Basic and Clinical Implications of Glucocorticoid Action – Focus on Development
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Glucocorticoids · Glucocorticoid receptor · Development · Stress

Introduction

Glucocorticoids regulate multiple metabolic and developmental processes and play a vital role in maintaining basal and stress-related homeostasis. During the last 50 years, pharmacologic doses of glucocorticoids have been used in the treatment of inflammatory, autoimmune, and lymphoproliferative diseases, and in the prevention of allograft rejection, while substitution doses have been employed in the management of adrenocortical insufficiency [1,2]. At the cellular level, the actions of glucocorticoids are mediated by an intracellular receptor protein, the glucocorticoid receptor (GR), which functions as a hormone-activated transcription factor that regulates the expression of glucocorticoid target genes [3–5].

The human (h) GR gene consists of 9 exons and is located on chromosome 5. Alternative splicing of the hGR gene in exon 9 generates two highly homologous receptor isoforms termed α and β. hGRα is ubiquitously expressed in almost all human tissues and cells, resides primarily in the cytoplasm, and represents the classic glucocorticoid receptor that functions as a ligand-dependent transcription factor. Upon hormone binding, hGRα translocates into the nucleus, where it modulates the transcription of target genes either by binding to glucocorticoid-response elements (GREs) [GRE-mediated actions] as a homodimer or by physically interacting as a monomer with other transcription factors such as activating protein-1 (AP-1) and nuclear factor-κB (NF-κB) [6].

The following abstracts were presented at the National Institutes of Health Conference on the “Basic and Clinical Implications of Glucocorticoid Action – Focus on Development” held at the NIH on June 17 – 18, 2003. These abstracts present the most recent advances in the basic and clinical aspects of glucocorticoid action, and in particular: i) The impact of maternal and early-life stress on stress-related gene regulation in the offspring [7]; ii) The importance of glucocorticoids and their receptors; iii) Further understanding of the mechanisms of GR action, including its effect on chromatin modulation, its interaction with co-activators and co-repressors, and genetic dissection of GR function in mice; iv) The interaction of GR with other transcription factors such as NF-κB, p53, transforming growth factor β (TGFβ) and chicken ovalbumin upstream promoter transcription factor I (COUP-TFI); v) Factors modulating GR function, including phosphorylation, recycling, ubiquitination and degradation of the receptor, actions of GRβ isofrom, a novel synthetic non-steroidal target gene-specific agonist, the importance of target tissue activity of 11β-hydroxysteroid dehydrogenase type 1 in glucocorticoid action in health and disease, the interaction of the receptor with the nutrient carnitine, anthrax products protective antigen (PA) and lethal factor (LF), and the Human Immunodeficiency Virus type-1 (HIV-1)-encoded molecules Vpr and Tat; vi) An update on the effects of glucocorticoids on the immune system; and vii) Clinical implications of glucocorticoid action, including glucocorticoid resistance/hypersensitivity, familial and sporadic glucocorticoid resistance, and effects of stress and depression.
The conference was organized in memory of the late Dr. Yukitaka Miyachi (March 2, 1939 – January 23, 2003), former Professor and Chairman of the Department of Internal Medicine at the Toho University School of Medicine, Tokyo, Japan.

The abstracts are listed in the order at which they were presented at the conference.

Contributors

Dr. M. J. Meaney: Maternal care permanently alters DNA structure and thus programs gene expression in brain regions that regulate stress responses
Dr. G. L. Hager: Nuclear receptor mobility on gene targets
Dr. S. S. Simons Jr.: Modulation of glucocorticoid receptor activities by coactivators and corepressors
Dr. J. I. Webster: Novel regulation of nuclear hormone action: Anthrax lethal factor represses glucocorticoid and progesterone receptor activity
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Dr. Dr. C. R. Yates: Effect of glucocorticoid administration on glucocorticoid receptor and NF-κB interaction in unresolved ARDS
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Dr. T. D. Gelehrter: Glucocorticoid and TGFβ crosstalk in the regulation of PAI-1 gene expression
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Dr. M. J. Garabedian: Modulation of glucocorticoid receptor function via phosphorylation
Dr. J. A. Cidlowski: The human glucocorticoid receptor: Molecular mechanisms controlling the termination of glucocorticoid responses
Dr. J. N. Miner: A non-steroidal, glucocorticoid receptor target gene-specific agonist
Dr. D. Franchimont: Panoramic view of glucocorticoid actions on the immune response: A good model to understand immunosuppression for new treatment strategies
Dr. I. J. Elenkov: Glucocorticoids, immunity and the T helper 1/T helper 2 balance
Dr. J. Seckl: 11β-Hydroxysteroid dehydrogenase type 1: An explanation of the metabolic syndrome – Cushing’s disease paradox?
Dr. S. Alesci: Carnitine: A nutritional modulator of glucocorticoid receptor function
Dr. T. Kino: Possible tissue glucocorticoid hypersensitivity in Human Immunodeficiency Virus type-1 (HIV-1) infection
Dr. E. Charmandari: Natural glucocorticoid receptor mutants causing generalized glucocorticoid resistance: Molecular genotype, genetic transmission and clinical phenotype
Dr. G. P. Chrousos: Glucocorticoid resistance and hypersensitivity states. Genetic and developmental aspects
Dr. P. Martinez: A prospective study of the offspring of depressed parents: An extension of the Barker hypothesis

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Maternal Care Permanently Alters DNA Structure and thus Programs Gene Expression in Brain Regions that Regulate Stress Responses

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Variations in maternal care in early postnatal life influence the development of individual differences in HPA responses to stress. Specifically, the adult offspring of mothers that exhibit increased levels of pup licking/grooming (HIGH LG) show increased hippocampal glucocorticoid receptor expression, enhanced corticosteroid negative feedback sensitivity, decreased hypothalamic CRF, and dampened pituitary-adrenal responses to stress. Maternal LG alters glucocorticoid receptor expression through a series of serotonin (5-HT) regulated pathways that involve the activation of cAMP/PKA signal transduction systems, with the subsequent increase in NGF-A expression. NGF-A binds to a consensus sequence on a neuron-specific promoter (exon 17 of the glucocorticoid receptor gene). This consensus sequence has two CpG dinucleotides (51 and 31) and in adult offspring of Low LG mothers the 51 CpG site is invariably methylated (on the cytosine), which blocks the ability of NGF-A to interact with its consensus sequence and thus activate glucocorticoid receptor expression. In the offspring of High LG mothers this same site is hypomethylated. Chromatin immunoprecipitation
assays confirm increased in vivo binding of NGFI-A to the exon 17 promoter in the adult offspring of High LG mothers. Methylation suppresses gene expression through a series of protein–protein interactions that involve histone deacetylase (HDAC). When infused into the brain of the adult offspring of Low LG mothers, trichostatin A (TSA) an HDAC inhibitor, permits an increase in glucocorticoid receptor expression, presumably by removing the blockade of expression, and renders HPA responses to stress indistinguishable from the adult offspring of High LG mothers. These findings clarify a pathway that leads from maternal care to gene function and, potentially, vulnerability/resistance to stress-induced illness.

**Nuclear Receptor Mobility on Gene Targets**

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Nuclear receptors exert their physiological effects largely through the modulation of gene expression rates for responsive genes in target tissues. Until recently, the mechanisms of transcription regulation have been modeled in terms of interactions between soluble members of the regulatory apparatus and the DNA template. We now know that a key feature of receptor action lies in the epigenetic modification of the nucleoprotein template. Receptors recruit many activities that change local chromatin structure, and these alterations in turn are central to the resulting activation or suppression of gene activity.

A central concept in endocrine biology has been that hormone-bound receptors bind their cognate response element and serve as a platform to recruit the many coactivators and corepressors that participate in modulating transcription rates. Through the use of novel imaging techniques, we observed direct steroid receptor binding to a tandem array of a hormone-responsive promoter in living cells. We unexpectedly found that the glucocorticoid receptor (GR) exchanges rapidly with regulatory elements in the continued presence of ligand [1]. Using fluorescence recovery after photobleaching (FRAP) we have now examined the dynamic behavior of several green fluorescence protein (GFP) tagged transcription factors at the promoter tandem array. The glucocorticoid receptor interacting protein 1 (GRIP1) exhibits a half maximal time for fluorescent recovery of 5 sec, the same rapid exchange as observed for GR. In contrast, the large subunit of RNA polymerase II (RPB1) shows a very slow exchange, requiring thirteen minutes for complete fluorescence recovery.

We have also reconstituted the GR-dependent nucleoprotein transition with chromatin assembled on promoter DNA. The remodeling event is ATP-dependent, and requires either a nuclear extract from HeLa cells or purified human SWI/SNF [2]. Through the use of a direct interaction assay (magnetic bead “pull-down”), we demonstrate recruitment of human SWI/SNF to promoter chromatin by GR [3]. We find that GR is actively displaced from the chromatin template during the remodeling process. Displacement requires the presence of ATP, can be reversed by the addition of apyrase, and is specific to chromatin templates. The disengagement reaction can be induced with purified human SWI/SNF, but the reaction occurs less efficiently, and is further stimulated by the inclusion of nuclear extract. Binding of the secondary transcription factor, NF-1, to the promoter is facilitated by GR-induced chromatin remodeling.

Using the in vitro template pull down assay, we also demonstrate that progesterone receptor (PR) binding is dynamic on MMTV chromatin. Both PR-A and PR-B liganded with R5020 are actively displaced from MMTV chromatin in the presence of either a HeLa nuclear extract or purified SWI/SNF and ATP. We have examined the effect of different classes of PR antagonists on this displacement reaction. We find that PR liganded to the agonist R5020 and the antagonist RU486 is actively displaced from chromatin during remodeling. However, PR liganded with the antagonist ZK98299 is not displaced from chromatin. We further examined the ability of PR bound to different ligands to recruit BRG1 MMTV chromatin, and our findings indicate that PR ligands exert a differential effect on the ability of the receptor to support dynamic exchange during chromatin remodeling.

Both the in vitro and in vivo results are consistent with a dynamic model (“hit and run”) in which GR first binds to chromatin after ligand activation, recruits a remodeling activity, and is simultaneously lost from the template. These findings suggest a new model for nuclear receptor function [4] in which the receptors reside on response elements in chromatin only for brief periods, then return to the template for successive binding events. We further observed that the template undergoes a cascade of modification events, which are triggered by the initial receptor binding event. We suggest that the receptor may recruit different co-factors during successive “return-to-template” events, and thus participate in biochemically distinct processes during each binding cycle. This view represents a major departure from classic endocrine models, which envisage the receptor as statically bound to responsive promoters in the continued presence of hormone.

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Modulation of Glucocorticoid Receptor Activities by Coactivators and Corepressors

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A major response of steroid receptors to steroid hormones is the induction of gene transcription. Two other physiologically relevant, but less studied, properties are the EC_{50} of receptor-agonist complexes and the partial agonist activity of receptor-antagonist complexes. Agonists are ligands that mimic the activity of endogenous steroid hormones. The dose-response curve of agonists gives the amount of gene induction by any concentration of steroid, with half of the maximal induction occurring at a value called the EC_{50}. The lower the EC_{50} of a given gene, the greater is the level of induction that is achieved with the circulating concentration of steroid in a cell or animal (Fig. 1). Antisteroids, or antagonists, block the action of agonist steroids. However, virtually all antisteroids display some partial agonist activity with selected genes. Recently, it has been appreciated that if one can selectively eliminate the trans-activation of a target gene, while reducing the expression of most of the other genes that are regulated by a given receptor (Fig. 1), then the number of undesirable side effects that usually result from the indiscriminate repression of all responsive genes will be greatly diminished.

Contrary to earlier expectations, accumulating experiments indicate that the value of neither the EC_{50} nor the partial agonist activity is constant for a given receptor-steroid complex. Our recent experiments with glucocorticoid (GR), progesterone (PR), and mineralocorticoid (MR) receptors in transiently transfected cells indicate that changing the concentrations of a variety of factors causes the position of the dose-response curve with agonists, and the amount of partial agonist activity of antagonists, to display a continuum of values over a broad range. Two sets of factors that have been extensively examined with GRs are the p160 coactivators (SRC-1, TIF2, and AIB1) and the corepressors (SMRT and NCoR). These studies have led to the two hypotheses (1) that coactivators and corepressors each bind to both GR-agonist and -antagonist complexes and (2) that the final biological response is influenced by the intracellular ratio of coactivators to corepressors (Fig. 2).

In an effort to obtain biochemical support for hypothesis #1 of our model, we examined the ability of coactivators and corepressors to interact with GR complexes in mammalian two-hybrid

Fig. 1. Variations in dose-response curves and partial agonist activity among different genes induced by the same receptor steroid complex.

Fig. 2. Equilibrium model for modulation of GR-agonist and -antagonist activities with varying concentrations of coactivators and corepressors.
and cell-free pulldown assays. Both agonist and antagonist complexes associate with the coactivator TIF2 in a manner that depends upon the presence of an intact receptor interacting domain (RID) in TIF2. Conversely, both agonist and antagonist complexes of GR also combine with the corepressors NCoR and SMRT. The ability of GR-agonist complexes to interact with corepressors was confirmed by the observation that mutating the RIDs of NCoR eliminated the mammalian two-hybrid interactions with both GR-agonist and antagonist complexes. Furthermore, the association of GR-agonist complexes was also seen with the wild type GR instead of the VP16/GR chimera that was used in the other two-hybrid experiments. Therefore, we conclude that GR-agonist complexes do bind to corepressors and that GR-antagonist complexes bind to coactivators.

In support of hypothesis #2 of our model, we found that overexpressed TIF2 and corepressors each competitively inhibit the association of GR with corepressors or coactivators in the mammalian two-hybrid assay. Furthermore, the position of the GR dose-response curve and the partial agonist activity of GR-antagonist complexes could be modulated simply by overexpressing thyroid receptor beta 2 thyroid hormone, thereby altering the ratio of endogenous coactivators to corepressors. In summary, we believe that our data support a model in which the dose-response curve and partial agonist activity of GR complexes can be modulated in an equilibrium manner by the competitive binding of coactivators and corepressors to both GR-agonist and antagonist complexes.

Novel Regulators of Nuclear Hormone Action: Anthrax Lethal Factor Represses Glucocorticoid and Progesterone Receptor Activity

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Death by anthrax is reported to result from systemic shock resembling lipopolysaccharide-induced shock, although the precise mechanisms are not understood. Anthrax lethal toxin (LeTx) is composed of two proteins, protective antigen (PA) and lethal factor (LF). PA binds to a cell surface receptor and allows entry of LF into the cell. This mechanism of entry into the cell is fairly well understood. However, the actions of LF once inside the cell are not so clear. LF has been shown to be a metalloprotease that cleaves members of the MAP kinase family. Much research has focused on this cleavage and inactivation of MAPKs, however, this MAPKK cleavage has been shown to be similar in LeTx sensitive and resistant cells.

Fischer (F344/N) rats have long been known to be extremely sensitive to LeTx with death occurring within 40 minutes after exposure to a lethal dose. F344/N rats are also known to exhibit a hyper-HPA axis and are resistant to autoimmune/inflammatory diseases. Intact glucocorticoid responses have been shown to be critical for survival from several proinflammatory or infectious insults, such as streptococcal cell walls (SCW), MCMV virus or Shiga toxin. Intervention of the HPA axis at any point results in increased mortality rates that can be subsequently reversed by glucocorticoid administration. We, therefore, hypothesized that the lethal toxin might act as a glucocorticoid repressor, thereby removing the protective anti-inflammatory properties of an intact glucocorticoid response.

We found that the fully active anthrax lethal toxin (LeTx), LF + PA, represses dexamethasone-induced glucocorticoid receptor (GR) transactivation in a transient transfection system at very low concentrations (Fig. 1). An LF mutant that is catalytically inactive was unable to repress GR, suggesting that the protease activity of LF may be required for GR repression. The activity of the endogenous GR-regulated gene, the liver enzyme tyrosine aminotransferase, was similarly repressed by LeTx in both a cellular system and in vivo in BALB/cj mice. This repression is non-competitive and does not affect ligand binding or DNA binding, suggesting that anthrax lethal toxin (LeTx) probably exerts its effects through a cofactor(s) involved in the interaction between GR and the basal transcription machinery.

This LeTx – nuclear hormone receptor repression is also selective, repressing GR, progesterone receptor B (PR-B) and estrogen receptor alpha (ERα), but not the mineralocorticoid receptor (MR) or ERβ. This is the first report of a bacterial product directly repressing the activity of GR. As known targets of LeTx are members of the MAPK family including MEK1/2 and MKK3/6 we tested the ability of inhibitors of the MAPK pathways to repress GR. Selective MEK/ERK inhibitors and JNK inhibitors had no effect on GR mediated transactivation. However, selected p38 MAP kinase inhibitors did repress GR in this transient transfection system, suggesting that the LeTx action may result in part from its known inactivation of MAP kinases.

A simultaneous loss of GR and other nuclear receptor activities could render an animal more susceptible to lethal or toxic effects of anthrax infection by removing the normally protective anti-inflammatory effects of these hormones, similar to the increased...
Glucocorticoids and mineralocorticoids are involved in numerous physiological processes important to maintain metabolic, cardiovascular, central nervous and immune system homeostasis. Germ line and somatic gene targeting allows the characterization of the various functions and molecular modes of action of these receptors. Most of the effects of the glucocorticoid receptor are mediated via activation and repression of gene expression. To separate activities of the GR, a point mutation was introduced which allowed to characterize and distinguish functions dependent on GR-binding to DNA from those mediated by protein/protein interaction leading to gene repression as well as gene activation. Cell/tissue-specific mutations of the glucocorticoid receptor is the basis for the evaluation of their cell-specific functions and characterization of target genes by expression profiling. The GR gene was inactivated in parenchymal cells of the liver, in thymocytes, monocytes/macrophages, skin and brain, respectively. The absence of GR in hepatocytes leads to a dramatic reduction of body size. We could show that growth hormone (GH) signalling mediated by STAT5 in hepatocytes is impaired in these mice leading to lower synthesis of IGF-1 and other STAT-dependent mRNAs. Interestingly, dimerization-defective GR mice show wild-type levels of these mRNAs and have a normal body size. These findings strongly suggest a model in which GR functions as a co-activator for STAT5 transcription upon GH stimulation and reveal an essential role of GC signalling through hepatic GR in the control of body growth. The MR is expressed in many regions of the brain with particular high levels in neurons of the hippocampus. Inactivation of MR in the forebrain leads to impaired learning and memory. A comparison of GR and MR functions in the brain will also be discussed.

**Effects of Glucocorticoid Administration on Glucocorticoid Receptor and NF-κB Interaction in Unresolving ARDS**

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Excessive systemic inflammation is the pathophysiologic hallmark of pulmonary and extrapulmonary organ dysfunction in patients with acute respiratory distress syndrome (ARDS), a frequent cause of hypoxemic respiratory failure associated with a 40–60% mortality. Failure to down-regulate inflammatory mediators is associated with maladaptive lung repair and inability to improve gas exchange and lung mechanics (clinically defined as unresolving ARDS). In immunohistochemical analyses of lung tissue obtained from patients with unresolving ARDS, we found that histologically severe ARDS was associated with predominant nuclear NF-κB uptake in resident cells, while mild ARDS was associated with predominant nuclear GRα uptake.

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NF-κB activation is critical to the experimental development of lung inflammation. NF-κB is found in essentially all cell types and is involved in activation of numerous target genes. Heterodimeric p65:p50 is the most abundant form of NF-κB in most cell types. NF-κB is maintained in an inactive form in the cytoplasm through interaction with inhibitory proteins IκBαs (most important being IκBα) (Fig. 1). In response to various inflammatory signals, the latent NF-κB/IκB complex is activated by phosphorylation and proteolytic degradation of IκB, with exposure of the NF-κB nuclear localization sequence (NSL). The liberated NF-κB then translocates to the nucleus and binds to promoter regions of target genes to initiate the transcription of numerous inflammatory genes including tumor necrosis factor-alpha (TNF-α) and the interleukins (IL) IL-1β, IL-2, IL-6, chemokines (e.g., IL-8), and cell adhesion molecules (e.g., intercellular adhesion molecule-1, E-selectin).

As a dimer and/or a monomer, GR-mediated transcriptional interference with NF-κB is achieved by five important mechanisms (Fig. 1): (i) physically interacting with the p65 subunit and formation of an inactive (GR-NF-κB) complex, (ii) by inducing the transcription of the inhibitory protein IκBα gene (iii) by blocking degradation of IκBα via enhanced synthesis of IL-10, (iv) by impairing TNF-α induced degradation of IκBα, and (v) by competing for limited amounts of GR co-activators such as CREB-binding protein (CBP) and steroid receptor coactivator-1 (SRC-1).

Using an ex vivo model of systemic inflammation, we investigated intracellular upstream and downstream events (cytosolic and nuclear) associated with DNA-binding of NF-κB and GRα in PBLs (naive cells) obtained from a healthy volunteer and incubated for 3 hours with plasma samples obtained longitudinally from patients with unresolving ARDS before and after randomization prolonged methylprednisolone (MP) vs. placebo administration. In the plasma, we measured the concentrations of TNF-α and IL-1β, ACTH and cortisol. In the observation period prior to randomization, the biologic and physiologic characteristics of the two groups were similar. Patients had persistent elevations in plasma TNF-α, IL-1β, ACTH and cortisol levels, and similar severity of organ dysfunction scores. The intracellular changes observed in exposed normal PBLs included escalating increases in NF-κB-mediated activities (NF-κB DNA-binding, p50 and p65 DNA-binding, and transcription of TNF-α and IL-1β) and modest changes in GRα-mediated activities. The reduction in cytoplasmic IκBα levels observed prior to randomization indicated that NF-κB-mediated IκBα degradation predominated over GRα-mediated IκBα formation.
Following randomization, MP-treated patients had progressive and sustained reductions of TNF-α, IL-1β, ACTH and cortisol levels over time. Normal PBL exposed to plasma exhibited significant progressive increases in all aspects of GR-mediated activity and significant reductions in NF-κB DNA-binding and transcription of TNF-α and IL-1β. Patients treated with MP had rapid, progressive and sustained reductions in plasma TNF-α, IL-1β, IL-6, ACTH and cortisol levels over time, which paralleled improvements in pulmonary and extrapulmonary organ dysfunction scores. Normal PBL exposed to plasma collected during MP vs. placebo treatment exhibited rapid, progressive significant increases in GRα-mediated activities (GRα binding to NF-κB, GRα binding to GRE DNA, stimulation of inhibitory protein IkBα, and stimulation of IL-10 transcription), and significant reductions in NF-κB binding and transcription of TNF-α and IL-1β (Fig. 2). With MP, the intracellular relations between the NF-κB and GRα signaling pathways changed from an initial NF-κB-driven and GR-resistant state to a GR-driven and GR-sensitive one. The responses observed during MP support the concept of inflammation-dependent acquired glucocorticoid resistance in patients with unresolved ARDS and underscore the central role played by the activated GRα in regulating NF-κB–driven inflammation.

Physiologic and Pathologic Consequences of the Interaction of Glucocorticoid Receptor with p53

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The tumour suppressor p53 and the glucocorticoid receptor (GR) respond to different types of stress. We found that dexamethasone-activated endogenous and exogenous GR inhibit p53 dependent functions, including trans-activation, up- (Bax and p21WAF1/CIP1) and down- (Bcl2) regulation of endogenous genes, cell cycle arrest and apoptosis. GR forms a complex with p53 in vivo, resulting in cytoplasmic sequestration of both p53 and GR.

In neuroblastoma (NB) cells, cytoplasmic retention and inactivation of wild-type p53 involves GR. p53 and GR form a complex that is dissociated by GR antagonists, resulting in accumulation of p53 in the nucleus, activation of p53 responsive genes, growth arrest and apoptosis. These results suggest that molecules that efficiently disrupt GR-p53 interactions would have a therapeutic potential for the treatment of neuroblastoma and perhaps other diseases in which p53 is sequestered by GR.

Using normal endothelial cells (HUVEC), we studied the mechanism of the mutual inhibition of GR and p53 in response to hypoxia, a physiological stress, and mitomycin C that damages DNA. Dexamethasone (Dex) stimulates the degradation of endogenous GR and p53 by the proteasome pathway in HUVEC under hypoxia and mitomycin C treatments, and also in hepatoma cells (HepG2) under normoxia. Dex inhibits the functions of p53 (apoptosis, Bax and p21WAF1/CIP1 expression) and GR (PEPCK and G-6-Pase expression). Endogenous p53 and GR form a ligand dependent trimeric complex with Hdm2 in the cytoplasm. Disruption of the p53-HDM2 interaction prevents Dex induced ubiquitylation of GR and p53. The ubiquitylation of GR requires p53, the interaction of p53 with Hdm2, and Hdm2’s E3 ligase activity. These results provide a mechanistic basis for GR and p53 acting as opposing forces in the decision between cell death and survival (Fig. 1).

p53 stimulates hematopoietic cell maturation and antagonises GR activity in hypoxia, suggesting that it may inhibit stress erythrocytosis. We report that mouse foetal liver cells that lack p53 proliferate better than wild type cells in the presence of the GR agonist dexamethasone. An important mediator of GR induced ebl self-renewal, the c-myb gene, is induced to higher levels in p53—/— ebls by dexamethasone. The stress response to anaemia is faster in the spleens of p53—/— mice, as shown by the higher levels of CFU-Es (colony forming units–erythroid) and the increase in the CD34/c-Kit double positive population. Our results show that p53 antagonises GR mediated ebl expansion and demonstrate for the first time that GR-p53 cross-talk is important in a physiological process in vivo, stress erythrocytosis.

Glucocorticoid and TGFβ Crossstalk in the Regulation of PAI-1 Gene Expression

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Transforming growth factor β (TGFβ) is a major regulator of extracellular matrix (ECM) deposition and a potent inducer of type-1 plasminogen activator inhibitor (PAI-1) gene expression. Glucocorticoids inhibit TGFβ-induced expression of ECM proteins including fibronectin, collagen, TIMPS and PAI-1, and thus have an important opposing role in wound healing and fibrosis. We have reported previously that liganded glucocorticoid receptor (GR) represses TGFβ transactivation of PAI-1 in Hep3B human hepatoma cells, and that it interacts functionally and physically with the C-terminal activation domain of Smad3, a major mediator of TGFβ signaling (Song et al. Proc Natl Acad Sci USA 96 : 11776, 1999).

To identify the domain(s) in the glucocorticoid receptor involved in repression of TGFβ signaling, we have examined the ability of various GR truncation, deletion, and substitution mutants to repress TGFβ transactivation in Hep3B human hepatoma cells, which lack functional endogenous GR. Partial deletions in the ligand binding domain (LBD), including the t2 and tC (AF2) re-
gions, greatly reduced or eliminated GR repression, while dele-
tion of the N-terminal AF1 (τ1) domain, and substitution muta-
tions in the DNA binding domain (DBD) had little or no effect. Li-
ganded androgen receptor repressed TGF-β transactivation as
strongly as GR, whereas mineralocorticoid receptor (MR) did
not. Studies with rat GR-MR chimeras confirmed that the GR C-
terminal domains, but not the N-terminal domains, were requir-
ed for repression. RU486, a strong antagonist of transactivation
by GR, only partially reversed repression by wild-type GR, and
was itself a partial agonist for repression.

Co-immunoprecipitation experiments in Hep3B cells demon-
strated that all GR mutants that repressed TGFβ transactivation
interacted physically, in vivo, with endogenous Smad3. However,
most GR mutants that did not repress also interacted with
Smad3, suggesting that physical interaction between GR and
Smad3 is necessary but not sufficient for repression. GST pull-
down assays confirmed these results and demonstrated that sev-
eral regions of the LBD were sufficient for GR-Smad3 physical in-
teraction in vitro. Interestingly, the physical interaction required
activation of Smad3 by TGFβ, but not dexamethasone binding to
GR. In contrast, glucocorticoid binding to GR was necessary for
transrepression. However, substitution of alanine for a conserved
serine in the s2 region of the LBD blocked transrepression even
though it did not interfere with dexamethasone binding; thus,
the s2 domain may play an important role in transrepression in-
dependent of its role in glucocorticoid binding.

We conclude that the LBD of GR is required for GR-mediated
transrepression of TGFβ transactivation. Unlike the GR transre-
pression of AP-1 or NFkB, in which the DBD of GR is essential,
the GR DBD is dispensable in GR transrepression of TGFβ signal-
ing. Ligand binding to GR is necessary but not sufficient for trans-
repression of TGFβ activation of PAI-1 gene expression. These re-
sults may have important implications for the development of
“dissociated steroids” for the therapy of chronic inflammatory
and fibrotic diseases.

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A New Regulator of Glucocorticoid Receptor Transcriptional
Activity: The Chicken Ovalbumin Upstream Promoter
Transcription Factor II (COUP-TFI)

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The Glucocorticoid Receptor (GR) is a transcription factor that
belongs to the nuclear receptor superfamily. The GR gene, locat-
ed in the chromosome 5, encodes for two isoforms, GRα and β,
depending on the alternative use of terminal exon 9α or 9β,
respectively. GRα is the classic GR that binds glucocorticoids
and transduces their biologic activities; in the GRβ, the last 50
amino acids of GRα are replaced with a unique 15 amino acid se-
quence that renders the molecule incapable of binding glucocor-
ticoids and therefore transcriptionally inactive. GRβ has domi-
nant negative activity upon GRα and unclear physiologic roles.
The ligand dependent activation of GRα modulates the transcrip-
tion of a large number of target genes whose coordinate expres-
sion plays important roles in the control of metabolic, cardio-
vascular, immune and behavioral homeostasis. Besides the classical
mode of action, via direct binding to GRE-DNA, the ligand
activated GRα affects the transcriptional events of other signal
transduction cascades via mutual protein-protein interaction
with several transcription factors, such as nuclear factor-κB (NF-
κB), activator protein-1 (AP-1) and Smad3 that transduces the
signal of transforming growth factor β (TGFβ). We identified the
chicken ovalbumin upstream promoter transcription factor II
(COUP-TFI) as a novel partner of GR isoforms in a yeast two hy-
brid screening assay using GR components as baits. COUP-TFI is
an orphan receptor, already known as a suppressor of the tran-
scriptional activity of several nuclear receptors such as retinoic
acid, thyroid hormone, vitamin D and others. This transcription
factor homodimerizes or heterodimerizes with nuclear hormone
receptors and binds a wide variety of response elements that
contain imperfect AGGTCA direct repeats separated by a variable
number of nucleotides. Moreover COUP-TFI, along with its
closely related protein COUP-TFI in humans, plays important
roles in glucose, fatty acids, cholesterol and xenobiotic metab-
olism as well as embryonic development. In a GST pull-down as-
say, COUP-TFI interacted with the hinge regions of both GRα and
GRβ via its DNA binding domain, while COUP-TFI formed a com-
plex with GRα, but not with GRβ on the COUP-TFI-responsive
cholesterol 7a-hydroxylase (CYP7A) promoter in a chromatin
immunoprecipitation assay. In functional reporter assays,
COUP-TFI repressed the transcriptional activity of ligand-
activated GRα on the glucocorticoid-responsive mouse mam-
ymur tumor virus promoter, while GRα, but not GRβ, enhanced
the transcriptional activity of COUP-TFI on the CYP7A promoter.
It is known that phosphoenolpyruvate carboxykinase (PEPCK), a
key enzyme of the gluconeogenic pathway and its promoter
activity, is regulated by both GR and COUP-TFI. We found that
COUP-TFI is necessary for the enhancement of this promoter ac-
tivity by glucocorticoids, using morpholino oligo-anitisense. We
conclude that COUP-TFI may mediate some part of glucocorti-
coid actions in the regulation of intermediary metabolism
through protein-protein interaction with GRα.

Modulation of Glucocorticoid Receptor Function
via Phosphorylation

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The glucocorticoid receptor (GR) is phosphorylated at multiple
serine residues in a hormone-dependent manner. It has been
suggested that GR phosphorylation modulates turnover, subcell-
ular trafficking, or transcriptional regulatory functions of the re-
sceptor, yet the contribution of individual GR phosphorylation
sites to the modulation of GR activity remains enigmatic. To
gain further insight into the function of GR phosphorylation in
vivo, we have produced antibodies that specifically recognize

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Our GR phosphorylation state-specific antibodies have allowed for the first time a detailed analysis of the localization of the GR phospho-isofoms in vivo. We found that differentially phosphorylated receptor species are located in unique subcellular compartments (Fig. 2). For example, the GR phospho-S211 form is found in the nucleus after hormone treatment, consistent with its agonist-dependent phosphorylation. Thus, the GR phospho-S211 form corresponds to the nuclear transcriptionally active subpopulation of the receptor. In contrast, in hormone-treated cells the GR phospho-S203 is predominantly cytoplasmic and remains largely confined to the perinuclear region of the cell, with only a small fraction of this receptor isofom transiently entering the nucleus. Thus, the GR phospho-S203 form may participate in receptor function(s) at the cytoplasmic/nuclear border, such as nuclear import or export.

We and others have proposed that GR phosphorylation modulates interaction with accessory proteins involved in transcriptional regulation, protein stability or subcellular trafficking, thereby affecting receptor function. Utilizing a yeast two-hybrid approach that allows transcriptional activators to be used as bait, we have previously identified the tumor susceptibility gene 101 (Tsg101) and the Vitamin D receptor-interacting protein 150 (DRIP150) as proteins that specifically interact with the GR AF-1 [2]. Interestingly, these protein:protein interactions appear sensitive to GR phosphorylation in a reciprocal manner: DRIP150 associates more efficiently with phospho-GR, whereas Tsg101 interacts more favorably with the non-phosphorylated receptor. Tsg101 shows significant sequence similarity to E2 ubiquitin ligases but is unable to catalyze ubiquitin transfer as it lacks the active site cysteine that forms the transient thioester bond with the C-terminus of ubiquitin. Interestingly, overexpression of Tsg101 stabilizes the non-phosphorylated form of GR in the absence of hormone, whereas hormone-dependent down-regulation of GR remains largely intact. Thus, Tsg101 is recruited to

(Fig. 1).
the GR AF-1 hypo-phosphorylated form in the absence of ligand, and protects GR from degradation. This may represent general mechanism for maintaining the steady state levels of steroid receptors in vivo in the absence of ligand. In contrast, upon ligand-binding when GR phosphorylation is high, DRIP150 is recruited to GR, which facilitates transcriptional enhancement. Indeed, we have shown differential occupancy of GR phospho-isomers at endogenous GR target genes in vivo by chromatin immunoprecipitation (ChIP) using GR phosphorylation site-specific antiserum (Fig. 3).

Thus, GR phosphorylation appears to be a versatile mechanism to modulate protein:protein interactions, which in turn stabilize the hypo-phosphorylated form of the receptor in the absence of ligand as well as facilitate transcriptional activation by the hyper-phosphorylated GR via cofactor recruitment upon ligand binding.

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A Non-Steroidal, Glucocorticoid Receptor Antagonist
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Selective intracellular receptor antagonists are used clinically to ameliorate hormone-dependent disease states. Patients with Cushing’s syndrome have high levels of the glucocorticoid, cortisol, and suffer significant consequences from this over-exposure. This hormone is also elevated under conditions of viral infection, stress and aging. We have identified the first selective, nonsteroidal, glucocorticoid receptor antagonist. This compound is characterized by a tri-arylmethane core chemical structure. This GR-specific antagonist binds with nanomolar affinity to the glucocorticoid receptor and has no detectable binding affinity for the highly related receptors for mineralocorticoids, androgens, estrogens and progestatones. We demonstrate that this antagonist binds to the receptor and inhibits glucocorticoid-mediated transcriptional regulation. This compound binds competitively with steroids, likely occupying a similar site within the ligand binding domain. Once bound however, the compound fails to induce critical conformational changes in the receptor necessary for agonist activity.

The Human Glucocorticoid Receptor: Molecular Mechanisms Controlling the Termination of Glucocorticoid Responses
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Glucocorticoids are a vital class of steroid hormones that regulate a diverse array of physiological actions in all vertebrates. The actions of glucocorticoids are mediated via the glucocorticoid receptor, a product of a single receptor gene. However, recent studies have shown that multiple receptor isoforms can be generated from this gene via alternative splicing and alternative translation initiation mechanisms. These isoforms, along with post translational modification of the receptor (i.e. phosphorylation, ubiquitination), may contribute to the diverse and tissue specific responses to glucocorticoids. Therapeutically both natural and synthetic glucocorticoids are widely used to control a variety of inflammatory diseases including asthma, inflammatory bowel disease, dermatitis and arthritis. The effectiveness of these drugs is often compromised by premature termination of steroid responsiveness and the development of resistance to glucocorticoids. In the lecture I will present new molecular data on three aspects of glucocorticoid resistance and the termination of GR signaling: 1) the role of NF-κB in repression of GR signaling; 2) the role of post translational modification of glucocorticoid receptors in targeting the receptor for degradation in the proteasome; and 3) the molecular mechanism of hGRβ as a dominant negative receptor and the development of steroid resistance.

Panoramic View of Glucocorticoids’ Actions on the Immune Response: A Good Model to Understand Immunosuppression for New Treatment Strategies
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Glucocorticoids have been used for over 50 years in the treatment of inflammatory and autoimmune diseases and in preventing graft rejection. Integrating molecular, cellular and pharmacological knowledge allows a better understanding of glucocorticoid – mediated immunosuppression. Gene profiling revealed that glucocorticoids exert both negative and positive effects at the same time with a dynamic and bi-directional spectrum of activities on various limbs and components of the immune response. Glucocorticoids modulate genes involved in the priming of the innate immune response, while their actions on the adaptive immune response are to suppress cellular (Th1-directed) immunity and promote humoral (Th2-directed) immunity. Indeed, this explains the beneficial and adverse effects of glucocorticoids on the immune response. They restrain inflammation and prevent tissue destruction, but can increase susceptibility to opportunistic infections, and trigger or worsen inflammatory diseases. Interestingly, glucocorticoids can also induce tolerance to specific antigens by influencing dendritic cell phenotype and promoting the development of regulatory high-IL-10 producing T cells. The ex-vivo therapeutic use of glucocorticoids could therefore represent an adjuvant treatment to cell therapy in autoimmune diseases, avoiding long-term deleterious adverse effects of glucocorticoids. Thus, a panoramic view of glucocorticoids’ actions on the immune system provides an interesting
model for characterizing immunomodulation pathways. This glucocorticoid-mediated immune profiling appears useful in order to find new gene targets for novel immunosuppressants and to facilitate the identification of new molecular pharmacogenomic markers that predict and monitor response to treatment.

**Glucocorticoids and the Th1/Th2 Balance**

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Immune responses are regulated by antigen-presenting cells (APC) -monocytes/macrophages and dendritic cells, and by natural killer (NK) cells that are components of innate immunity, and by the recently described T helper (Th) lymphocyte subclasses Th1 and Th2, that are components of adaptive (acquired) immunity. Th1 cells primarily secrete IFN-γ, IL-2 and TNFβ, which promote cellular immunity, whereas Th2 cells secrete a different set of cytokines, primarily IL-4, IL-10 and IL-13 which promote humoral immunity. Naive CD4+ (antigen-inexperienced) Th0 cells are clearly bipotential and serve as precursors of Th1 and Th2 cells. Among the factors currently known to influence the differentiation of these cells towards Th1 or Th2, cytokines produced by cells of the innate immune system are the most important. Thus, IL-12, produced by activated monocytes/macrophages or other APCs, is a major inducer of Th1 differentiation and hence cellular immunity. This cytokine acts in concert with NK-derived IFN-γ to further promote Th1 responses. APC-derived IL-12 and TNFα in concert with natural killer (NK) cell and Th1-derived IFN-γ stimulate the functional activity of T cytotoxic cells (Tc), NK cells and activated macrophages, which are the major components of cellular immunity. The type 1 cytokines IL-12, TNFα and IFN-γ also stimulate the synthesis of nitric oxide (NO) and other inflammatory mediators that drive chronic delayed type inflammatory responses. Because of these crucial and synergistic roles in inflammation IL-12, TNFα and IFN-γ are considered the major pro-inflammatory cytokines. Th1 and Th2 responses are mutually inhibitory. Thus, IL-12 and IFN-γ inhibit Th2 cells activities, while IL-4 and IL-10 inhibit Th1 responses. IL-4 and IL-10 promote humoral immunity by stimulating the growth and activation of mast cells and eosinophils, the differentiation of B cells into antibody-secreting B cells, and B cell immunoglobulin switching to IgE. Importantly, these cytokines also inhibit macrophage activation, T-cell proliferation and the production of pro-inflammatory cytokines. Therefore, the Th2 (type 2) cytokines IL-4 and IL-10 are the major anti-inflammatory cytokines.

Previous studies have shown that glucocorticoids suppress the production of TNFα, IFN-γ and IL-2 in vitro and in vivo in animals and humans. These early observations, in the context of the broad clinical use of glucocorticoids, initially led to the conclusion that stress hormones are, in general, immunosuppressive. Recent evidence indicates, however, that systemically, glucocorticoids cause selective suppression of Th1 responses and a shift towards Th2-mediated humoral immunity, rather than generalized immunosuppression. Thus, recent evidence indicates that glucocorticoids also act through their classic cytoplasmic/nuclear receptors on APCs to suppress the production of the main inducer of Th1 responses IL-12 in vitro and ex vivo. Since IL-12 is extremely potent in enhancing IFN-γ and inhibiting IL-4 synthesis by T cells, the inhibition of IL-12 production by APCs may represent a major mechanism by which glucocorticoids affect the Th1/Th2 balance. Thus, glucocorticoid-treated monocytes/macrophages produce significantly less IL-12, leading to their decreased capacity to induce IFN-γ production by antigen-primed CD4+ T cells. This is also associated with an increased production of IL-4 by T cells, probably resulting from disinhibition from the suppressive effects of IL-12 on Th2 activity. Furthermore, glucocorticoids potently downregulate the expression of IL-12 receptors on T and NK cells. This explains why human peripheral blood mononuclear cells (PBMCs) stimulated with immobilized anti-CD3 lose their ability to produce IFN-γ in the presence of glucocorticoids. Thus, although glucocorticoids may have a direct suppressive effect on Th1 cells, the overall inhibition of IFN-γ production by these cells appears to result mainly from the inhibition of IL-12 production by APCs and from the loss of IL-12 responsiveness of NK and Th1 cells.

It is particularly noteworthy that glucocorticoids have no effect on the production of the potent anti-inflammatory cytokine IL-10 by monocytes; yet, lymphocyte-derived IL-10 production appears to be upregulated by glucocorticoids. Thus, rat CD4+ T cells pretreated with dexamethasone exhibit increased levels of mRNA for IL-10. Similarly, during experimental endotoxemia or cardiopulmonary bypass, or in multiple sclerosis patients having an acute relapse, treatment with glucocorticoids is associated with increased plasma IL-10 secretion. This could be the result of a direct stimulatory effect of glucocorticoids on T cell IL-10 production and/or a block on the restraining inputs of IL-12 and IFN-γ on monocyte/lymphocyte IL-10 production. In contrast to catecholamines, glucocorticoids have a direct effect on Th2 cells by upregulating their IL-4, IL-10 and IL-13 production.

Although interest in the Th2 response was initially directed at its protective role in helminthic infections and its pathogenic role in allergy, this response may have important regulatory functions in countering the tissue-damaging effects of macrophages and Th1 cells. Thus, systemically, an excessive immune response, when circulating pro-inflammatory cytokines are high, or local inflammation through stimulation of the afferent vagus nerve fibers, stimulate the hypothalamic-pituitary-adrenal (HPA) axis. The subsequent release of glucocorticoids may trigger a mechanism that inhibits Th1 but potentiates Th2 responses. This important feed-back mechanism may protect the organism from “overshooting” with type 1/pro-inflammatory cytokines and other products of activated macrophages with tissue damaging potential.
11β-Hydroxysteroid Dehydrogenase Type 1: An Explanation of the Metabolic Syndrome – Cushing’s Disease Paradox?

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Conventional wisdom states that glucocorticoid action is determined by the levels of steroid in the circulation and the density of receptors in target cells. Recently however, a novel level of control of glucocorticoid action upon target tissues has been elucidated, cellular metabolism by intracellular enzymes, notably 11beta-hydroxysteroid dehydrogenases (11β-HSDs). There are 2 isoforms, the products of distinct and only distantly related genes. The function of 11β-HSD-2, a potent dehydrogenase, is to protect receptors from active glucocorticoids (cortisol in humans, corticosterone in rats and mice) by rapidly inactivating them to inert 11-keto forms (cortisone, 11-dehydrocorticosterone). Thus 11β-HSD-2 prevents glucocorticoid access to intrinsically non-selective mineralocorticoid receptors in the distal nephron, producing aldosterone selectivity in vivo. In contrast, the function of the ‘cinderella’ type 1 isozyme (11β-HSD-1), though the first to be isolated and cloned, was obscure until recent studies showed that in intact cells and organs it catalyses the reverse or reductase reaction. This suggests that 11β-HSD-1 regenerates active glucocorticoids from inert keto-steroids, notably in glucocorticoid receptor rich tissues such as liver, adipose and the brain.

A key example of the importance of 11β-HSD-1 has come from recent studies of the prevalent Metabolic Syndrome of visceral obesity, insulin resistance, hyperglycaemia, dyslipidaemia and hypertension. The phenotype closely resembles the rare Cushing’s syndrome of cortisol excess, but in simple obesity plasma cortisol levels are not elevated. Recent evidence has shown an adipose-selective 2 – 3-fold increase in 11β-HSD-1 activity and mRNA levels in rodent models (Zucker rats, ob/ob mice) and human populations with the metabolic syndrome or abdominal obesity. We and others hypothesised that, by locally increasing glucocorticoid action, this might produce a localised ‘Cushing’s syndrome of adipose tissue’. To address causation transgenic mice with –2.5 fold overexpression of 11β-HSD-1 only in adipose tissue (under the aP2 promoter) were generated. These mice have elevated adipose corticosterone levels, whilst systemic plasma levels of the glucocorticoid remain unaltered. The transgenic mice show 3-fold visceral obesity, hyperglycaemia, insulin resistance, dyslipidaemia and hypertension. The latter is apparently driven by angiotensinover over-production (4-fold) selectively in adipose tissue and is normalised by low doses of an angiotensin 2 receptor antagonist. This mouse also has elevated portal blood levels of corticosterone, implicating excess liver glucocorticoid exposure in its phenotype. To dissect this, we recently made liver-specific 11β-HSD-1 transgenic mice lines driven by the apoE promoter. These mice have intrahepatic fat accumulation and hyperlipidaemia, modest insulin resistance and hypertension, but have normal body weight and fat distribution and normal glucose tolerance. Thus the effects of adipose overexpression alone can model the metabolic syndrome fully.

To explore the physiological importance and therapeutic potential of 11β-HSD-1 inhibition we made 11β-HSD-1 knock-out mice on both obesity-prone (C57BL/6J) and obesity-resistant (MF-1) strain backgrounds. The null mice have improved glucose tolerance and lower plasma triglycerides driven, in part, by reduced hepatic gluconeogenesis and increased liver beta-lipid oxidation and insulin sensitivity. Adipose tissue is also important in the 11β-HSD-1/– phenotype. Thus, 11β-HSD-1/– mice have 67% lower intra-adipose corticosterone levels. Both strains of 11β-HSD-1/– mice have reduced fat pad weights. Intriguingly, with chronic high fat (HF) feeding, both strains exhibit reduced visceral fat accumulation, redistributing fat to metabolically safer peripheral depots (Fig. 1). On the C57BL/6J obesity-prone background 11β-HSD-1/– mice had 15% lower weight gain despite consuming 12% more calories than wild types. The favourably altered fat distribution was associated with improved glucose tolerance and insulin sensitivity in vivo. The null mice also had reduced free fatty acids and a ‘cardioprotective’ serum lipid profile (low LDL, high HDL-cholesterol). Isolated adipocytes from 11β-HSD-1/– mice exhibited higher basal and insulin-stimulated glucose uptake indicating insulin sensitisation. The biology appears conserved, at least in part, since administration of the 11β-HSD...
inhibitor carbenoxolone to humans also increases insulin sensitivity.

Unexpectedly, wild type mice down-regulated 11β-HSD-1 in white and brown fat in response to HF feeding. Moreover, obesity-resistant A/J mice had both lower basal levels and down-regulated adipose 11β-HSD-1 more markedly than obesity-prone C57BL/6j mice. Down-regulation of adipose 11β-HSD-1 in response to dietary fat represents a novel adaptive mechanism, more pronounced in obesity-resistant animals, that counteracts central adiposity and its metabolic consequences.

11β-HSD-1 is also highly expressed in the brain, notably in hippocampus and cerebellum. 11β-HSD-1 null mice show modestly elevated plasma corticosterone levels at the diurnal nadir compatible with blunted feedback upon the hypothalamic-pituitary-adrenal axis. Despite this, 11β-HSD-1/- mice have lower intrahippocampal corticosterone levels and resist glucocorticoid-associated impairments of cognitive (hippocampal) function with ageing. Again the biology may be conserved as 11β-HSD-1 is expressed in the human hippocampus and carbenoxolone improves aspects of cognitive performance in healthy elderly humans. Thus 11β-HSD-1 represents a novel control of intracellular glucocorticoid action prior to glucocorticoid receptor binding and may be a useful therapeutic target in the Metabolic Syndrome and age-related cognitive disorders.

Carnitine: A Nutritional Modulator of Glucocorticoid Receptor Function

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Background: L-Carnitine (3-hydroxy-4-N, N, N-trimethylamino- butyrate) is a nutrient that plays a major role in energy metabolism. Human plasma level of L-carnitine (LCAR) is approximately 0.05 mM, as opposed to tissue concentrations of 1 to 100 mM, depending on the active uptake of this compound from the extracellular space. Currently, the only indication to the use of pharmacologic doses of LCAR (up to 600 mg/kg body weight/day) is the treatment of primary and secondary LCAR deficiencies. However, evidence from animal and human studies suggests that, at pharmacologic doses, LCAR may mimic some of the biologic actions of glucocorticoids (GCs), including their well known immunosuppressive effect. We hypothesized that these effects could be mediated through activation of the glucocorticoid receptor (GR) by LCAR. Therefore, we investigated the influence of LCAR on the various activities of this steroid receptor.

Major findings: At millimolar concentrations, which were not cytotoxic in vitro, LCAR significantly reduced the specific whole cell binding (SWCB) of 3H-dexamethasone (3H-Dex) to GR in HeLa cells in a dose dependent fashion (Fig. 1). The reduced binding was accompanied by a significant increase in the Kd of GR for Dex, with no change in the Bmax. Moreover, at the same doses, LCAR triggered the nuclear translocation of green fluorescent protein (GFP)-fused human GR alpha and transactivated the GC-responsive MMTV and TAT3 promoters in transiently transfected HeLa (Fig. 2a,b) and CV-1 cells (Fig. 2c). Finally, similarly to GCs, LCAR suppressed TNF alpha and IL-12 release from human primary monocytes stimulated with lipopolysaccharide and primed with IFN gamma ex vivo (Fig. 3). Both the transcriptional and the cytokine suppressive effect of LCAR were abolished by the well known GR-antagonist mifepristone (RU 486), further supporting the GR dependence of these actions.
Discussion: LCAR is one of a few compounds that are sold in the United States both as a prescription drug and as an over-the-counter nutritional supplement. In the present study we found that, similarly to the synthetic GC Dex, LCAR at millimolar concentrations, is able to: i) trigger nuclear translocation of human GR; ii) stimulate GR-mediated transactivation of known GC-responsive promoters in vitro; iii) suppress in a GR-dependent fashion the ex vivo release of proinflammatory cytokines from human monocytes. In addition, LCAR can compete with Dex for binding to the GR. Taken together, these findings suggest that LCAR, a nutrient structurally diverse from the classic GC Dex, may stimulate GR-mediated transactivation by functioning as an allosteric regulator of this steroid receptor. Indeed, the decreased affinity of GR for Dex in the presence of LCAR might be explained by the ability of this nutrient to interact with a portion of the receptor outside the GC binding pocket and modify the allosteric structure of the latter. This structural modification could, at the same time, reduce the affinity of the binding pocket for Dex, and create conformational changes similar to those induced by the native ligand, ultimately resulting in GR activation (Fig. 4). The ability of LCAR to reduce GR affinity for Dex, combined with its weaker transactivating effect compared to Dex, brings also up the possibility that this compound may act as a partial GC agonist/antagonist, able to both transactivate GR in the absence of the native ligand, and antagonize GR activation in its presence. The agonist/antagonist effect may depend on the responsive promoter and be cell-specific.

Conclusions: In summary, we provide novel evidence that pharmacologic doses of LCAR, a popular widely available nutritional supplement, can activate GR and modulate the transcription of GC-responsive genes in vitro, potentially sharing and/or influencing some of the biologic and pharmacologic actions of these hormones. It was recently reported that, at high concentrations, LCAR has also a positive effect on the bone. Indeed, this invites the speculation that pharmacologic doses of LCAR might share the beneficial immunomodulatory properties of GCs, but not their deleterious effects on the bone. More generally, the modulatory effects of LCAR on GR functions may be tissue- and/or gene-specific, being influenced by receptor abundance and distribution, and/or by transcription regulatory or co-regulatory molecules, such as transcription factors, co-activators and/or co-repressors. The clinical and therapeutic implications of these findings should be evaluated in controlled trials.

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Possible Tissue Glucocorticoid Hypersensitivity in Human Immunodeficiency Virus type-1 (HIV-1) Infection

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Acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus type 1 (HIV-1) infection, develops profound deterioration of the host immune system. AIDS patients also present with a variety of manifestations including myopathy/ muscle wasting, osteoporosis, dementia and growth retardation in children as well as dyslipidemia and insulin resistance/overt diabetes mellitus, accompanied by a characteristic lipodystrophy phenotype [1,2]. In addition to the suppression of host immune function, many of these changes can be induced by exogenously administered glucocorticoids and are seen in hypercortisolemic patients with endogenous Cushing syndrome. It is, therefore, possible that some unknown factor(s) might up-regulate glucocorticoid actions in certain tissues of AIDS patients.

Based on the above clinical evidence, we explored the possibility of glucocorticoid hypersensitivity in AIDS/HIV-1 infection, focusing on one of the HIV-1 accessory proteins, Vpr. This viral molecule is a 96-amino acid virion-associated protein and has multiple functions. Vpr is known to enhance the replication of HIV-1 virus in lymphocyte- and monocyte-derived cell lines. It also behaves as a transcriptional activator of several viral promoters and as an enhancer of HIV-1 long terminal repeat (LTR) promoter activated by Tat. Vpr also causes host cell arrest in the G2/M phase of the cell cycle, and induces terminal differentiation in some cell lines. Furthermore, Vpr increases the translocation of the HIV-1 pre-integration complex into the nucleus, and promotes efficient infection of non-dividing macrophages. Since Vpr circulates at detectable levels in HIV-1-infected individuals and is able to penetrate the cell membrane, its effects may be extended to cells not infected by HIV-1.

We found that this small HIV-1 protein enhances transcriptional activity of the glucocorticoid receptor (GR) on its responsive promoters by functioning as a coactivator of steroid hormone receptors [3]. Indeed, Vpr contains a nuclear receptor signature motif LXXLL at amino acids 64 to 68, which is used by host nuclear receptor coactivators to bind the receptors. Vpr mimics host p160 nuclear receptor coactivators, functioning as an adaptor molecule between promoter-bound GR and p300/CPBP coactivators in a glucocorticoid-responsive promoter by binding to amino acids 2045 to 2191 of p300, which also physically interact with host p160 coactivators [4]. To address Vpr effect on endogenous glucocorticoid-responsive molecules, we created the Vpr peptide in a baculovirus system and tested it on the glucocorticoid-responsive cytokine production by human peripheral monocytes/macrophages. We found that extracellularly administered Vpr enhanced the suppressive actions of the ligand-activated glucocorticoid receptor on Interleukin (IL)-12 secretion and its mRNA expression by these cells, a well-known cytokine that plays a critical role in the development of the T-helper 1 lymphocytes and are regulated by glucocorticoids [5]. The GR antagonist RU486 inhibited this effect. Moreover, Vpr changed the expression of an additional 5 glucocorticoid-responsive genes in the same direction as dexamethasone.

In a separate experiments, we also found that Tat, another key HIV-1 accessory protein, moderately potentiates GR activity [6]. Indeed, the coactivator complexes of the HIV-1 LTR and the glucocorticoid-responsive promoters both include p160 coactivators and use similar coactivator and elongation complexes for their transcription (Fig. 1). Tat may function as an adaptor molecule efficiently stimulating the processes of transcription initiation and elongation, through potentiation of the coupling of p160

Fig. 1  Schematic models of HIV-1-LTR and glucocorticoid-responsive mouse mammary tumor virus (MMTV)-LTR transactivation modulation by coactivators and p-TEFb. These models do not show the dynamic, sequential interactions between the reactants, which would be essential to explain the presence of multiple, overlapping binding sites that these proteins have for each other.

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coactivators and the positive-acting transcription elongation factor b (p-TEFb) complex.

Therefore, through its encoding proteins, Vpr and Tat, HIV-1 may facilitate the transcription of genes encoding its own proteins by directly stimulating viral proliferation. On the other hand, by inducing hypersensitivity to glucocorticoids, these proteins contribute to the proliferation of the virus indirectly by suppressing the host immune system (Fig. 2). Extensive further clinical and basic investigations are crucial to examine the clinical importance of glucocorticoid hypersensitivity and to develop novel effective therapeutic approaches in AIDS.

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Natural Glucocorticoid Receptor Mutants Causing Generalized Glucocorticoid Resistance: Molecular Genotype, Genetic Transmission and Clinical Phenotype

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Glucocorticoid resistance is a rare, familial or sporadic condition characterized by generalized, partial end-organ insensitivity to physiologic glucocorticoid concentrations. Patients have compensatory elevations in circulating cortisol and adrenocorticotropic hormone (ACTH) concentrations, and resistance of the hypothalamic-pituitary-adrenal (HPA) axis to dexamethasone suppression, but no clinical evidence of hypo- or hypercortisolism. The clinical spectrum of the condition is broad, ranging from completely asymptomatic to severe cases of hyperandrogenism, fatigue and/or mineralocorticoid excess. The molecular basis of glucocorticoid resistance in several families and sporadic cases has been ascribed to mutations in the human glucocorticoid receptor-α (hGRα) gene, which impair one or more of the molecular mechanisms of glucocorticoid receptor function, thus altering tissue sensitivity to glucocorticoids (Fig. 1, Table 1).

We systematically investigated the molecular mechanisms through which natural, ligand-binding domain (LBD) hGRα mutants, including hGRα559N, hGRα571A, hGRα641V, hGRα729I and hGRα747M, produce a defective signal, and determined whether their differential effects on hGRα function might account for the type of genetic transmission of the disorder and the variable clinical phenotype of the affected subjects.
Table 1 Mutations of the human glucocorticoid receptor gene causing glucocorticoid resistance

| Mutation Position | Amino acid | Biochemical Phenotype | Present Study | Genotype | Transmission | Phenotype |
|-------------------|------------|-----------------------|---------------|----------|-------------|----------|
| cDNA              | cDNA       |                       |               |          |             |          |
| 2054 (A→T)       | 641 (D→V) | Affinity for ligand ↓  | Transcriptional activity of LBD ↓ Transdominance ↓ Nuclear translocation ↓ DNA binding (+) Abnormal interaction with GRIP1 | Homozygous | Autosomal Recessive | Hypertension Hypokalemic alkalosis |
| 4 bp deletion in exon 6 | hGRαx number: 50% of control | Inactivation of the affected allele | Heterozygous | Autosomal Dominant | Hirsutism Male-pattern hair-loss Menstrual irregularities |
| 2317 (G→A)       | 729 (V→I) | Affinity for ligand ↓  | Transcriptional activity of LBD ↓ Transdominance ↓ Nuclear translocation ↓ DNA binding (+) Abnormal interaction with GRIP1 | Homozygous | Autosomal Recessive | Precocious puberty Hyperandrogenism |
| 1808 (T→A)       | 559 (I→N) | Affinity for ligand ↓  | Transcriptional activity of LBD ↓ DNA binding (+) Abnormal interaction with GRIP1 | Heterozygous | Sporadic | Hypertension Oligosperma, Infertility |
| 1844 (C→T)       | 571 (V→A) | Affinity for ligand ↓  | Transcriptional activity of LBD ↓ DNA binding (+) Normal interaction with GRIP1 | Homozygous | Autosomal Recessive | Ambiguous genitalia Hypertension Hyperandrogenism Hypokalemia |
| 2373 (T→G)       | 747 (I→M) | Affinity for ligand ↓  | Transcriptional activity of LBD ↓ DNA binding (+) Normal interaction with GRIP1 | Heterozygous | Autosomal Dominant | Cystic acne Hirsutism Oligoamenorrhea |
| 1430 (G→A)       | 477 (R→H) | Transactivation ↓      | Heterozygous | Sporadic | Hirsutism, Fatigue Hypertension |
| 2035 (G→A)       | 679 (G→S) | Affinity for ligand ↓  | Transactivation ↓ | Heterozygous | Sporadic | Hirsutism, Fatigue, Hypertension |

Our findings suggest that all five mutant receptors studied have LBDs with decreased intrinsic transcriptional activity. Unlike hGRαI559N and hGRαI747M, which exert a dominant negative effect upon the transcriptional activity of hGRα, hGRαV571A, hGRαD641V and hGRαV729I do not have such an effect. All five mutants studied demonstrate varying degrees of decreased affinity for the ligand in a standard dexamethasone-binding assay, and preserve their ability to bind DNA. Fusion of the wild type and mutant receptors with GFP enabled the study of their subcellular localization in HeLa cells in the absence or presence of ligand. In the absence of dexamethasone, GFP-fused-hGRα was primarily localized in the cytoplasm. Addition of 10⁻⁶ M dexamethasone resulted in translocation of the wild-type receptor into the nucleus within 12 min. The pathologic mutant receptors GFP-hGRαI559N, GFP-hGRαV571A, GFP-hGRαD641V and GFP-hGRαI747M were predominantly observed in the cytoplasm in the absence of ligand. Exposure to 10⁻⁶ M dexamethasone induced a slow translocation of these mutant receptors into the

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nucleus, which took 180, 25, 22 and 30 min, respectively. The mutant receptor GFP-hGRαV729I was observed predominantly in the nucleus in the absence of ligand, while further translocation from the cytoplasm into the nucleus required longer exposure (120 min) to the same concentration of dexamethasone. Finally, all five mutants studied displayed an abnormal interaction with the GRIP1 coactivator in vitro.

We conclude that each of the above hGRα mutations imparts different functional defects upon the glucocorticoid signal transduction pathway, which explain the recessive or dominant transmission of the disorder, but might only in part explain its variable clinical phenotype.

**Glucocorticoid Resistance and Hypersensitivity States. Genetic and Developmental Aspects**

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Glucocorticoids have been in the treatment of inflammatory and autoimmune diseases and to prevent graft rejection for over 50 years. These hormones exert their effects through cytoplasmic, heat shock protein-bound glucocorticoid receptors. Upon binding with the hormone, these receptors translocate into the nucleus where they modulate the transcription rates of responsive genes in either of two ways: by binding to specific promoter DNA sequences, the glucocorticoid-responsive elements (GRE-mediated actions), or by interacting with and altering the activity of transcription factors that regulate genes of importance to their actions (non-GRE-mediated actions). The latter include NF-κB, AP-1 and several STATs. Recently described glucocorticoid receptor coactivators and corepressors, respectively, enhance or diminish the actions of glucocorticoids on the promoter activities of glucocorticoid-responsive genes. Thus, in addition to the glucocorticoid receptor, multiple factors modulate the cell sensitivity to glucocorticoids, and may explain their gene-, cell-, tissue- and activity-dependent actions. DNA microarray analysis revealed that up to 20 percent of the human genome responds to glucocorticoids. Glucocorticoids stimulate new families of genes, such as toll-like receptors, scavengers and thrombospondins, which are involved in the early phase of the innate immune response, while their actions on the adaptive immune response are to promote humoral (Th2-directed) and to suppress cellular (Th1-directed) immunity. They do this by strongly inhibiting Th1- and by enhancing secretion of some Th2-cytokines, and by differentially regulating the signaling of these cytokines toward the same end-effect. By limiting the inflammatory reaction, glucocorticoids prevent tissue damage. Inflammatory mediators, however, decrease the responsiveness of inflamed tissues to glucocorticoids by neutralizing the glucocorticoid receptor via phosphorylation, nitrosylation, and oxidation, by sequestering important limiting coactivators, and by overwhelming it with increased quantities of activated interacting proinflammatory transcription factors, such as NF-κB.

The last decade has produced new insights into the mechanisms of glucocorticoid sensitivity and resistance among the various inflammatory, autoimmune, allergic, and infectious diseases, and into the different expression of glucocorticoid sensitivity, dependency, and resistance in patients suffering from the same disease. Both the quality and severity of the inflammatory stimulus, as well as the genetics and constitution of the patient, play key roles in defining the therapeutic efficacy and side-effects of glucocorticoids. Although glucocorticoids increase susceptibility to intracellular and opportunistic infections, they are also highly beneficial in the presence of serious systemic inflammation, when administered in a sustained fashion throughout the course of the disease. This is observed in the systemic inflammatory syndrome, acute respiratory distress syndrome (ARDS) and septic shock, and in other conditions, such as meningococcal meningitis. New studies provide support for the presence of inadequate endogenous glucocorticoid secretion, as well as of systemic inflammation-induced peripheral glucocorticoid resistance in these states. They also demonstrate that prolonged administration of moderate doses of glucocorticoids accelerates the resolution of both systemic inflammation and peripheral glucocorticoid resistance. Glucocorticoids produce side effects through their mostly GRE-mediated cardiovascular and metabolic actions, while they influence immunity primarily through non-GRE-mediated mechanisms. A better understanding of their use (administration, dose, time-course and type of glucocorticoid) might enhance their efficacy and decrease their adverse effects. Fortunately, the first generation of tissue- and immune vs. cardiovascular/metabolic effect-selective glucocorticoids is available for study and further improvement. “Designer” glucocorticoids promise to be a great new advance in the therapy of inflammatory diseases.

Tissue-specific glucocorticoid resistance becomes clinically apparent with clinical manifestations from the hypersensitive tissue, because it is not compensated for by increased cortisol levels. This type of resistance may result in pathophysiologic processes and/or may contribute to such processes by not allowing glucocorticoids to exert their physiologic effects, e.g., anti-inflammatory effects; these processes include glucocorticoid-resistant asthma, rheumatoid arthritis, osteoarthritis, Crohn’s disease and ulcerative colitis. Tissue-specific glucocorticoid resistance has also been observed in lymphoid tumor cells, which fail to respond to the lytic effects of glucocorticoids as the disease progresses. Finally, tissue-specific glucocorticoid resistance has been observed in ACTH-producing adenomatous corticotroph cells. This is characterized by impaired negative feedback regulation and, thus, uninhibited ACTH-production by pituitary adenomas (Cushing’s disease) and ectopic ACTH-secreting tumors. Tissue specific glucocorticoid hyper-sensitivity on the other hand may lead to abnormalities in systems that are hypersensitive to normal levels of glucocorticoids under physiologic conditions. The regulation of blood pressure and adipose tissue distribution represent such systems. The higher frequency of particular RFLPs of the GR or the 11β-dehydrogenase type 1 genes in patients with familial hypertension or central obesity may reflect an abnormality of the glucocorticoid transduction system in these diseases. Similarly, expression of AIDS virus proteins with GR coactivator activity, such as Vpr and Tat, may explain the insulin resistance and lipodystrophy syndrome that is seen in a large pro-
portion of AIDS patients. Finally, a third system that might be affected by tissue-specific glucocorticoid hypersensitivity is the central nervous system. Up to 70% of patients with chronic hypercortisolism present with depression of the atypical type, the most common cause of depression in the general population. Similarly, if the CNS targets were sensing excessive glucocorticoid effects one could expect depression as a result.

**A Prospective Study of the Offspring of Depressed Parents: An Extension of the Barker Hypothesis?**

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**Background:** Individuals with major depression have an attenuated ACTH response to exogenous CRH infusion, compared to normal controls. A similar response has been found in severely depressed children. Animal studies have demonstrated the long-term effects of rearing practices on the HPA axis function in the offspring. To date similar studies have not been carried out in humans. It has been shown that the genetic and behavioral contributions of parents with affective disorders, increase the vulnerability of the offspring to psychopathology. However, little is known about the development of the HPA axis function in offspring of mothers with mood disorders.

**Methods:** We performed a CRH infusion test in 63 offspring (ages 9 to 18 years) participating in a longitudinal study of mothers with major depression, bipolar illness or no psychiatric illness. Psychiatric and observational assessments of these families were done over the course of 15 years.

**Results:** Adolescents in late puberty (Tanner 4 and 5) had lower ACTH and cortisol responses to CRH infusion than those in early puberty (p < 0.02) (Fig. 1). Offspring who experienced major depression subsequent to the CRH challenge, had an attenuated ACTH response during the CRH challenge (p < 0.03) (Fig. 2). Offspring of mothers with avoidant personality disorders had an exaggerated ACTH but normal cortisol response (p < 0.001) (Fig. 3). Chronic high family stress was associated with reduced cortisol responses (p < 0.02).
Conclusions: Our findings suggest mechanisms through which maternal psychopathology can be transmitted to her offspring. The present study highlights the relationship between early childhood experiences and differential HPA axis responses. Maternal characteristics and environmental factors may contribute to specific HPA axis patterns. Post CRH infusion patterns displayed by adults with depression appear to develop with repeated depressive episodes and may be mediated by puberty. Attenuated ACTH response to the CRH infusion could be an early indicator of susceptibility to the development of depression later in life.

In Memory of Yukitaka Miyachi

Dr. Yukitaka Miyachi graduated from the Medical School of the University of Tokyo, Japan, in 1963; four years later he received his Ph.D. degree from the Graduate School of the same University. He subsequently did his residency in Medicine and fellowship in Endocrinology and Metabolism at the Third Department of Internal Medicine, University of Tokyo Medical School. He joined the National Institutes of Health as a visiting fellow in 1971, and continued his research in the field of the hypothalamic-pituitary-adrenal axis under the supervision of the late Dr. M. B. Lipsett for two years, before returning to his native country as an Associate Professor of Internal Medicine.

Dr. Miyachi became Chief and Director of Internal Medicine at Shizuoka General Hospital in 1983, Associate and Full Professor of Internal Medicine at the University of Toho in 1987 and 1990, respectively, and Chairman of the Department of Internal Medicine at the Toho University School of Medicine, Tokyo, Japan, in 1998. He was a leader of the Japanese Society of Endocrinology for many years and founded the Japanese Society on Hormonal Steroids. He made major research contributions to the fields of peptide and steroid radio-immuno-assays and performed a number of excellent studies in Clinical Endocrinology. He maintained close contact with his former mentor and other colleagues at the NIH, and collaborated with scientists worldwide.

Colleagues will remember Dr. Miyachi for his ebullient personality, his humor, his dedication to research and clinical practice of Endocrinology, his kindness and his generosity. He trained many young colleagues who will always be grateful for his unwavering support, encouragement and guidance, and for giving them the opportunity to undertake clinical and/or research training in Endocrinology under his supervision.