Methylprednisolone inhibits uptake of Ca^{2+} and Na^{+} ions into concanavalin A-stimulated thymocytes

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The glucocorticoid drug methylprednisolone inhibits respiration in concanavalin A-stimulated rat thymocytes at concentrations that are relevant to its acute clinical efficacy against autoimmune diseases and spinal cord injury. Methylprednisolone affects several processes, including ion cycling, substrate oxidation reactions and RNA/DNA synthesis. The inhibition of respiration used to drive ATP-consuming cycles of Ca^{2+} and Na^{+} ions across the plasma membrane has been proposed to be either primary or secondary to restriction of cellular ATP supply. By comparing

the effects of methylprednisolone with those of myxothiazol, an inhibitor of the mitochondrial electron transport chain, we show that the effects of methylprednisolone on Ca^{2+} and Na^{+} cycling are primary. We propose that methylprednisolone acts by affecting membrane properties to inhibit Ca^{2+} and Na^{+} uptake across the plasma membrane and to increase H^{+} uptake across the mitochondrial membrane, and that other effects are secondary.

INTRODUCTION

We have recently shown a clear hierarchy of the responses of different energy-consuming reactions to changes in energy supply for concanavalin A-stimulated thymocytes, a model in which more than 80 % of the consumption of ATP can be accounted for [1]. In these experiments mitochondrial ATP production was restricted by progressively inhibiting the electron transport chain with myxothiazol, an inhibitor of complex III, to show that processes not essential for the immediate needs of the cell are given up before those that are more critical for ionic integrity. Pathways of macromolecule biosynthesis (protein synthesis and RNA/DNA synthesis) are the most sensitive to ATP supply, followed by Na^{+} ion cycling and then by Ca^{2+} ion cycling across the plasma membrane. Mitochondrial proton leak is the process least sensitive to energy supply [1].

In the present study we use the same model system to investigate further the short-term effects of glucocorticoids on cellular energy metabolism. We previously showed that the glucocorticoid drug methylprednisolone at clinically relevant concentrations inhibited Ca^{2+} and Na^{+} cycling across the plasma membrane and decreased intracellular free Ca^{2+} concentrations but had little effect on protein synthesis [2]. Recently we described the significant kinetic effects of treatment with methylprednisolone on the different subsystems within oxidative phosphorylation in concanavalin A-stimulated thymocytes [3]. Using top-down elasticity analysis (a method to identify effector targets [4]) we found that methylprednisolone at 0.2 mg per 10^7 cells greatly inhibited the reactions of substrate oxidation and increased mitochondrial proton leak but did not seem to affect significantly the synthesis and turnover of ATP by the phosphorylating system. The lack of effect on the reactions of ATP turnover was rather surprising because the rates of some ATP-consuming processes are decreased when methylprednisolone is added, as described above. This could either indicate that the inhibition of these ATP consumers is secondary to a decrease in ATP production after inhibition of substrate oxidation and stimulation of proton leak or that the overall direct inhibitory effects of methylprednisolone on ATP consumers were too small to be detected in these experiments.

In the work described in the present paper, our objective was to distinguish between these two possibilities, to complete our description of the mechanism of the acute effects of methylprednisolone megadoses on thymocytes and to evaluate further our model system and techniques for investigating short-term effects on energy metabolism. We show that the inhibition of cation cycling is caused by direct effects and not by a decrease in ATP production.

MATERIALS AND METHODS

Preparation and incubation of cells

Thymocytes were prepared from 4–6-week-old female Wistar rats as described previously [3]. The thymus was disaggregated by pressing it through nylon mesh. The cells were centrifuged once at 1000 *g* for 5 min and resuspended at $(5\text{--}6) \times 10^7$ cells/ml at 37 °C. The isolation and incubation medium was RPMI 1640 with 10 mM glucose (unless stated otherwise) and 2 mM glutamine (obtained powdered from Flow Laboratories), buffered at pH 7.4 with 10 mM Hepes/24 mM NaHCO_3 , supplemented with 20 $\mu\text{g}/\text{ml}$ gentamicin and filtered through a filter (0.2 μm pore size) to remove undissolved particles. Cells were stored and incubated for up to 4 h in plastic flasks at 37 °C with gentle shaking (80 cycles/min) under an atmosphere of air/ CO_2 (19:1). The viability of freshly isolated cells was greater than 95 % as determined by Trypan Blue exclusion.

Measurement of respiration rate

Oxygen consumption was measured in 1.0 ml of cell suspension with a Clark electrode for up to 15 min as described previously [1]. The cell suspensions in the Perspex incubation chamber of the electrode were magnetically stirred and thermostatically maintained at 37 °C. Myxothiazol was dissolved in DMSO; other inhibitors were dissolved in water. Concanavalin A (ICN Immuno-Biologicals, Lisle, Israel) was dissolved in water and in all experiments was added to cell suspensions at 25 $\mu\text{g}/\text{ml}$.

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Methylprednisolone hemisuccinate-Na, METYPRED (Orion Pharmaceutica, Helsinki, Finland) was used. As described previously [1–3] we applied inhibitors of protein synthesis (1 mM cycloheximide), Na^+/K^+ -ATPase (1 mM ouabain), Ca^{2+} -ATPase (2 mM $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$), RNA/DNA synthesis (0.6 mM actinomycin D) (all from Sigma Chemical Co., Poole, Dorset, U.K.) and mitochondrial electron transport (7.5–17.5 nM myxothiazol) (Boehringer, Mannheim, Germany). Respiration rates were measured 1–3 min after each addition of concanavalin A or inhibitor.

ATP measurements

Cell suspension (1 ml) was incubated with 25 $\mu\text{g}/\text{ml}$ concanavalin A in a plastic vial and stirred magnetically for 2 min under an atmosphere of air/ CO_2 (19:1). Myxothiazol (10 nM), methylprednisolone (0.2 mg per 10^7 cells) or no inhibitor (control) was added and the cells were stirred for a further 5 min. Cell suspension (0.5 ml) was transferred into the oxygen electrode chamber and oxygen consumption was measured immediately. Respiration rates were stable for at least 5 min after the start of measurement. A 0.1 ml portion of the remaining cell suspension was taken and assayed for ATP. From each preparation of thymocytes ($n = 4$), two incubations with inhibitors as described above were processed in parallel and the oxygen and ATP measurement duplicates from each preparation were then averaged.

Total thymocyte ATP concentrations were measured in a luciferin–luciferase assay with an ATP assay kit supplied by BioOrbit (Turku, Finland). Briefly, 0.1 ml of ice-cold 2.5% (w/v) trichloroacetic acid was added to 0.1 ml of cell suspension and left on ice for 10 min. After centrifugation, the supernatant was diluted 10-fold in buffer containing 0.1 M Tris acetate/2 mM EDTA (pH 7.75). ATP measurements were then performed in a Labtech luminometer.

RESULTS

The basal respiration rate of quiescent thymocytes was 4.1 ± 0.2 nmol of O_2/min per 5×10^7 cells. Addition of concanavalin A stimulated this within seconds by approx. 30% to 5.4 ± 0.2 nmol of O_2/min per 5×10^7 cells (means $\pm$ S.E.M. for 16 cell preparations). These values agree with our previous results in this and other media [1–3,5]. We investigated concanavalin A-stimulated thymocytes in the present paper as a model for cells activated in disease and because we can account for more than 80% of the energy-consuming pathways in these concanavalin A-stimulated cells [1–3]. The rates of these pathways can be conveniently monitored by measuring oxygen consumption.

This technique using specific inhibitors has been used successfully in several different model systems [1,2,6–9] and has been tested for validity in different ways. Thus we have previously quantified protein synthesis directly and shown that it gives the same numerical result as the inhibition method [7]. We have shown that the inhibition of one ATP-consuming process does not significantly affect the rates of the others [1]. Decreased respiration caused by secondary effects of the inhibitors could be a potential problem. However, the effects of the specific inhibitors used are very rapid (less than 30 s) and then stable for at least 10 min, a pattern that is not compatible with secondary effects. There is direct evidence for the specificity of ouabain in the time frame we used: Felber and Brand [10] showed in pig lymphocytes that ouabain causes a step decrease of 7 mV in plasma membrane potential that was stable for at least another 12 min, suggesting that Na^+ and K^+ gradients must have been stable for at least 12 min after ouabain inhibition. In hepatocytes [11], secondary

Figure 1 Effects of methylprednisolone on respiration in concanavalin A-induced thymocytes

Thymocytes were incubated in RPMI 1640 medium with 25 $\mu\text{g}/\text{ml}$ concanavalin A, followed by application of 0.1–1.0 mg of methylprednisolone per 10^7 cells (1–10 mM). Respiration rate was assayed as described in the Materials and methods section. Results are means $\pm$ S.E.M. for 11 cell preparations.

effects of ouabain do occur, but only after 15–20 min. Finally, in our experiments the inhibitions sum to approx. 100%, which would not be expected if they had overlapping secondary effects.

We first performed a series of titrations of respiration rate with methylprednisolone and, for comparison, with the electron transport chain inhibitor myxothiazol, which inhibits complex III of the respiratory chain [12]. Myxothiazol is very specific, and all of its effects on ATP-consuming reactions can be ascribed to its primary inhibition of electron transport followed by secondary inhibition of ATP synthesis. Figure 1 shows the inhibition of respiration by methylprednisolone in concanavalin A-stimulated thymocytes. These results and those obtained with myxothiazol [1] are similar to those found previously [2,3].

To determine which ATP-consuming processes were direct targets of methylprednisolone action and which were inhibited indirectly by restriction of ATP availability, we compared the inhibition of different ATP-consuming processes by methylprednisolone and by myxothiazol. We measured the rates of different ATP-consuming reactions by measuring the effects on respiration rate of completely inhibiting them one by one with specific inhibitors. We determined the rates of protein synthesis (Figure 2a), plasma membrane Na^+ cycling (Figure 2b), plasma membrane Ca^{2+} cycling (Figure 2c) and RNA/DNA synthesis (Figure 2d) at different degrees of inhibition by methylprednisolone or myxothiazol.

The results in Figure 2 show that protein synthesis was much less sensitive to methylprednisolone than it was to myxothiazol. This suggests that methylprednisolone does not decrease ATP levels within the cells (at a given rate of respiration) as much as myxothiazol does. Na^+ and Ca^{2+} cycling across the plasma membrane were much more sensitive to methylprednisolone than to myxothiazol, showing that the inhibition of these cation cycles cannot have been secondary to the inhibition of ATP production but must have been caused by a more direct effect. RNA/DNA synthesis was as sensitive to methylprednisolone as it was to myxothiazol; because methylprednisolone probably lowered ATP less effectively than myxothiazol did, this suggests that methyl-

Figure 2 Effects of methylprednisolone- and myxothiazol-produced inhibition of respiration on different ATP-consuming processes

Thymocytes were incubated in RPMI 1640 medium with 25 μ g/ml concanavalin A and pretreated with different concentrations of methylprednisolone or myxothiazol to give the different values of inhibition of respiration reported on the horizontal axis. The rate of each ATP-consuming process in each condition was assayed by measuring the extra inhibition caused by addition of the appropriate inhibitor as described in the Materials and methods section. Inhibitors were (a) cycloheximide, (b) ouabain, (c) lanthanum and (d) actinomycin. Results are means $\pm$ S.E.M. for five to nine cell preparations. S.E.M. values for the horizontal axis (not shown, for clarity) were between 2% and 5.8%. Symbols: $\circ$, respiration progressively inhibited by myxothiazol; $\blacksquare$, respiration progressively inhibited by methylprednisolone.

prednisolone also had an effect on RNA/DNA synthesis that was not secondary to the inhibition of ATP supply.

Direct measurement of total thymocyte ATP showed that the decrease in ATP was the same or less with methylprednisolone than with myxothiazol. Myxothiazol (10 nM) inhibited respiration to $73.4 \pm 5.2\%$ (mean $\pm$ S.E.M., $n = 4$) and lowered cellular ATP to $91.5 \pm 2.9\%$ compared with control, whereas methylprednisolone (0.2 mg per 10^7 cells) inhibited respiration to $72.7 \pm 1.2\%$ and lowered ATP to $94.3 \pm 2.2\%$ compared with control.

To investigate the influence of the medium we performed identical experiments with RPMI 1640 medium lacking glucose. The results were the same as with the complete medium (results not shown), indicating that glycolytic ATP production was not a complicating factor in these results.

DISCUSSION

Although low concentrations of glucocorticoids have important effects on cells through receptor-mediated activation of gene expression, there is growing evidence that at high concentrations there are rapid direct effects that are not mediated by the induction or repression of specific genes. Experimental results

and clinical experience suggest that fast-response mechanisms of glucocorticoid action are involved in the therapeutic effects of glucocorticoid megadoses [13–21]. In general, methylprednisolone at very high doses has clinically relevant short-term effects on cells such as those of the activated immune system. Because of the timescale, these effects do not seem to be mediated by nuclear events but are likely to be due in part to physico-chemical effects on membranes.

The clinical relevance of these effects can be estimated from the relationship between the concentrations of methylprednisolone that we have used *in vitro* and those that are achieved in clinical practice calculated with the equations given in [22]. For autoimmune disorders up to 15 mg/kg body weight and for neurotraumatic conditions 30 mg/kg are used, giving a maximal plasma concentration of up to 0.5 g/l. These concentrations are equal to 0.1 mg per 10^7 cells and above under the conditions we have used in the present paper, so the effects that we observe *in vitro* (Figure 1) with 0.2 mg per 10^7 cells seem to be in the clinically relevant concentration range. Even higher concentrations might be achieved locally for short periods during the intravenous injection of bolus megadoses.

The results presented in this paper provide evidence that the glucocorticoid strongly inhibits plasma membrane cycling of

both Ca^{2+} and Na^{+} independently of any decrease in ATP supply. The sensitivities of the two processes to methylprednisolone did not differ significantly. RNA/DNA synthesis also seemed to be directly inhibited by methylprednisolone, but to a distinctly smaller extent than the cation cycles, and protein synthesis was very insensitive to methylprednisolone. This pattern is completely different from the hierarchy of ATP consumers previously found with an authentic inhibitor of ATP production, the respiratory chain inhibitor myxothiazol. In that case protein synthesis shuts off at first, followed by RNA/DNA synthesis and finally by cation cycling [1]. The lack of significant inhibition of the overall phosphorylation reactions in [3] might therefore reflect the relatively high experimental errors in the measurements of the kinetics of this subsystem, which masked the effect on the specific ATP consumers observed here, or it might reflect differences in the times of measurement in two types of experiment.

Does methylprednisolone prevent the cation cycles by inhibiting ion uptake or by inhibiting the Ca^{2+} -ATPase and the $\text{Na}^{+}/\text{K}^{+}$ -ATPase? The most likely explanation is that it inhibits ion uptake and as a result the ATPases are inhibited indirectly through changes in intracellular Ca^{2+} or Na^{+} concentrations. A direct inhibition of the Ca^{2+} -ATPase is unlikely because we have shown that methylprednisolone causes a decrease in intracellular Ca^{2+} concentration in thymocytes [2]. This could either mean that the Ca^{2+} -ATPase is stimulated or that the influx of Ca^{2+} is decreased. Because the present and previous results show a clear inhibition of the Ca^{2+} cycle, the results support the inhibition of ion uptake. This inhibition could be a direct effect on ion channels or it could be due to a physicochemical membrane-stabilizing effect of methylprednisolone [13]. It is not due to an inhibition of the transduction of the concanavalin A signal because the effect of methylprednisolone on intracellular Ca^{2+} concentration is apparent even in the absence of the mitogen.

Summarizing our results, we can now explain the effects of methylprednisolone on isolated concanavalin A-stimulated thymocytes. Methylprednisolone inhibits plasma membrane Ca^{2+} uptake, plasma membrane Na^{+} uptake, RNA/DNA synthesis and substrate oxidation reactions, and stimulates the leak of protons across the mitochondrial inner membrane. It lowers cytoplasmic free Ca^{2+} concentrations and abolishes the Ca^{2+} rise and the respiratory stimulation caused by concanavalin A. The simplest explanation of these effects is that methylprednisolone dissolves in membranes and, at the high concentrations employed here, affects physicochemical membrane properties and the activities of membrane-associated proteins. The inhibition of Ca^{2+} and Na^{+} entry across the plasma membrane would explain the decrease in ATP use for plasma membrane ion cycling and the decrease in cytosolic free Ca^{2+} concentration, and a direct effect on the mitochondrial inner membrane would explain the observed increase in H^{+} permeability and the consequent partial uncoupling of oxidative phosphorylation. Concanavalin A activates substrate oxidation pathways and RNA/DNA synthesis in thymocytes, so the inhibition of these processes by

methylprednisolone could be secondary to its inhibition of the mitogenic rise in cytoplasmic Ca^{2+} concentration. Protein synthesis is much less activated by concanavalin A and seems to be relatively unaffected by methylprednisolone. Because these effects of methylprednisolone *in vitro* are seen at concentrations that might be experienced by cells of the immune system during clinical use of the glucocorticoid and would have the effect of diminishing and preventing the acute immune response, we suggest that they underlie the beneficial clinical effects of the drug.

In general, the use of top-down elasticity analysis in combination with quantification of ATP-consuming processes turned out to be powerful in identifying short-term effects of substances on energy metabolism. For this aim, concanavalin A-stimulated thymocytes represent a complex model especially useful for those studies.

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