Nitric oxide (NO), produced by alveolar macrophages (AM) is used as a marker of respiratory tract inflammation. Lipocortin 1 (Lc-1) is an anti-inflammatory, glucocorticoid-inducible protein. The current aims were to determine whether (a) an Lc-1-derived peptide, Ac2–26, inhibited lipopolysaccharide (LPS)-induced NO release by primary AM in vitro and (b) the inhibitory action of dexamethasone was Lc-1-dependent. LPS treatment stimulated NO release from rat AM. Ac2–26 had little effect on unstimulated release, but suppressed LPS-stimulated release at concentrations ≥320 nM (320 nM, 10 ± 3%; 3.2 μM, 15 ± 3%; 32 μM, 27 ± 4% NO inhibited, mean ± SEM, n = 6). Inhibition by dexamethasone of NO release was unaffected by neutralizing anti-Lc-1 indicating that this action is Lc-1-independent in primary AM. Nevertheless inhibition of NO release by Ac2–26 (80 μM) was similar to that of 1 μM dexamethasone (Ac2–26, 40 ± 6%; dexamethasone, 48 ± 6% NO inhibited, mean ± SEM, n = 6).

Key words: lipocortin peptide, glucocorticoid, nitric oxide, alveolar macrophage, dexamethasone

Introduction

Lipocortin-1 (Lc-1) is a 37 kDa anti-inflammatory, glucocorticoid-inducible member of the annexin superfamily of proteins. Its synthesis is upregulated by glucocorticoids both in vitro and in vivo in a number of species, including man. There is considerable evidence to suggest that intact Lc-1 and synthetic peptides derived from its primary sequence possess intrinsic anti-inflammatory properties in a range of experimental models. Lipocortin-1 is a potential mediator of the anti-inflammatory action of glucocorticoids. Increased levels of cellular Lc-1 protein have been observed in vivo in human alveolar macrophages (AM) exposed to oral glucocorticoid and in human mononuclear cells following in vitro administration of hydrocortisone. Studies on adrenalectomized rats have shown a decrease in Lc-1 mRNA and protein in the lung, spleen, liver and kidney, suggesting not only a pharmacological modulation, but endogenous physiological steroid control on Lc-1 expression and turnover. Immuno-neutralization studies have confirmed that a number of the anti-inflammatory actions of exogenous glucocorticoids are, in part, dependent on Lc-1, both in vivo and in vitro. Previous studies have demonstrated that the lung is a rich source of Lc-1 which is detectable in a number of different cell types, including human AM and epithelial cells. Lc-1 is present in bronchoalveolar lavage fluid, in which its levels are increased by oral glucocorticoids in healthy volunteers, patients with lung disease, and control and carrageenin-treated rats. Increased Lc-1 concentrations in lung lavage also occur in response to non-specific stimuli, such as cigarette smoke and LPS; this may reflect increased expression of Lc-1, but may also be a result of increased cell number, cell death or injury.

Inflammation in the respiratory tract is associated with the release of numerous inflammatory mediators, including cytokines and eicosanoids. Recently, nitric oxide (NO) has received much attention as a potential modulator of lung function in health and disease. Both constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS) are present in the respiratory tract. The former is found in endothelial cells and peripheral nerves, where it acts as a smooth muscle relaxant and neurotransmitter and the latter, which is probably involved in lung defence, is localized to AM and epithelial cells and is increased in acute and chronic inflammation. Systemic administration of LPS or generalized sepsis results in high levels of exhaled NO in the lungs of rats. Wood smoke-exposed rats have elevated concentrations of NO metabolites in the plasma which correlates with increased lung epithelial permeability, a process reversible with NOS inhibitors. This suggests that smoke-induced damage may be due to reactive nitrogen radicals in the lung. Intra-tracheal instillation of LPS to rats also causes an increase in epithelial leak, elevated levels of NO in respiratory tract secretions and increased NO release by AM and...
is associated with induction of iNOS mRNA.\textsuperscript{36} Raised iNOS activity and NO release also occur in rat macrophages and epithelial cells in response to exposure to cytokines, such as IFN-γ, TNF-α and IL-1.\textsuperscript{31} Unlike cNOS, which produces NO at a constant low level, activation of iNOS results in high output NO production.\textsuperscript{35} Thus, many pulmonary inflammatory conditions are accompanied by increased NO release. Although it is unclear whether the NO is damaging or protective, NO release is used as a marker of lung inflammation.

Glucocorticoids inhibit NOS activity in the J774.2 macrophage-like cell line,\textsuperscript{38} an effect which is Lc-1 dependent in these cells.\textsuperscript{18} Similarly, inhibition by glucocorticoid of LPS-induced iNOS activity in rat lung homogenates is Lc-1-dependent, the AM being suggested as a potential target for the glucocorticoid action.\textsuperscript{12} Therefore, we hypothesized that NO release from AM in vitro could be inhibited by glucocorticoids in a Lc-1-dependent manner. Primary AM represent a useful in vitro model system for examining the relationship between glucocorticoids, Lc-1 and pulmonary NO, since they possess functional glucocorticoid receptors,\textsuperscript{39} release NO via the activity of iNOS and express Lc-1.\textsuperscript{21} In addition they are the predominant cell type in the airspaces with potential anti-and pro-inflammatory functions. The AM therefore encapsulates all the components under examination and, furthermore, is easily accessible and readily isolated.

Thus the aims of the current study were to determine the effect of a synthetic peptide derived from the N-terminal of Lc-1 (Ac2–26),\textsuperscript{4,6,40,41} on NO release by primary rat AM in vitro and to examine whether inhibition of NO release by glucocorticoids in this system is Lc-1 dependent.

**Methods**

**Animals and removal of lungs**

Pathogen-free male Wistar rats (Charles River), weighing 200–250 g were killed by a lethal intra-peritoneal injection of pentobarbitone (1 g/kg body weight) and heparin (1000 U/kg body weight). Prior to excision, the trachea was cannulated and the lungs perfused free of blood via the right ventricle and pulmonary artery using a gravity feed of sterile 0.15 M NaCl.

**Isolation and culture of alveolar macrophages (AM)**

Excised lungs were fully inflated via the tracheal cannula with 10 ml aliquots of sterile 0.15 M NaCl, emptied, and lavage repeated until approximately 50 ml of fluid had been recovered. The lavage fluid was pooled and centrifuged at 300 × g for 10 min at 4°C. Pelleted cells were resuspended in Hanks’s balanced salt solution, without Ca\textsuperscript{2+} or Mg\textsuperscript{2+} (Sigma) and centrifuged as before. BAL cells (approximately 95% AM) were resuspended in low protein hybridoma medium, containing 2% foetal calf serum, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were counted and seeded in to 96-well plates (Nunc) at a density of 1 × 10\textsuperscript{5} cells per well. AM were allowed to adhere for 3 h in a humidified chamber at 37°C in 5% CO\textsubscript{2} in air. All cell culture reagents were obtained from Gibco.

**Effect of dexamethasone and peptide Ac2–26 on NO release**

Following the 3-h adherence period, non-adherent cells and conditioned media were aspirated and replaced by fresh medium containing graded concentrations of Lc-1 peptide, Ac2–26 (3.2 nM–80 μM), or dexamethasone (10 nM–1 μM). Adherent cells were pre-incubated for 1 h, following which lipopolysaccharide (0.1–10 μg/ml, serotype 055:B5 from *Escherichia coli*, Sigma) was added to all wells except controls and the cells incubated for a further 24 h. Conditioned media were harvested and analysed for nitrite. The competitive NOS inhibitor N\textsuperscript{G}-monomethyl L-arginine (L-NMMA 4 mM, Sigma) was added to selected wells to determine the proportion of measured nitrite which originated from the activity of NOS.

**Effect of Lc-1 immuno-neutralization on dexamethasone activity**

Non-adherent cells and conditioned media were removed as above and replaced with fresh medium containing a sheep polyclonal neutralising antibody to Lc-1 (LC81, the generous gift of Professor R. J. Flower, William Harvey Research Institute, London) used at 1:50 dilution. The cells were pre-treated with this anti-serum for 3 h, then incubated with graded doses of dexamethasone for 1 h and stimulated with lipopolysaccharide for 24 h as described above.

**Measurement of nitrite**

Spontaneous oxidation of the NO radical in aqueous solutions leads to the formation of nitrite (NO\textsubscript{2}–) as the predominant stable breakdown product.\textsuperscript{29,42} Nitrite accumulation in conditioned media was therefore used as an index of NO release by AM, 24 h post-LPS. This was measured using the colorimetric Griess reaction.\textsuperscript{43} Nitrite concentrations were determined by comparison with standard solutions of sodium nitrite (0.78–100 μM) prepared in culture medium.

**Data analysis**

Data were analysed by the Wilcoxon’s signed rank test for paired data; a probability of *P* < 0.05 was regarded as statistically significant. Percentage inhibition of
**Inhibition of NO by a lipocortin peptide**

**Results**

**Effect of lipopolysaccharide on NO release**

Median basal release of NO was 56, range 47–61 nmol NO/10^6 cells/24 h in culture (Fig. 1, n = 4); 86% was inhibited by 4 mM L-NMMA. Stimulation of AM with LPS produced a concentration-dependent increase in nitrite accumulation in the culture medium comparable with that observed in previous studies\(^{31,38,44}\) (Fig. 1). At a concentration of 10 μg LPS/ml, 85% of NO release was inhibited by L-NMMA. Based on these observations, an LPS dose range of 0.1–10 μg/ml was used in all subsequent experiments.

**Effect of peptide Ac2–26 on NO release**

The peptide had no significant effect on basal NO release in unstimulated cells (Fig. 2) except at the highest concentrations of Ac2–26 tested (32 μM and 80 μM) where basal release was significantly inhibited (31% and 28% respectively). Ac2–26 significantly inhibited LPS-induced NO release by AM at concentrations of 320 nM or more (Fig. 3). The maximum inhibition observed was 40% by a concentration of 80 μM Ac2–26. The action of Ac2–26 was similar irrespective of the LPS concentration used (Table 1) and therefore only data for LPS at 10 μg/ml are illustrated.

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**Figure 1.** Effect of increasing concentrations of LPS on NO release from rat AM in vitro. Data are presented as mean ± SEM, n = 4; Open squares represent LPS-stimulated NO release; Open diamond represents basal (unstimulated) NO release.

**Figure 2.** Effect of Ac2–26 and dexamethasone on basal NO release by rat AM in vitro. Percentage NO remaining following L-NMMA, peptide or dexamethasone treatment was calculated using the following formula: (NO release by unstimulated AM + peptide(NO release by unstimulated AM) × 100 Data are presented as mean ± SEM, n = 6, *P < 0.05, Wilcoxon's signed rank test for paired data; open squares represent NO release in the presence of Ac2–26; open diamonds represent NO release in the presence of dexamethasone; open circle represents NO release in the presence of L-NMMA.

**Figure 3.** Effect of Ac2–26 and dexamethasone on NO release by LPS-stimulated rat AM in vitro (10 μg/ml LPS). Percentage NO remaining following L-NMMA, peptide or dexamethasone treatment was calculated using the following formula: (NO release by LPS-stimulated AM + peptide(NO release by LPS-stimulated AM) × 100 Data are presented as mean ± SEM, n = 6, *P < 0.05, Wilcoxon signed rank test for paired data; open squares represent NO release in the presence of Ac2–26; open diamonds represent NO release in the presence of dexamethasone; open circle represents NO release in the presence of L-NMMA.
Effect of dexamethasone on NO release

Dexamethasone significantly inhibited basal NO release at all concentrations of drug and LPS-stimulated release at concentrations above 10 nM dexamethasone (Table 1 and Figs 2 and 3). Maximal inhibition by dexamethasone was 48%.

Effect of anti-Lc-1 neutralizing antibody on NO release

Pre-treatment of rat AM with a neutralizing antibody to Lc-1, had no effect on dexamethasone-mediated inhibition of NO release, irrespective of the concentration of dexamethasone used (Fig. 4).

Discussion

This study demonstrates that the N-terminal peptide of Lc-1, Ac2–26, inhibits LPS-induced NO release from rat AM in a concentration-dependent manner. To our knowledge, this is the first such in vitro demonstration in a primary cell with a known pro-inflammatory function. By increasing the dose of Ac2–26 to 80 μM it was possible to achieve the same degree of inhibition as with 1 μM dexamethasone. Interestingly use of a neutralizing anti-Lc-1 antiserum indicated that the inhibitory action of dexamethasone itself on NO release was independent of endogenous Lc-1. Downregulation of LPS-induced NO release by a longer Lc-1 peptide, Lc-11–188, at a concentration of 20 μg/ml has been reported in the J774.2 cell line. The observation that it was effective at a substantially lower concentration than Ac2–26 may reflect differences between the peptides themselves, as shown previously with murine neutrophils; it is possible that the Ac2–26 does not activate the signal transduction machinery needed to trigger the cell response as effectively as does the larger molecule. The optimal size for inhibition of NO release by Lc-1-derived...
peptides remains to be established. Provided that the peptides are as effective as dexamethasone and low in toxicity, the absolute concentration required to achieve inhibition is probably not a major consideration for their use in vivo.

Dexamethasone was an effective inhibitor of NO release by AM in this study, although interestingly, use of neutralizing antiserum indicated that its action was Lc-1-independent. Pulmonary AM treated with dexamethasone are capable of increasing their Lc-1 expression and release. However, studies in our laboratory indicate that at the highest concentration of dexamethasone used in the current investigation (1 μM) the increase in cellular Lc-1 above baseline is likely to be modest in rat AM.

In summary, we have shown that Ac2–26 causes the same degree of inhibition of LPS-induced NO release by AM as dexamethasone, but at a much higher concentration and by a different mechanism. This and other peptides derived from Lc-1 may have therapeutic potential for suppression of pulmonary inflammation, since they could be delivered topically to the target organ.

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ACKNOWLEDGEMENTS. This work was supported by the Trustees Research Committee of Charing Cross & Westminster Medical School. A.M.K. thanks the Sir Jules Thorne Charitable Trust for personal support.

Received 22 December 1997; accepted 9 January 1998