Neutrophil Respiratory Burst

Influence of taurine and a substituted taurine on the respiratory burst pathway in the inflammatory response
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Introduction
Psoriasis is a common skin disease and provides a useful model to study the pathogenic significance of chemical mediators of inflammation both in vivo and in vitro. The histological features of this condition include increased epidermal cell proliferation and infiltration of neutrophils, macrophages and lymphocytes into the lesions. In fact, neutrophil infiltrates have been reported to be one of the earliest phenomena in pre-pinpoint lesions [1]. Elevated levels of leukotriene B4 (LTB4) have been reported in extracts of psoriatic skin lesions [2], suggesting a pathogenic role for this chemoattractant lipoxigenase product. On the other hand, prostaglandin E2 (PGE2) levels in psoriatic skin lesions do not differ significantly from clinically uninvolved skin [3] and are not believed to play a significant pro-inflammatory role in psoriasis. Support for a pathogenic role of LTB4 derives in part from studies demonstrating that benoxaprofen, a 5-lipoxygenase (E.C. 1.13.11.12) inhibitor, causes marked improvement in patients with severe psoriasis [4]. Conversely, specific cyclo-oxygenase (E.C. 1.14.99.1) inhibitors, such as indomethacin, may stimulate the lipoxigenase pathway by diversion of substrate and have been reported to exacerbate the condition [5]. The generation of all arachidonic acid-derived products is inhibited by corticosteroids, which are phospholipase A2 inhibitors. However, these drugs have undesirable side-effects which limit their use. Therefore, the development of non-toxic anti-psoriatic drugs would provide new therapeutic approaches to this disease.

Activation of oxidative metabolism in leucocytes leads to generation of reactive oxidants (RO) in a process designated the respiratory burst [6, 7]. Such production of RO is believed to play a role in the pathogenesis of inflammatory diseases by causing 'auto-oxidative damage' at the site of inflammation [8]. Hydrogen peroxide (H2O2) produced during the respiratory burst is detoxified by catalase or further metabolism to form hypochlorous acid (HOCl) by myeloperoxidase (MPO). Measurement of chemiluminescence allows quantification of RO production by leucocytes in response to a soluble [9] or particulate [10] challenge. It also provides a rapid and sensitive method for studying the effect on non-steroidal anti-inflammatory drugs (NSAID) on cell function [11]. Stimulation of the inflammatory process also leads to the production of leukotrienes [12, 13] including LTB4, which is one of the most potent natural inflammatory mediators known [14, 15]. It is understood that the MPO–H2O2–halide system plays an important role in the catabolism of active leukotrienes (C4, D4 and E4) to inactive chiral sulphones and diastereoisomers of 6-trans-LTB4 [16]. This effect has been attributed to the action of HOCl produced by leucocytes through the MPO–H2O2–halide system, on the active leukotrienes. LTB4 has also been demonstrated to be inactivated by this system, possibly by oxidative cleavage or halogenation [17]. Such inactivation of LTB4 would have a modulatory effect on the inflammatory response and hence the MPO–H2O2–halide system is believed to play an important role in the control of the inflammatory process [18–20].

N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (Hepes) is a substituted taurine derivative and is well known for its properties as a biological buffer [21]. We studied the effect of taurine and Hepes on neutrophil function, using luminol-amplified chemiluminescence in response to calcium ionophore (A23187). We also measured secreted LTB4 in the supernatants of A23187-stimulated neutrophils which were preincubated in the presence and absence of taurine or Hepes. Since we showed that neutrophil MPO activity was significantly enhanced in the presence of taurine or Hepes, we describe herein observations in peripheral neutrophils which indicate that taurine and Hepes possess anti-inflammatory properties in vitro, and a hypothesis of the mechanism of action is proposed.

Methods
Peripheral blood from normal subjects, patients with psoriasis and other skin diseases (acne, dermatitis herpetiformis, non-specific and atopic derma-
titis) were taken into sodium heparin vacutainers (Becton Dickinson, Rutherford, NJ, U.S.A.) and subjected to dextran sedimentation using Dextraven 150 (Fisons, Loughborough, U.K.), followed by Hypaque–Ficoll gradient centrifugation of the leucocyte-rich plasma (Ficoll-Paque, Pharmacia, Uppsala, Sweden). The neutrophils and residual erythrocytes sedimented to the base of the gradient. Erythrocyte contamination was removed from the neutrophil fraction by hypotonic lysis. Identification and counting of cells was done by fluorescence microscopy using ethidium bromide and Acridine Orange staining. The neutrophils were suspended in 0.9% (w/v) saline containing 1 mM-glucose. Freshly isolated cells were shown to have a viability of >98%. Unless otherwise stated, in all experiments the cells were preincubated with Dulbecco's (Gibco, Paisley, Scotland) phosphate-buffered saline containing 1 mM-glucose (PBS) at the appropriate pH (non-treated), 40 mM-taurine in PBS pH 8.0 (taurine-treated) or 10 mM-Hepes (Sigma, Poole, U.K.) in PBS pH 6.0 (Hepes-treated) for 1 min at 37°C. The pH was then adjusted to pH 7.3 by addition of incubation medium for each experiment.

**Chemiluminescence**

Chemiluminescence activity was measured using $5 \times 10^5$ cells in the presence and absence of taurine or Hepes, stimulated with 2 $\mu$m-A23187 in the presence of 0.1 mM-Luminol (Lumac, St Paul, MN, U.S.A.), in a total volume of 0.3 ml, and measured in a luminometer (Lumac M2010 Biocounter). Peak responses obtained after approximately 10 min incubation at ambient temperature are reported.

**Degranulation, LTB$_4$ and PGE$_2$**

Degranulation was measured by incubation of 0.5 ml of non-treated, taurine- or Hepes-treated cells at $5 \times 10^6$ cells/ml with 0.5 ml A23187 (2 $\mu$m) for 10 min at 37°C. The supernatants were assayed for $\beta$-glucuronidase (E.C. 3.2.1.3.1.) by the fluorimetric method of Peters et al. [22]. LTB$_4$ and PGE$_2$ levels in the supernatants of stimulated cells were determined by the radioimmunoassay procedures of Salmon et al. [23] and Bauminger et al. [24], respectively.

**DNA, MPO and HOCI**

Isolated neutrophils were sonicated and DNA measured by fluorimetry [22]. Sonicated samples were preincubated with either PBS, taurine or Hepes for 1 min at 37°C prior to assay of MPO and HOCI. MPO (E.C. 1.11.17) was assayed spectrophotometrically by the o-dianisidine method [25]. HOCI was determined by measuring the decrease in absorbance at 290 nm of monochlorodimedon (0.025 mM) using the method of Hager et al. [26].

**Chloramines**

Formation of taurine-chloramine and Hepes-chloramine was measured by the method of Weiss et al. [27]. The absorption spectra of taurine and hypochlorite (OCI$^-$) and a mixture of taurine and OCI$^-$ were determined in a total volume of 1.0 ml PBS, pH 7.3, using a Pye-Unicam SP6-450 spectrophotometer. To each was then added 10 $\mu$l of 1.26 M-H$_2$O$_2$, and the samples were then incubated for 5 min at ambient temperature before re-reading over the same range (230-350 nm).

**Statistical analysis**

Within each subject group, the effect of taurine or Hepes was assessed statistically using the Wilcoxon signed rank test. For inter-group analysis (normal versus psoriatics and normal versus disease control) the Mann-Whitney U-test for unpaired data was employed. P values <0.05 were considered to be significant.

**Results**

Table 1 shows that there was a significant reduction in chemiluminescence activity in normal, psoriatic and disease control neutrophils in the presence of taurine or Hepes compared with non-treated cells. There was no significant difference between non-treated, taurine- or Hepes-treated cells in each subject group. Fluorescence microscopy using ethidium bromide and Acridine Orange demonstrated no adverse effect on viability of cells by preincubation with taurine or Hepes.

Extracellular degranulation of the lysosomal enzyme, $\beta$-glucuronidase, was measured in the supernatants of A23187-stimulated neutrophils from each group. It was demonstrated that there was no significant difference in degranulation by neutrophils preincubated with Hepes compared to non-treated cells. In addition there was no significant difference in degranulation by normal compared with psoriatic cells and disease control cells. It was not possible to assess the effect of taurine on degranulation as it caused a concentration-dependent increase in enzyme activity, which was verified using purified $\beta$-glucuronidase (data not shown). This suggests that this enzyme is unsuitable as a marker of degranulation in studies using taurine.

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taurine or Hepes was observed. There was no significant difference between non-treated, taurine- or Hepes-treated cells of normal subjects compared to psoriatic or disease control subjects.

A significant increase in HOCl production was noted in each subject group in the Hepes-treated neutrophils. In addition, non-treated cells produced greater amounts of HOCl in the psoriatic and disease control groups compared with normal subjects. However, there was no significant difference between the Hepes-treated cells in each subject group. In the presence of taurine there was no observable HOCl production. Further experiments demonstrated no measurable HOCl when hypochlorite was incubated with excess taurine (data not shown). It was concluded that hypochlorite was rapidly sequestered by taurine to form taurine-chloramine.

We showed a significant reduction of LTB₄ levels secreted from taurine- or Hepes-treated cells in each subject group compared with controls in the supernatants of A23187-stimulated cells. There was no significant difference in secreted LTB₄ from non-treated normal, psoriatic or disease control cells. In the Hepes-treated samples, while there was no significant difference in LTB₄ levels by normal and psoriatic cells, a significant difference was observed between normal and disease control samples.

We demonstrated no significant difference in secreted PGE₂ levels in the supernatants of A23187-stimulated cells from all subjects treated with taurine or Hepes compared with non-treated cells.

We compared the absorption spectrum of taurine-chloramine to that obtained by addition of Hepes to HOCl (Fig. 1). Fig. 1(a) shows that there was no significant absorption of taurine over the range measured, whereas OCI⁻ showed an absorption at 290–300 nm. A mixture of taurine and OCI⁻ showed an absorption at 250–260 nm. This peak was also demonstrated in studies by Weiss et al. [27] and was attributed to the formation of taurine-chloramine. The addition of H₂O₂ reduced OCI⁻ with no effect on taurine-chloramine. Fig. 1(b) demonstrates no observable absorption of Hepes, whereas there was a significant absorption peak at 250–260 nm in the presence of OCI⁻, which was also unaffected by the addition of H₂O₂.

Discussion

In this study, it was observed that there was a significant reduction in chemiluminescence activity in taurine- or Hepes-treated neutrophils from normal subjects, and patients with psoriasis and other skin diseases compared with non-treated cells (Table 1). Thus, a 1 min preincubation at 37°C of non-treated, taurine- or Hepes-treated cells followed by assay of chemiluminescence resulted in a significantly reduced response, suggesting that there was a taurine- or Hepes-dependent reduction of chemiluminescence in all subjects tested.

Stimulation of neutrophils by A23187 not only activates the respiratory burst, but is also associated with release of granular enzymes from the cells [28]. Our data showing no significant effect of Hepes on degranulation of β-glucuronidase suggested that Hepes did not act as an inhibitor of neutrophil function. Thus, the reduction in chemiluminescence observed in the presence of Hepes was unlikely to have been due to inhibition of the inflammatory response. It was also observed that taurine caused a concentration-dependent increase in β-glucuronidase activity which was confirmed.

Fig. 1

Absorption spectra of OCI⁻, taurine, Hepes and their chloramine derivatives

(a) (- - -) 4 mM-taurine; (- - -) 5 mM-OCI⁻; (--- - -) 4 mM-taurine/5 mM-OCI⁻/12.6 mM-H₂O₂. (b) (----) 4 mM-Hepes; (-----) 5 mM-OCI⁻; (---- - -) 4 mM-Hepes/5 mM-OCI⁻; (-----) 4 mM-Hepes/5 mM-OCI⁻/12.6 mM-H₂O₂.
Table I

<table>
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<th>Chemiluminescence (Counts/5 x 10⁶ cells)</th>
<th>β-Glucuronidase (Units/10⁶ cells)</th>
<th>MPO (Units/μg DNA)</th>
<th>HOCI (nmol/5 x 10⁶ cells)</th>
<th>LTB₄ (ng/10⁶ cells)</th>
<th>PGE₂ (ng/10⁶ cells)</th>
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<td>Normal</td>
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<tr>
<td>(b)</td>
<td>4045 ± 941†</td>
<td>10.2 ± 1.0</td>
<td>9.42 ± 1.13‡</td>
<td>82.53 ± 6.82‡</td>
<td>1.72 ± 0.33‡</td>
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<td>(b)</td>
<td>4827 ± 924†</td>
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<td>(b)</td>
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<td>3.58 ± 0.40*</td>
<td>0.67 ± 0.08</td>
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<td>5702 ± 1609†</td>
<td>(12)</td>
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Results expressed as activity of non-treated neutrophils (a) and taurine- or Hepes-treated neutrophils (b) for each subject group. Mean ± S.E.M. of averages of duplicate determinations are shown; number of subjects in parentheses. Statistical significance: (Wilcoxon signed rank test), *P < 0.05, †P < 0.005, ‡P < 0.001 compared with controls; (Mann–Whitney U-test), §P < 0.05 compared with normal subjects. Disease controls: acne, dermatitis herpetiformis, non-specific and atopic dermatitis.

using purified β-glucuronidase (data not shown). Therefore, degranulation cannot be studied in the presence of taurine using β-glucuronidase as a marker enzyme.

MPO converts H₂O₂ to HOCl via a two-step pathway [29]. The first step is the reaction of MPO with H₂O₂, where native MPO is converted to Compound I, and the haem group of the enzyme now contains two oxidizing equivalents more than the native enzyme (Fig. 2). The unstable Compound I can be reconverted into the native form via a single two-electron reduction step with the release of HOCl. An inactive intermediate (Compound II) is formed in the presence of excess H₂O₂ during reconversion, containing one oxidizing equivalent more than the active enzyme. Compound III is an oxyperoxidase containing three oxidizing equivalents above the native enzyme, and can be formed by reaction of Compound II with H₂O₂, by aerobic oxidation of NADH, by reaction of ferrous MPO with oxygen or by reaction of ferric MPO with superoxide.

Our studies demonstrated that the activity of MPO was significantly increased in those samples containing taurine or Hepes. In addition, the MPO activity of both non-treated and treated cells was
totally inhibited by the presence of 1 mM-sodium azide (data not shown). On the other hand, catalase (E.C. 1.11.1.6) activity, measured by the method of Peters et al. [22], was unaffected by the presence of taurine or Hepes (data not shown). Therefore, our results indicated that there was enhanced metabolism of $H_2O_2$ through the MPO-$H_2O_2$-halide system in the presence of taurine or Hepes which might account for the observed reduction in chemiluminescence. However, there was no correlation between enhanced enzyme activity and reduced chemiluminescence response in the presence of taurine or Hepes.

Increased production of HOCl by chlorination of $H_2O_2$ was also demonstrated in the presence of Hepes. We were unable to demonstrate HOCl production in the presence of taurine, and believe that sequestration of HOCl by taurine prevented it from being assayed. We believe that taurine- or Hepes-mediated manipulation of HOCl production through the MPO-$H_2O_2$-halide system could play an important role in the modulating of the inflammatory process.

We showed a significant reduction of LTB$_4$ in the supernatants of cells preincubated with taurine or Hepes. The reduced levels of LTB$_4$ in these studies were believed to be due to increased catalolism mediated through the MPO-$H_2O_2$-halide system. In further studies, we observed that cells preincubated with taurine or Hepes in the presence of 1 mM-potassium iodide showed no measurable LTB$_4$ in the supernatants demonstrating an increased susceptibility of active LTB$_4$ to hypoiodous acid (data not shown). These observations support the concept that other halide cofactors may be more effective than chloride in this system. Clark & Klebanoff observed this phenomenon in the oxidation of the chemotactic factor C5a and synthetic chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine [18].

It has been observed that neutrophils can transform prostaglandins by the MPO-$H_2O_2$-halide system [30]. Our results did not confirm previous reports of PGE$_2$ transformation by the MPO-$H_2O_2$-halide system. We showed no effect of taurine or Hepes on secreted PGE$_2$ by cells from normal, psoriatic or disease control subjects.

Taurine has been proposed to modulate the inflammatory process by scavenging HOCl and is believed to prevent feedback inhibition of HOCl on MPO [31]. Fig. 1(a) demonstrates that the chlorination of taurine by OCI$^-$ leads to a significant absorption peak at 250 nm which was attributed to the formation of taurine-chloramine [27]. We believe that the absorption peak at 250–260 nm in the presence of Hepes and OCI$^-$ was due to the formation of Hepes-chloramine (Fig. 1b). Since we also demonstrated that taurine- or Hepes-chloramine, as well as HOCl, have the capacity to cause a reduction in LTB$_4$ (data not shown), taurine or Hepes would appear to have significant anti-inflammatory properties, i.e. by scavenging HOCl and thereby preventing feedback inhibition of HOCl on MPO. This results in enhanced MPO activity which in turn increases metabolism of RO and also increases further production of HOCl.

The concept of the MPO-$H_2O_2$-halide system playing a modulatory role in the control of the inflammatory process is not new, but our studies highlight the significance of stimulating the system by the addition of exogenous taurine or Hepes, and suggest that the metabolic pathway involved could be manipulated to yield reduced levels of inflammatory RO and LTB$_4$. Such control of this pathway would have significant implications in the management of inflammatory diseases. We are currently assessing the effect of other substituted taurine derivatives on RO, HOCl and LTB$_4$ production in addition to their effect on MPO. We believe that these studies will give further insight into the natural control of the inflammatory process. We also believe that care of interpretation of results should be taken in studies on the inflammatory response where the 'Good' buffers [21] have been utilized.

It is well recognized that RO and LTB$_4$ production by neutrophils are central to the pathogenesis of some inflammatory diseases. We chose psoriasis as a model of cutaneous inflammatory disease, since neutrophils are prominent in its pathogenesis. In this study, we demonstrated that both RO and LTB$_4$ levels may be reduced by manipulation of the MPO-$H_2O_2$-halide system in vitro by the addition of taurine or Hepes. Therefore, the use of these structurally related compounds may provide new therapeutic strategies for treatment of inflammatory diseases.

Received 10 September 1990