

The water-soluble components of the essential oil of *Melaleuca alternifolia* (tea tree oil) suppress the production of superoxide by human monocytes, but not neutrophils, activated in vitro

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Abstract. *Objective:* To evaluate the regulatory properties of the essential oil of *Melaleuca alternifolia* (tea tree oil) on the production of oxygen derived reactive species by human peripheral blood leukocytes activated in vitro.

Materials and methods: The ability of tea tree oil to reduce superoxide production by neutrophils and monocytes stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA) was examined.

Results: The water-soluble fraction of tea tree oil had no significant effect on agonist-stimulated superoxide production by neutrophils, but significantly and dose-dependently suppressed agonist-stimulated superoxide production by monocytes. This suppression was not due to cell death. Chemical analysis identified the water-soluble components to be terpinen-4-ol, α -terpineol and 1,8-cineole. When examined individually, terpinen-4-ol significantly suppressed fMLP- and LPS- but not PMA-stimulated superoxide production; α -terpineol significantly suppressed fMLP-, LPS- and PMA-stimulated superoxide production; 1,8-cineole was without effect.

Conclusion: Tea tree oil components suppress the production of superoxide by monocytes, but not neutrophils, suggesting the potential for selective regulation of cell types by these components during inflammation.

Key words: Tea tree oil – Monocytes – Neutrophils – Oxygen derived reactive species – Superoxide production

Introduction

Tea tree oil (TTO) is the essential oil steam distilled from the Australian native plant, *Melaleuca alternifolia*. It is a com-

plex mixture of approximately 100 terpenes and hydrocarbons, the main component being terpinen-4-ol which comprises at least 30% of the oil. Besides anecdotal evidence for the anti-inflammatory properties of TTO, components of the oil have been demonstrated to show anti-inflammatory activity in experimental inflammation in animals. For example, in a carageenan-induced hind paw oedema model in rats, terpinen-4-ol had anti-inflammatory activity when applied topically in mg amounts [1]. In the same model, α -terpineol (a minor component of TTO comprising approximately 3% of the oil) was anti-inflammatory when administered subcutaneously as a 7.5% mixture with linalool [2]. However, the mechanisms of the anti-inflammatory effects of TTO remain undefined.

The inflammatory reaction involves a network of mediators which signal a variety of cell types, including lymphocytes, macrophages and neutrophils, to release products important in the killing of micro-organisms but which also cause tissue damage and pathology. Recently, this laboratory showed that the water-soluble TTO component, terpinen-4-ol, suppressed the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF) in cultured human monocytes stimulated with lipopolysaccharide (LPS) [3].

Oxygen derived reactive species (ODRS) such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radical, as well as hypochlorous acid and various chloramines [4], are formed when macrophages and neutrophils are stimulated and components of the NADPH oxidase are assembled on the plasma membrane of the phagocytic cell. Activation of the oxidase promotes the reduction of oxygen in the extracellular milieu. These ODRS play an important role in immunological host defence, providing anti-microbial, anti-viral and anti-tumour activity, as well as being involved in apoptosis and cell survival [4, 5]. There is evidence that they also have a role in the action and secretion of some cytokines, growth factors and hormones, and in the regulation of intracellular signalling pathways [4, 5]. However, increased levels

of ODRS (such as those generated during chronic and acute inflammatory diseases) are cytotoxic and may cause tissue damage through lipid peroxidation, oxidation of amino acid side chains, protein cross-linking and fragmentation, and DNA damage [6–8].

To further define the mechanisms of the anti-inflammatory properties of TTO, we examined the effects of this oil on the production of ODRS (superoxide) in monocytes and neutrophils stimulated *in vitro*. The results show that the water-soluble components of TTO, specifically terpinen-4-ol and α -terpineol, significantly suppress agonist stimulated-superoxide production by monocytes, but not by neutrophils, suggesting that TTO may selectively regulate monocyte function during an inflammatory response.

Materials and methods

Tea tree oil and its major components

Tea tree oil (batch 97/03) was kindly provided by Australian Plantations (Wyrallah, NSW, Australia) and fulfilled the criteria of the Australian Standard [9] with a terpinen-4-ol level of 41.6% and 1,8-cineole level of 2.0% as determined by gas chromatography-mass spectrometry (GC-MS). For individual study, terpinen-4-ol and α -terpineol were obtained from Fluka, Buchs, Switzerland and 1,8-cineole from Sigma Chemical Co., St Louis, MO.

Neutrophil isolation

Neutrophils were prepared from blood of healthy volunteers by a rapid single-step method [10]. Briefly, freshly collected whole heparinized blood was layered onto hypaque-ficoll medium of density 1.114 g/ml and centrifuged at $400 \times g$ for 30 min. With centrifugation, the leucocytes resolved into 2 bands allowing distinction between mononuclear cells and neutrophils. The neutrophils were harvested from the lower band and washed in Medium 199 (Gibco, New York, NY) to yield a final purity of > 96% and viability of > 99% as determined by exclusion of trypan blue dye (0.1%) and used immediately for assessment of superoxide production.

Monocyte isolation and culture

Human monocytes were isolated from peripheral blood as published [11, 12] to > 93% purity by countercurrent centrifugal elutriation and cultured in RPMI 1640 medium (Cytosystems, Castle Hill, Australia) supplemented with 13.3 mM NaHCO₃, 2 mM glutamine, 50 μ M β -mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM 3-(N-morpholino)propanesulphonic acid with an osmolality of 290 mmol/kg ('complete RPMI'). During isolation and subsequent culture of all cells, extreme care was taken to limit LPS contamination of buffers and culture fluids [11, 12]. Freshly isolated monocytes (10^6 cells/ml) were cultured overnight (16 h) in complete RPMI supplemented with 5% heat inactivated (56 °C for 30 min) fetal calf serum (FCS) at 37 °C in 5% CO₂ in 40 ml Teflon pots (Savillex, Minnetonka, MN) prior to assessment of superoxide production.

Chemiluminescence and superoxide production

Superoxide production was measured as chemiluminescence (CL) resulting from the oxidation of the fluorescent probe lucigenin (bis-*N*-methylacridinium nitrate). This assay provided a direct and specific measure of superoxide production and could be inhibited totally by superoxide dismutase [13].

Neutrophils (10^6 in 100 μ l of Hanks balanced salt solution (HBSS) supplemented with 1% heat inactivated autologous serum (AS) or FCS, pH 7.3) or non-adherent monocytes (10^6 in 100 μ l of HBSS supplemented with 1% heat inactivated FCS, pH 7.3) were transferred into luminometer tubes and pre-incubated for 30 min at 37 °C in 5% CO₂ with TTO, its water-soluble components or HBSS before addition of agonist (5×10^{-5} M *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), 500 ng/ml lipopolysaccharide from *Escherichia coli* K-235 (LPS) or 10^{-6} M phorbol 12-myristate 13-acetate (PMA)) and HBSS for a total volume of 500 μ l. Lucigenin (500 μ l, 127.5 μ g/ml) was then added. fMLP, LPS, PMA and lucigenin were obtained from Sigma. The resulting CL was monitored in a water-jacketed (37 °C) luminometer chamber (Model 1250, LKB, Wallac, Turku, Finland) and recorded in millivolts (mV) as the peak initial rate of superoxide production. For fMLP this usually occurred within 1 to 2 minutes of agonist addition producing a response of intermediate size, for LPS within 60 minutes producing the weakest response and for PMA within 15 to 20 minutes producing the strongest response.

Measurement of toxicity of tea tree oil and its water-soluble components

The viability of neutrophils or monocytes incubated with TTO or its water-soluble components was determined by exclusion of trypan blue dye (0.1%) after recording the CL response. Total assay time ranged from 30 min for measurement of fMLP-stimulated superoxide production to 1.5 h for measurement of LPS-stimulated superoxide production.

Measurement of cellular uptake of water-soluble TTO components by neutrophils and monocytes

Neutrophils or monocytes (10^7 in 900 l HBSS supplemented with 1% heat inactivated FCS) were transferred into luminometer tubes and incubated for 1 h at 37 °C in 5% CO₂ with 100 μ l of the water-soluble fraction of TTO (0.125% v/v). HBSS supplemented with 1% heat inactivated FCS was incubated with or without TTO under identical conditions. The cultures were centrifuged at $400 \times g$ for 5 minutes and the supernatants harvested. A 1 μ l aliquot of each supernatant was injected into a Varian Saturn 4D gas chromatograph-mass spectrometer with a J & W DB5MS capillary column (5% phenylmethylpolysiloxane 30 m \times 0.25 mm id), oven 80 °C isothermal, injector 200 °C. All peaks were unambiguously characterised by a mass-spectral library matching service, and the readings for terpinen-4-ol, α -terpineol and 1,8-cineole present in the supernatants integrated to give corresponding peak areas. These have been converted to a percentage of total product (%).

Expression of results and statistical analysis

Measurement of superoxide production was performed on duplicate or triplicate samples and results were normalised to the agonist-stimulated level in the absence of TTO at 100%. The mean values from each set of replicates were used to determine the mean \pm SEM for *n* donors. For responses by cell populations from a number of different donors, a multiple comparison procedure employing a one-way analysis of variance and Fisher's test was used to determine the statistical significance of differences between experimental and control groups. Probabilities less than 0.05 were considered significant.

Results

Preparation of tea tree oil

To investigate the effect of TTO on superoxide production by neutrophils and monocytes, it was necessary to prepare emulsions in HBSS, as dissolving in propylene glycol, di-

Table 1. Separation of major TTO components under different conditions.

Component	Concentration in TTO (%)	Glass + Sonication 10% FCS		Plastic 0% FCS	
		Aqueous (%)	Oil (%)	Aqueous (%)	Oil (%)
1. α -pinene	2.4	0.2	2.8	–	2.8
2. sabinene	0.4	–	0.9	–	0.9
3. α -terpinene	10.0	0.8	9.1	–	9.3
4. limonene	0.9	–	1.1	–	1.0
5. p-cymene	1.8	0.7	6.1	–	6.8
6. 1,8-cineole	2.0	2.8	1.7	3.3	1.8
7. γ -terpinene	21.5	2.3	23.0	–	21.0
8. terpinolene	3.5	–	3.8	–	3.5
9. terpinen-4-ol	41.6	80.4	34.3	83.8	37.0
10. α -terpineol	3.1	6.3	2.7	6.5	2.9
11. aromadendrene	1.1	–	1.4	–	1.3
12. ledene	0.9	–	1.2	–	1.1
13. δ -cadinene	1.0	–	1.2	–	1.1
14. globulol	0.5	–	0.5	–	0.5
15. viridiflorol	0.2	–	0.4	–	0.4

methylsulfoxide or ethanol followed by dilution to a non-toxic concentration resulted in separation of TTO from its diluent. TTO was either emulsified into HBSS containing 10% FCS by sonication in a glass tube or prepared in serum-free buffer in a polystyrene plastic tube followed immediately by vortexing for 1 minute (designated water-soluble TTO fraction). The latter allowed TTO to be separated broadly into its water-soluble and hydrophobic components (Table 1: aqueous and oil, respectively). GC-MS analysis of TTO components that had partitioned into the serum-free buffer when diluted in plastic tubes showed they were identical to those in the aqueous phase when 500 mg TTO was rapidly mixed with 50 ml water in a glass vessel. These were identified to be terpinen-4-ol, α -terpineol and 1,8-cineole. The solubility of TTO when mixed in water was determined to be 1.6 g/l or 0.16% v/v [3].

Effect of TTO on superoxide production by neutrophils

TTO emulsified by sonication in HBSS containing serum was toxic for neutrophils at concentrations above 0.016% v/v. This concentration of TTO significantly induced the basal and fMLP-stimulated CL response but was without significant effect at half this concentration of TTO (Fig. 1). For LPS- and PMA-stimulated CL responses, no significant effect of TTO at these concentrations (0.016% and 0.008%) was detected (data not shown).

Effect of the water-soluble TTO fraction on superoxide production by neutrophils

The effect of the water-soluble TTO fraction at concentrations of 0.1% v/v and 0.05% v/v on superoxide production by neutrophils in 1% AS in the absence and presence of agonist (fMLP, LPS and PMA) was investigated. There was no significant effect of the water-soluble TTO components on responses measured (Fig. 2). At a concentration of TTO of

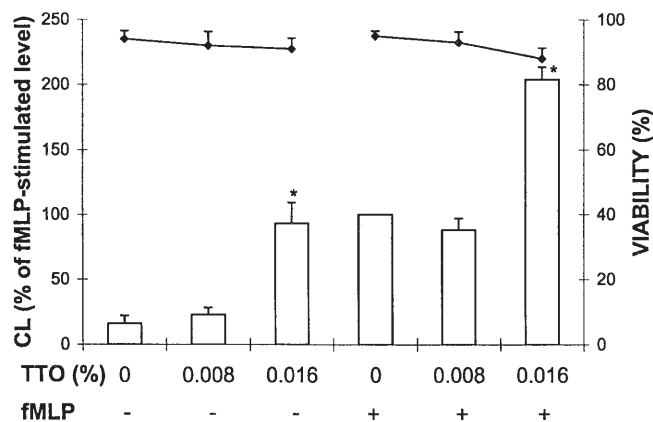


Fig. 1. The effect on superoxide production by neutrophils of TTO emulsified into buffer containing 10% FCS by sonication in glass tubes (sum of oil and water-soluble fractions). Neutrophils from 3 donors were incubated for 30 min with increasing amounts of TTO prior to stimulation with fMLP (5×10^{-7} M). Regulation of lucigenin-dependent superoxide production is shown by histograms (left axis; CL); the fMLP-stimulated superoxide level was normalised to 100% and the mean result from each experiment used to calculate the mean \pm SEM. The mean percentage \pm SEM of viable neutrophils is shown by the line (right axis). An asterisk indicates a significant enhancement in superoxide production compared with the absence of TTO.

0.25% v/v, any decrease was associated with significant toxicity (data not shown). Addition of FCS instead of AS did not change the inability of the water-soluble TTO fraction to suppress fMLP-stimulated superoxide production by neutrophils (data not shown).

Effect of TTO on superoxide production by monocytes

As for neutrophils, TTO at concentrations greater than 0.016% was toxic for monocytes. However, at non-toxic levels tested (less than, or equal to 0.016%), it significantly induced superoxide production by unstimulated monocytes.

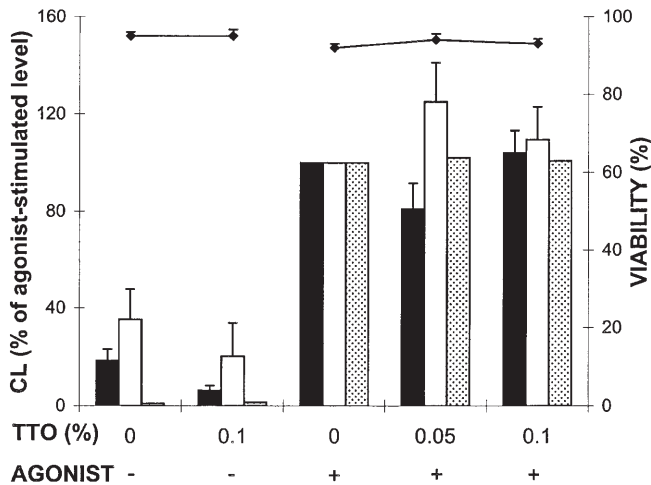


Fig. 2. The effect on superoxide production by neutrophils of TTO prepared in serum-free buffer in polystyrene plastic tubes (water-soluble fraction). Neutrophils were incubated for 30 min with increasing amounts of TTO prior to stimulation with fMLP (5×10^{-7} M, $n=8$), LPS (100 ng/ml, $n=3$) or PMA (10^{-8} M, $n=1$). Regulation of lucigenin-dependent superoxide production is shown by histograms (left axis, CL; fMLP, solid bars; LPS, open bars; PMA, hatched bars); the agonist-stimulated superoxide level was normalised to 100% and the mean result from each experiment used to calculate the mean \pm SEM. The mean percentage \pm SEM of viable neutrophils is shown by the solid line (reference: right axis).

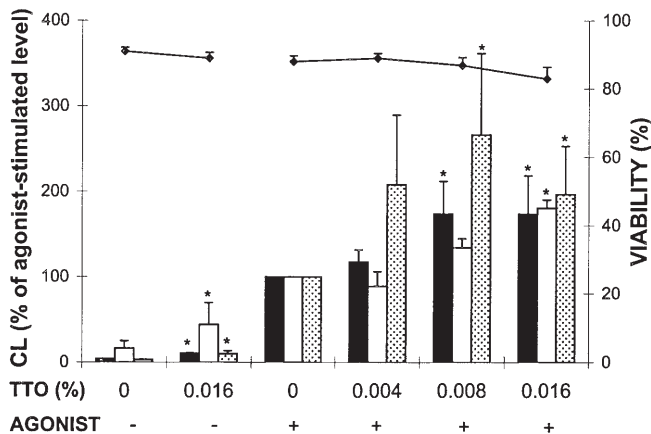


Fig. 3. The effect on superoxide production by monocytes of TTO emulsified into buffer containing 10% FCS by sonication in glass tubes (sum of oil and water-soluble fractions). Monocytes were incubated for 30 min with increasing amounts of TTO prior to stimulation with fMLP (5×10^{-7} M, $n=3$), LPS (100 ng/ml, $n=3$) or PMA (10^{-8} M, $n=3$). Regulation of lucigenin-dependent superoxide production is shown by histograms (left axis, CL; fMLP, solid bars; LPS, open bars; PMA, hatched bars). The mean percentage \pm SEM of viable monocytes is shown by the line (right axis). An asterisk indicates a significant enhancement in superoxide production.

Pooled results from 3 donors indicated that TTO significantly induced fMLP-stimulated superoxide production at a concentration greater than, or equal to 0.008%; significantly induced LPS-stimulated superoxide production at a concentration of 0.016%; and significantly induced PMA-stimulated superoxide production at a concentration greater than, or equal to 0.008% (Fig. 3).

Effect of the water-soluble TTO fraction on superoxide production by monocytes

The effect of the water-soluble TTO fraction at concentrations ranging from 0.125% v/v to 0.008% v/v on monocyte superoxide production was investigated. Monocyte viability assessed by trypan blue exclusion was unaffected by the water-soluble TTO fraction at any of the concentrations investigated (Fig. 4). In the absence of agonist (fMLP, LPS or PMA), superoxide production was significantly suppressed at a concentration of TTO of 0.125%. Significant and dose-dependent suppression of superoxide production was seen at a concentration greater than, or equal to 0.031% by fMLP- and LPS-stimulated monocytes from 5 and 4 donors respectively. PMA-stimulated superoxide production was suppressed at a concentration greater than, or equal to 0.008% from 4 donors (Fig. 4). This suppressive effect was very potent and was removed, for cells from one donor, when TTO was diluted approximately one million-fold (data not shown).

Effects of terpinen-4-ol, α -terpineol and 1,8-cineole on superoxide production by monocytes

The water-soluble components of TTO, terpinen-4-ol, α -terpineol and 1,8-cineole (42, 3 and 2% of TTO respectively), were initially examined at concentrations equivalent to those found in 0.125% v/v, 0.031% v/v and 0.008% v/v TTO. Dilutions were sonicated in glass tubes and serum-free buffer to maximise the amount of terpinen-4-ol, α -terpineol and 1,8-cineole that remained in the aqueous phase. No effect was seen on superoxide production by unstimulated monocytes for any component, and viability remained unaffected.

Terpinen-4-ol at a concentration greater than, or equal to 0.013% (equivalent to 0.031% TTO) significantly and dose-dependently suppressed superoxide production by fMLP-

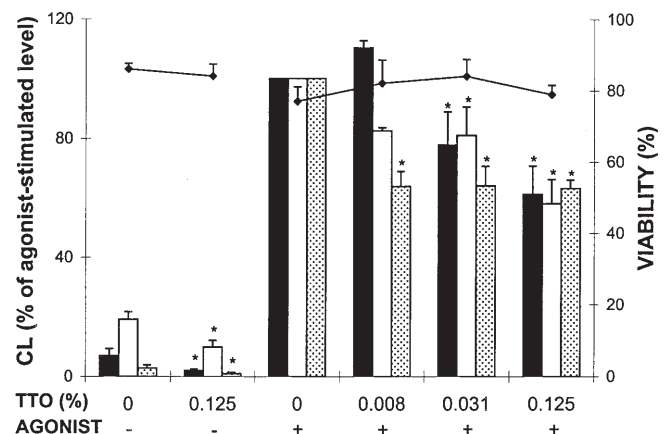


Fig. 4. The effect on superoxide production by monocytes of TTO prepared in serum-free buffer in polystyrene plastic tubes (water-soluble fraction). Monocytes were incubated for 30 min with increasing concentrations of TTO prior to stimulation with fMLP (5×10^{-7} M, $n=5$), LPS (100 ng/ml, $n=4$) or PMA (10^{-8} M, $n=4$). Regulation of lucigenin-dependent superoxide production is shown by histograms (left axis, CL; fMLP, solid bars; LPS, open bars; PMA, hatched bars). The mean percentage \pm SEM of viable monocytes is shown by the line (right axis). An asterisk indicates a significant reduction in superoxide production.

stimulated (n=5) and LPS-stimulated (n=4) monocytes. No effect was seen on PMA-stimulated superoxide production by monocytes from 4 donors (Fig. 5A).

α -Terpineol significantly and dose-dependently suppressed fMLP- and LPS-stimulated superoxide production by monocytes from 3 donors at a concentration greater than, or equal to 0.001% (equivalent to 0.031% TTO) and PMA-stimulated superoxide production by monocytes from 5 donors at a concentration greater than, or equal to 0.00025% (equivalent to 0.008% TTO) (Fig. 5B). Although for the aggregated data

the suppression of superoxide production by monocytes stimulated with PMA appeared to be dose-responsive, 2 distinct patterns were observed. Inhibition was experienced by all donors. However only some responded in a concentration dependent fashion, while the remainder failed to dose respond in a manner similar to that seen with the water-soluble TTO fraction.

There was no significant effect of 1,8-cineole on superoxide production by monocytes stimulated with fMLP, LPS or PMA (Fig. 5C).

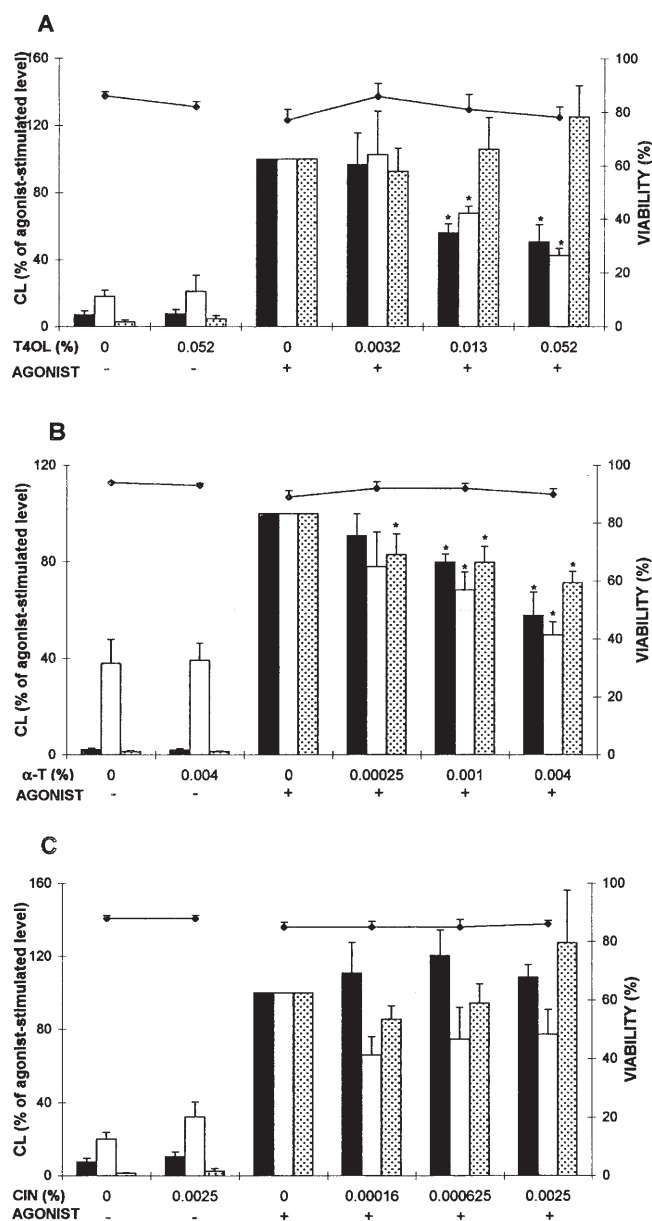


Fig. 5. The effects of terpinen-4-ol (A), α -terpineol (B) and 1,8-cineole (C) prepared in serum-free buffer in glass tubes on superoxide production by monocytes. Monocytes were incubated for 30 min with increasing amounts of each component prior to stimulation with fMLP (5×10^{-7} M), LPS (100 ng/ml) or PMA (10^{-8} M). Regulation of lucigenin-dependent superoxide production is shown by histograms (left axis, CL; fMLP, solid bars; LPS, open bars; PMA, hatched bars). The mean percentage \pm SEM of viable monocytes is shown by the line (right axis). An asterisk indicates a significant reduction in superoxide production.

Uptake of terpinen-4-ol, α -terpineol and 1,8-cineole by neutrophils and monocytes

GC-MS analysis showed no significant differential uptake of any one component by monocytes compared with neutrophils following incubation for 1 h at 37 °C (Table 2).

Discussion

Separation of TTO into its hydrophobic and water-soluble components was encouraged by dilution of TTO in polystyrene plastic tubes with serum-free buffer. Using this method, the hydrophobic TTO components adsorbed to the plastic wall of the tube, while the water-soluble TTO components remained in the aqueous phase. This was confirmed by analysis based on GC-MS where the water-soluble TTO fraction was shown to contain terpinen-4-ol (84%), α -terpineol (7%) and 1,8-cineole (3%) (Table 1).

When examined on monocytes at concentrations equivalent to those found in TTO, terpinen-4-ol suppressed superoxide production by monocytes stimulated with fMLP and LPS but not with PMA (data summarized in Table 3). Since fMLP and LPS bind to surface receptors and act intracellularly on protein kinase C (PKC), terpinen-4-ol probably acts on signals upstream from PKC, but not at the surface receptor level because the water-soluble components of TTO did not modulate CD14 levels on monocytes during the 30 min incubation prior to LPS addition (flow cytometry data not shown).

Previously, we demonstrated that terpinen-4-ol also suppressed TNF α , IL-1 β , IL-8, IL-10 and PGE $_2$ production by human peripheral blood monocytes stimulated in vitro with LPS [3]. Thus, it is evident that this component of TTO affects the production of two major products of mononuclear phagocytes, pro-inflammatory cytokines and ODRS. These mediators have been established as important contributors to

Table 2. Water-soluble components of TTO remaining in 1 ml medium after incubation with cells (10^7) and TTO (0.125% v/v, prepared in plastic tubes) for 1 hour.

Incubation with	Terpinen-4-ol (mean \pm SD, %) ^a	α -Terpineol (mean \pm SD, %)	1,8-Cineole (mean \pm SD, %)
No cells	91.1 \pm 2.0	6.8 \pm 1.8	2.1 \pm 0.4
Monocytes	91.1 \pm 0.6	6.8 \pm 0.4	2.1 \pm 0.3
Neutrophils	90.2 \pm 0.5	7.7 \pm 0.6	2.1 \pm 0.2

^a = triplicate cultures.

Table 3. Effect of the water-soluble components of TTO on superoxide production by agonist-stimulated monocytes.

	Water-soluble TTO fraction (0.052% v/v)	Terpinen-4-ol (0.004% v/v)	α -Terpineol (0.125% v/v)	1,8-Cineole (0.0025% v/v)	
fMLP+	39% ↓ (5)	50% ↓ (5)	42% ↓ (3)	–	(5)
LPS+	42% ↓ (4)	58% ↓ (4)	50% ↓ (3)	–	(4)
PMA+	37% ↓ (4)	–	29% ↓ (5)	–	(4)

– = no significant effect ($P > 0.05$).

(n) = number of donors.

the inflammatory reaction and the tissue damage which occurs in chronic inflammation [3, 4].

Interestingly, α -terpineol could suppress fMLP-, LPS- and PMA-stimulated superoxide production (data summarized in Table 3) but not LPS-stimulated inflammatory mediator production by monocytes [3]. This difference was further confirmed on cells from each of 2 donors whereby α -terpineol suppressed LPS-induced superoxide production but was without effect on LPS-induced TNF α production (data not shown). This suggested α -terpineol was a potent inhibitor of superoxide production, as it comprises only 3% (compared to 42% for terpinen-4-ol) of TTO but was able to produce a strong effect. Furthermore, α -terpineol regulated NADPH oxidase activity and subsequent production of ODRS by targeting PKC or downstream molecules. The ability of α -terpineol to inhibit LPS-induced superoxide production but not cytokine production suggests that these two monocyte functions are independently controlled and that the mechanism by which terpinen-4-ol inhibits monocyte function is different to that mediated by α -terpineol.

In contrast to the effects on monocytes, the water-soluble TTO components had no significant effect on agonist-stimulated superoxide production by neutrophils. This response could not be explained by differential cellular uptake of terpinen-4-ol, α -terpineol or 1,8-cineole by neutrophils and monocytes. The influence of the water-soluble TTO components on neutrophil and monocyte adherence to fibronectin-coated plates, another function shared by these two cell types, was examined. However, no effect of the water-soluble components of TTO was seen on either cell type (data not shown).

Several studies have suggested an anti-inflammatory activity of TTO and its components [1, 2]. However, TTO is also associated with some cytotoxicity to mammalian cells *in vitro* [14, 15]. It was reported that TTO at a concentration of 0.05% v/v caused greater than 85% suppression of superoxide production by neutrophils stimulated *in vitro* [16]. However in our studies, 0.05% TTO was very toxic, and it was necessary to dilute TTO to 0.016% v/v to avoid toxicity to neutrophils and monocytes during short term exposure. This concentration increased fMLP-stimulated superoxide production but as we have shown previously, was significantly toxic for monocytes after 20 h [3]. This suggested TTO contains components that can activate NADPH oxidase and therefore production of ODRS in phagocytic leukocytes, and enhance subsequent responses to other agonists, but may be responsible for the toxicity evident during longer exposure. However, it is likely that neutrophils and monocytes would

not be exposed to all TTO components *in vivo*. The *stratum corneum* may act as a selective barrier allowing penetration of the water-soluble components into the vascularised dermis but differentially retaining the hydrophobic components. The selective effect on monocyte but not neutrophil superoxide production implies TTO components do not scavenge oxygen radicals.

In our study 1,8-cineole was inactive, demonstrating no significant effect on cell viability or agonist-stimulated superoxide production at the concentrations tested (data summarized in Table 3). Despite contrary reports [17, 18], 1,8-cineole was also unable to regulate LPS-stimulated inflammatory mediator production by monocytes [3]. However, another study has demonstrated that 1,8-cineole possesses local irritant properties, inducing oedema following subplantar injection into the hind paw of rats and causing rat peritoneal mast cell degranulation and release of histamine and serotonin *in vitro* [19]. 1,8-Cineole and other components of TTO including limonene, terpinen-4-ol and α -terpineol [20–22], demonstrate percutaneous penetration enhancing properties. As such, they may increase penetration of potential anti-inflammatory components such as terpinen-4-ol and α -terpineol, beyond the *stratum corneum* into the vascularised dermis resulting in levels that would be biologically significant following topical application. Nevertheless, studies measuring the actual concentration of TTO components penetrating into the skin and their effect on inflammatory cells and mediators *in vivo* are required. Typical protocols for usage of TTO include application of a preparation of up to 100% to skin. In this study we used the highest concentration of TTO that was not toxic to mammalian cells *in vitro*. It represented approximately one thousandth of the recommended dosage and is a level less than the water-solubility of TTO (1.6 g/l).

The physiological relevance of this study is high as it implies TTO has potential as an anti-inflammatory agent. The results suggest TTO contains water-soluble components, specifically terpinen-4-ol and α -terpineol, that may selectively regulate cell function during inflammation, in particular monocyte activity, and following topical application may control inflammatory responses to foreign antigens in the skin. TTO may enable neutrophils to be fully active in an acute inflammatory response and eliminate foreign antigens, while suppressing monocyte superoxide production and thereby preventing oxidative tissue damage that may be seen in more chronic inflammatory states.

The anti-microbial activity of TTO is already well established [23–31]. If its potential anti-inflammatory properties (including prevention of oxidative tissue damage and inhibition of inflammatory mediator production) can be further elucidated, acceptability of TTO for treatment of acne, eczema, burns and periodontal disease will be increased.

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