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COMMENTARY

Therapeutically Targeting Lymphocyte Energy Metabolism by High-Dose Glucocorticoids

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ABSTRACT. Lymphocytes use a considerable amount of energy, mainly in the form of ATP, especially when they become stimulated following activation by antibodies or mitogens. Cellular respiration is the major energy source, and in quiescent cells the ATP produced is used to drive protein synthesis and sodium transport. In stimulated cells there is significantly higher ATP production to balance the higher ATP demand of specific processes resulting from activation. The major ATP-consuming processes under these conditions are protein synthesis and Na^+,K^+-ATPase (about 20% each), while Ca^{2+}-ATPase and RNA/DNA syntheses contribute about 10% each. There is a wealth of available information about glucocorticoid effects on lymphocytes, but here we focus on the extent to which this lymphocyte bioenergetic machinery is targeted by glucocorticoids when they are used therapeutically at high doses. High-dose glucocorticoids have been shown recently to interfere with processes that are essential for the activation and maintenance of lymphocytes, such as sodium and potassium transport. Therefore, in this article we describe the bioenergetics of lymphocytes in resting, activated, and glucocorticoid-treated states and present a concept for discussion to describe the relationship among these states in fundamental and clinical terms. BIOCHEM PHARMACOL **59**;6:597–603, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. quiescent and stimulated lymphocytes; energy metabolism; concanavalin A; mitogenic stimulation; high-dose glucocorticoids; therapeutic effects

As in all living cells, general housekeeping functions and specialised activities in lymphocytes depend directly or indirectly on the cellular energy supply, which is provided mainly in the form of ATP. This is true for the quiescent state, but is even more important when the cells become stimulated following activation by antibodies and lectins. The purpose of this commentary was not to collect all the data that have been published about lymphocyte activation (as has been done in excellent reviews [1–3]), but instead to focus on the reactions of cellular energy metabolism in this regard and to discuss the potential relevance of glucocorticoid effects on this machinery.

ATP-PRODUCING AND -CONSUMING PATHWAYS

Metabolism can be conceptually divided into reactions that provide or use energy. Free energy is released by either glycolysis or respiration and then distributed to energy-requiring reactions using intermediates such as ATP or other nucleoside triphosphates. Glycolysis produces relatively small amounts of ATP. In contrast, cellular respiration (oxidation of fuel molecules to drive oxidative phosphorylation) is the major energy source in aerobic organisms. Oxidative phosphorylation is the synthesis of ATP from ADP and P_i by mitochondria, driven by electron flow from a reduced substrate to oxygen. Electron transport is coupled to pumping of protons from the mitochondrial matrix, forming an electrochemical potential difference for protons across the mitochondrial inner membrane, known as the protonmotive force (Δp). Protonmotive force drives the protons back into the matrix through ATP synthase, forming ATP [4]. The main ATP consumers are the transport of cations and the synthesis of macromolecules [5–9]. The first accounting of ATP production and consumption in a respiring cell was made in rabbit reticulocytes [10], which lack DNA and RNA syntheses. About 70% of ATP consumption was accounted for by protein synthesis, Na^+,K^+-ATPase, and ATP- and ubiquitin-dependent proteolysis. In a more complex cell, the Ehrlich ascites tumour cell, about 80% of the total ATP consumption could be assigned, mostly to protein synthesis (30%) and Na^+,K^+-ATPase (20%), with RNA synthesis, ATP-dependent proteolysis, and Ca^{2+}-ATPase each contributing about 10% [11].

MITOCHONDRIAL PROTON LEAK

Not all mitochondrial oxygen consumption is coupled to ATP synthesis, since mitochondria show a significant

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TABLE 1. Oxygen consuming processes of quiescent and Con A-stimulated thymocytes

Oxygen consumption	Quiescent thymocytes	Con A-stimulated thymocytes
Total (nmol/min per 5×10^7 cells)	6.6 ± 1.0 (41)*	8.8 ± 0.6 (43)
Assigned to (%):		
Na^+K^+-ATPase	13.8 ± 2.0 (7)	19.3 ± 1.6 (6)
Ca^{2+}-ATPase	NS†	14.5 ± 1.9 (6)
RNA/DNA syntheses	NS	10.9 ± 1.3 (6)
Protein synthesis	15.1 ± 1.8 (6)	19.3 ± 1.9 (6)
ATP-dependent proteolysis	NS	NS
Mitochondrial proton leak	22.5 ± 5.2 (2)	18.1 ± 3.2 (3)

In quiescent lymphocytes, only protein synthesis and cation transport consume significant amounts of oxygen. In contrast to stimulated cells, oxygen consumption for RNA/DNA syntheses, ATP-dependent proteolysis, and Ca^{2+}-ATPase is not measurable. This, however, significantly comes into play a few seconds after mitogenic activation. The oxygen consumption to drive the mitochondrial proton leak is about the same under both conditions. Data from Ref. 7.

*Mean ± SEM; the number of cell preparations is given in parentheses.

†Not significant.

passive permeability to protons (termed "proton leak"), which is not an artifact of mitochondrial isolation since it has been demonstrated in mitochondria within isolated cells [12]. It is interesting that, first, the proton leak is higher in smaller mammals [13] and, second, mitochondrial proton permeability and leak flux depend on thyroid hormones [14]. Mitochondrial proton leak is an important component of cellular metabolism; its possible functions include thermogenesis, protection against reactive oxygen species, endowment of metabolic sensitivity, and maintenance of carbon fluxes [15].

ENERGY METABOLISM OF QUIESCENT LYMPHOCYTES

The energy metabolism of quiescent lymphocytes is not very complex. In quiescent thymocytes of the rat, only 50% of the coupled oxygen consumption could be assigned to specific processes [7]. Oxygen is used mainly to drive mitochondrial proton leak and to provide ATP for protein synthesis and cation transport, whereas oxygen consumption to provide ATP for RNA/DNA syntheses, ATP-dependent proteolysis, and Ca^{2+}-ATPase was not measurable (Table 1). The sink for the ATP produced by the remaining 50% of oxygen consumption remains unidentified, but might include futile cycling reactions. Similar results have been found for human peripheral blood lymphocytes (unpublished).

MITOGENIC ACTIVATION OF ENERGY METABOLISM

Several signal-transducing pathways have been described for lymphocyte activation. Antigens, mitogens, and other ligands can initiate a complex cascade of transmembrane signalling events involving different second messenger systems. These include pathways dependent on phospholipase C, protein kinase C, tyrosine kinases, and perhaps reactive oxygen species [16–18]. The lectin acts as a mitogen that preferentially activates T-cells. It stimulates the energy metabolism of thymocytes within seconds of exposure [19]. In general, lymphocyte activation includes energy-requiring events such as the early rise in intracellular calcium or the final increase of macromolecule synthesis.

How is energy metabolism in lymphocytes affected by these events? In rat thymocytes [7] and in human peripheral blood lymphocytes (unpublished), Con A* produces a persistent increase in oxygen consumption of up to 35% within seconds (Fig. 1). This reflects higher ATP production (via respiration) to balance the higher ATP demand of specific processes resulting from activation (Table 1).

But what, in detail, are the processes that demand ATP after exposure to Con A? The stimulation immediately allows more than 80% of respiration to be assigned to specific processes. The major oxygen-consuming processes of Con A-stimulated thymocytes are mitochondrial proton leak, protein synthesis, and Na^+,K^+-ATPase (about 20% each), while Ca^{2+}-ATPase and RNA/DNA syntheses contribute about 10% each [7] (Table 1). Quiescent thymocytes, like resting hepatocytes [20] and quiescent human peripheral blood lymphocytes (unpublished), have major unidentified ATP-consuming pathways that account for 50% or more of their oxygen consumption rate. In contrast, Con A-stimulated thymocytes [7], Con A-stimulated human peripheral lymphocytes (unpublished), Ehrlich ascites tumour cells [11], and (mechanically stimulated) splenocytes [6] substantially decrease these unidentified pathways, and 80% or more of their oxygen consumption drives known processes.

These results allow us to discuss mitogenic activation from an energetic point of view. They also spotlight events that are rather surprising in terms of their rapid onset following mitogenic activation.

Cation Transport

Primary activation by antigen receptors or mitogens is potentiated or modified by secondary signals. Lymphocytes become activated, and there are complex changes in ion

* *Abbreviation*: Con A, concanavalin A.

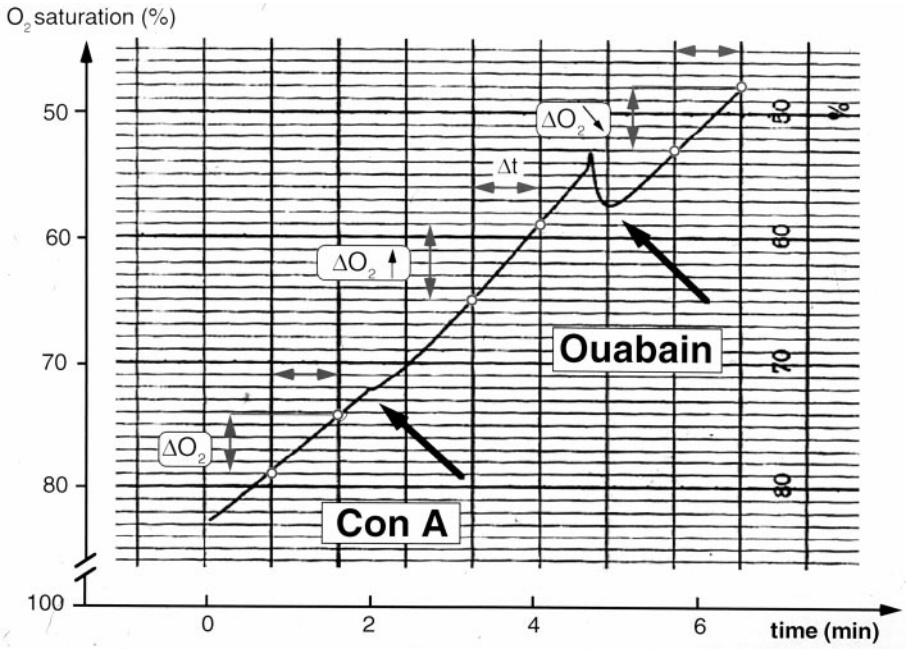

FIG. 1. Oxygen consumption of lymphocytes as stimulated by Con A and inhibited by ouabain (original trace measured using a Clark electrode). This figure shows an original trace of oxygen consumption in thymocytes measured amperometrically with a Clark electrode. The first part of the curve reflects the basal rate of oxygen consumption, calculated by dividing the change in oxygen saturation (ΔO_2) by the time (Δt). Addition of Con A leads to a significantly greater rate of oxygen consumption within seconds as reflected by the changed slope of the line. Addition of ouabain is accompanied by an artifact due to simultaneous addition of oxygen dissolved in the solvent, immediately followed by a slower steady rate of oxygen consumption. Since ouabain is a specific inhibitor of Na^+, K^+-ATPase, the decrease in oxygen consumption is a measure of the oxygen used in the previous state to provide the ATP for use by this enzyme.

permeabilities and fluxes across the plasma membrane and intracellular membranes and alterations in the intracellular concentrations of ions and their binding to membrane components and intracellular proteins. The underlying mechanism is the stimulus-induced opening or closing of ion channels, resulting in an increase in intracellular sodium concentration from 20 to 50 mM. This is necessarily followed by activation of energy-consuming ion pumps to attempt to restore the sodium and potassium gradients, since the pumps are sensitive to changes in the ionic composition of the cell and its environment [21]. Voltage-dependent ion transport systems and ion exchange antiport mechanisms also come into play to counter or amplify ionic processes [22, 23].

Although lymphocytes have this complex machinery to maintain ionic homeostasis at or near the state of the resting cell, the significant and immediate energy demands of the ATPases are of special interest. The Na^+,K^+-ATPase utilises ATP to pump sodium out of and potassium into the cell, maintaining the intracellular potassium concentration high compared with the extracellular concentration (roughly 120:5 mM) and the intracellular sodium concentration low (roughly 20:120 mM). Pump activity is stimulated by extracellular potassium and intracellular sodium. When lymphocytes are stimulated to proliferate, there is a rapid 2- to 3-fold increase in the pump rate [22] of the Na^+,K^+-ATPase, detectable within 3 min of stimulation with Con A. It lasts for 3 hr and is followed (by about 5 hr) by an increase in the number of Na^+,K^+-ATPase pumps in the membrane [22]. The initial increased energy demand of this enzyme can be estimated by specifically inhibiting it and measuring the immediate change in oxygen consumption (Fig. 1). This technique has been used successfully in several model systems and tested for validity in different ways [6, 7, 9, 11, 24–26]. The oxygen consumption to drive this enzyme in thymocytes is 0.9 units in the absence of the mitogen, but 1.7 units about 3 min after Con A addition [7] (Table 1).

Similar findings have been seen for Ca^{2+}-ATPase. No significant oxygen consumption can be assigned to this enzyme in quiescent cells, but after Con A stimulation it is responsible for about 15% of total oxygen consumption [7], showing that the pumping of calcium across the plasma membrane requires significant amounts of ATP only in the activated state. We will not discuss this issue in detail, since excellent reviews on the major role of increases in the concentration of cytoplasmic free calcium in the activation of lymphocytes are available [23, 27]. The key bioenergetic message is that only with higher cytoplasmic calcium concentrations following cellular activation is there a significant ATP requirement for calcium ion pumping.

Macromolecule Synthesis

Surprisingly, a significant increase in ATP use by macromolecule synthesis appears immediately after Con A stim-

ulation. Oxygen consumption for protein synthesis rises from 1.0 to 1.7 units, and oxygen consumption for RNA/DNA syntheses rises from undetectable to 1.0 unit within 3 min of Con A addition [7]. It is well known that mitogenic stimulation is followed by increased rates of macromolecule synthesis, but the reported effects are much later than those described here. Obviously, there is an immediate ATP demand by precursor processes (e.g. transport of amino acids across the plasma membrane) that are directly linked to the basic steps of transcription, translation, and replication.

Proton Leak

The proton leak is not changed significantly by Con A activation [7]. The relative invariance of this process can also be seen when ATP production is restricted, when proton leak turns out to be least sensitive [9]. This may reflect the importance the cell attaches to homeostasis of the protonmotive force (and the resulting constancy of the proton leak) despite changes in energy flow.

Other Processes

Even in stimulated cells about 20% of oxygen consumption remains uncharacterised. It comprises many minor ATP-consuming reactions, including those in signalling and activation (e.g. activities of kinases, phosphoinositide, and cyclic nucleotide metabolism, and arachidonate metabolism), futile cycles, and about 2% extramitochondrial oxygen consumption. Although phosphoinositide metabolism is of great importance in lymphocyte activation [28], its ATP requirement is obviously relatively low.

Hierarchy of ATP-Consuming Pathways

There is a hierarchy of energy-consuming reactions in Con A-stimulated cells: macromolecule biosynthesis (protein synthesis and RNA/DNA syntheses) is most sensitive to the ATP supply, followed by sodium cycling and then by calcium cycling across the plasma membrane. Mitochondrial proton leak is least sensitive to the energy supply [9]. This hierarchy is mostly as predicted by Atkinson [29], showing that if the ATP supply is compromised, processes not essential for the immediate needs of the cell will be given up before those that are more critical for ionic integrity.

GLUCOCORTICOID EFFECTS ON ENERGY METABOLISM

Glucocorticoids have profound anti-inflammatory and immunosuppressive actions when used therapeutically. The therapeutic dose is quite variable and depends on the disease, but ranges from very low (e.g. basal immunosuppressive low-dose treatment in autoimmune diseases) to extremely high (e.g. pulse therapy used to treat flares of autoimmune diseases). In general, the more severe the underlying disorder, the higher the dose of glucocorticoids.

What is the rationale for the use of various dosage regimens for specific clinical indications? There is evidence that glucocorticoids have three distinct therapeutically relevant effects: genomic, specific nongenomic, and unspecific nongenomic [30]. Genomic effects are mediated by cytosolic receptors that alter expression of specific genes (e.g. those encoding lipocortin-1, cyclooxygenase-2, and proinflammatory cytokines) after at least 30 min [31–33]. Specific nongenomic effects on second messenger systems occur within a few minutes and are mediated by steroid-selective membrane receptors [34]. Unspecific nongenomic effects occur within seconds, but only at high glucocorticoid dosages, and seem to result from direct interactions with biological membranes. We recently proposed that the additional therapeutic benefit of higher doses is obtained via these nongenomic effects [30].

We will focus here on the unspecific nongenomic effects on cellular energy metabolism that may be of clinical relevance in high-dose glucocorticoid therapy [8, 24, 26]. Clinically relevant concentrations of methylprednisolone (the glucocorticoid most commonly used for high-dose therapy) inhibit calcium and sodium cycling across the plasma membrane and decrease intracellular free calcium concentrations, but have little effect on protein synthesis [24]. The inhibition of cation cycling in Con A-stimulated thymocytes by the glucocorticoid is caused by direct effects and not by a reduction in ATP production [26], even though methylprednisolone reduces ATP availability to some extent by inhibiting the reactions of substrate oxidation and by increasing mitochondrial proton leak [8, 35].

The simplest explanation for these effects is that methylprednisolone dissolves in membranes and affects physicochemical membrane properties and the activities of membrane-associated proteins. The resulting inhibition of calcium and sodium entry across the plasma membrane would explain the decrease in ATP use for plasma membrane ion cycling and the drop in cytosolic free calcium, while a direct effect on the mitochondrial inner membrane would explain the observed increase in proton permeability and the consequent partial uncoupling of oxidative phosphorylation. Alternatively, fast-responding steroid-selective membrane receptors may be involved to mediate these glucocorticoid effects.

The effects of methylprednisolone are seen *in vitro* at concentrations that may be experienced by cells of the immune system during clinical use. They could diminish or prevent the acute immune response by interfering with processes, such as the rise in intracellular Ca^{2+} concentration, that are essential for the immediate and sustained activation of lymphocytes. These facts provide one possible explanation for the clinical observation that generally only high doses of glucocorticoids are successful in acute exacerbations of immunologically mediated diseases. The immediate effects produced by high doses could be additive to the effects mediated by nuclear receptors. The additional

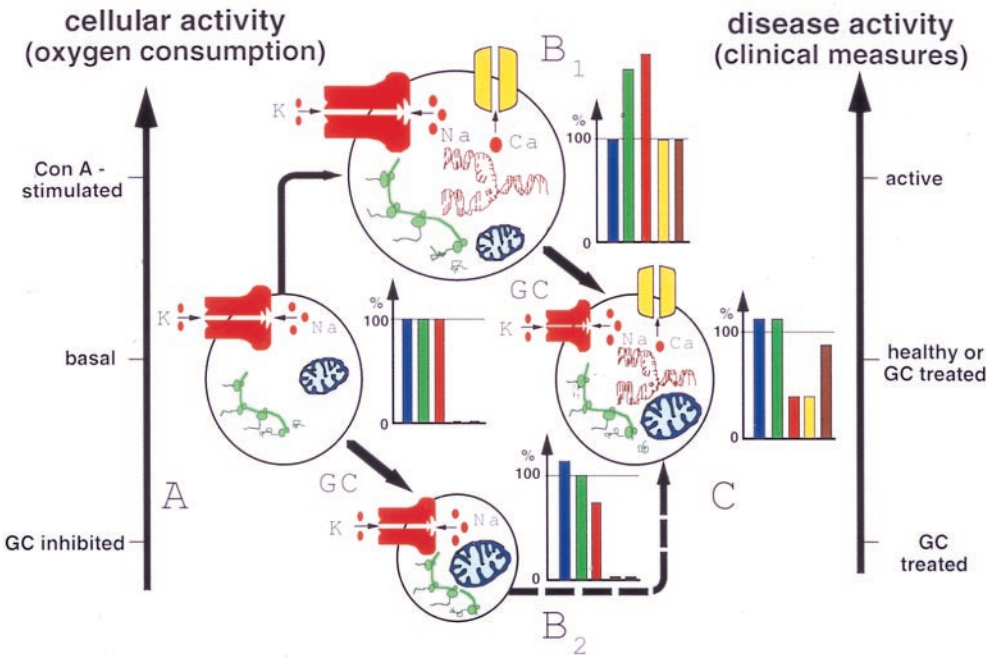

FIG. 2. Energy metabolism in quiescent, stimulated, and glucocorticoid-treated lymphocytes. This figure equates the bioenergetics of lymphocytes artificially stimulated by the mitogen Con A (left y-axis) to those that become activated during the pathogenesis of (auto)immunologically mediated diseases (right y-axis). (A) Quiescent cells = healthy state or the quiescent cells in a disease state; (B_1) mitogenically stimulated cells = disease activity; (B_2 and C) glucocorticoid-treated cells = therapy in case of disease activity. The inserted graphs show the activity of proton leak (blue), protein synthesis (green), Na^+, K^+-ATPase (red), Ca^{2+}-ATPase (yellow), and RNA/DNA syntheses (brown) in each state. GC: glucocorticoid.

quantum of inhibition of the excessive immune processes could make a crucial contribution to the therapeutic effect by helping to terminate the acute exacerbation [30]. In rheumatology and clinical immunology, acute phases or particularly severe forms of rheumatic diseases such as systemic lupus erythematosus, vasculitis, polymyositis, and rheumatoid arthritis are examples of the successful use of high-dose glucocorticoid therapy. Pulse therapy results in termination of the exacerbation or regression of a severe form of disease in a high proportion of cases with a relatively low incidence of side-effects. The mechanisms of action described here also provide an explanation for the beneficial therapeutic effect of high-dose glucocorticoid therapy for many other indications. These include the treatment of acute spinal injuries and use in immune thrombocytopenia, juvenile dermatomyositis, juvenile chronic arthritis, optic neuritis, rapid progressive glomerulonephritis, and pemphigus vulgaris.

CLINICAL IMPLICATIONS

We would like to give our personal view of the whole story of quiescent, stimulated, and glucocorticoid-treated lymphocytes by using a descriptive model that summarises and interprets in clinical terms (Fig. 2). At first glance the figure may look rather complicated, but what is its message?

Lymphocytes artificially stimulated by the mitogen Con A (left y-axis) are equated bioenergetically to those that become activated during the pathogenesis of (auto)immunologically mediated diseases (right y-axis). Quiescent cells (A) represent either the healthy state or the quiescent cell population in a diseased state. Activation by mitogen is equated to activation in disease, so mitogenically stimulated cells (B_1) are a model for the activated cell population in a diseased state. If high doses of glucocorticoid are applied therapeutically in the case of disease activity, or artificially *in vitro,* than both the activated (C) and the quiescent cell populations (B_2) are affected. The mitogen-activated, glucocorticoid-inhibited cells in (C) represent cells in the diseased state during high-dose glucocorticoid therapy.

What are the effects of high-dose glucocorticoid treatment on lymphocytes in patients with immunologically mediated disorders? If activated cells (B_1) are treated with high doses of glucocorticoids, their activity is strongly reduced, which is the therapeutically desired effect. However, the treated state (C) is not the same as the untreated, resting state (A) even if the oxygen consumption is the same: processes critical for the initiation and maintenance of lymphocytic activation are partially inhibited in (C). This inhibition is compensated for bioenergetically by relatively higher rates of protein synthesis and proton leak. These two processes may be of less clinical relevance, but they could be the reason for some side-effects observed in patients who have received very large doses of a glucocorticoid intravenously. The same arguments are relevant for patients with spinal cord injury, where high-dose glucocor-

ticoid effects may stabilise cellular membranes, preventing edema and secondary destructive mechanisms.

What is the consequence of treating quiescent cells with high doses of glucocorticoids (B_2)? This is the usual clinical situation, since all immune cells, regardless of their level of activation, experience a drug that has been administered. We have shown in thymocytes [29] that glucocorticoid pretreatment prevents the Con A-induced increase of oxygen consumption and rise in cytosolic Ca^{2+}. Consequently, a crucial effect may be that quiescent glucocorticoid-treated cells are less likely to become activated during the course of the disease.

Thus it appears that high-dose glucocorticoids can antagonise, but not completely reverse, the changes in energy metabolism induced by activation of lymphocytes. This might explain the beneficial therapeutic effects. On the other hand, these observations may explain why glucocorticoids are sometimes only partially effective, and may explain the side-effects that occur. In bioenergetic terms it may not matter for a single cell whether the activation or the glucocorticoid treatment comes first; the same changes are evident at the end. This might be why glucocorticoids are successful in therapy despite their lack of selectivity for activated cells.

These considerations also apply to high-dose glucocorticoid therapy with other glucocorticoids. We have shown that the glucocorticoids prednylidene, dexamethasone, prednisolone, and betamethasone produce the same effects as methylprednisolone, but with huge differences in their potencies [36].

OPEN QUESTIONS

Of course, there are important questions open for further investigating high-dose glucocorticoid therapy, and experimental effort is being made currently to solve them.

First, the mechanisms of nongenomic effects of glucocorticoids need to be defined further. This would include the detection and cloning of the proposed membrane receptors as well as the definition of the physico-chemical interaction of high-dose glucocorticoids with membranes.

A second issue that is being examined currently in this regard is: How long do these acute effects last? At the moment, these effects are considered to be evident only in the presence of high doses, and they probably fade away if the dose is tapered. This conclusion comes from clinical experience rather than from experimental work, since high-dose therapy is used only in temporary and time-limited ways, e.g. in spinal cord injury to prevent edema or in acute immunologically mediated disorders to produce additional effects and/or to bridge the gap until genomic effects are maximal, as we have discussed above.

Third, apart from apoptotic events less is known currently about long-term effects of high glucocorticoid doses on immunity. Are there any immune parameters that are affected significantly once immune cells have been exposed to glucocorticoids in a time- and dose-dependent manner that is produced by high-dose therapy? Are there any differences in this regard from cells being treated with low-dose glucocorticoids?

Finally, are all cells influenced equally? The preliminary answer is yes, as we have shown these effects not only in thymocytes, but also very similarly in Ehrlich ascites tumour cells [25] and human lymphocytes (unpublished). Also, different groups have shown that high doses of glucocorticoids increase the resistance of red cells to hypoosmotic hemolysis or decrease granulocyte membrane fluidity, to name a few other examples. These effects need to be further evaluated in terms of their clinical relevance. This may apply to clinically relevant side-effects (cardiac arrhythmias due to effects on cation transport?) as well as to therapeutically beneficial effects. As a recent example to illustrate the broad spectrum in this regard, it has been shown that antepartum administration of high-dose dexamethasone in pregnancies complicated by the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets) is therapeutically helpful, and nongenomic effects may well be involved [37].

CONCLUSIONS

Lymphocytes require sufficient energy to maintain cellular integrity and basal metabolism. This energy supply is crucial for lymphocytes that enter the activated state following stimulation by antibodies and lectins, when a significantly increased ATP requirement for cation transport and macromolecule synthesis becomes evident. In addition to their well-known genomic effects, high doses of glucocorticoids interfere, via nongenomic pathways, with processes of energy metabolism crucial for the immediate and sustained activation of lymphocytes. Problems that need to be sorted out in this regard in the future involve the mechanistic background, the time course, the long-term impacts on immunity, and the cellular specificity of these nongenomic glucocorticoid effects.

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