

# Regulation of Wheal and Flare by Tea Tree Oil: Complementary Human and Rodent Studies

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**When applied 20 min after injection of histamine into human forearm skin, tea tree oil (TTO) reduces the developing cutaneous vascular response. In this study, the effect of TTO on inflammatory microvascular changes was dissected at the base of an experimental blister on rat skin. 1,8-Cineole, representing 2% of TTO, reduced vascular changes induced by sensory neuropeptides released when the distal portion of a cut sciatic nerve was electrically stimulated. The pre-terminal modulatory effect of 1,8-Cineole was confirmed in tests in sensory-denervated rats. Terpinen-4-ol (approximately 40% TTO) reduced substance P-induced microvascular changes and protein extravasation by a direct nitric oxide-mediated effect on the microvasculature, without sensory nerve involvement.  $\alpha$ -Terpineol (3% of TTO) regulated both pre- and post-sensory nerve terminals. In human skin, terpinen-4-ol applied 10 min after histamine injection, but not  $\alpha$ -terpineol or 1,8-cineole, regulated the developing wheal and flare suggesting that the histamine-induced responses in humans (at the dose used in this study, 50  $\mu$ L of 330  $\mu$ M histamine) are in large part determined by histamine directly affecting the vasculature via post-terminal-mediated events. The underlying strength of these studies is the use of a well-established rat physiologic model to differentiate the mechanism of regulation of microvascular changes by modulatory agents.**

Key words: sensory nerves/skin blisters/skin inflammation/substance P/terpenes  
J Invest Dermatol 123:683–690, 2004

Tea tree oil (TTO) is the essential oil steam-distilled from *Melaleuca alternifolia*, an Australian native plant. There are now many reports of the susceptibility of a range of bacteria, yeasts, and fungi to the antimicrobial properties of TTO that support the increasing popularity of TTO as an antimicrobial agent for the treatment of conditions such as tinea pedis and acne (Bassett *et al*, 1990; Tong *et al*, 1992; Carson and Riley, 1994; Nenoff *et al*, 1996; Concha *et al*, 1998). There have also been reports of TTO having anti-inflammatory properties. In the mouse, TTO reduced the edema but not the influx of inflammatory cells associated with the efferent phase of a contact hypersensitivity response but was without effect on an irritant response or the edema associated with exposure to UVB radiation (Brand *et al*, 2002a). Furthermore, TTO reduced immediate type hypersensitivities in the mouse, i.e., topical application of TTO significantly suppressed histamine-induced ear swelling in murine ears (Brand *et al*, 2002b).

Topically applied TTO also reduced the wheal induced by intradermal injection of histamine in human skin (Koh *et al*, 2002). In that study, 21 volunteers were injected intradermally in each forearm with histamine. After 20 min, TTO was applied topically to one forearm, leaving the other as a

control. Flare and wheal diameters, and double skin fold thickness were measured every 10 min for an hour. Mean wheal volume, but not flare area, significantly decreased 10 min after TTO application (Koh *et al*, 2002).

It was important to determine the mechanism by which TTO reduced histamine-induced skin inflammation and to characterize the component of TTO responsible. For the TTO used, gas chromatography/mass spectrometry identified terpinen-4-ol (42% of TTO),  $\alpha$ -terpineol (3%), and 1,8-cineole (2%) as the main water-soluble components of TTO (Hart *et al*, 2000). These are the components of TTO that would penetrate into the dermis and regulate both the vasculature and peripheral nervous systems. In this study, we investigated the regulatory properties of TTO, as well as terpinen-4-ol,  $\alpha$ -terpineol, and 1,8-cineole, on peripheral inflammatory responses in rat skin.

Histamine receptors have been identified on both endothelial cells and sensory nerves (Greaves and Wall, 1996; Clough *et al*, 1998; Clough, 1999) and it has been documented that histamine causes vasodilation and an increase in microvascular permeability leading to increased tissue perfusion and plasma extravasation (wheal and flare responses) by direct effects on the former cells or indirect effects on the activity of sensory nerves. To determine how TTO was regulatory, we employed a well-validated rat physiological model to differentiate between regulation of pre- and post-sensory nerve terminal events. This involved

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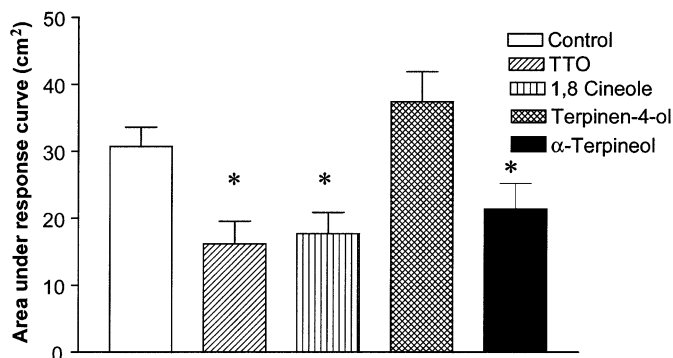
Abbreviations: L-NAME, N<sup>G</sup>-nitro-L-arginine; TTO, tea tree oil

assessing the inflammatory response at the base of blisters raised on the rat hind footpad after superfusing inflammatory mediators over the blister base or electrically stimulating the distal portion of a cut sciatic nerve to release sensory neuropeptides (Khalil *et al*, 1994; Khalil, 1999). These responses were also studied in sensory-denervated rats (Lembeck and Holzer, 1979). Finally, the components of TTO that modulate histamine-induced wheal and flare in human skin were characterized and their mechanism of action interpreted in the light of investigations of the activity of TTO on vascular changes in the rat hind footpad.

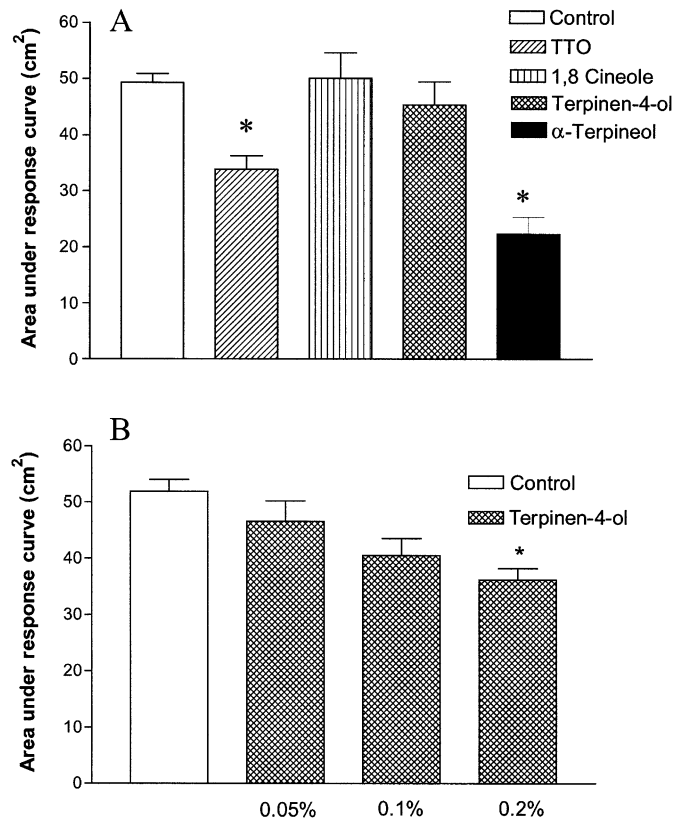
## Results

**Effect of TTO and its components on the vascular response to sensory nerve stimulation** As in models previously described (Khalil *et al*, 1994), selective electrical stimulation of C fibers by antidromic stimulation of the sciatic nerve caused the release of neuropeptides and an increase in the microvascular blood flow at the base of an experimentally induced blister on a rat hind footpad. It is generally accepted that the regulation of the vasodilator response to electrical stimulation primarily reflects control of pre-terminal events with an additional post-terminal component (Khalil *et al*, 2001). When an unfractionated preparation of the water-soluble components of TTO (0.125%) was perfused for 10 min prior to, during, and for 20 min subsequent to the 1 min electrical stimulation, there was a significant reduction in the area under the response curve (Fig 1). A significant reduction (approximately 50%) in the microvascular blood flow was also measured after perfusion with 1,8-cineole (0.0025%, i.e., 2% of 0.125%) or  $\alpha$ -terpineol (0.0038%, i.e., 3% of 0.125%), but not after perfusion with terpinen-4-ol (0.053%, i.e., 42% of 0.125%).

**Effect of TTO and its components on vascular responses to substance P** Perfusion of substance P over the base of an experimentally induced blister causes a vasodilator



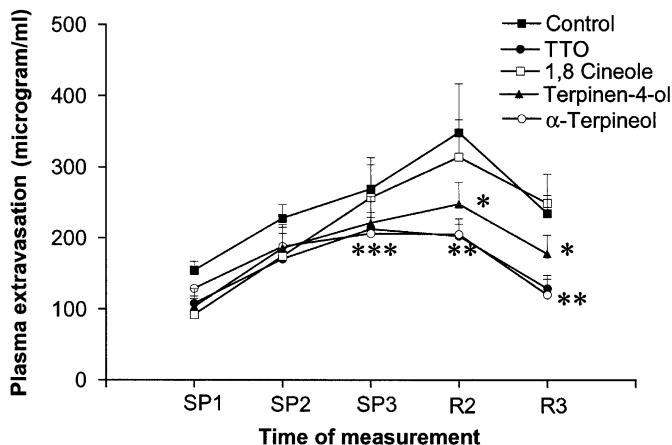
**Figure 1** Effect of 1,8-cineole, terpinen-4-ol, and  $\alpha$ -terpineol, as well as an unfractionated preparation of the water-soluble components of tea tree oil (TTO), on microvascular blood flow following sensory nerve stimulation. The test reagents were perfused over the base of a blister raised on the hind footpad of naive rats at concentrations of 0.0025%, 0.053%, 0.0038%, and 0.125% for six to eight rats per group. An asterisk denotes significant difference from control. Mean  $\pm$  SEM.



**Figure 2** Effect of 1,8-cineole, terpinen-4-ol, and  $\alpha$ -terpineol, as well as an unfractionated preparation of the water-soluble components of tea tree oil (TTO), on microvascular blood flow induced by substance P. In (A), the test reagents were perfused over the base of a blister raised on the hind footpad of naive rats at concentrations of 0.0025%, 0.053%, 0.0038%, and 0.125%. In (B), terpinen-4-ol was tested at 0.05%, 0.1%, and 0.2%. An asterisk denotes significant difference from control. Mean  $\pm$  SEM for six to eight rats per group.

response. It is known that the vasodilation response to substance P is the outcome of a combined post-terminal component with a significant pre-terminal component (Khalil and Helme, 1989). When perfused for 10 min prior to, and 30 min together with substance P, an unfractionated preparation of the water-soluble components of TTO (0.125%) significantly reduced the substance P-induced increase in microvascular flow by approximately 30% (Fig 2A).  $\alpha$ -Terpineol (0.0038%), but not 1,8-cineole (0.0025%), or terpinen-4-ol (0.053%), significantly suppressed substance P-induced increases in microvascular flow (Fig 2A). A significant inhibitory effect of terpinen-4-ol was detected only at a concentration of 0.2% (Fig 2B).

**Effect of TTO and its components on plasma extravasation induced by substance P** Regulation of substance P-induced plasma extravasation was also investigated by measurement of protein in the perfusates every 10 min during the 30 min perfusion with substance P, and then twice at 10 min intervals during the post-stimulation period of perfusion with Ringer's solution. As the plasma extravasation response to substance P is the outcome of only a post-terminal effect on neurokinin-1 receptors on post-capillary venules (Khalil and Helme, 1989), regulators of this response

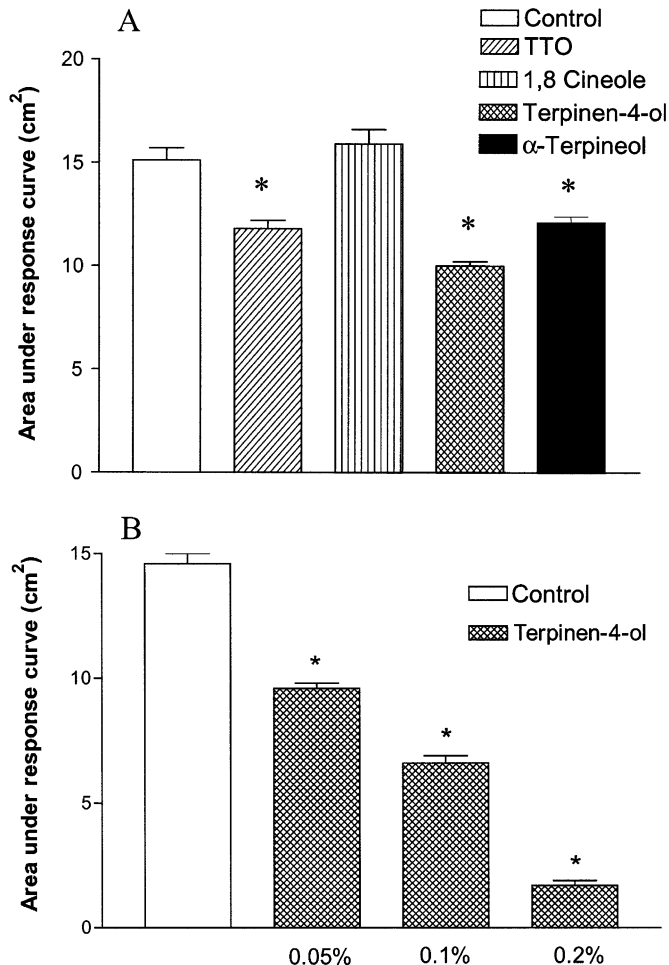


**Figure 3**  
Effect of 1,8-cineole, terpinen-4-ol, and  $\alpha$ -terpineol, as well as an unfractionated preparation of the water-soluble components of tea tree oil (TTO), on plasma extravasation induced by substance P. Protein levels in plasma were measured every 10 min during perfusion with substance P (SP1, SP2, SP3), as well as during two 10 min periods of perfusion with buffer (R2, R3). The test reagents were perfused over the base of a blister raised on the hind footpad of naïve rats at concentrations of 0.0025%, 0.053%, 0.0038%, and 0.125%, with six to eight rats per group. An asterisk denotes significant difference from control at that time point. Mean  $\pm$  SEM.

must be modulating a vascular response unrelated to sensory nerve involvement. An unfractionated preparation of the water-soluble components of TTO (0.125%), terpinen-4-ol (0.053%), and  $\alpha$ -terpineol (0.0038%), but not 1,8-cineole (0.0025%), significantly reduced substance P-induced plasma extravasation. Significant inhibition was detected in the last 10 min of perfusion with substance P and in the perfusates after substance P was removed (Fig 3).

**Effect of TTO and its components on vascular responses to substance P in sensory-denervated animals** To further clarify whether (a) the regulatory effects of an unfractionated preparation of the water-soluble components of TTO (0.125%), terpinen-4-ol (0.053%), and  $\alpha$ -terpineol (0.0038%) on microvascular changes were principally mediated by direct action on endothelial cells, and (b) the regulatory effect of 1,8-cineole (0.0025%) on microvascular changes was principally indirect and was by an effect on sensory nerves, blisters were induced in sensory-denervated rats and substance P-induced vasodilation was examined. In sensory-denervated rats, perfusion with an unfractionated preparation of the water-soluble components of TTO (0.125%) significantly suppressed the smaller substance P-induced increases in microvascular blood flow by 25% (Fig 4A) and also suppressed plasma extravasation (Fig 5). A significant reduction in both parameters of inflammation was also detected for terpinen-4-ol (0.053%) and  $\alpha$ -terpineol (0.0038%), but not 1,8-cineole (0.0025%) (Fig 4A and Fig 5). The effect of terpinen-4-ol was concentration-dependent with 90% inhibition of the substance P-induced microvascular blood flow at 0.2% (Fig 4B).

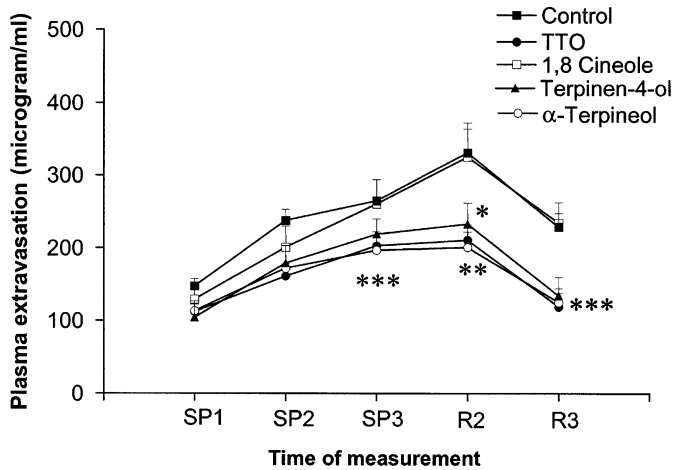
**Role of endothelial nitric oxide in the vascular response to terpinen-4-ol** Nitric oxide (NO) production constitutes the main mechanism by which substance P induces vaso-



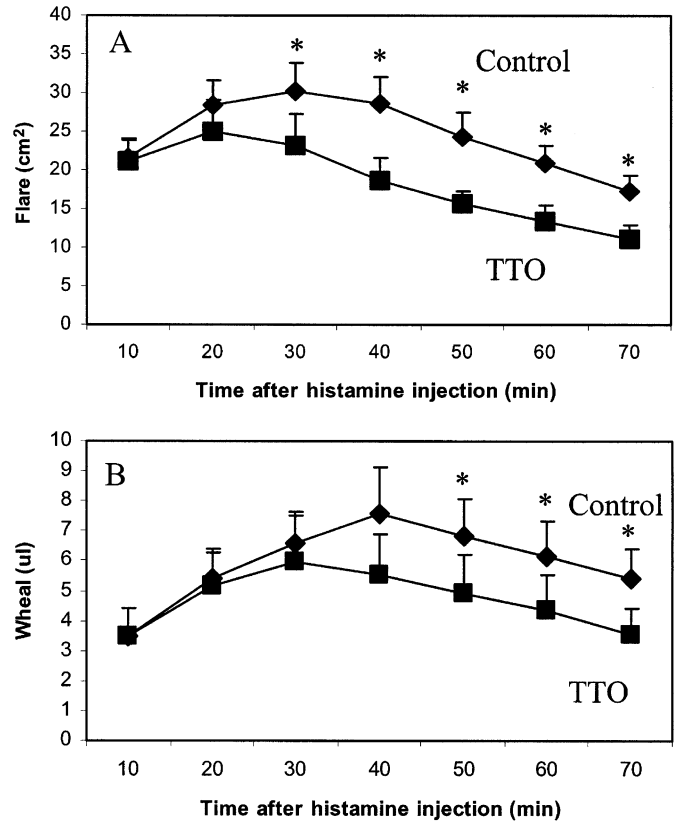
**Figure 4**  
Effect of 1,8-cineole, terpinen-4-ol, and  $\alpha$ -terpineol, as well as an unfractionated preparation of the water-soluble components of tea tree oil (TTO), on substance P-induced microvascular blood flow in sensory-denervated rats. For six to eight rats per group, vascular responses were recorded from the base of blisters raised on the hind footpad of capsaicin-pre-treated rats. In (A), the test reagents were perfused at concentrations of 0.0025%, 0.053%, 0.0038%, and 0.125%. In (B), terpinen-4-ol was perfused at 0.05%, 0.1%, and 0.2%. An asterisk denotes significant difference from control. Mean  $\pm$  SEM for six to eight rats per group.

dilatation at a post-terminal level (Ralevic *et al*, 1995). N<sup>G</sup>-Nitro-L-arginine (L-NAME), an inhibitor of NO biosynthesis, at the submaximal concentration of 100  $\mu$ M inhibited the vascular response to substance P by 44% (Fig 6). Although terpinen-4-ol at 0.2% inhibited the vascular response to substance P by 30%, when combined with L-NAME, the overall inhibition (40%) was not additive (Fig 6).

**Effect of undiluted TTO, terpinen-4-ol,  $\alpha$ -terpineol, and 1,8-cineole on histamine-induced wheal and flare in human skin** We have previously reported that when applied 20 min after histamine injection, TTO (100%) reduced the developing wheal, but not flare response (Koh *et al*, 2002). But when TTO (100%) was applied at both 10 and 20 min after histamine injection, TTO significantly reduced both the flare and wheal response (Fig 7). The effect of terpinen-4-ol (100%), 1,8-cineole (100%), and  $\alpha$ -terpineol (100%) was also examined with application (25  $\mu$ L) of the TTO



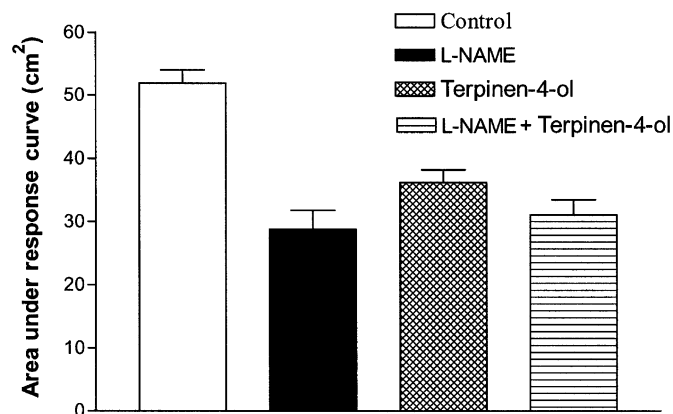
**Figure 5**  
Effect of 1,8-cineole, terpinen-4-ol, and  $\alpha$ -terpineol, as well as an unrefractionated preparation of the water-soluble components of tea tree oil (TTO), on substance P-induced protein extravasation in sensory-denervated rats. Protein levels in the perfusate were measured every 10 min during perfusion with substance P (SP1, SP2, SP3), as well as during two 10 min periods of perfusion with buffer (R2, R3). Plasma extravasation was measured for six to eight capsaicin-pre-treated rats per group. The test reagents were perfused at concentrations of 0.0025%, 0.053%, 0.0038%, and 0.125%. An asterisk denotes significant difference from control at that time point. Mean  $\pm$  SEM.



**Figure 7**  
Effect of tea tree oil (TTO) on histamine-induced (A) flare and (B) wheal in 18 volunteers. In (A), the mean flare area and (B), the mean wheal volume for the control (triangles) and study arms (squares) with increasing time after histamine injection is shown. TTO was applied at 10 and 20 min after histamine administration. Mean  $\pm$  SEM. An asterisk indicates a significant difference between control and TTO-treated arms at that time point.

components at both 10 and 20 min after histamine injection. The components were tested undiluted to prevent an interfering effect of a diluent. No adverse effect was measured in response to the undiluted chemicals. Terpinen-4-ol (Fig 8), but not 1,8-cineole or  $\alpha$ -terpineol (data not shown), significantly reduced the histamine-induced wheal and flare response. The intensity of the erythema induced by histamine injection was also measured using a fiberoptic tissue spectrum analyzer (Sumitomo Electrics, Tokyo, Japan); the erythema index was determined by subtracting the mean reading of normal skin (adjacent to the reaction site) from the mean reading of the reaction site. Terpinen-4-ol, but not 1,8-

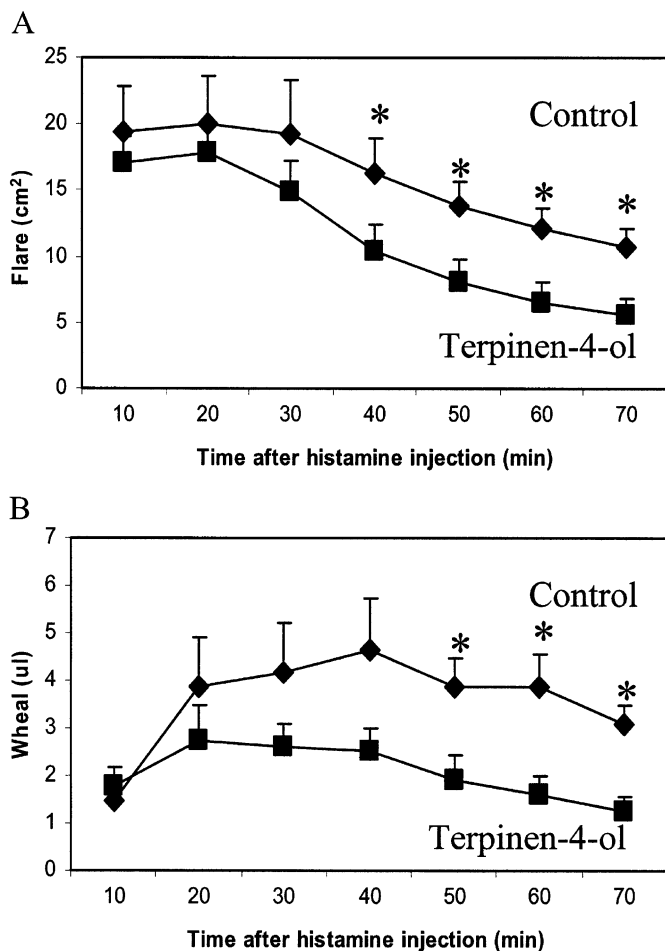
cineole, or  $\alpha$ -terpineol, also reduced the histamine-induced erythema index (data not shown).



**Figure 6**  
Role of NO in the regulation by terpinen-4-ol of substance P-induced microvascular blood flow. N<sup>G</sup>-Nitro-L-arginine (L-NAME) (100  $\mu$ M), terpinen-4-ol (0.2%) or both reagents together were perfused for 10 min prior to, and for 30 min together with substance P (1  $\mu$ M), over the base of a blister raised on the hind footpad of naïve rats. Mean  $\pm$  SEM for six to eight rats per group.

## Discussion

This study illustrates the ability of complementary rodent and human studies to determine the mode of action of immunoregulatory preparations on skin inflammation. We had previously shown that topical TTO could reduce skin inflammation experimentally induced by histamine. The study of microvascular changes in a blister base suggested that TTO was regulating both the sensory-nerve-associated and endothelial components of vasodilation. Further, the pre-terminal effect was due to 1,8-cineole and  $\alpha$ -terpineol that comprise approximately 2% and 3%, respectively, of TTO. The post-terminal effects of TTO on microvascular flow were due to the actions of terpinen-4-ol (approximately 42% of TTO) and  $\alpha$ -terpineol. Having determined the mode of action of the TTO components in this well-validated rat model that parallels the microvascular changes that take place in human skin, the component of TTO responsible for the immunoregulatory effects of TTO on human skin was examined. Terpinen-4-ol, but not 1,8-cineole or  $\alpha$ -terpineol, significantly reduced both the wheal



**Figure 8**  
**Effect of terpinen-4-ol on histamine-induced (A) flare and (B) wheal in 10 volunteers.** In (A), the mean flare area and (B) the mean wheal volume for the control (triangles) and study arms (squares) with increasing time after histamine injection are shown. Terpinen-4-ol was applied 10 and 20 min after histamine administration. Mean  $\pm$  SEM. An asterisk indicates a significant difference between control and terpinen-4-ol-treated arms at that time point.

and flare following histamine injection. Thus, under conditions similar to those used in our human study, the effects of TTO on human skin involve regulation of post-terminal events, without involvement of sensory nerves. This study suggested that the histamine-induced responses in human skin > 10 min after administration and at the dose used in this study (50  $\mu$ L of 330  $\mu$ M histamine) are in large part determined by histamine effects on endothelial cells (i.e., via post-terminal mediated events). This is supported by evidence that histamine is only able to activate sensory nerve terminals at much higher concentrations at the mM range (Khalil and Helme, 1989).

Solubility tests of TTO in water in which the aqueous layer is filtered through filter paper, extracted with ether, and assessed by gas chromatography/mass spectrometry, demonstrated that the three main water-soluble components of TTO are terpinen-4-ol,  $\alpha$ -terpineol, and 1,8-cineole (Hart *et al*, 2000). It has however, been shown that the water-soluble components of TTO can be separated without difficulty by their ability to remain in serum-free tissue culture medium although the cell-toxic oil-soluble components

adhere to the side of polystyrene plastic tubes (Hart *et al*, 2000). The use of this simple separation procedure for identification of an active fraction was validated when terpinen-4-ol,  $\alpha$ -terpineol, and 1,8-cineole were tested as pure substances. In the rat experiments, the plastic-non-adherent, water-soluble components (in this study called an unfractionated preparation of the water-soluble components of TTO) were examined at a concentration of TTO of 0.125%. This concentration was found in studies with human monocytes and neutrophils to be immunoregulatory but not toxic (Hart *et al*, 2000; Brand *et al*, 2001). As it is recommended that a 100% solution of TTO is applied to skin, this concentration represents approximately one-thousandth of that used and is a level less than the water solubility of TTO (1.6 g per liter) (Hart *et al*, 2000). The ability of TTO components to reach the epidermal and dermal tissues and enter the systemic circulation has not been measured. TTO contains several components known to enhance skin penetration of other compounds, e.g., 1,8-cineole (Obata *et al*, 1991), limonene (Okabe *et al*, 1990), terpinen-4-ol (Magnusson *et al*, 1997), and  $\alpha$ -terpineol (Magnusson *et al*, 1997). To test the activity of terpinen-4-ol,  $\alpha$ -terpineol, and 1,8-cineole in isolation, calculations were made according to their concentration in whole TTO and therefore their approximate level in TTO solutions of 0.125%. For testing on human skin, pure compounds were used undiluted. No control oil was included in this study. Application of liquid paraffin has been used previously on human skin as a control for TTO and was without effect on the histamine-induced wheal and flare (Koh *et al*, 2002). In this study, the non-reactive components of TTO provided a control for the activity of TTO and terpinen-4-ol on histamine-induced responses in human skin.

This study highlights the complexity of bioactivity of natural plant oils. The three main water-soluble components of TTO had different, but readily dissected, modulatory effects on the vascular responses in an experimentally induced blister. Terpinen-4-ol, the main water-soluble component of TTO, was without effect on sensory nerves but instead modulated vasodilation and plasma extravasation. In a recent report, another property of terpinen-4-ol was identified; it induced vascular smooth muscle relaxation in the deoxycorticosterone-acetate-salt hypertensive rat, without any effect on sympathetic nervous system activity (Lahlou *et al*, 2003). In contrast, in our skin models of inflammation in rat and human, the inhibitory effects of terpinen-4-ol were attributed to modulation of a post-terminal endothelium-mediated vasodilatation. In our animal experiments, a small vasodilatory response to terpinen-4-ol was observed upon its perfusion over the blister base; however, this effect was subsequently masked by its inhibitory effect on the inflammatory response to substance P. The inhibitory effect of terpinen-4-ol on the inflammatory response was then confirmed by the data demonstrating its inhibitory effect on histamine-induced inflammation in human skin. The possibility that terpinen-4-ol has an inhibitory effect on the vasodilatation response when applied locally in an inflammatory environment (this study), as well as a smooth muscle relaxant effect when applied systemically in a hypertensive environment (Lahlou *et al*, 2003), is an intriguing possibility that needs further investigation. In our study, 1,8-cineole

acted directly on sensory nerves, highlighting its potential anesthetic properties. This supports a study of oral feeding of 1,8-cineole to rats and mice resulting in decreased chemical nociception induced by intraplantar formalin or intraperitoneal acetic acid (Santos and Rao, 2000). The third water-soluble component of TTO,  $\alpha$ -terpineol, had both anti-edema and anesthetic properties with both a direct and indirect effect on the microvascular responses of the blister base.

As terpinen-4-ol, the main water-soluble component of TTO was the most active component in regulating the wheal and flare response in human skin; its anti-inflammatory potential and possible mechanism of action were further investigated. In sensory-intact animals (where the response to substance P is mainly pre-terminal with some post-terminal component), terpinen-4-ol reduced microvascular blood flow by 10%, 22%, and 30% (at 0.05%, 0.1%, and 0.2% concentration, respectively). In sensory-denervated animals (where the response to substance P is mainly post-terminal), terpinen-4-ol inhibited the response by 35%, 55%, and 90%, respectively. These data together with the ability of terpinen-4-ol to inhibit the plasma extravasation response to substance P (a post-terminal mediated event) and its inability to inhibit the vascular response to sensory nerve stimulation (mainly a pre-terminal-mediated event) further support our argument that terpinen-4-ol is mainly acting via a post-terminal-mediated mechanism. NO is involved in the vascular response to substance P (Ralevic *et al*, 1995). There was no additive effect between L-NAME and terpinen-4-ol in reducing substance P-induced microvascular blood flow and this suggests that terpinen-4-ol could be acting via a mechanism that involves modulating the ability of substance P to release endothelial NO. The involvement of other mechanisms in the anti-inflammatory actions of terpinen-4-ol, however cannot be excluded.

The study of physiologic models in rats contributes to an understanding of the bioactivity of TTO in other models of skin inflammation. In studies of histamine-induced edema in murine ears, terpinen-4-ol was equivalent in potency to TTO in suppressing the swelling and thus, could be interpreted as TTO acting on components of the vasculature, and not on sensory nerves (Brand *et al*, 2002b). This was supported by the unaltered ability of TTO to reduce histamine-induced ear swelling in sensory neuropeptide-depleted mice (Brand *et al*, 2002b). In contrast, TTO, as well as terpinen-4-ol and  $\alpha$ -terpineol, could reduce the edema associated with the efferent phase of a contact hypersensitivity response suggesting some sensory nerve control of this response (Brand *et al*, 2002a).

In summary, terpinen-4-ol is the active component of TTO in the regulation of histamine-induced wheal and flare in human skin. The underlying strength of these studies is, however, the use of a well-established rat physiologic model to dissect the mechanism of regulation of microvascular changes by modulatory agents.

## Materials and Methods

**TTO** TTO was provided by Novasel Australia Pty Ltd (Mudgeeraba, Queensland, Australia) and fulfilled the criteria of the Australian Standard (ISO-4370, 1996 International Organisation for

Standardisation, Geneva, Switzerland). Gas chromatographic analysis of the TTO used in this study by Wollongbar Agricultural Institute, Wollongbar, Australia, showed the following proportions: terpinen-4-ol, 41.6%;  $\gamma$ -terpinene, 21.5%;  $\alpha$ -terpinene, 10.0%; terpinolene, 3.5%;  $\alpha$ -terpineol, 3.1%;  $\alpha$ -pinene, 2.4%; 1,8-cineole, 2.0%; *p*-cymene, 1.8%; aromadendrene, 1.1%;  $\delta$ -cadinene, 1.0%; limonene, 0.9%; ledene, 0.9%; globulol, 0.5%; sabinene, 0.4%; and viridiflorol, 0.2%. TTO was kept in 10 mL aliquots in brown glass bottles to minimize oxidation and discarded after 1 mo.

An unfractionated preparation of the water-soluble components of TTO was obtained as previously described (Hart *et al*, 2000; Brand *et al*, 2001). Briefly, TTO preparations of 1.25% (vol/vol) were prepared in polystyrene plastic tubes in serum-free culture medium, mixed well, and left to stand for 10 min. Gas chromatography/mass spectrometry confirmed that the water-insoluble components adhered to the side of the plastic tubes, whereas the water-soluble components remained in the culture medium (Hart *et al*, 2000). For individual study, terpinen-4-ol and  $\alpha$ -terpineol were obtained from Fluka (Buchs, Switzerland) and 1,8-cineole from Sigma Chemical Co. (St Louis, Missouri).

**Animals** Outbred male Sprague–Dawley rats (3 mo of age) were used. Anesthesia was induced with sodium pentobarbitone (60 mg per kg, i.p.) and maintained by supplementary injections (15 mg per kg, when needed). All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia.

**Blister induction and substance P perfusion** A blister of 0.25 cm<sup>2</sup> was induced on the hind footpad of the anesthetized rat by applying a vacuum pressure of  $-40$  kPa to the glabrous skin for approximately 30 min, using a metal suction cap heated to 40°C by an attached heating element (Khalil and Helme, 1989; Khalil *et al*, 1994; Khalil, 1999). When a blister was established, the surface epithelium was removed and a perspex chamber with inlet and outlet ports was fixed over the blister base. Perfusion of the drugs over the blister was maintained at 4 mL per h by a peristaltic pump (Microperpex S, LKB, Bromma, Sweden). Both perfusion temperature and body temperature were kept at 37°C. The experimental protocol consisted of an initial 20 min equilibration with Ringer's solution to establish a stable baseline. Sodium nitroprusside, a direct smooth muscle vasodilator, was perfused at 100  $\mu$ M for 10 min. The latter is used to control for the variability in smooth muscle reactivity between rats. This was followed by perfusion of Ringer's solution to re-stabilize the baseline. An unfractionated preparation of the water-soluble components of TTO, or purified preparations of 1,8-cineole,  $\alpha$ -terpineol, or terpinen-4-ol, was prepared in Ringer's solution, then perfused at concentrations of 0.125%, 0.0025% (equivalent to 2% of 0.125%), 0.0038% (equivalent to 3% of 0.125%), and 0.053% (equivalent to 42% of 0.125%), respectively. Concentrations of terpinen-4-ol of 0.1% and 0.2% were also tested, as was L-NAME (100  $\mu$ M in Ringer's solution, Sigma) with or without terpinen-4-ol. The compounds were perfused for 10 min prior to and for 30 min together with substance P (1  $\mu$ M). Finally, there was a 20 min post-stimulation period with perfusion of Ringer's solution.

**Antidromic stimulation of sciatic nerve** Immediately after blister induction but prior to removal of the epidermis, the hair was trimmed at the right mid thigh region and a small incision was made in the skin (Khalil *et al*, 2001). The sciatic nerve was carefully exposed using blunt-end dissection. It was then cleared and mobilized from the surrounding connective tissue, and cut as proximally as possible. The distal portion of the cut nerve was placed over bipolar platinum electrodes and immersed in a paraffin oil pool formed using the skin flaps of the wound. The paraffin oil was preheated to 37°C. The electrodes were fixed in such a position that electrical leakage to adjacent nerve structures was minimized. The blister and the perfusions were as described above, except that the TTO compounds were perfused for 10 min prior to, during and for

20 min subsequent to electrical stimulation whereby the distal portion of the sciatic nerve was stimulated with a Grass S48 stimulator (Grass Instrument Company, Quinay, Massachusetts) using parameters of 20 V, 5 Hz, and 2 ms square waves, for 1 min duration.

**Sensory neuropeptide depletion in rats** Neonatal rats were pre-treated on the second day of life with a single subcutaneous injection of 50 mg per kg capsaicin. Blisters were induced and peptides perfused in these rats at the age of 3 mo. Efficacy of this treatment to permanently destroy the majority of sensory nerve fibers was confirmed as previously described (Khalil *et al*, 1994; Khalil, 1999).

**Measurement of cutaneous blood flow** A laser Doppler flowmeter probe (Periflux, PF2B, Perimed, Sweden) was positioned vertically over the exposed blister in the hind paw via the perspex chamber (Khalil *et al*, 2000). The flux output of the laser Doppler monitor is a function of the concentration and the velocity of the red blood cells moving in the tissue penetrated by the laser light. The changes in relative blood flow (as determined by changes in red cell flux) were continuously displayed on a chart recorder. Raw data were evaluated by calculating the area under the response curve (cm<sup>2</sup>). All measurements were made relative to a stable baseline obtained prior to drug perfusion. The baselines did not differ between control and capsaicin-pre-treated rats or control and any other acute treatment groups.

**Measurement of plasma extravasation** The perfusate was collected during the 30 min perfusion with substance P in three 10 min intervals (SP1, SP2, SP3) and during two 10 min intervals post substance P perfusion (R1, R2). Plasma extravasation was determined by assaying the protein content of the perfusate using the Bradford method (Bradford, 1976).

**Participants in human studies** Forty healthy people in total (some on more than one occasion) were tested for the regulatory effects of TTO ( $n = 18$ ), terpinen-4-ol ( $n = 10$ ), 1,8-cineole ( $n = 10$ ), or  $\alpha$ -terpineol ( $n = 10$ ). Seventy percent (28 of 40) had previously used a TTO product on their skin. Participants had no severe generalized skin conditions such as eczema or psoriasis, atopy (eczema, hayfever, or asthma), or previous skin or systemic sensitivity to TTO and had no severe allergic reactions in the past. The participants were not on systemic immunosuppressant therapy and had not taken oral anti-histamines or topical corticosteroids in the preceding 2 wk. This study was approved by the Clinical Investigation Committee of Flinders Medical Centre, Adelaide, Australia.

**Induction of wheal and flare** Histamine (50  $\mu$ L of 100  $\mu$ g per mL solution (330  $\mu$ M)) was injected intradermally into the inner forearm skin (approximately midway along the volar aspect) of both arms and the resulting wheal and flare measured at 10 min intervals for 60 min (Koh *et al*, 2002). After 10 min and again after 20 min, 25  $\mu$ L of undiluted TTO, terpinen-4-ol,  $\alpha$ -terpineol, or 1,8-cineole were applied topically with a pipette to cover the flare and wheal on the experimental arm. Study arms (TTO or components) and control arms were assigned in an alternating fashion from subject to subject. In this way, each subject acted as his or her own control. Wheal and flare diameters (cm) were measured with calipers (Mitutoyo Corp., Tokyo, Japan). Wheal skin double thickness (mm) was measured by lightly pinching the skin and measuring with a spring-loaded gauge (Mitutoyo). Flare area (cm<sup>2</sup>) and wheal volume ( $\mu$ L) were calculated as previously described (Marshman *et al*, 1996; Koh *et al*, 2002).

**Expression of data and statistical analysis** Vasodilator responses in rat skin were measured as the area under the response curve (cm<sup>2</sup>) using a digital planimeter (Tamaya, Ohta-Ku, Japan). Results are expressed as mean  $\pm$  SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls *post hoc* test. Sodium nitroprusside

responses were used as a covariate in the analysis. For comparison of wheal and flare responses, the readings for control and test arms for a single time point were compared by a paired Student's *t* test. Significance was set at  $p < 0.05$ .

We are grateful to Novasel Australia Pty Ltd for support of this project and to all the subjects who volunteered to participate. Dr A. Pearce was supported by a scholarship from the Flinders Foundation, Adelaide, Australia.

DOI: 10.1111/j.0022-202X.2004.23407.x

Manuscript received February 11, 2004; revised April 23, 2004; accepted for publication May 16, 2004

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