=3$ ). PE slope and  $pED_{50}$  values did not change over time ( $pED_{50} = 7.85 \pm 0.09$  vs.  $7.95 \pm 0.19$ ;  $p>0.05$ , ns;  $nH = 1.08 \pm 0.10$  vs.  $1.24 \pm 0.10$ ;  $p>0.05$ , ns). Part D shows cold immersion did not affect PE slope or  $pED_{50}$  values ( $n=4$ ) ( $pED_{50} = 7.70 \pm 0.15$  vs.  $8.30 \pm 0.11$ ;  $p<0.05^*$ ;  $nH = 1.05 \pm 0.08$  vs.  $1.16 \pm 0.27$ ;  $p>0.05$ , ns).

In the presence of L-NAME, cold water immersion unmasked a depressor effect on vasoconstrictor reactivity to PE, possibly due to a direct inhibitory effect of cold immersion on vascular smooth muscle contractility. Ischaemia *per se* decreased contractile activity of vascular smooth muscle to PE, while cold ischaemia seems to afford some protection to vascular smooth muscle, such as is the case in cold organ preservation.

In preparations precontracted with 6 $\mu$ M PE, a 1 hour cold water immersion significantly attenuated vasodilation to acetylcholine (ACh), histamine (HA), bradykinin, and calcium ionophore A23187 relative to a standard vasodilator response to a 300nmol dose of the directly acting vasodilator papaverine. Conversely, vasodilator responses to the nitric oxide (NO) donor sodium nitroprusside were augmented following cold immersion, and in response to NOS inhibition with L-NAME (300 $\mu$ M), possibly suggesting that any defect in NO signalling may relate to the synthesis rather than utilisation of NO. Vasodilation to ACh and HA were confirmed to be at least partially endothelium-dependent, with responses being significantly attenuated following NOS inhibition and endothelial denudation with distilled water. Remaining vasodilation to ACh and HA may indicate a NO-independent component.

Cold water immersion for 1 hour was associated with a significant increase in rat tail artery mRNA expression of NOS III. NOS III mRNA expression was also increased following rewarming. However, NOS II mRNA expression was not significantly affected following cold immersion, or by subsequent rewarming. Cold immersion significantly increased mRNA levels of COX-1, HO-2, and the  $\alpha_{2A}$  adrenoceptor subtype relative to time-matched room temperature controls. With regard to the additional role of HO-1 as a heat shock protein, HO-1 mRNA expression was found to be decreased by cold water immersion, with expression being significantly increased on rewarming. This suggests possible initiation of the heat shock response by a relative heat stress following cold immersion. Furthermore, indirect immunofluorescence studies showed a reduction in fluorescent staining for NOS III following a 1 hour cold immersion, while staining for vWF showed an apparent redistribution of vWF from the cytoplasm to the cell surface membrane following cold immersion.

## CONCLUSIONS

Previous studies have suggested that acute cold exposure such as a 1-hour cold-water immersion is sufficient to cause damage to the vascular endothelium (Endrich *et al.*, 1982; Svanes, 1964). The present data suggest that acute cold water immersion is associated with a loss of NO-dependent endothelial function, with endothelial activation/inflammation in the *in vitro* perfused rat tail preparation. Cold water immersion also appears to produce a defect in vascular smooth muscle contractility. Furthermore, cold-induced activation of the Rho kinase pathway may play a role in mediating cold-induced vascular injury, such as in the case of acute NFCI.

It is concluded that Rho kinase may present a novel pharmacological target for the prevention of cold-induced endothelial dysfunction, such as occurs in NFCI.

The present study also validates the cold-immersed, perfused rat tail preparation as a suitable model for the *in vitro* study of NFCI and other vascular conditions.

## ACKNOWLEDGEMENTS

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## REFERENCES

- Endrich, B., Laprell-Moschner, C., Brendel, W. & Messmer, R. (1982) Effects of prolonged cold injury on the subcutaneous microcirculation of the hamster. *Res. Exp. Med. (Berl.)* 181: 49-61.
- Francis, T. J. & Golden, F. St.C. (1985) Non-freezing cold injury: the pathogenesis. *J. Roy. Nav. Med. Serv.* 71:3-8.
- Francis, T. J. & Oakley, E. H. (1996) Col Injury. In: Tooke, J. E. & Lowe, G. D. O. (Eds) *A textbook of vascular medicine.* pp353-370.
- Golden, F. St.C., Eglin, C. M., Inns, R., Laight, D., Martin, L. & Tipton, M. (2003) Assessment and management of non-freezing cold injury. University of Portsmouth Report to UK Gov dstl/tr/06470.
- Montgomery, H., Horwitz, O., Pierce, G. & Sayen, A. (1954) Experimental immersion foot i. The effects of prolonged exposure to water at 3 degrees C on the oxygen tension and temperature of the rabbit leg. *J. Clin. Invest.* 33: 361-9.
- Svanes, K. (1964) Studies in hypothermia. The influence of prolonged deep hypothermia and of barbiturate anaesthesia on the blood leukocytes in mice. *Acta Anaesthesiol Scand.* 89: 167-79.
- Ungley, C. C., Channell, G. D. & Richards, R. K. (1945) The immersion foot syndrome. *Br. J. Surg.* 33: 17-31.