Modulation of cytokine production by human mononuclear cells following impairment of Na,K-ATPase activity

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Abstract

Cytokines, including TNFα and IL-1β, are central to the chronic inflammatory process and tissue damage that characterises diseases such as rheumatoid arthritis. The mechanisms responsible for long-term generation of these molecules are poorly understood. We have previously demonstrated impaired activity of Na,K-ATPase, a key enzyme regulating intracellular cation levels, on rheumatoid mononuclear cells. Mimicking this ‘defect’ on normal mononuclear cells with ouabain has been shown to induce TNFα and, in particular, IL-1β production, whereas IL-6 synthesis was suppressed. A similar pattern of cytokine generation was noted when mononuclear cells were treated with the sodium ionophore, monensin. Induction of cytokine production was related to up-regulation of the appropriate mRNA, although enhanced secretion of processed IL-1β was also observed. The mechanism underlying these cellular responses appears to involve sodium/calcium exchange across the cell membrane. Impaired Na,K-ATPase activity might promote pro-inflammatory cytokine secretion in patients with rheumatoid arthritis.

Keywords: ATPase, Na+/K−; Mononuclear cell; Cytokine; (Human)

1. Introduction

Cytokines are potent regulators of cell function relevant to virtually all aspects of tissue physiology and pathology. Chronic inflammation, such as exemplified by the disease rheumatoid arthritis (RA), is one process in which a wide range of cytokines, including IL-1, TNFα and IL-6, are critically involved in promoting tissue injury [1]. Reviews of the biological properties of these cytokines [2–5], and in particular of IL-1 and TNFα, show a close correlation with observed changes in the rheumatoid joint.

Numerous studies have demonstrated local synthesis of IL-1α and β [6–8], TNFα [6,9] and IL-6 [6,10] in this tissue. Recently, both chimeric anti-TNFα monoclonal antibody [11] and IL-1 receptor antagonist [12] have been shown to exert beneficial therapeutic effects in rheumatoid patients.

The mechanisms underlying the long-term generation of pro-inflammatory cytokines within chronically inflamed tissue are unclear, but are likely to be complex and multifactorial. Little is known about the functional consequences of biochemical changes occurring within cells through exposure to the chronic inflammatory process. Recent observations have identified an impairment of Na,K-ATPase activity (the sodium pump, EC 3.6.1.37) in rheumatoid erythro-
cyte membranes [13], and we have shown similar results in studies of peripheral blood mononuclear cells (MNC) [14]. The Na,K-ATPase is the key ion-motive enzyme in all eukaryotic cells, pumping potassium into and sodium out of the cell at the expense of hydrolysing ATP and, through secondary cation exchangers and antiporters (e.g. Na/Ca, Na/H), effectively controlling intracellular calcium and other cation levels [15]. The electrogenic action of the pump helps to maintain the cell’s membrane potential, and the ion gradients generated are used to regulate many aspects of cell function, including glucose and aminoacid transport, osmotic balance and cell volume. The enzyme is specifically inhibited by ouabain and other cardiac glycosides. Given its vital role in regulating intracellular cation levels and the importance of such cations in cell signalling pathways, it seemed likely that impairment of the Na,K-ATPase activity would have functional consequences in MNC that might be relevant to the chronic inflammatory process. In this report, nanomolar concentrations of ouabain have been shown to exert marked but differential effects on cytokine generation by healthy MNC.

2. Materials and methods

2.1. MNC preparation

MNC from peripheral blood of healthy volunteers were isolated by density gradient centrifugation on Lymphoprep (density = 1.077 g/ml, Oxoid, Bristol, UK). After washing in PBS, the cells were suspended at the required density in RPMI 1640 medium containing glutamine, penicillin-streptomycin and 5% v/v heat-inactivated fetal calf serum (Life Technologies, Paisley, Scotland, UK).

2.2. Induction and assay of cytokine proteins

Mononuclear cells were incubated at 37°C in polypropylene tubes (Falcon, Becton Dickinson, Oxford, UK) to minimise false positive cytokine profiles due to adherence activation and were stimulated with ouabain (Sigma, Poole, Dorset, UK), a specific Na,K-ATPase inhibitor, monensin (Sigma), a sodium ionophore, or the calcium ionophore A23187 (Sigma) over titration ranges of 1 nM to 10 μM and time courses up to 24 h. Some cultures also contained 3,4-dichlorobenzamil (0.3–30 μM, DCB), a selective sodium/calcium exchange inhibitor [16]. Unless stated otherwise, all incubations were carried out in RPMI 1640 medium supplemented with 5% v/v heat-inactivated FCS. Supernatants were harvested by centrifugation at 1500 rpm for 5 minutes at room temperature and careful aspiration from the cell pellets. These supernatants were then stored at −20°C until cytokine content was quantified by immunoassay (Medgenix, High Wycombe, Bucks, UK) following the instructions supplied with the kits.

2.3. Induction and detection of cytokine messenger RNAs

Levels of mRNA encoding the TNFα, IL-1β and IL-6 proteins were determined by Northern blot analysis [17] of total cellular RNA [18] using cDNA oligonucleotide probes labelled by random sequence hexanucleotide priming using the Klenow fragment and [α-32P]dATP (Multiprime, Promega, Southampton, UK). IL-1β mRNA was detected using a 320 bp EcoRI-PstI fragment from pGem1 IL-1β [19], and the cDNA probe for TNFα was a 1200 bp molecule derived from PAT-153 using PstI [20]. IL-6 mRNA was identified using a 300 bp SalI-EcoRI fragment from pSP-64 [21], and the control probe used was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, a 1000 bp molecule derived from pG3PCR-4 using BamHI [22].

2.4. Determination of intracellular IL-1β by flow cytometry

Total intracellular IL-1β (31 kDa precursor plus 17 kDa mature form) was quantified in control and ouabain-stimulated MNC by flow cytometry (Becton Dickinson FacStar Plus with air-cooled argon ion laser). Cells were fixed and permeabilised with paraformaldehyde and saponin [23]. Specific protein was detected using a primary mouse monoclonal antibody (Genzyme Diagnostics, West Malling, Kent, UK) and secondary FITC-conjugated anti-mouse IgG reagents (Sigma). Control replicates included both an irrelevant isotype-matched primary antibody, and secondary antibody alone.
3. Results

From measurements of $^{3}$H-ouabain binding and functional enzyme inhibition experiments (data not shown), it was estimated that the degree of Na,K-ATPase inhibition detected on rheumatoid MNC could be reproduced by incubating normal cells with ouabain concentrations of $1\times 10^{-8}$ M. Accordingly, functional studies involving time courses and dose response curves to ouabain were performed around this range. The most dramatic outcome of inhibiting Na,K-ATPase was to increase production of IL-1β from low levels to a mean value of over 10 ng/$10^6$ cells after 24 h. The results shown in Fig. 1A indicate that production of IL-1β was just detectable after 2 h incubation at 37°C with the optimal concentration of ouabain (100 nM), and reached a maximum after 12 h culture. This represents extracellular secretion of mature 17 kD IL-1β, since this is the only molecular species detected by the immunoassay kit used (Medgenix). The induction of TNFα production was slightly faster than that of IL-1β, being apparent within 1 h and peaking after 4 h incubation with ouabain (Fig. 1B). However, production of TNFα was consistently up to ten-fold less than that of IL-1β. Production of IL-6 was, in most cases, readily detectable in unstimulated MNC (Fig. 1C), increasing with time over 24 h. In the presence of ouabain, IL-6 production paralleled that in control cultures for the first 2 h of incubation, and then ceased completely. This argues against the notion that ouabain might be enhancing cytokine secretion non-specifically, either by overt toxicity or some other mechanism. This pattern of results was unaffected by adding polymyxin B (up to 10 μM) to the cultures to inactivate any endotoxin present. In contrast, preincubating the MNC with an inhibitor of protein synthesis, cycloheximide (10 μM for 12 h), abolished all detectable cytokine synthesis in response to subsequent stimulation by ouabain (data not shown).

Since the initial biochemical change within ouabain-treated cells will be an increase in intracellular sodium ion concentration, functional outcomes mediated by ouabain might also occur in cells responding to the sodium ionophore monensin. Indeed, the pattern of cytokine responses to this compound was very similar to that observed for ouabain (Fig. 2). Both induced a marked increase in the production of IL-1β and TNFα by MNC, with a fall in the generation of IL-6. For comparative purposes, cytokine production was determined in MNC responding to the calcium ionophore A23187. This compound also caused induction of IL-1β and TNFα synthesis but suppressed IL-6 secretion (Fig. 2). The similarity between the patterns of cytokine production by MNC responding to ouabain, monensin and A23187 indicated that all three reagents could be functioning through increasing intracellular calcium.

Fig. 1. Time course of cytokine induction in healthy MNC by ouabain (100 nM). Data are presented as mean secreted protein levels + S.D. of 4–6 individual experiments. Filled bars are results from control cultures, hatched bars are plus ouabain. Panel A, IL-1β; Panel B, TNFα; Panel C, IL-6.
ion concentrations. To test this, MNC were stimulated with ouabain in the presence of 3,4-dichlorobenzamil (DCB), a selective inhibitor of sodium/calcium exchange [16]. These experiments show a dose-dependent inhibition of IL-1β and TNFα production by the ouabain-stimulated MNC (Fig. 3). In all experiments, cell viability as measured by trypan blue exclusion was unaffected by any of the compounds (>95% at 24 h) except at the highest concentrations tested (viability 75–85%).

The time-course of the ouabain-mediated changes in cytokine production was consistent with an effect on mRNA transcription, and direct evidence supporting this was obtained by Northern blot analysis of total cellular RNA extracted from control and ouabain-treated MNC. A representative experiment illustrating dose-dependent responses to ouabain in cells treated for 2 h at 37°C is shown in Fig. 4. The data indicate that some IL-1β mRNA is detectable with only 1 nM ouabain, but maximum induction of IL-1β mRNA was obtained at a concentration of 100 nM. The induction of TNFα mRNA showed a similar response profile, with maximal levels being detected at 100 nM ouabain. Significant expression of IL-6 mRNA was detected in control cultures, which was markedly reduced by low (1–10 nM) ouabain con-

Fig. 2. Dose response of cytokine induction in healthy MNC incubated for 24 h with ouabain, monensin or A23187. Data are presented as percentage cytokine secretion relative to control unstimulated cultures (=100%) and represent mean ± S.D. of 4–7 experiments. Absolute levels of cytokine production in control cultures were as illustrated in Figure 1. Filled bars are results from ouabain-, close hatched bars are from monensin- and light hatched bars are from A23187-stimulated MNC. Panel A, IL-1β, Panel B, TNFα, Panel C, IL-6.

Fig. 3. Effect of 3,4-dichlorobenzamil (DCB) on IL-1β and TNFα production by healthy MNC incubated for 24 h with ouabain (100 nM). Data are the mean of 3 experiments and represent % cytokine production ± S.D. relative to values in the absence of DCB (=100%). Mean absolute cytokine production in control ouabain-stimulated cultures was 7000 pg/ml IL-1β and 1680 pg/ml TNFα.
centrations. Interestingly, some IL-6 mRNA remained in the cells even in the presence of high ouabain levels.

In addition to the above effects on transcription of cytokine mRNAs, ouabain was also investigated as a potential modulator of the secretion of IL-1β following processing from its intracellular 31 kDa precursor. Data in Table 1 demonstrate a dose-dependent decrease both in the proportion of cells staining positive for intracellular IL-1β (31 kDa plus 17 kDa) and in the Mean Fluorescence intensity of those positive cells following 24 h incubation with increasing concentrations of ouabain. Levels of secreted IL-1β showed a concomitant increase over the same dose range.

4. Discussion

Incubation of normal MNC with non-toxic, nanomolar concentrations of ouabain caused both a marked stimulation of TNFα, and more especially IL-1β, production, but suppression of IL-6 synthesis. An earlier report has also demonstrated stimulation of IL-1β production by ouabain [24], and our data are consistent with other reports showing enhanced IL-1β processing and secretion following an induced fall in intracellular potassium levels [25,26], or following increases in intracellular sodium levels [27]. A sodium-dependent modulation of TNFα production by human monocytes has also been reported [28]. The major action of ouabain on cytokine production by MNC in this study is clearly mediated at the gene level through modulating transcription of the relevant mRNAs. The time course of these effects suggested that mRNA stability and half-life were not markedly influenced by inhibiting Na,K-ATPase data not shown, although direct measurements of these parameters were not performed. The time courses and concentration dependency of changes in cytokine mRNA expression were in close agreement with the measurements of cytokine protein production. Up-regulation of TNFα mRNA occurred first, with IL-1β mRNA being induced slightly more slowly. Perhaps surprisingly, it was possible to demonstrate the appearance of IL-6 mRNA in the ouabain-stimulated MNC, although no secreted protein product was detectable following the initial 2 h of the culture period. This requires further investigation of events downstream from transcription, such as processing and secretion of IL-6 protein, which may be modulated by inhibition of Na,K-ATPase.

The significance of these acute observations of ouabain-treated healthy MNC to the chronic overproduction of pro-inflammatory cytokines in rheumatoid synovial tissue remains unproven. It is clear from data generated in both functional and ligand-binding

Table 1

Effect of ouabain on levels of intracellular IL-1β after 24 h incubation

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>% positive cells</th>
<th>MFI (positive cells)</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>25.0±4.1</td>
<td>543±60</td>
</tr>
<tr>
<td>Ouabain:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁹ M</td>
<td>17.0±5.1</td>
<td>432±57</td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td>4.0±1.0</td>
<td>297±47</td>
</tr>
<tr>
<td>10⁻⁷ M</td>
<td>2.0±0.4</td>
<td>286±34</td>
</tr>
</tbody>
</table>

Data are presented as mean±S.D. of 3 experiments, showing % positive cells staining for intracellular IL-1β and the mean fluorescence intensity (MFI) of those positive cells.
assays that Na,K-ATPase enzyme activity is depressed on rheumatoid peripheral blood MNC. These observations support other results showing a similar ‘defect’ on rheumatoid erythrocytes [13] and suggest that this biochemical abnormality may be detectable in a wide range of cells from these patients. A more detailed examination of cation-dependent biochemistry in rheumatoid cells would seem to be justified given the profound effects of inhibiting Na,K-ATPase on cytokine production by healthy MNC. The nanomolar concentrations of ouabain achieving these effects in vitro induce a degree of Na,K-ATPase inhibition that is similar to the depressed enzyme activity on rheumatoid MNC observed ex vivo. Biochemical changes following impairment of the sodium pump include a rise in intracellular sodium and, through sodium-calcium exchange, calcium ion concentrations. Data presented in this report strongly suggest that the effects on cytokine production of inhibiting the Na,K-ATPase are mediated through changes in intracellular calcium ion concentration. DCB, a selective sodium-calcium exchange inhibitor [16] suppressed IL-1β and TNFα generation with an IC₅₀ of approximately 3 μM. This potency is similar to that observed for DCB-mediated suppression of T cell proliferation and IL-2 secretion [29]. Increased levels of sodium ions have been measured in rheumatoid erythrocytes [13], and elevated intracellular calcium levels have been determined in rheumatoid red blood cells and granulocytes [30]. Further studies are justified to ascertain the significance of this biochemical abnormality as a contributor to cytokine-mediated tissue damage in rheumatoid joints.

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