Review

Novel role for vitamin B_6 in steroid hormone action: a link between nutrition and the endocrine system

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Introduction

Vitamin B_6 is a water soluble vitamin necessary for normal growth, development, and biological function.[1,2] The physiologically active form of the vitamin, synthesized from precursors obtained through the diet, is historically known for its role as a cofactor in many enzymatic reactions regulating amino acid metabolism[3] and nervous system function.[4-7] In addition, the vitamin is also involved in modulating lipid metabolism, gluconeogenesis,[8] and immune function.[9,10] While these enzymatic reactions represent the best-characterized functions of vitamin B_6, more recent evidence suggests a role for this vitamin in a nontraditional aspect of metabolism, steroid hormone action. This novel and provocative aspect of vitamin B_6 function has been suggested through indirect results obtained in several laboratories[11] and elucidated through more direct molecular studies of the glucocorticoid hormone receptor.[12] The purpose of this review is to present the information currently available pertaining to the relationship between vitamin B_6 and steroid hormone action. We will begin with a synopsis of the synthesis and distribution of vitamin B_6 throughout the body, followed by a brief discussion of the role of the vitamin in growth and development, neural function, and immune surveillance. We will then concentrate on the role for vitamin B_6 in steroid hormone action, ending with a speculative discussion on the future directions and significance of research in this area.

Synthesis and distribution of vitamin

The dietary forms of vitamin B_6 are three naturally occurring compounds which are biologically inactive: pyridoxal, pyridoxine, and pyridoxamine.[13,14] These vitamers are absorbed in the intestinal tract and enter the circulation, where a substantial portion is transported to the liver. Once in the liver, the inactive forms are phosphorylated by pyridoxal kinase, yielding pyridoxal phosphate, pyridoxine phosphate, and pyridoxamine phosphate. The latter two forms are then oxidized by an FMN-dependent oxidase to yield pyridoxal-5′-phosphate, the physiologically active form of vitamin B_6.[15] The newly synthesized pyridoxal phosphate is then released into the circulation. Plasma pyridoxal phosphate is found in association with albumin,[16,17] and this association is thought to protect the active form of the vitamin from degradation or metabolism while it is in circulation.[18] Studies have demonstrated that the liver is the primary site of synthesis of pyridoxal phosphate and, further, that the liver possesses an apparently unique transport mechanism that provides for the efflux of pyridoxal phosphate into the circulation.[19] Given that the liver is also the primary site of albumin synthesis, high affinity association of pyridoxal phosphate with albumin may represent the as-yet undefined mechanism for efflux of pyridoxal phosphate from the liver into general circulation for distribution to tissues throughout the body.

The phosphorylated form of the vitamin found in plasma is not able to cross cell membranes and enter cells; thus, at target tissues, pyridoxal phosphate must be dephosphorylated by a membrane-associated alkaline phosphatase(s) to regenerate the inactive form of pyridoxal, which readily enters cells.[20] Biologically active pyridoxal phosphate is then regenerated through the action of pyridoxal kinase within the target tissue or cells.[18] It is interesting that these target tissues do not release the newly resynthesized pyridoxal phosphate. This may result from the lack of albumin synthesis in these other tissues, offering further support for the idea that association with albumin is the mechanism by which pyridoxal phosphate is extruded from the liver into the circulation. This mechanism would thereby serve to prevent newly synthesized pyridoxal phosphate within target tissue from being lost into the

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general circulation and undergoing redistribution to other tissues.

Most precursors in excess of target tissue need are oxidized to 4-pyridoxic acid, which is not a substrate for reconversion and, as such, represents a metabolically inactive end-product which is excreted.[21] Control of intracellular concentrations of the biologically active form of the vitamin is additionally regulated through a negative feedback loop in the liver, by which intracellular pyridoxal phosphate acts to inhibit the oxidase enzyme, thereby preventing the initial synthesis step.

Maintenance of tissue culture cells in medium devoid of the precursor pyridoxine does not produce a rapid decrease in intracellular concentration of pyridoxal phosphate.[12] Interestingly, Meisler and Thanassi have demonstrated that intracellular pyridoxal phosphate levels are maintained in a hepatoma-derived HTC cell line despite the absence of the oxidase enzyme.[22] Their work importantly demonstrates that alternate mechanism(s) for synthesizing or maintaining pyridoxal phosphate may well exist in cells. Thus, it seems that liver, and perhaps other tissues or cells as well, can recycle metabolites to prevent vitamin deficiency and may develop or utilize alternate pathways for generation of active forms of vitamins; this underscores the importance of vitamin B_6 in the maintenance of biological function and homeostasis.

In the body, the majority of pyridoxal phosphate (75–80%) is located in muscle, primarily in association with glycogen phosphorylase.[18] This large pool of vitamin is relatively unavailable to other tissues, except through muscle destruction. Interestingly, pyridoxal phosphate does not act as a classic cofactor for the glycogen phosphorylase reaction, and a role in the catalytic mechanism of the enzyme has not been clearly established.[23,24] However, pyridoxal phosphate is known to serve as a structural component of the enzyme, acting to stabilize its quaternary structure.[25] The second largest pool of pyridoxal phosphate in the body is in liver, which accounts for 5–10% of the vitamin. Within the liver, the majority (66%) of pyridoxal phosphate is found in the cytosolic compartment,[26] where the metabolic conversion of dietary precursors to pyridoxal phosphate takes place. Smaller amounts are found in the kidney, where pyridoxine is absorbed from plasma against a concentration gradient and converted intracellularly to pyridoxal phosphate.[27] Pyridoxal phosphate is also found in the brain, where it is distributed non-uniformly in discrete regions of the brain;[28] the physiological significance, if any, of this uneven distribution is not currently known.

Role in growth and development

Vitamin B_6 serves as a cofactor for over 100 different enzymes involved in almost all aspects of cellular biochemistry and metabolism.[1,2] Because of its involvement with such a diverse array of metabolic enzymes, this vitamin is crucial for normal growth, development, and homeostasis. An absolute requirement for pyridoxal phosphate as an enzymatic cofactor has been established for many enzymes involved in amino acid and protein metabolism, including transaminases, racemaces, aldolases, dehydratases, cystathyionases, and decarboxylases.[29,30] While it is outside the scope of this review to discuss the role of vitamin B_6 in each of these enzyme groups, it is fruitful to consider a few examples which demonstrate the importance of this cofactor role of the vitamin.

Depending on the nutritional and hormonal status of the body, amino acids are utilized for protein synthesis or gluconeogenesis, the synthesis of glucose from amino acids. Amino acids are metabolized through aminotransferase enzymes, which catalyze the removal or addition of amino groups, thus permitting synthesis, catabolism, interconversion, and recycling of amino acids. Virtually all of the 20 amino acids present in proteins undergo aminotransferase reactions during metabolic breakdown,[31] and all of the known aminotransferases are pyridoxal phosphate-dependent enzymes. The requirement for adequate levels of pyridoxal phosphate becomes overtly apparent when one considers that cellular levels of the 10 nonessential amino acids are achieved and maintained through transaminase reactions. Indeed, in vitamin B_6 deficiency, the nonessential amino acids are synthesized only poorly and, therefore, protein formation does not proceed normally.[31] The enzyme aspartate aminotransferase is one of the best characterized enzymes involved in pyridoxal phosphate-dependent amino acid metabolism. Extensive X-ray crystallographic experiments have demonstrated the specific amino acids involved in tethering the vitamin to the active site of the enzyme, and have further identified the amino acids involved in electron transport which are central to enzymatic activity.[32–35] The C4-carboxaldehyde group of pyridoxal phosphate is crucial, as it is this moiety which physically interacts with the enzyme, through Schiff's base formation with a specific lysine residue, initiating electron transfer and ensuring amino group transfer.

On a more general note, for all enzymes for which the pyridoxal phosphate binding site is known, interaction with a specific lysine serves as the mechanism for initiation of cofactor action.[36] Proteins typically contain a number of lysine residues, and any lysine is, in principle, capable of interaction with pyridoxal phosphate and Schiff's base formation. Thus, association of pyridoxal phosphate with a single particular lysine residue indicates that there is a specific domain structure of the enzyme which favors stabilization of the enzyme-cofactor interaction, which would otherwise be readily hydrolyzed.

A very important role for vitamin B_6 has also been implicated in the regulation of both cell replication and cancer.[37] Here again, the vitamin mediates its effects through its involvement as a cofactor for the enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase. These enzymes are required for biosynthesis of the polyamines putrescine, spermidine, and spermine which are known to have impor-

tant, though not mechanistically clear, roles in cell replication and in cancer promotion.[38,39] Furthermore, cellular levels of thymine, one of the four fundamental DNA bases, are indirectly dependent on pyridoxal phosphate.[40] Thymine synthesis requires N5, N10-methylene tetrahydrofolic acid (THFA), the production of which is vitamin B_6 dependent. Thus, vitamin B_6 deficiency could lead to reduced levels of cellular thymine, with concomitant deleterious effects on DNA synthesis and cell replication.

Role in neural function

There are many neurotransmitters, for example, dopamine, norepinephrine, serotonin, γ-amino butyric acid (GABA), taurine, and histamine, whose synthesis and metabolism are mediated through the action of vitamin B_6 dependent enzymes,[41] and it has been clearly documented that vitamin B_6 deficiency has detrimental effects on neural development and function.[42] For example, Parkinson's disease, a non-genetic disorder characterized by a rhythmic tremor, a unique form of increased muscle rigidity, and slowness in the initiation and execution of movement, has been clearly associated with loss of the neurotransmitters dopamine, norepinephrine, and serotonin in the midbrain.[43] The loss of dopamine is further correlated with a deficiency of the vitamin B_6-dependent enzyme dopa-decarboxylase, which catalyzes the formation of dopamine from the precursor dopa.[44] Serotonin, also invoked in Parkinson's disease, is derived from tryptophan through the enzymatic reaction of another pyridoxal phosphate dependent enzyme, tryptophan decarboxylase.[45] Thus, the mental deterioration observed in cases of vitamin B_6 deficiency could be manifest through effect on synthesis of these as well as other neurotransmitters.

The inhibitory neurotransmitter γ-amino butyric acid (GABA) is synthesized from the amino acid glutamic acid in a reaction catalyzed by the vitamin B_6-dependent enzyme glutamic acid decarboxylase,[46,47] an enzyme which has been postulated to serve as a regulator of neural excitability.[48] It is well known that B_6 deficiency induces seizures, convulsions and abnormal EEG tracings, and investigations suggest that pyridoxal phosphate may affect the central nervous system by modulation of glutamic acid decarboxylase activity.[44] Specifically, insufficient levels of pyridoxal phosphate may lead to excitability of the central nervous system as a result of decreased synthesis of the inhibitory neurotransmitter GABA.

Role in immune function

Studies have shown that vitamin B_6 plays an important role in the development and maintenance of a competent immune system.[49] It has been demonstrated that vitamin deficiency in animals results in a suppression of cellular immune response, with a reduction in the levels of circulating antibodies produced in response to antigen exposure.[50,51] In addition, vitamin deficiency produces a severe atrophy of the thymus gland in several animal species, in which the thymuses are virtually depleted of lymphocytes and consist only of epithelial cells and stroma.[49] It is not completely clear what effect thymus atrophy has on the immune function, but there is evidence that a thymic humoral factor which is required for formation and maintenance of a subclass of lymphocytes is lost upon vitamin deficiency-mediated thymus atrophy.[49] In any case, it is clear that compromised immune protection or surveillance, brought about by vitamin B_6 deficiency, may have serious consequences on normal immune function. In support of this conclusion, it has been demonstrated recently that pyridoxine supplementation can prevent stress-induced immunosuppression in human subjects.[52]

Influence of vitamin on steroid receptors

Evidence has accumulated from several laboratories over the past decade suggesting that, in addition to its well-known and classical effects on overall cellular metabolism and homeostasis, vitamin B_6 may also play an important regulatory role in steroid hormone action. The steroid hormones, glucocorticoid, estrogen, progesterone, and androgen, are involved in the regulation of growth, development, reproduction, and metabolism in many tissues throughout the body. Each of these hormones exerts its effects in target tissues through direct interaction with specific intracellular receptor proteins. The physiological effects of steroid hormones are achieved through the binding of receptors to specific sites in DNA and the concomitant changes in gene expression which occur.[53-56]

The glucocorticoid hormones are synthesized in the adrenal glands, in a diurnal rhythm as well as in response to stress, and have many effects on protein, nucleic acid, fat, and carbohydrate metabolism. In mediating its effects, the glucocorticoid hormone binds with high affinity to the glucocorticoid hormone receptor located in the cytoplasm of target cells. The unliganded receptor is characterized as having little affinity for nuclei or DNA; binding of hormone initiates the poorly-understood process of transformation or activation, through which the receptor gains affinity for nuclei and DNA.[57] The activated receptor translocates to the nuclear compartment of cells and binds to specific DNA sequences. These DNA sequences, termed glucocorticoid regulatory elements or GRE's, were first identified in the long terminal repeat of the mouse mammary tumor virus genome.[58-60] GRE's are found near, or occasionally within, hormonally-regulated genes, and binding of the glucocorticoid receptor to these sequences results in altered levels of gene expression. The changes in gene expression lead to changes in mRNA and protein levels, and the resultant alterations in cellular phenotype.[57,61-63]

Despite the wealth of information which has accumulated over the past several years concerning the mechanism by which steroid hormones act to elicit their effects, surprisingly little is known regarding how

the receptors regulate gene expression and much remains to be elucidated. Over the past decade, we and others have shown that several properties of the steroid hormone receptors are altered by in vitro treatment with pyridoxal phosphate, including molecular conformation, surface charge, susceptibility to exogenous proteolysis, and DNA binding capacity and subcellular localization.[11] Further, in vivo alterations in pyridoxal phosphate concentration have been shown to modulate the ability of the glucocorticoid receptor to induce target gene expression.[12] These in vitro and in vivo effects of pyridoxal phosphate have suggested a physiological role for vitamin B_6 in the regulation of steroid hormone action.

Effect of pyridoxal phosphate on interaction of steroid receptors with DNA and nuclei

In 1978, Nishigori et al. first demonstrated that binding of partially purified progesterone receptor to ATP-Sepharose could be inhibited by treatment of the receptor preparation with pyridoxal phosphate.[64] Subsequent studies of Cake et al. indicated that pyridoxal phosphate could inhibit the in vitro association of partially purified rat liver glucocorticoid receptor with DNA cellulose, phosphocellulose, and isolated liver nuclei.[65] However, it is not known if complexes formed under in vitro conditions between glucocorticoid receptors and these artificial matrices or isolated nuclei are representative of the physiological association of glucocorticoid receptor with chromatin in the intact cell. Further, these studies did not differentiate between two possible effects of the vitamin: reversal of the process of receptor activation or inhibition of receptor association with artificial matrices. Early work from our laboratory addressed both of these concerns using intact thymic lymphocytes, which contain specific, high affinity, saturable receptors for glucocorticoids.[66] Exposure of thymocytes to the synthetic glucocorticoid hormone dexamethasone results in activation and nuclear localization of glucocorticoid receptors. This work demonstrated that the glucocorticoid receptor, localized to the nucleus under physiological conditions of hormone treatment, was very efficiently extracted by treatment with pyridoxal phosphate. The specificity of extraction of glucocorticoid receptors from intact rat thymocyte nuclei is shown in *Figure 1*. Only the physiologically active form of the vitamin, pyridoxal phosphate, is effective; neither precursors (pyridoxine, pyridoxamine phosphate), metabolites (pyridoxal), nor nonphysiological analogs (5-deoxypyridoxal) were capable of extracting receptors previously localized to the nuclear compartment of cells. Thus, pyridoxal phosphate need not exert its effects through the process of receptor activation, but may instead act to cause release of receptor previously bound to nuclei. This study also documented the importance of the C4-carboxaldehyde group of pyridoxal phosphate in mediating this extraction; as discussed earlier, it is through the C4-carboxaldehyde group that pyridoxal phosphate is known to

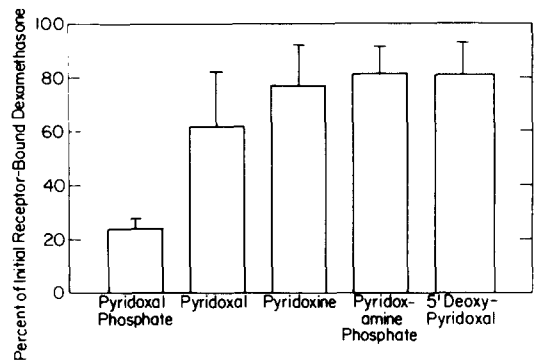

Figure 1 Extraction of nuclear dexamethasone-receptor complexes with pyridoxal phosphate and analogues. Rat thymocyte nuclei were exposed to 20 nM [^{3}H]dexamethasone followed by extraction with the indicated analogues of pyridoxal phosphate at a final concentration of 5 mM. Values shown are the mean ± SD of determinations from two separate experiments. The data are expressed as a percentage of the value obtained relative to control. (Reproduced with permission from Cidlowski and Thanassi, (1978). *Biochem. Biophys. Res. Commun.* **82,** 1140–1146)

interact with the enzymes for which it serves as cofactor. Reduction of pyridoxal phosphate with either hydroxylamine or semicarbizide produces derivatives of pyridoxal phosphate which are also incapable of extracting receptors from nuclei. These data were the first to demonstrate a direct correlation between the ability of pyridoxal phosphate to interact with other proteins, through Schiff's base formation, and its ability to extract glucocorticoid receptors from nuclei. Importantly, this observation suggests that the actions of pyridoxal phosphate on the glucocorticoid receptor may be mediated through a direct interaction of pyridoxal phosphate with the glucocorticoid receptor, or perhaps through interaction with an auxiliary protein involved in hormone action.

In other studies, pyridoxal phosphate treatment *in vitro* has also been shown to inhibit association of other steroid receptors, including the progesterone,[67] estrogen,[68] and androgen[69] receptors, with DNA and nuclei. Further, animals with elevated levels of vitamin B_6 exhibit decreased nuclear localization of uterine estrogen receptor,[70] while vitamin B_6 deficiency has been shown to enhance nuclear localization of the uterine estrogen receptor[71,72] as well as the prostatic androgen receptor.[73] Thus, it appears that pyridoxal phosphate acts to modulate at least two properties common to the steroid receptors, DNA binding and nuclear localization.

Influence of pyridoxal phosphate on the conformation of the glucocorticoid receptor

The effects of vitamin B_6 concentration on subcellular localization of steroid hormone receptors prompted further investigation into the mechanisms(s) by which pyridoxal phosphate acts. To that end, the effect of in vitro pyridoxal phosphate treatment on the conformation of the glucocorticoid receptor prepared from HeLa S_3 cells was examined.[74] These cells are of hu-

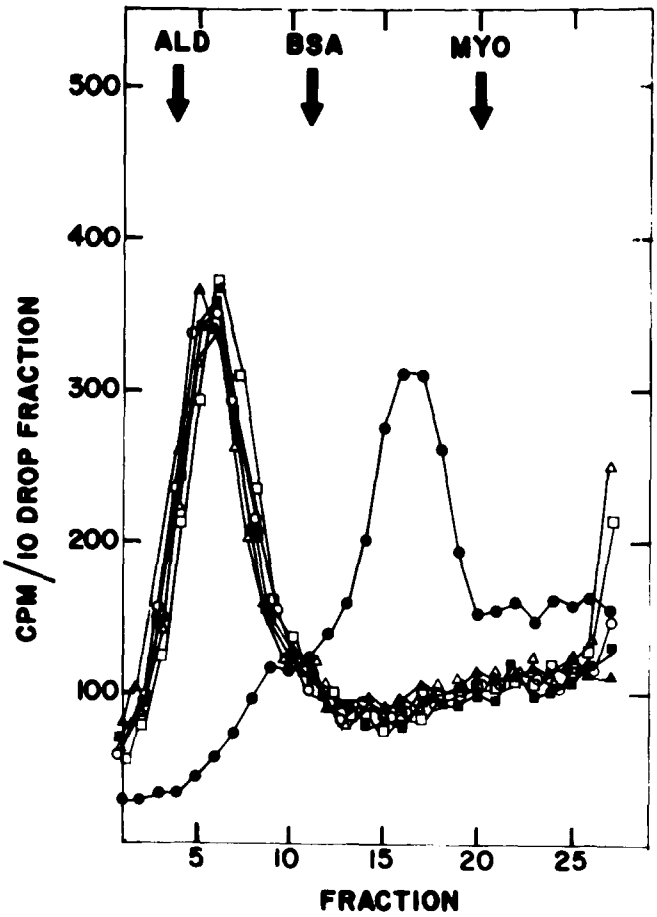

Figure 2 Specificity of pyridoxal phosphate on sucrose density gradient sedimentation coefficient of glucocorticoid receptors. HeLa S_3 cells were incubated with 20 nM [^{3}H]dexamethasone at 0°C. Cytosols were prepared and incubated for two hours with pyridoxal phosphate analogues at a final concentration of 5 mM as indicated: pyridoxine □- - -□; pyridoxal ■- - -■; pyridoxamine △- - -△; pyridoxamine 5'-phosphate ▲- - -▲; pyridoxal phosphate ●- - -●. Cytosols were then reduced with $NaBH_4$ and subjected to sucrose density gradient centrifugation. (Reproduced with permission from O'Brien and Cidlowski, (1981). *J. Ster. Biochem.* **14,** 9–18)

man origin and contain high-affinity, saturable, specific glucocorticoid receptors.[75] Early studies with this cell line demonstrated that the unactivated form of the glucocorticoid receptor migrates on sucrose density gradients with a sedimentation coefficient of 8S, while the nuclear or activated receptor exists as an approximately 4S form. Pyridoxal phosphate treatment produces a shift in the sedimentation profile of the unactivated receptor to an approximately 6S, nonproteolyzed form which is reflective of a change in the conformation of the receptor. Reduction with sodium borohydride after exposure of the receptor preparation to pyridoxal phosphate produces a receptor which sediments as an approximately 4S form. The data shown in *Figure 2* demonstrates that this reduction in sedimentation value is specific for the biologically active form of the vitamin, pyridoxal phosphate, since neither precursors, metabolites, nor nonphysiological analogs were capable of eliciting a change in the sedimentation coefficient of the glucocorticoid receptor.

These studies further demonstrated that vitamin analogs lacking the C4-carboxaldehyde group, pyridoxine and pyridoxamine phosphate, did not alter the sedimentation of the receptor. As discussed earlier, pyridoxal phosphate directly interacts with lysine residues in the enzymes for which it serves as a cofactor by Schiff's base formation through the C4-carboxaldehyde group. The effect of pyridoxal phosphate on the sedimentation of the receptor and the requirement for the carboxaldehyde group suggest that the vitamin may also interact with the glucocorticoid receptor through a similar association with lysine residues. This prompted an examination of the influence on receptor conformation of other compounds known to modify lysine residues. Such studies demonstrated that treatment of glucocorticoid receptors prepared from HeLa cells with a series of lysine-reactive compounds, including dinitrofluorobenzene, picryl sulfonic acid, succinate, or p-nitrophenyl acetate, had no effect on the sucrose density gradient sedimentation properties.[74] Thus, pyridoxal phosphate is specific in its effects on the glucocorticoid receptor, while nonspecific modification of lysine residues within the receptor by other reagents has no detectable effect on the sedimentation properties of the glucocorticoid receptor.

Together, these observations demonstrate that the glucocorticoid receptor exhibits an alteration in conformation or subunit composition as a result of exposure specifically to the physiological form of vitamin B_6. A requirement for the carboxaldehyde moiety of the vitamin has been established, supporting the idea of a direct interaction between pyridoxal phosphate and the glucocorticoid receptor. Pyridoxal phosphate treatment has also been shown to affect both progesterone[67] and estrogen[68] receptors, with a similar reduction in sedimentation coefficient on sucrose gradients, suggesting that pyridoxal phosphate may act through a single, common mechanism to exert its effects on members of the steroid receptor family of proteins.

Interaction of pyridoxal phosphate with the glucocorticoid receptor: in vitro studies

The effects of vitamin B_6 on glucocorticoid receptor conformation and nuclear location evoked by the physiologically active form of vitamin B_6, and the absolute requirement for the C4-carboxaldehyde group of the vitamin in mediating these effects leads to an interesting hypothesis: pyridoxal phosphate may directly interact with the glucocorticoid receptor to mediate its effects on the physical properties of the receptor. To investigate further this idea, the effect of pyridoxal phosphate on site-specific, limited exogenous proteolytic digestion of the rat thymic glucocorticoid receptor was examined.[76] The thymic glucocorticoid receptor elutes from S-200 Sephacryl as a 56–60A form when in the unactivated state, and treatment with exogenous trypsin generates a much smaller, 18–20A form of receptor. Not unexpectedly, treatment of un-

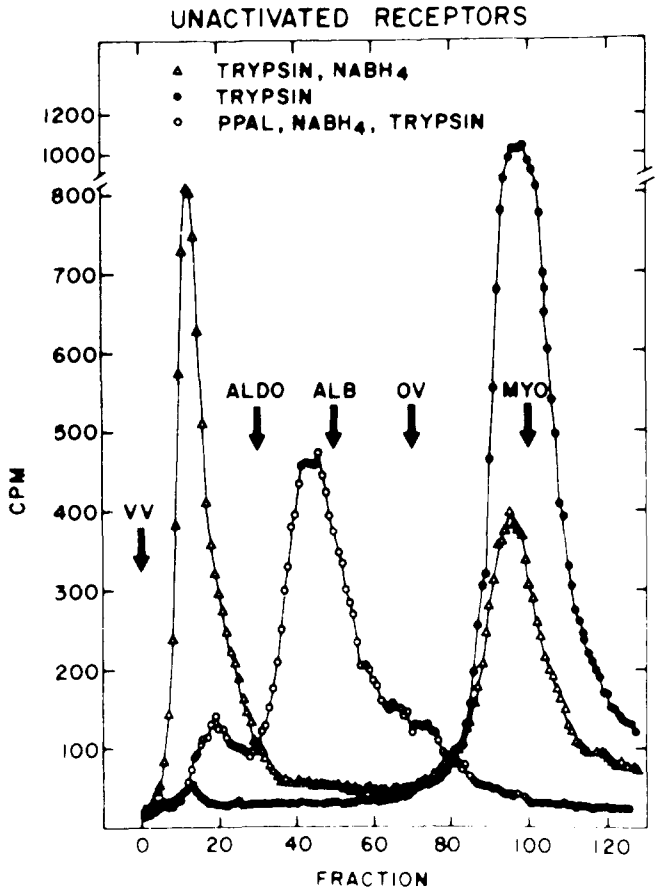

Figure 3 Influence of pyridoxal phosphate on site-specific, limited proteolytic degradation of glucocorticoid receptors. Cytosols prepared from rat thymocytes were incubated with 20 nM [^{3}H]dexamethasone at 0°C and then treated with trypsin at a concentration of 10 μg/L for 30 min at 4°C. The trypsin digestion was stopped by the addition of 1 mg soybean trypsin inhibitor before chromatography on Sephacryl S 200. The closed circles represent unactivated samples treated with trypsin, and the open triangles are from samples reduced with $NaBH_4$ before trypsin treatment. The open circles represent samples of cytosol treated with 5 mM pyridoxal phosphate for three hours at 0–4°C, reduced with $NaBH_4$, and then treated with trypsin. The profiles shown are representative of three experiments. (Reproduced with permission from Cidlowski, (1980). *Biochemistry* **19**, 6162–6170; copyright American Chemical Society)

activated receptor preparations with pyridoxal phosphate generates an intermediately sized receptor, a 42A form which is consistent with the effects of pyridoxal phosphate on the sucrose density gradient sedimentation of glucocorticoid receptor. Surprisingly, however, pretreatment of unactivated receptor preparations with pyridoxal phosphate prior to exposure to trypsin protects the receptor from proteolytic digestion, as shown in *Figure 3*. Protection from proteolytic degradation was specific for pyridoxal phosphate, since neither pyridoxine nor pyridoxal could prevent generation of the small, proteolytic fragment of receptor. These observations suggest that pyridoxal phosphate physically binds to the glucocorticoid receptor at or near a site which is susceptible to cleavage by trypsin, and thereby prevents proteolytic digestion.

The availability of an antibody which specifically recognizes pyridoxal phosphate has allowed further investigation of this provocative notion of a direct interaction between vitamin B_6 and the glucocorticoid receptor. In one series of experiments, this antibody was demonstrated to further alter the sucrose density gradient sedimentation properties of glucocorticoid receptor pretreated with pyridoxal phosphate, causing it to sediment as a much larger complex.[77] By two-dimensional gel analysis, Kitler, Thanassi, and Cidlowski (unpublished observations) have demonstrated that the same protein isolated from HeLa cells which binds radiolabelled steroid is also recognized specifically by the antibody against pyridoxal phosphate.

The observations made with this antibody directed against pyridoxal phosphate, coupled with the data on protection of receptor from proteolytic digestion, offer the most direct evidence to date that pyridoxal phosphate can directly interact with the glucocorticoid receptor. These data clearly support the notion that pyridoxal phosphate affects many properties of steroid hormone receptors (DNA binding, nuclear localization, and conformation) through a direct interaction of the vitamin with the receptor itself and not through receptor-associated proteins or factors.

Subsequent experiments have demonstrated that one possible site of interaction of pyridoxal phosphate exists within the carboxyl-terminal fourth of the glucocorticoid receptor.[78] This receptor fragment, the glucocorticoid mero-receptor, is a proteolytic fragment which retains steroid binding capacity. Several properties of the mero-receptor are affected by treatment with pyridoxal phosphate, including both chromatographic elution from DEAE Bio-Gel A and isoelectric focusing. Thus, at least one site of interaction of pyridoxal phosphate can be localized to this portion of the receptor. However, it remains to be determined if this represents the only site of interaction between the vitamin and the glucocorticoid receptor, and if the effects of the vitamin on the receptor are mediated through this site.

Interaction of pyridoxal phosphate with the glucocorticoid receptor: in vivo studies

The studies which have been described to this point have examined effects of in vitro exposure of receptor preparations to pyridoxal phosphate. However, at least one characteristic of the glucocorticoid receptor, its isoelectric focusing point, is similarly affected by either in vivo or in vitro exposure to elevated levels of pyridoxal phosphate.[11] This treatment produces more acidic receptor forms, which may have significant physiological ramifications. The shift in isoelectric point to more acidic forms offers support for the hypothesis that pyridoxal phosphate interacts with a basic amino acid(s) of the glucocorticoid receptor, such as lysine, masking its positive charge and, perhaps more importantly, substituting a negative charge derived from the phosphate moiety. This type of reaction

would render the receptor protein itself more acidic, and may thereby result in less favorable or stable interactions between receptor and other acidic species, such as DNA. Thus, the specific alteration in surface charge that would result upon interaction with pyridoxal phosphate may serve as the mechanism by which DNA binding and nuclear localization of the glucocorticoid receptor is inhibited or decreased by exposure to pyridoxal phosphate.

In addition to clearly demonstrating an effect of pyridoxal phosphate on the surface charge of the receptor, this data also documents that the physical properties of the glucocorticoid receptor in whole cells can be affected by in vivo modulation of vitamin B_6 concentration and thus further suggests that this vitamin may serve to modulate steroid hormone receptor function. These observations, however, have not permitted assessment of the possible effects of vitamin B_6 on steroid hormone action at the biological level.

Effects of pyridoxal phosphate on physiological aspects of steroid hormone action

This uncertainty has provided the impetus for investigations into the effects of vitamin B_6 on physiological functions of the steroid receptors. Because steroid hormones are known to exert their physiological effects through regulation of target gene expression, such studies have all involved examining the effect of vitamin concentration on induction of hormonally-responsive genes. For example, DiSorbo and Litwack have reported that induction of tyrosine aminotransferase by glucocorticoid treatment is increased after restriction of the vitamin and decreased following exposure to pharmacological doses of pyridoxine.[79] Interpretation of these data is complicated, however, by the fact that tyrosine aminotransferase itself is a vitamin B_6 dependent enzyme[80] and their studies relied on enzymatic activity as the measure of hormone responsiveness. Studies in our laboratory have demonstrated that the level of glucocorticoid-induced alkaline phosphatase activity in HeLa S_3 cells is reduced under conditions of vitamin excess.[77] However, because induction of alkaline phosphatase activity may be regulated through multiple factors or pathways in addition to glucocorticoid hormones,[81] it has not been possible to clearly determine if vitamin B_6 directly influences the physiological process of hormonal regulation of gene expression in these studies. Subsequent experiments were designed specifically to overcome these limitations. In these studies,[12] a glucocorticoid-responsive reporter plasmid was introduced into HeLa S_3 cells. The promoter directing expression of the target reporter gene was chosen so that any effects which were detected at the reporter gene level were, in fact, due to changes at the level of target gene transcription. Specifically, a plasmid containing the chloramphenicol acetyltransferase (CAT) gene under transcriptional control of the glucocorticoid-responsive mouse mammary tumor virus (MMTV) long terminal repeat promoter was introduced into the cells. This DNA sequence contains the glucocorticoid regulatory elements known to confer glucocorticoid responsiveness upon heterologous genes, with changes in the level of heterologous gene expression directly reflective of alterations in the level of glucocorticoid-mediated transcription from the MMTV promoter.[82] Thus, it was possible to determine if changes in vitamin concentration directly affected the ability of the glucocorticoid receptor to induce expression of the CAT reporter gene.

Preliminary studies demonstrated that glucocorticoid treatment of HeLa cells transfected with this plasmid resulted in an increased level of CAT activity. Induction of CAT activity in this system was restricted to the glucocorticoid class of steroid hormones, presumably due to the absence of receptors for the other hormones (estrogen, androgen, and progesterone), and was mediated through the high-affinity glucocorticoid receptor present in these cells.

The effect of intracellular vitamin B_6 concentration on glucocorticoid-mediated induction of CAT activity was then examined. Results of these experiments, demonstrated in *Figure 4*, revealed that when cells transfected with the reporter plasmid were grown in medium supplemented with the biosynthetic precursor pyridoxine to elevate the intracellular concentration of pyridoxal phosphate, the level of glucocorticoid-induced CAT activity was diminished. While simple removal of pyridoxine from the culture medium did not affect intracellular levels of pyridoxal phosphate, a condition of mild vitamin deficiency was achieved by culturing cells in the presence of a pyridoxal phosphate synthesis inhibitor, 4-deoxypyridoxine.[83] This reagent specifically inhibits the kinase enzyme involved in the first stage of pyridoxal phosphate synthesis. When transfected cells grown under conditions of mild vitamin deficiency were treated with hormone, the level of glucocorticoid-induced CAT activity was enhanced.

To determine if the effects of altered intracellular pyridoxal phosphate concentrations on induction of CAT gene expression were specific for glucocorticoid receptor-mediated gene expression, the regulation of CAT gene expression derived from other transfected reporter plasmids was also examined. For these studies, two plasmids from which CAT gene expression is constitutive and glucocorticoid-independent were used. HeLa cells were transfected with either of the constitutive plasmids or the glucocorticoid-responsive plasmid, the intracellular concentration of pyridoxal phosphate was subsequently modulated, and resultant effects on CAT gene expression were monitored. Results of these studies, shown in *Figure 5*, clearly demonstrated that under vitamin conditions which modulate glucocorticoid-induced gene expression, the level of CAT activity derived from either of the glucocorticoid-insensitive plasmids was unaffected. This observation strongly suggests that vitamin B_6 does not influence gene expression non-specifically but acts se-

lectively to modulate glucocorticoid receptor-mediated gene expression. It will be of interest to determine if vitamin B_6 similarly affects gene expression mediated through the other steroid hormone receptors or if the modulatory effect of the vitamin is specific for the glucocorticoid hormone receptor.

The degree of hormone responsiveness of a target cell or tissue is known to be regulated, at least in part, by the concentration of hormone receptors.[84] Thus, pyridoxal phosphate-induced alterations in the level of cellular glucocorticoid receptor number or its ligand binding capacity could mediate the observed effects on hormone-induced gene expression. However, several different analyses, including measurement of glucocorticoid receptor protein and mRNA levels as well as hormone binding assays, demonstrated that alterations in intracellular pyridoxal phosphate concentration have no effect on either glucocorticoid receptor protein or mRNA levels, or on binding of ligand.[12] Thus, the modulation of glucocorticoid receptor-mediated gene expression which is observed under conditions of altered intracellular pyridoxal phosphate concentrations must occur by a mechanism(s) that does not involve changes in glucocorticoid receptor number or hormone binding capacity.

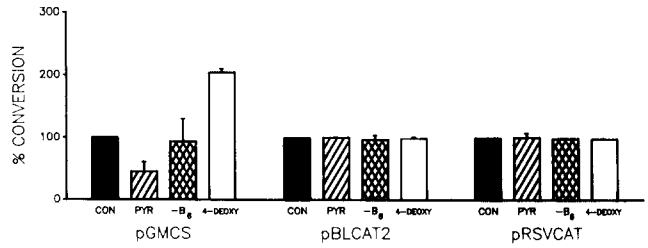

Figure 5 Effect of alterations in intracellular pyridoxal phosphate concentration on CAT activity derived from glucocorticoid-responsive and non-responsive plasmids in transfected HeLa S_3 cells. Following transfection with either the glucocorticoid-responsive plasmid pGMCS or a glucocorticoid-insensitive plasmid, pBLCAT2 or pRSVCAT, HeLa cells were cultured in unaltered medium (CON), pyridoxine-deficient medium ($-B_6$), medium supplemented with 1 mM pyridoxine (PYR), or medium supplemented with 5 mM 4-deoxypyridoxine (4-DEOXY) for 48 h. Cells transfected with pGMCS were treated with 100 nM dexamethasone for 8 h prior to determination of CAT activity. The values shown are representative of the mean ± SD from two independent experiments, with CAT activity (% conversion of substrate chloramphenicol to its acetylated derivatives) from cells grown in unaltered medium assigned a value of 100, and CAT activity from cells grown under altered medium conditions expressed as a fraction of control. (Reproduced with permission from Allgood, Powell-Oliver, and Cidlowski, (1990). *J. Biol. Chem.* **265,** 12424–12433)

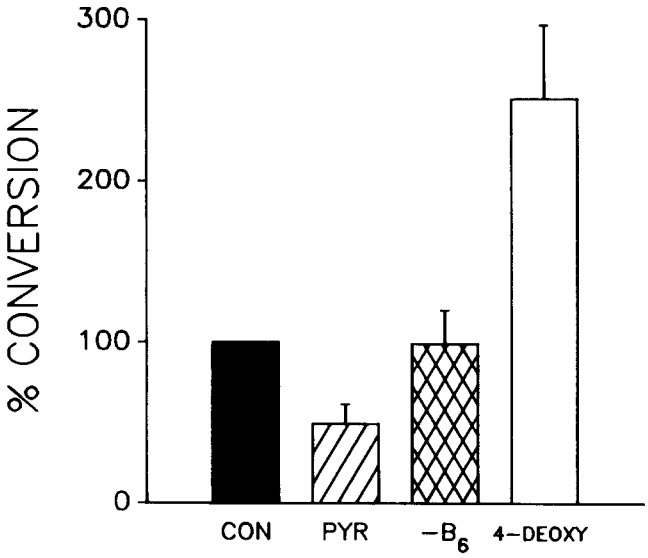

Figure 4 Effect of alterations in intracellular pyridoxal phosphate concentration on glucocorticoid receptor-induced CAT activity in transfected HeLa cells. After transfection with the glucocorticoid-responsive reporter plasmid pGMCS, cells were introduced into the following media in order to alter intracellular pyridoxal phosphate levels: unaltered medium (CON); medium prepared without pyridoxine ($-B_6$); medium supplemented with 1 mM pyridoxine (PYR); or medium supplemented with 5 mM 4-deoxypyridoxine (4-DEOXY). After culture for 48 h under these conditions, cells were stimulated with 100 nM dexamethasone or no hormone for 8 h and the amount of CAT activity determined. The values shown represent the mean ± SD from three independent transfection experiments. CAT activity (% conversion of substrate chloramphenicol to its acetylated derivatives) obtained from dexamethasone-stimulated cells grown in unaltered medium is assigned a value of 100, and the level of CAT activity derived from dexamethasone-stimulated cells grown in altered media conditions is expressed as a fraction of the control value. (Reproduced with permission from Allgood, Powell-Oliver, and Cidlowski, (1990). *J. Biol. Chem.* **265,** 12424–12433)

These data are consistent with a series of somewhat indirect but extremely provocative reports on the effects of vitamin B_6 nutriture and hormone responsiveness. Majumder et al. have shown that increased concentrations of vitamin B_6 inhibit glucocorticoid-induced casein mRNA accumulation in mouse mammary gland.[85] Early work of Symes *et al.* demonstrated that the stimulation of rat prostate growth by androgen is enhanced in animals maintained on a vitamin B_6-deficient diet.[73] Similarly, the estrogen stimulation of rat uterine growth and peroxidase activity is enhanced under conditions of vitamin B_6 deficiency.[71] In addition, administration of pyridoxine during gestation has been shown to reduce the incidence of glucocorticoid-induced cleft palate in rats.[86] These in vivo, physiological studies all support the notion that steroid hormone responsiveness is modulated in response to alterations in vitamin B_6 concentration. However, they do not allow determination or examination of the mechanism through which alterations in vitamin concentration modulate these hormone-dependent events; it is not possible to distinguish between direct and indirect effects of the vitamin on steroid hormone action in these studies. When considered in conjunction with the studies on regulation of specific gene expression, a very compelling hypothesis emerges: vitamin B_6 may affect steroid hormone action by modulating the ability of the steroid hormone receptor to regulate target gene expression. It is clear that further studies are required to elucidate the precise mechanism through which pyridoxal phosphate affects the transcriptional regulatory function of the receptor.

Speculation

There is clear evidence in support of a direct interaction between pyridoxal phosphate and the glucocorticoid receptor, including the protection of receptor from proteolytic degradation and association of pyridoxal phosphate-treated receptor with an antibody directed against pyridoxal phosphate. Thus, it is possible that the effects of pyridoxal phosphate on both the physical properties (subunit composition, conformation, and surface charge) and transcriptional activation function of the receptor are mediated through this putative direct interaction. However, it bears emphasis that effects on the physical properties of the receptor and the interaction of receptor with the anti-pyridoxal phosphate antibody are reflective of an in vitro interaction of receptor with pyridoxal phosphate. While it is likely that these in vitro observations are indicative of in vivo events and, as such, are physiologically relevant, it remains formally possible that such a direct interaction between the receptor and pyridoxal phosphate does not exist in vivo or that an association does occur but is not responsible or sufficient to mediate the effects observed at the gene expression level. Identification of a site on the receptor with which pyridoxal phosphate interacts to mediate its effects on the glucocorticoid receptor function may distinguish between these possibilities.

An interesting possibility is that pyridoxal phosphate may interact with another protein involved in regulation of hormone-induced gene expression. It is becoming increasingly apparent that gene expression is regulated through a complex array of DNA elements which are recognized by different and diverse transacting transcription factors.[87] Reports in the literature indicate that, at least in some cases, gene expression is regulated through interaction among the different transcription factors.[88,89] In fact, the glucocorticoid receptor has been reported to act in concert or synergistically with other known transcription factors.[90-97] Thus, it is possible that pyridoxal phosphate may exert its effects through another transcription factor involved in glucocorticoid-regulated gene expression. Alternately, pyridoxal phosphate may interact directly with the receptor but act to modulate a subsequent interaction between the receptor and another auxiliary transcription factor. In extension of these ideas, it is possible that pyridoxal phosphate itself is a ligand for an as yet undefined transcription factor which is involved in hormone-mediated gene expression. This idea is supported by the recent demonstration that two other known vitamins, vitamins A and D, bind to specific cellular proteins, the retinoic acid[98] and vitamin D_3[99] receptors respectively, and regulate target gene expression. Thus, these nutrients serve as ligands for specific transcription factors. An observation reported by Meisler and Thanassi[100] offers the most direct support for the existence of a specific pyridoxal phosphate-binding protein. They have demonstrated the presence of a non-histone protein present in liver nuclei which specifically binds pyridoxal phosphate. This protein may well represent a transcription factor for which pyridoxal phosphate serves as an activator ligand; that pyridoxal phosphate is the predominant vitamin form present in liver nuclei[100] supports this theory.

Conclusion

Available evidence pertaining to the relationship between vitamin B_6 and the glucocorticoid hormone receptor indicates that pyridoxal phosphate affects many physical properties of the receptor, including the capacity for the receptor to mediate hormonally-regulated gene expression. There is evidence to support the existence of a direct interaction between the glucocorticoid receptor and the physiologically active form of vitamin B_6, pyridoxal phosphate. This interaction may serve as the mechanism through which pyridoxal phosphate exerts its effects on the receptor. However, as we have speculated, other possible mechanisms also exist. A role for vitamin B_6 in the physiological modulation of glucocorticoid receptor function is strongly implicated, and investigations over the coming years will undoubtedly elucidate the mechanism(s) through which glucocorticoid hormone action is modulated by this important vitamin.

Where possible, information relating vitamin B_6 to the other steroid hormone receptors has also been presented. It is clear that in vitro treatment with pyridoxal phosphate produces similar effects on other members of the steroid hormone receptor group, and it is possible, even likely, that the vitamin acts through a common pathway to achieve similar effects on the members of this family of proteins. This raises the possibility that vitamin B_6 may also act through the endocrine system to mediate its classical effects on growth, development, and normal biological function. This idea is supported by the knowledge that steroid hormones exert a variety of effects in a wide range of target cells and tissues, and some of these effects overlap with the known actions of vitamin B_6. For example, in promoting gluconeogenesis, the glucocorticoid hormones regulate metabolism of proteins and amino acids. The immune system is regulated to a significant extent by glucocorticoid hormones, in that immune responses and cell-mediated immunity are suppressed by glucocorticoids. Normal growth and development is affected by glucocorticoid hormones, in part through glucocorticoid-modulated growth hormone synthesis. Lastly, synthesis of at least one neurotransmitter, epinephrine, is regulated through a glucocorticoid-dependent enzyme. In each of these instances—amino acid metabolism, immune function, normal growth, and neural function—a known role for vitamin B_6 has been clearly and historically defined without invoking a requirement for steroid hormones. However, it is now possible, and perhaps necessary, to speculate that the effects of the vitamin may be mediated additionally through the steroid hormone receptor. It is possible that by allowing multiple roles or mechanistic pathways for the vitamin, both as an

enzymatic cofactor and as a modulator of the endocrine system, an added dimension of diversity or control may be afforded. Thus, at least some of the diverse actions of vitamin B_6 may be mediated through non-classical effects on the endocrine system.

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