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Impact on Public Health

Influence of Body Weight, Diet, and Stress on Aging, Survival and Pathological Endpoints in Rodents: Implications for Toxicity Testing and Risk Assessment

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Abstract: Dietary restriction in rodents has been repeatedly shown to increase lifespan while reducing the severity and retarding the onset of both spontaneous and chemically induced neoplasms. These effects of dietary restriction are associated with a spectrum of biochemical and physiological changes that characterize the organism's adaptation to reduced caloric intake and provide the mechanistic basis for dietary restriction's effect on longevity. Evidence suggests that the primary adaptation appears to be a rhythmic hypercorticism in the absence of elevated ACTH levels. This characteristic hypercorticism evokes a spectrum of responses including: decreased glucose uptake and metabolism by peripheral tissues, decreased mitogenic response coupled with increased rates of apoptosis, reduced inflammatory response, reduced oxidative damage to proteins and DNA, reduced reproductive capacity, and altered drug metabolizing enzyme expression. The net effect of these changes is to: (1) decrease growth and metabolism in peripheral tissues to spare energy for central functions and (2) increase the organism's capacity to withstand stress and chemical toxicity. These adaptations suggest an evolutionary mechanism that provides rodents with an adaptive advantage in conditions of fluctuating food supply. During periods of abundance, body growth and fecundity are favored over endurance and longevity. Conversely, during periods of famine, reproductive performance and growth are sacrificed to ensure survival of individuals to breed in better times. This phenomenon has been observed in rodent populations that are used in toxicity testing. Improvements in animal husbandry and nutrition, coupled with selective breeding for growth and fecundity, resulted in several strains exhibiting larger animals with reduced survival and increased incidence of background lesions. Mechanistic data from dietary restriction studies suggest that these large animals will also be more susceptible to chemically induced toxicity, thus creating problems in comparing tests performed on animals of different weights and in comparing data generated today with the historical database. The rational use of dietary restriction to control body weight to within preset guidelines was proposed as a possible way of alleviating this problem. Recent data from studies testing this paradigm have demonstrated that dietary control not only can increase animal survival in two-year studies but also can increase bioassay sensitivity.

Introduction

Dietary restriction has been repeatedly shown in rodents to increase maximally achievable life span and to decrease the incidence and proliferative rate of spontaneous and chemically induced neoplasia (1,2). During the last decade significant new information became available on the biochemical and molecular mechanisms through which dietary restriction influences cancer rates and aging (3-8). Nutrient stress, which is characterized by elevated glucocorticoid levels in the absence of elevated ACTH or inflammatory cytokines, appears to play a central role in mediating the effects of dietary restriction (3,9-11) and has certain similarities to the stress response elicited by many chemicals when administered to rodents at their maximally tolerated dose (3).

While it has been known for over 60 years that survival and neoplasm incidence in laboratory rodents is influenced profoundly by caloric intake and body weight (12), it was only during the last decade that diet and body weight have become major issues in the design and interpretation of animal toxicity and carcinogenicity studies. Attention to these issues was precipitated by the observation, during the early 1990s, that mean life span of rodents, which were commonly used in cancer bioassays had been steadily decreasing, concurrently with an increase in mean body weight and in the background incidence of neoplastic and other degenerative diseases. For several strains it had reached a point where assay interpretation was being compromised due to insufficient animals surviving to the end of the study (13-15). Various symposia addressed this issue and generally recommended that some form of dietary control, either through dietary restriction or new diet formulations, be used to maintain animals within a healthy weight range during toxicity testing (16,16-20). Several approaches to dietary control have now been tested, and there has been a trend among rodent breeding companies to select for smaller animals. This paper reviews and updates the current state of knowledge on how dietary restriction evokes its beneficial effects on aging and disease and describes the relative success of dietary control techniques in increasing survival while decreasing variability and background neoplasia rates in laboratory rodents.

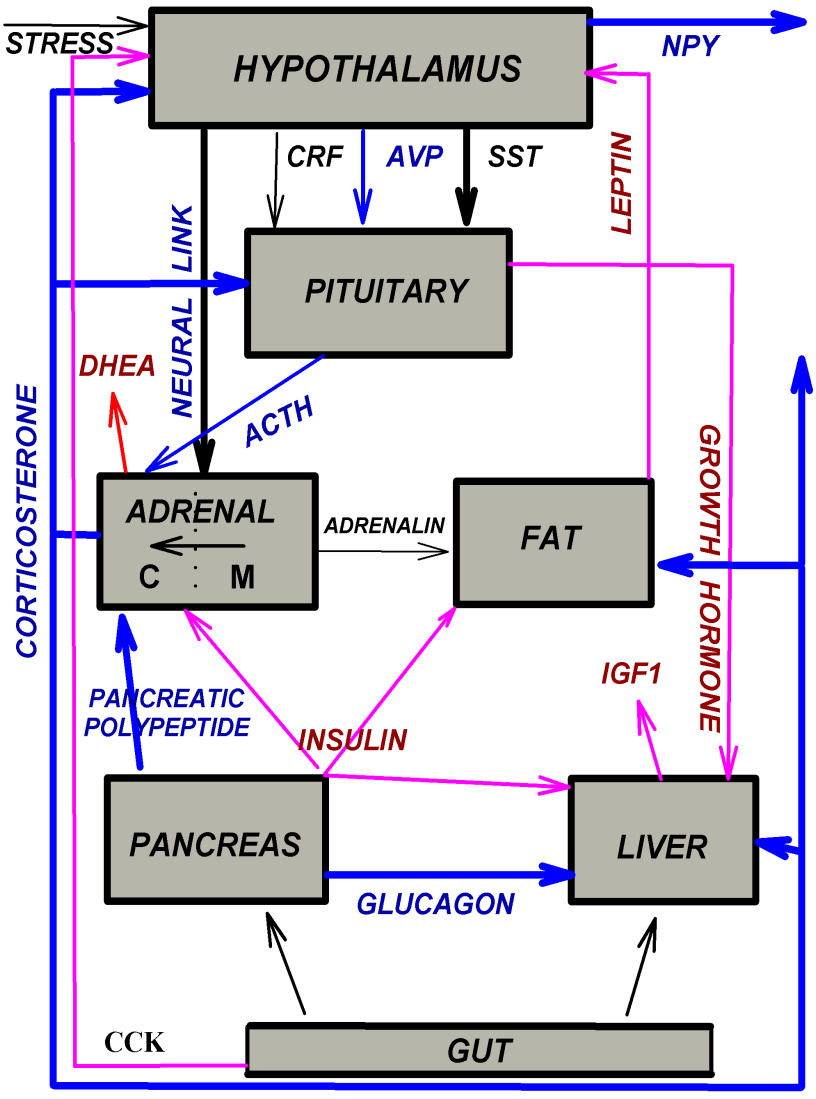

Figure 1. Hormonal Control of Glucose and Energy Homeostasis

Energy homeostasis and physiological blood glucose levels are maintained predominantly by the reciprocal actions of glucocorticoids and insulin. These hormones in turn regulate mitogenesis and growth rate via growth hormone, IGF1 and DHEA. See text and (31) for references. Blue lines = response to caloric deficit, red lines = response to caloric excess, black lines = classic stress response. AVP = arginine vasopressin, SST = somatostatin, NPY = neuropeptide Y, CRF = corticotropin releasing factor, CCK = cholecystokinin, DHEA = dehydroepiandrosterone, and IGF1 = insulin-like growth factor 1.

Hypercorticism - An Adaptive Response to Nutrient Stress

Although the precise mechanisms by which dietary restriction

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evokes its beneficial effects on disease and longevity have not been fully determined, it is becoming evident that glucocorticoid hormones play a significant role in mediating these effects (10,21). It was first reported over fifty years ago that caloric restriction resulted in adrenal hypertrophy (22), and during the last few years a number of laboratories have demonstrated increased corticosterone concentrations in serum from dietary restricted rats and mice (10,21,23-30).

The mechanism controlling the adaptive response to reduced caloric intake involves the complex, dynamic interplay between the hormones that control energy balance, appetite, cell proliferation and apoptosis, stress response, metabolic rate, inflammation, and repair systems (3,21) [Figure 1]. Glucocorticoids and insulin appear to play a reciprocal role as the major mediators of energy balance and glucose homeostasis in mammals (31-33). Serum corticosterone levels rise in response to hypoglycemia and increase blood glucose levels by inhibiting glucose transport into peripheral tissues while increasing gluconeogenesis and glucose output by the liver. In the hypoglycemic state, corticosterone also stimulates appetite by inducing neuropeptide Y production in the arcuate nucleus of the hypothalamus (34) and stimulates lipolysis in adipose tissue, while reducing energy expenditure in other peripheral tissues by decreasing thermogenesis and inhibiting the effects of mitogenic and excitatory hormones (21,32). Conversely, in the hyperglycemic state insulin levels rise and decrease blood glucose levels by stimulating glucose uptake and glycogen synthesis in liver and muscle and by increasing glucose uptake and lipogenesis in adipose tissue (31,32). Insulin also stimulates leptin production in adipose tissue, which in turn decreases appetite and increases metabolism and energy expenditure in peripheral tissues (35-38). Glucocorticoid treatment also stimulates leptin production; but this is possibly an indirect effect resulting from increased insulin levels, increased insulin sensitivity or functional maturity of adipocytes (39). The leptin gene promoter region does not contain glucocorticoid response elements; and during fasting conditions, where glucocorticoid levels increase, while those of insulin decrease plasma leptin levels also decrease (39,40). Thus, under normal physiological conditions, a balanced opposing relationship exists between insulin and corticosterone, which maintains blood glucose levels within the normal physiological range (31).

Nutrient stress, such as fasting, starvation or insulin-induced hypoglycemia results in elevated glucocorticoid levels, but unlike classic stress, hypothalamic release of corticotropin-releasing factor (CRF) does not appear to play a major role in initiating the glucocorticoid response (41-43). Rather arginine vasopressin (AVP) plays the major role in the hypothalamus, and the adrenal response to ACTH appears to be amplified by pancreatic polypeptide, which is secreted by the pancreas during periods of hypoglycemic stress (44-46). In addition, adrenal corticosterone secretion may be further increased by neural stimulation via the adrenal medulla (47,48). This results in elevated corticosterone concentrations in the absence of elevated ACTH (and, by inference, CRF) in both starved (41) and calorically restricted (10,43) rats.

Under normal physiological conditions, once the hypoglycemic crisis has been rectified, insulin levels will rise and, as suggested by *in vitro* experiments (49), may down-regulate adrenal corticosterone secretion in favor of dehydroepiandrosterone (DHEA) secretion. DHEA, like insulin is generally anabolic in function, and it is reported to antagonize many of the effects of glucocorticoids (50-53).

During pathological conditions such as Cushing's syndrome or prolonged, excessive glucocorticoid therapy, natural feedback regulation is bypassed, and a pathological hyperglycemia develops, which is characterized by concurrent elevated insulin and glucocorticoid levels. Such conditions of hypercorticism concurrent with hyperinsulinemia, if prolonged, would be expected to result in pathological conditions such as atherosclerosis and mature-onset diabetes (54). Classic stress appears to be primarily controlled by the hypothalamic-pituitary-adrenal axis (HPA). CRF and AVP secretion from the hypothalamus increase in response to interleukins or neuropeptides and stimulate ACTH secretion by the pituitary (55). Thus, plasma concentrations of both CRF and ACTH are increased in addition to serum corticosterone levels. CRF decreases hyperphagia (56) and is pyrogenic and an inflammatory mediator (57).

Anti-Neoplastic Effects of Glucocorticoids

The net effects of hypercorticism resulting from nutrient stress are, therefore, a reduction of glucose uptake and energy metabolism in peripheral tissues. This, in itself, may provide a beneficial effect on aging and carcinogenesis by reducing rates of intracellular glycoxidation and oxidative damage from respiratory chain enzymes (58,59). However, the primary mechanism by which glucocorticoids impact upon aging and degenerative disease may be through their anti-mitotic and anti-inflammatory functions.

Anti-mitotic effects: Growth hormone and glucocorticoids are mutually antagonistic in their effects on body growth (60) and wound healing (61), and some of the anti-mitogenic effects of glucocorticoids are mediated through changes in the hypo-

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thalamic-pituitary-liver growth hormone/IGF_1 axis. Glucocorticoids disrupt the pulsatile secretory profiles of growth hormone (62,63) in rats and decrease hepatic IGF_1 expression (64). They also antagonize the proliferative effects of EGF and PDGF in various cell culture systems (65,66) and antagonize the stimulatory effects of Luteinizing Hormone (LH) on the adenyl cyclase/cAMP system in Leydig cells and possibly other endocrine tissues (67,68). Although high glucocorticoid levels can cause atrophy of skeletal muscle, they stimulate hypertrophy in cardiac muscle (69). This effect is associated with alterations in expression of myosin isoforms resulting in the high efficiency V_3 isoform being favored over the low efficiency V_1 isoform (69,70). Thus, the anti-mitogenic effects of glucocorticoids appear to be selective.

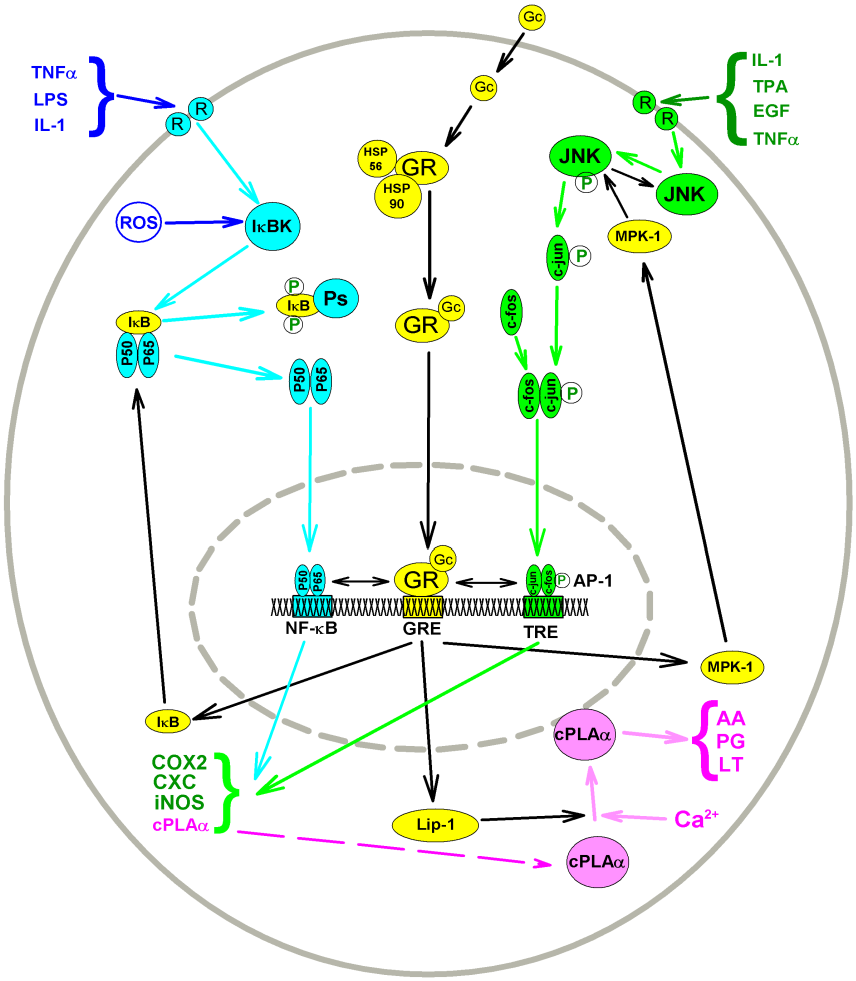

Figure 2. Intracellular Actions of Glucocorticoids on Inflammatory and Mitogenic Signal Transduction Pathways

Glucocorticoids downregulate NF-kB and AP-1 signal transduction pathways at multiple levels. In many cell types these pathways activate inflammatory or mitogenic responses and inhibit apoptosis. In addition to transrepressing the interaction of activated NF-kB and AP-1 complexes to their DNA response elements, glucocorticoids induce proteins, which inhibit the activation of these complexes.

Abbreviations used: AA = arachidonic acid, AP-1 = activator protein 1 complex (the active complex is composed of a dimer of a c-fos protein with a phosphorylated c-jun protein), cPLAα = cytosolic phospholipase $A_2\alpha$, COX2 = prostaglandin synthetase (cyclooxygenase) 2, CXC = chemokines, EGF = epidermal growth factor, GC = glucocorticoid, GR = glucocorticoid receptor, GRE = glucocorticoid response element, IκB = NFκB complex inhibitor protein, IκBK = IκB kinase, IL-1 = interleukin 1, iNOS = inducible nitric oxide synthase, JNK = Jun N-terminal kinase, LP-1 = lipocortin 1, LPS = lipopolysaccharide, LT = leukotrienes, MPK-1 = mitogen activated protein kinase phosphatase 1 (inactivates JNK by dephosphorylation), NF-κB = nuclear factor kappa B (the active complex is composed of a dimer of the P-50 and P65 proteins), PG = prostaglandins, Ps = proteasome complex which degrades phosphorylated IκB, R = plasma membrane receptors for cytokines and other inflammatory molecules, ROS = reactive oxygen species, TNFα = tumor necrosis factor alpha, TPA = 12-*O*-tetradecanoylphorbol-12-acetate, TRE = TPA response element.

Apoptosis plays an important role in inhibiting tumor development by eliminating damaged and genetically transformed cells from tumor susceptible tissues (71-76). Apoptosis is characterized as differing from tissue necrosis in that only selected cells are eliminated, and the resulting cell debris is immediately phagocytized by adjacent cells so that an inflammatory response is not initiated (77,78). Glucocorticoids induce apoptosis in lymphatic tissues (72), fibroblasts (79) and, possibly, in mammary epithelium (77,80). Glucocorticoids may also selectively mediate the effects of TGFß in stimulating apoptosis in preneoplastic hepatocytes (58,66).

Anti-inflammatory effects: When used therapeutically, glucocorticoids are extremely potent anti-inflammatory agents, which interact with practically every stage of the inflammatory response (81). Although it was once proposed that physiological levels of endogenous glucocorticoids stimulated the inflammatory response, as part of the general adaptation to stress (82), it now appears their physiological role during stress is to protect the organism from an overstimulated inflammatory response (81,83). Glucocorticoids achieve this by inhibiting the

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production of, or antagonizing the actions of, inflammatory mediators such as prostaglandins, leukotrienes, interleukins, and atrial natriuretic factor (3,21,81) [Figure 2]. Many of the anti-inflammatory and anti-mitotic effects of glucocorticoids appear to be mediated by the glucocorticoid-inducible protein, lipocortin 1 (81,84-87). Lipocortin 1 (also known as annexin 1) is a glycosylated 37 kDa Ca^{2+}-dependent phospholipid binding protein, which inhibits its phospholipase A_2, a key enzyme in the synthesis of inflammatory prostaglandins and leukotrienes from arachidonic acid (81). In addition to directly inhibiting phospholipase A_2 activity, lipocortin 1 recently has been shown to inhibit the EGF-mediated phosphorylation of the cytosolic form of this enzyme (cPLAα) (88,89). cPLAα is activated by phosphorylation as part of a G-protein dependent, EGF-mediated mitogenic response (88). Lipocortin 1 also mediates glucocorticoid feedback effects on the HPA-axis by inhibiting both basal and interleukin-induced release of CRF and AVP by the hypothalamus (55,84,90). Glucocorticoids also down-regulate mRNA expression of several key inflammatory enzymes. These include 12-lipoxygenase (91) and the inducible, but not constitutive, forms of prostaglandin synthase (COX2) (92-95), nitric oxide synthase (iNOS) (95-97) and intestinal phospholipase A_2 (PLA_2II) (98). These enzymes generally are induced by endotoxins, tumor necrosis factor, interleukins, phorbol esters, or growth factors. Although it is not known whether glucocorticoids directly or indirectly repress transcription of these enzymes, lipocortin 1 appears to mediate glucocorticoid-mediated down-regulation of iNOS (99), but not COX2 (100).

Lipocortin 1 has been proposed to be a mediator of glucocorticoid-induced apoptosis. It is induced in apoptotic cells where it has been proposed to inhibit recognition of the dying cells by macrophages (77). Lipocortin 1 is also a substrate for transglutaminase. This enzyme is induced in apoptopic cells where it catalyzes the covalent linkage of proteins. Covalently linked lipocortin dimers can form polymers with other proteins during apoptosis potentially enhancing phagocytic uptake by adjacent cells (77,101,102). Lipocortin 1 was shown to protect cultured rat thymocytes from H_2O_2-elicited necrosis. Glucocorticoid treatment, which induced lipocortin 1, stimulated apoptosis while treatment with an anti-lipocortin 1 antibody enhanced necrosis (103).

Glucocorticoids also mediate inflammation through interactions with the nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) signal transduction pathways (104-106). Both of these pathways play a major role in the inflammatory and mitogenic responses in many cell types and in general protect cells from apoptosis when stimulated by cytokines, such as tumor necrosis factor α (TNFα) or oxidative stress (107-110). The NF-κB transcription factor complex is usually retained in the cell cytosol in an unstimulated state by an inhibitory protein (named IκB), which binds to the cytoplasmic NF-κB complex and inhibits its translocation into the nucleus. TNFα and other cytokines stimulate the phosphorylation of IκB, which targets the inhibitor protein for proteolytic degradation, which then frees the NF-κB complex for nuclear translocation (Figure 2). Glucocorticoids induce the expression of IκB, thereby down regulating NF-κB activation, whereas insulin also stimulates IκB phosphorylation (111-114). This suggests that whether or not a cell will initiate apoptosis in response to TNFα will depend on the insulin: glucocorticoid ratio in its interstitial environment.

Activated glucocorticoid receptor complexes are also able to inhibit inflammatory and mitogenic transcription factors by direct protein-protein interaction. These interactions have been demonstrated for the NFκB complex, the AP-1 ligands c-fos and c-jun, and several STAT proteins, and are independent of glucocorticoid-mediated transcription (106,115).

Despite their global anti-inflammatory effects, glucocorticoids have been shown to potentiate certain aspects of the host defense system. For example, they have been reported to induce expression of heat shock proteins such as HSP_{70} (116) and increase activity of the DNA repair enzyme O^6-methylguarnine-DNA methyltransferase (117) in certain tissues. They also potentiate the effects of interleukin-6 and hepatocyte-stimulating factor in inducing hepatic acute phase proteins, such as Mn-superoxide dismutase and α_2-macroglobulin (118-122). Although both glucocorticoids and lymphocyte stimulatory agents that are mediated via intracellular Ca^{2+} or protein kinase c (e.g., calcium ionophors/phorbol esters, antibodies to the T-cell antigen receptor) initiate apoptosis in maturing lymphocytes, they are mutually antagonistic to the extent that glucocorticoids protect lymphocytes from activation-induced apoptosis (123,124). Thus, the effects of glucocorticoids on the inflammatory and immune systems are modulatory rather than simply suppressive.

Inflammation, necrosis, oxidative damage and regenerative hyperplasia all play a significant role in chemically induced tumor promotion, and glucocorticoids have been shown to inhibit hyperplasia and neoplasia in a number of systems. For example, glucocorticoids are used therapeutically as antineoplastic agents in several types of leukemia and lymphoma (52,125), and they suppress growth of certain lung or mammary adenocarcinomas (80,126-128). Dexamethasone has been reported to inhibit both peroxisome proliferator-induced and lead

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nitrate-induced proliferative hyperplasia in rat liver (129,130). Glucocorticoids have also been shown to induce connexin expression and stimulate gap junction formation in cultured hepatocytes and embryonic cells (131-133). Inflammatory agents, such as phorbol esters, promote, and glucocorticoids inhibit papilloma formation in mouse skin (134).

Toxic Effects of Glucocorticoids

Chronic and excessive elevation of glucocorticoid levels increases the risk of developing hypertension, hyperkalemia, diabetes, atherosclerosis, osteoporosis, glaucoma, and impairment of the immune and reproductive systems (135,136). The organ most susceptible to glucocorticoid toxicity appears to be the hippocampus. High doses of corticosterone administered to adrenalectomized rats resulted in neuronal atrophy in the hippocampus, but not in other areas of the brain (54,137,138). Because the hippocampus, in conjunction with the hypothalamus, controls feedback regulation of the HPA, it was suggested by Sapolsky and coworkers (138), in what has become known as the *glucocorticoid cascade hypothesis*, that glucocorticoid-evoked hippocampal damage impairs the feedback regulation of adrenal glucocorticoid output, which could result in further increases in glucocorticoid levels and additional hippocampal damage. Over a lifetime, such an effect may result in premature aging of the brain. Evidence supporting this hypothesis includes *in vitro* studies, which have demonstrated that glucocorticoids impair the ability of cultured hippocampal cells to withstand neurotoxic stresses (138). The proposed mechanisms responsible for these effects include inhibition of glucose transport and disruption of Ca^{2+} homeostasis (138-140). In humans, patients with Cushing's syndrome have been reported to exhibit memory impairment, which correlated with serum cortisol levels (141), and dexamethasone treatment has been reported to impair declarative memory performance (142). However, although hypercorticism is often manifested in Alzheimer's patients (139), long-term treatment with glucocorticoids is associated with delay in the onset of Alzheimer's disease (143). Lipocortin 1 is expressed throughout the brain, including the hippocampus, and has been shown to protect against neuronal damage resulting from either ischemia or NMDA receptor agonists (144,145).

Exposure of adult rats to stress, hypercorticism or glucocorticoid therapy reduces reproductive hormone levels in both sexes (3). In males, for example, glucocorticoids appear to inhibit LH-mediated testosterone synthesis by cultured rat Leydig cells (146) and dexamethasone treatment decreases, while adrenalectomy increases serum testosterone levels *in vivo* (147,148). In females, glucocorticoids decrease FSH-stimulated aromatase activity and estrogen production by ovarian granulosa cells (149), suppress ovulation and inhibit ovarian prostaglandin metabolism (150). They also inhibit the preovulatory pituitary LH surge in female rats (151) and estradiol- and gonadotropin releasing hor-

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Table 1. Major Effects of Caloric Restriction

Direct Effects	Aging-dependent Effects
Blood glucose - unchanged or decreased.	Neoplasia - delayed.
Pancreas, insulin secretion - decreased.	Nephropathy - delayed.
Serum corticosterone - increased.	Cardiopathy - delayed.
Plasma ACTH - decreased.	Hyperinsulinemia - decreased.
Body temperature - decreased.	Cognitive defects - decreased.
Hepatic gluconeogenic enzymes -increased.	Reproductive senescence - delayed.
Cardiac muscle, myosin V_1 - decreased, myosin V_3 - increased.	Antioxidant enzymes - increased.
Pulsatile growth hormone - inhibited.	*DNA repair - increased.*
Hepatic IGF_1 synthesis - decreased.	Pulsatile growth hormone - maintained.
Hepatic sex-specific drug metabolism - decreased.	Hepatic IGF1 synthesis - increased.
Cell proliferation - decreased.	Hepatic sex-specific drug metabolism - increased.
Apoptosis - increased.	Cell proliferation - increased.
Blood - leukopenia.	*Blood - leukopenia.*
Inflammatory response - decreased.	
Lipocortin production - increased.	
12-lipoxygenase - decreased.	
Male gonadal steroids - feminized.	
Reproductive function - decreased.	
HSP70 - increased.	

Direct effects of caloric restriction are those occurring immediately after restriction is initiated and result directly from the organism's response to caloric deficit. Aging-dependent effects of caloric restriction are those occurring in response to the delay in physiological aging that results from caloric restriction. Effects in *italics* are those which are consistent with hypercorticism. References given in text or in references (3, 21, 266).

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mone-induced LH production in cultured rat pituitary cells (152). In male rats, glucocorticoids inhibit pituitary secretion of prolactin (153), but not mean LH levels (154). However, CRF and stress inhibit pituitary LH secretion in both sexes (155,156).

Glucocorticoid-Mediated Effects of Dietary Restriction

Dietary restriction not only evokes anti-inflammatory and antineoplastic effects that are consistent with chronic hypercorticism, but also protects the aging rodent against insulin resistant diabetes (29,157-159), impaired tissue growth and regeneration (160,161), certain neurological impairments (162,163), and reproductive senescence (164,165) [Table 1]. Although these latter effects appear at first sight to be inconsistent with hypercorticism, on further analysis they appear to be the natural consequence of the nutrient stress that is produced by caloric restriction under the conditions used for most experimental paradigms.

There are several factors that differentiate the nutrient stress produced by dietary restriction from other stress situations or glucocorticoid therapy (3). Firstly, unlike treatment with pharmacological doses of synthetic glucocorticoids, hypercorticism resulting from nutrient stress involves the natural glucocorticoids, corticosterone or cortisol. The effects of these natural glucocorticoids are mediated by serum transcortin and 11ß-hydroxysteroid dehydrogenase, which may protect tissues from extreme hypercorticism (3). Furthermore, unlike synthetic steroids such as dexamethasone, corticosterone and cortisol bind to both Type I and Type II glucocorticoid receptors so that the Type I receptor response is not inhibited concurrently with an excessive Type II receptor response (166).

Secondly, the hypercorticism exhibited by dietary restricted rodents differs from the continuously elevated

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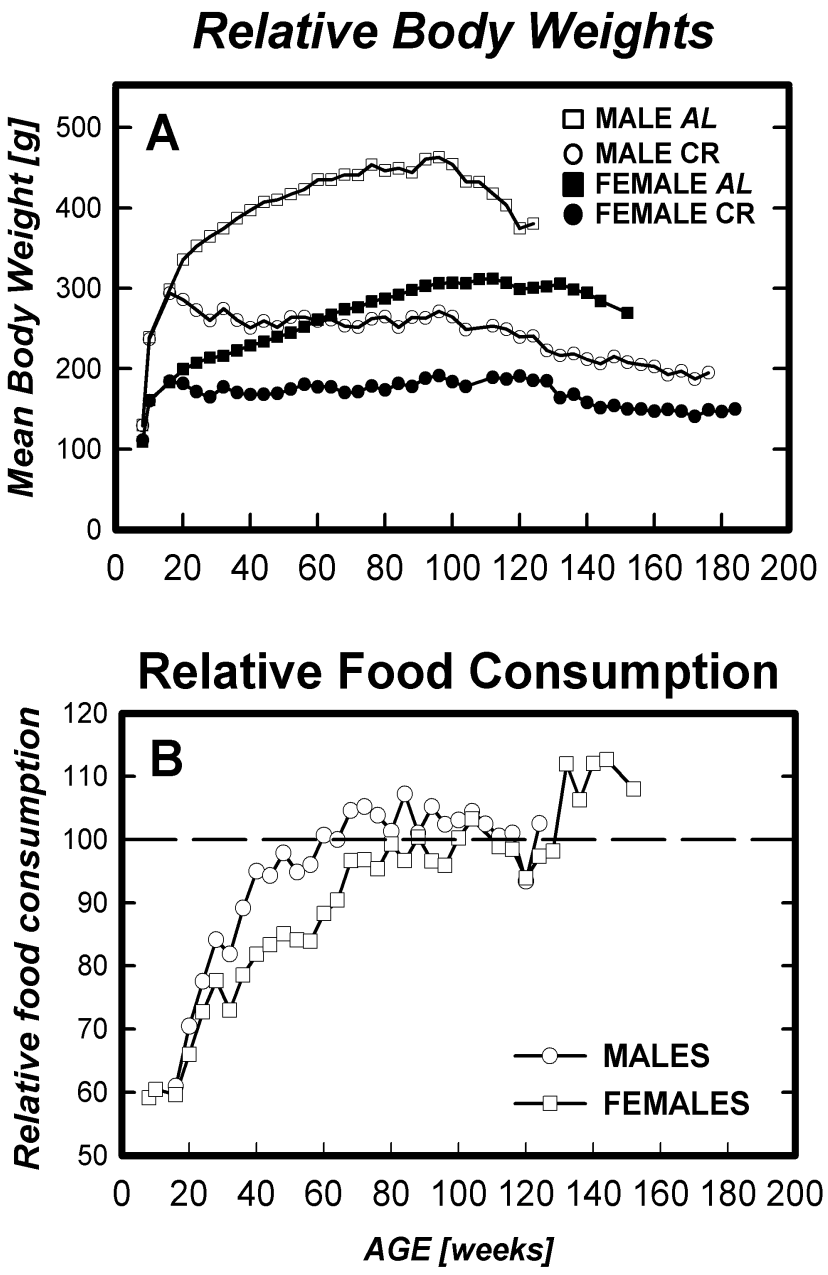

Figure 3. Influence of Body Weight on Caloric Consumption in Calorically Restricted Fischer 344 Rats

Fischer 344 rats, housed in a specific pathogen free barrier facility at NCTR, were placed on a vitamin fortified NIH-31 diet at 60% of *ad libitum* food consumption as described by Duffy *et al* (177). **A**, the weight curves for male and female rats. **B**, relative food consumption (expressed as food consumed per gram body weight by the calorically restricted rats as a percentage of that consumed per gram body weight by the *ad libitum*-fed rats) as a function of age. By 50 weeks for the males and 70 weeks for the females the calorically restricted rats consume equivalent amounts of food per gram body weight as their *ad libitum*-fed counterparts.

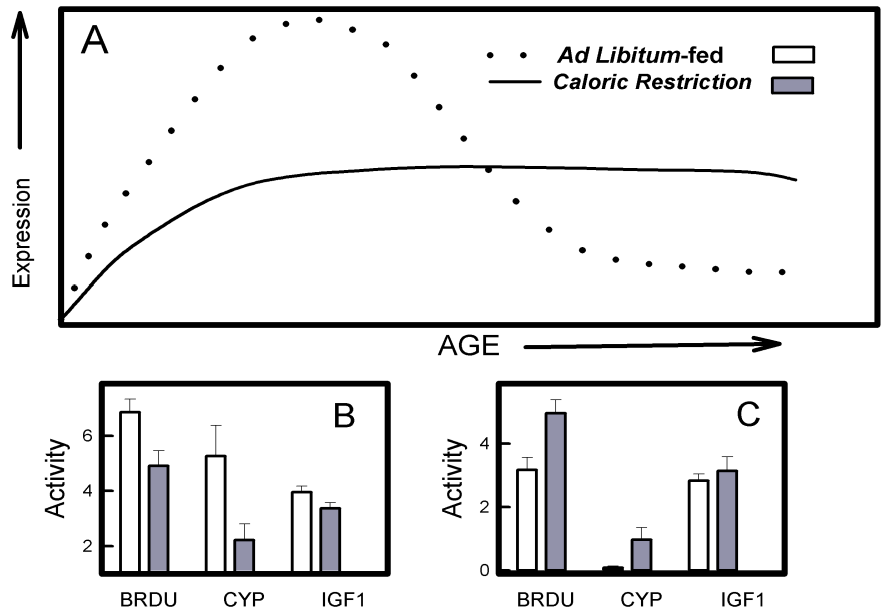

Figure 4. Age-dependent Effects of Caloric Restriction

(**A**) Schematic representation of the effects of caloric restriction on mitogenic endpoints and reproductive function. Reduction in the early burst of activity delays the degradation of these systems in old age. Examples include: [BRDU], cell proliferation in kidney tubule cells from B6D2F$_1$ mice, as measured by *in vivo* labeling with BrdU (161); [CYP] expression of hepatic cytochrome P450 2C11 (CYP2C11) and its dependent activity, testosterone 16"-hydroxylase in male Fischer 344 rats (183); [IGF] expression of hepatic IGF1 mRNA in male Fischer 344 rats (182). Caloric restriction decreases these parameters in young rats (**B**) but maintains them in old rats (**C**).

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serum corticosterone levels exhibited by starved or chronically stressed rodents in that corticosterone levels are increased, above those of their *ad libitum*-fed counterparts, only during a limited circadian period that is prior to and coincident with feeding activity (167). This type of intermittent hypercorticism appears to be less damaging to mitogenic processes than continuously elevated glucocorticoid levels (3).

Thirdly, because the hypercorticism is a response to caloric deficit and potential hypoglycemia and occurs in conjunction with normal feedback regulatory systems, it is not associated with chronic hyperglycemia or hyperinsulinemia (21,29,168). Thus, insulin resistance and protein glycation, which are the usual pathological consequences of glucocorticoid-induced hyperglycemia, should not occur. Instead, rates of intracellular glycation and oxidation of protein would be expected to decrease in peripheral tissues due to reduced glucose incorporation. Reduced collagen glycoxidation has been observed in skin from calorically restricted rats (169), and accumulative oxidative damage to both protein and DNA is reduced by dietary restriction in a number of tissues (59,170-176).

Fourthly, under the usual conditions that are used for dietary restriction experiments, significant hypercorticism only occurs during the early stages of restricted feeding (25,28). In most strains of rodents used in caloric restriction experiments, body weight gain is reduced in the restricted animals to an extent where the body weight difference between the restricted and *ad libitum*-fed animals equals or exceeds the caloric deficit (177) [Figure 3]. Thus, during the latter half of a calorically restricted rat's life span its caloric consumption per gram body weight is equal to or greater than that of its *ad libitum*-fed counterpart. Under these conditions significant hypercorticism would not be required to protect the animal from potential hypoglycemia. As a consequence, during senescence, when rodents are most susceptible to tissue degeneration due to reduced capacity for cellular proliferation and reduced output of mitogenic hormones (161,178); serum corticosterone levels are normally no longer significantly increased in chronically calorically restricted animals (3,25,28).

The effects of dietary restriction on biomarkers of mitogenesis are generally consistent with the occurrence of hypercorticism during the early, but not the late stages of caloric restriction. For example, caloric restriction from 16 weeks of age abolishes growth hormone pulsatility in six month-old male Brown Norway rats, but pulsatility is restored in older animals (179). In male rats, pulsatile growth hormone controls hepatic expression of both IGF$_1$ and sex-specific drug metabolizing enzymes such as cytochrome P450 2C11 (CYP2C11) (180,181). As expected from its effects on pulsatile growth hormone, caloric restriction decreases hepatic expression of both IGF$_1$ and CYP2C11 in young male rats (182,183). However, as the rats age, hepatic IGF$_1$ and CYP2C11 expression decreases in the *ad libitum*-fed rats, but is maintained by caloric restriction animals so that in old rats hepatic IGF$_1$ and CYP2C11 expression is greater in the calorically restricted animals (182,183). This age-dependent biphasic effect of caloric restriction is illustrated in Figure 4 and is a common feature of

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several of the reported effects of caloric restriction in rodents. These include: cell proliferation rates in kidney, pancreas and possibly liver from B6D2F$_1$ mice (161), serum DHEA levels in Fischer 344 rats (184), and reproductive function in both rats and mice.

The effects of caloric restriction on female reproductive function include delayed puberty (185,186), inhibition of LH pulsatility concurrent with hypercorticism (26), inhibition of ovulation (187), decreased litter size (188,189), increased lactational diestrus (190), and reduced milk production (191) during the initial period of caloric restriction and delayed reproductive senescence during the later stage (165,188). In males, the initial effects of caloric restriction include decreased LH pulsatility (192), reduced ratios of serum testosterone to estradiol (193), decreased sperm motility in rats (194,195), and decreased prostate weight, testicular sperm density and fertility in mice (189). Long-term caloric restriction reduces testicular hyperplasia and delays Leydig cell adenoma formation in old male rats (193,196), whereas chronic feeding of a high caloric diet reduced reproductive performance in old male CF-1 mice (197).

The anti-inflammatory effects of caloric restriction are also generally consistent with effects resulting from hypercorticism. For example, caloric restriction has been reported to induce lipocortin 1 immunoreactive proteins in rat liver (21), to inhibit carrageenan-induced inflammation in mice (30), to decrease 12-lipooxygenase activity in rat liver and testes (3), to delay the onset of autoimmunity in autoimmune-prone mice (198), and to inhibit promotion of mouse skin papillomas by phorbol esters (199,200). In the last case, adrenalectomy reversed the effect of caloric restriction whereas the effect was enhanced by glucocorticoid replacement (11). Interestingly, caloric restriction both potentiates regenerative hepatocyte proliferation in partially hepatectomized rats (201) and reduces cell proliferation while stimulating apoptosis in preneoplastic liver (202,203). Such an effect is consistent with the reported dual synergistic and antagonistic effects of glucocorticoids on TGFß in neoplastic and non-neoplastic hepatocytes (3,66). Dietary restriction also reduces lung inflammation in rats exposed to ozone (204,205) and enhances resistance to gram-positive bacteria, while lowering the production of pro-inflammatory mediators elicited by endotoxin, a component of gram-negative bacteria.

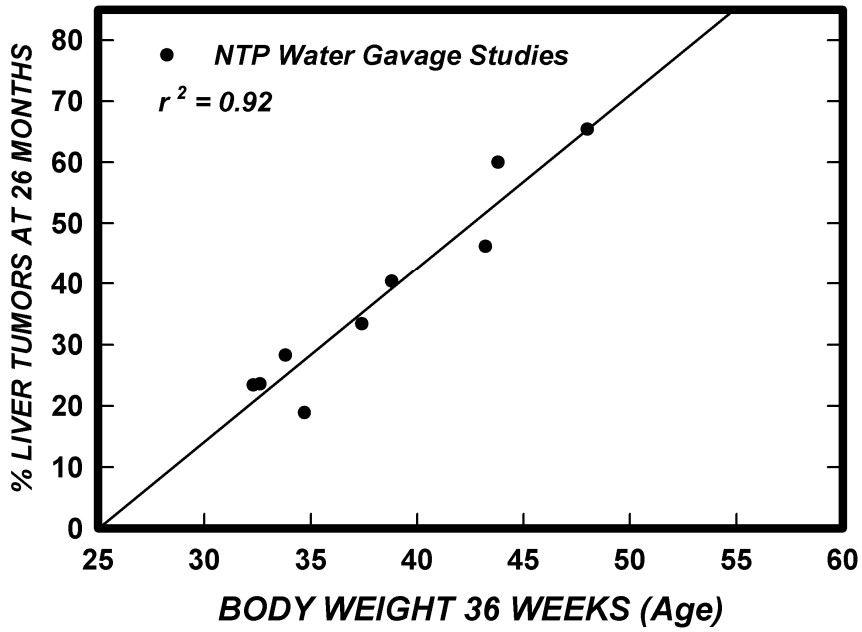

Figure 5. Association between Mean Body Weight and Liver Neoplasm Incidence in Male B6C3F$_1$ Mice

Data from control groups from nine NTP studies conducted during the 1980s and early 1990s that used water-based gavage for dosing. The percent liver neoplasm values are the survival-adjusted rates of hepatocellular adenoma or carcinoma. The individual studies used are listed in (251).

While old dietary restricted mice exhibited improved cognitive function, motor performance, and reduced oxidative damage in the brain (162,163), dietary restriction neither inhibited hippocampal aging in rats, nor appeared to be overtly detrimental to the hippocampus (140,206). However, dieting and dietary restriction have been reported to impair cognitive function in humans (207). Despite potential endangerment to the hippocampus, hypercorticism during nutrient stress would be expected to be beneficial, since the alternative, hypoglycemia in conjunction with increased inflammatory activity, would pose a greater threat to the entire central nervous system.

Taken together, dietary restriction in rodents appears to produce a series of pleiotropic biochemical and physiological effects that are consistent with a condition hypercorticism that is more severe in the early stages of caloric restriction than in the later stages and that occurs without concurrent hyperglycemia. The overall effect of this condition is to conserve energy by minimizing metabolism, proliferation and nones-

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sential functions in peripheral tissues. This in turn appears to minimize damage to the affected tissues so that the progression of degenerative or neoplastic lesions is delayed.

Dietary Restriction, Hypercorticism and Chemical Toxicity

Dietary restriction has been reported to increase the maximum tolerated dose or LD_{50} of a number of chemicals (208,209) and to cause isoform-selective alterations in drug-metabolizing enzyme expression (183). While relatively large changes occur in sex-specific isoforms that are regulated by growth hormone, other isoforms are either unaffected or show moderate, circadian-dependent alterations (183). For example, 40% caloric restriction increased hepatic CYP1A-selective 7-ethoxyresorufin *O*-deethylase and CYP2B-selective 7-pentoxyresorufin *O*-dealkylase activities and immunoreactive protein in both male and female 18 week-old Fischer 344 rats, but only at specific circadian time-points (183,210). Conversely, caloric restriction decreased and eliminated the circadian variation of testicular CYP2A1-dependent testosterone 7α--hydroxylase activity (193). In addition to altering drug metabolism, it is probable that caloric restriction may also stimulate the renal clearance of drugs since caloric restriction or fasting may cause polydipsia and increase diuresis and natriuresis consistent with elevated ANP levels (177,211-213). It is possible that both these effects of fasting on serum ANP levels and of caloric restriction on polydipsia and hepatic CYP1A and CYP2B expression result from hypercorticism, since glucocorticoids induce ANP levels (214), cause polydipsia (215), and stimulate induction of CYP1A1 and CYP2B isoforms (216-218). In a recent study (219), caloric restriction was shown to enhance the induction of hepatic peroxisomal marker enzymes in $B6C3F_1$ mice treated with chloral hydrate. This was also consistent with restriction-induced hypercorticism because glucocorticoids induce the hepatic peroxisome proliferator activated receptor PPARα.

In several cases, the effects of dietary restriction on the metabolic activation of genotoxic chemicals have been shown to correlate with specific isoform expression. For example, *in vivo* and *in vitro* binding of aflatoxin B_1 to DNA was decreased in liver from caloric restricted rats concurrently with decreased CYP2C11, whereas binding of benzo(a)pyrene to DNA was increased concurrently with increased 7-ethoxyresorufin *O*-deethylase activity (183,220,221). Dietary restriction has also been reported to reduce endogenous DNA damage in liver, mammary gland and other tissues (59,170,171). However, although there are several reports demonstrating that caloric restriction increases DNA repair activity in a number of cell systems, the effect is confined mostly to old animals (222).

Dietary restriction has been shown to reduce the severity or delay the onset of carcinogenesis in rodents exposed to a number of chemical carcinogens including aflatoxin B_1, polycyclic aromatic hydrocarbons (PAH) and nitosamines (220,223-226). Although dietary restriction clearly alters the initiation stage of chemical carcinogenesis (220), it is now apparent that the major beneficial effects of caloric restriction are associated with the promotion and progression stages. This is best illustrated by experiments involving neonatal exposure of male mice to PAH (227). When mice were injected (ip) with 6-nitrochysene at 8 and 15 days *post partum,* they exhibited a 100% incidence liver adenomas and carcinomas when necropsied at 12 months of age. Dietary restriction (40%), initiated at 14

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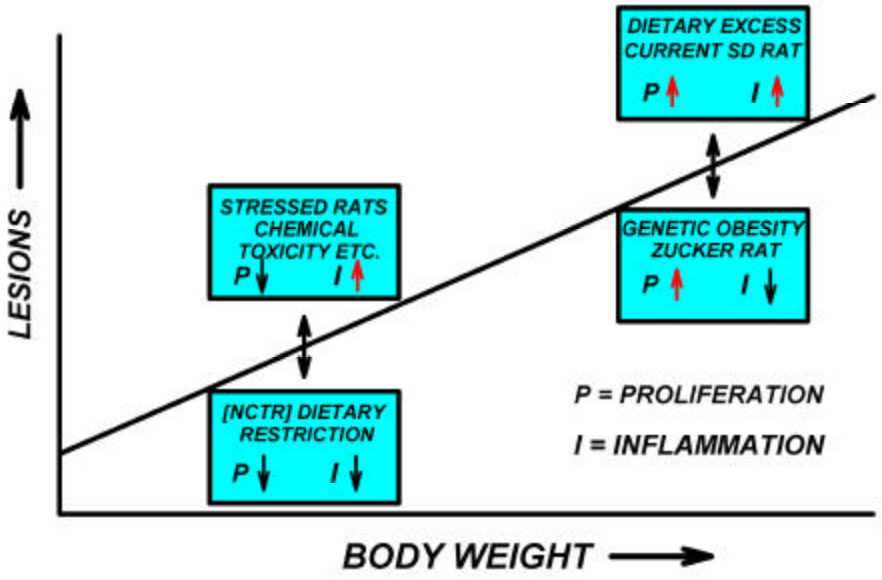

Figure 6. Factors Affecting the Relationship Between Body Weight and Pathological Endpoints in Chronic Cancer Bioassays

The development of pathological lesions in rodents used in chronic bioassays is influenced by both mitogenic and inflammatory effects. Conditions, which increase inflammation in addition to increasing mitogenesis, would be expected to increase the tumor risk to a greater extent than predicted by body weight alone.

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weeks of age, completely inhibited liver tumor formation even though the restriction was not started until after the initiation and early promotion stages of the carcinogenesis process were complete (227). Such effects are consistent with the antimitotic and antiproliferative effects of caloric restriction and hypercorticism that are described above.

The observation that the body weight of rodents used in cancer bioassays directly correlates with terminal incidence of background tumors (228-231) is also consistent with effects on growth and cell proliferation, playing a major role in mediating the antineoplastic effects of caloric restriction. These body weight-tumor correlations were demonstrated from analysis of the control animals from cancer bioassays conducted by the National Toxicology Program (NTP). In $B6C3F_1$ mice, terminal lung tumor incidence exhibited a positive correlation with body weight at nine months on test. Conversely, terminal liver tumor incidence correlated optimally with body weight at 12 months on test (229,232). A typical correlation graph for liver tumors in male mice is shown in Figure 5. In Fischer 344 rats, terminal pituitary tumor incidence exhibited a positive correlation with body weight at 13 months on test, whereas terminal leukemia incidence exhibited a positive correlation with body weight at 14 weeks (233). Interestingly, caloric restriction initiated at six weeks of age inhibited leukemia to a much greater extent than restriction initiated at 14 weeks, whereas pituitary adenoma formation was affected equally by both caloric restriction paradigms (233). This suggests that critical periods exist when rodents are most susceptible to subsequent development of specific cancer endpoints. This effect can also be demonstrated for background liver tumors in $B6C3F_1$ mice (231).

It would appear, therefore, that the rate of growth during the early adult period of an organism's life determines its subsequent susceptibility to neoplastic or degenerative diseases, and rates of growth are in part dependent on glucocorticoid status and caloric intake. Glucocorticoids are a major component of the stress and inflammatory responses, where their primary functions appear to be: [1] to globally reduce energy consumption so that energy may be channeled to the site of trauma or inflammation, and [2] to prevent excessive tissue damage due to overexpression of the inflammatory response (83). During severe nutrient stress, hypercorticism allows an organism to conserve energy so that it may survive, but in the process,

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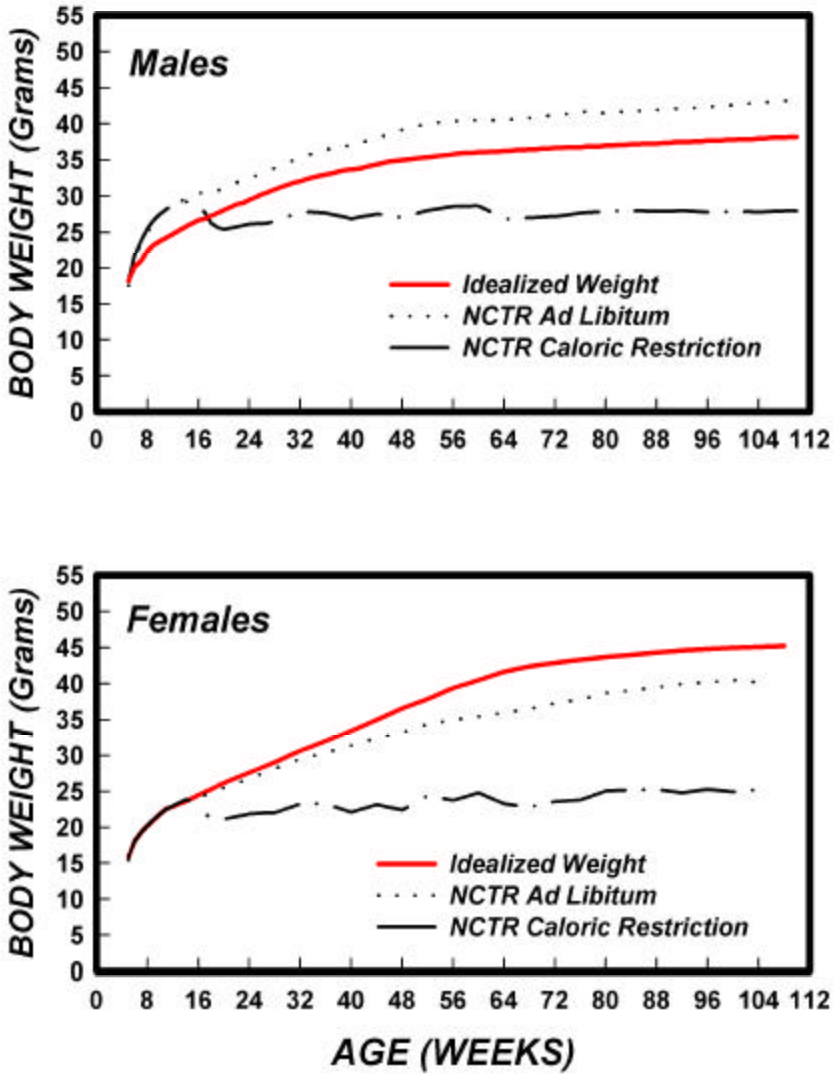

Figure 7. Idealized Body Weight Curves for Male and Female $B6C3F_1$ Mice

Body weights for *ad libitum*-fed and 40% calorically restricted mice from caloric restriction studies performed at NCTR are shown for comparison. Food consumption data from these mice were used to construct a feeding schedule used for manipulating the animals' weights to fit the idealized body weight curve. Taken from Leakey *et al*. (231), full details are given in this reference.

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growth and reproductive immune and cognitive functions may be compromised. However caloric excess may be equally detrimental resulting in overstimulated growth, uncontrolled cell proliferation, autoimmunity, inflammatory diseases, and neoplasia. Between these two extremes lies a physiological window where health and longevity is maximized. Hypercorticism, as a hormonal response to nutrient stress, appears to be common to most mammalian species and most probably evolved as a mechanism to ensure survival of the species through periods of famine (234-237). In times of abundant food supply, rapid growth and fecundity are favored over endurance and longevity. Conversely, when food becomes scarce reproductive performance and growth are sacrificed in favor of extended total and reproductive lifespans, thus increasing the probability that sufficient individuals will survive to restore the population when conditions improve. This phenomena can be observed in the human population, where rising living standards are correlated with obesity and increased cancer rates (4), as well as in rodent strains that are used in toxicology testing.

Consequences for Chronic Toxicity Testing

Over the last three decades, improvements in diet formulations and animal husbandry techniques and commercial breeding considerations have resulted in a general drift towards heavier animals for all the major rodent strains used in toxicity testing (13,14,229,230). Increase in body weight in these strains is frequently associated with decreased survival and increased susceptibility to neoplastic and degenerative diseases (13,14,165). Furthermore, interlaboratory variations in mean body weights and tumor incidence complicate comparison between studies (18).

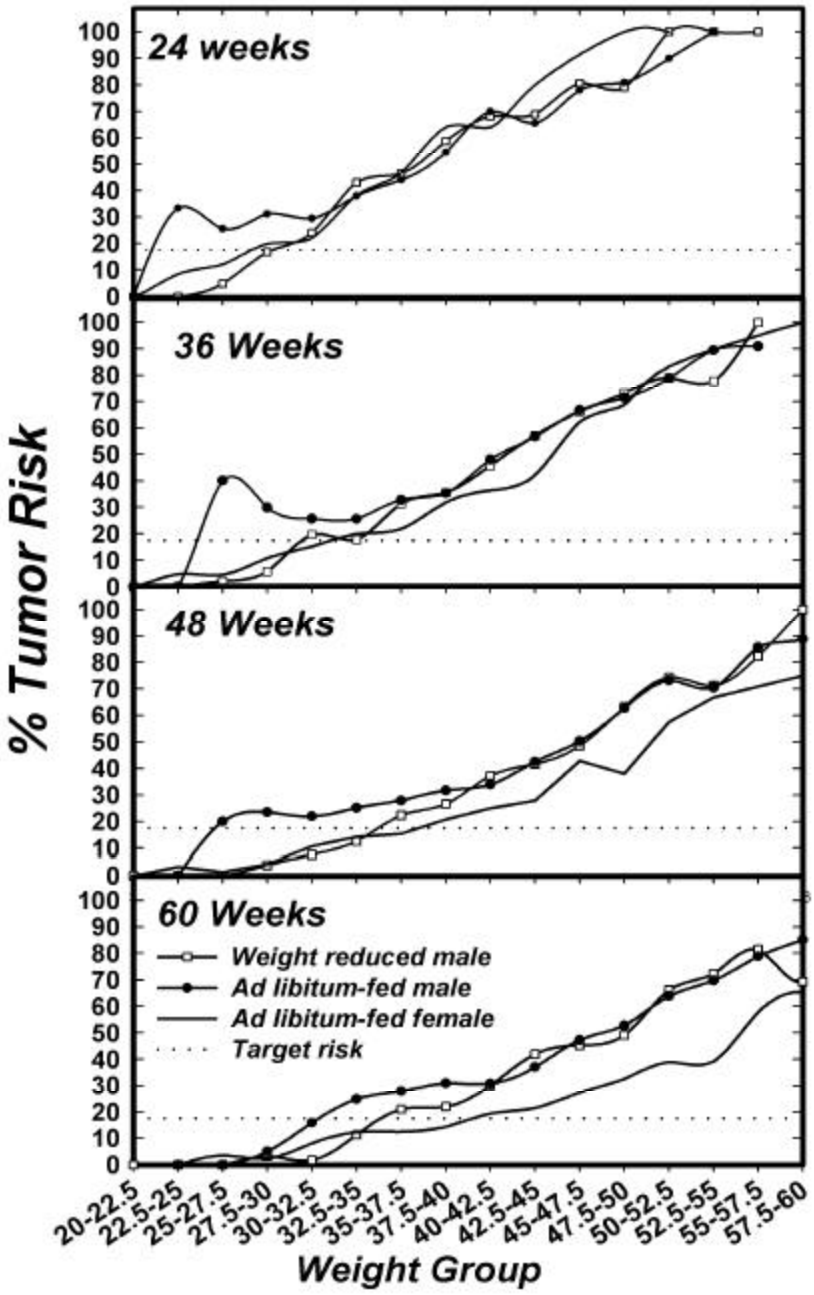

Figure 8. Liver Tumor Risk Curves for *Ad Libitum*-fed Male and Female, and *Weight-Reduced* Male B6C3F$_1$ Mice at Various Ages

Liver tumor risk curves were constructed as described in Leakey *et al* (231) from body weight values corresponding to the ages shown on each graph. They are plotted as spline curves rather than bar graphs. The dotted line represents the target tumor risk (17.5%) for the idealized weight curve.

This effect can create problems for the interpretation of chronic cancer bioassays. For example, the incidence of background liver tumors in control B6C3F$_1$ mice used for chronic bioassays, conducted by the NTP, has been shown to vary be-

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Table 2. Liver Neoplasms in *Ad Libitum*-Fed and Dietary-Controlled Male Mice in the Two-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Hepatocellular Adenoma				
Ad Libitum-Fed				
Overall rate	12/48 (25%)	19/48 (40%)	17/47 (36%)	17/48 (35%)
Adjusted rate	25.2%	40.8%	37.8%	36.2%
Terminal rate	9/41 (22%)	14/37 (38%)	15/36 (42%)	16/44 (36%)
First incidence (days)	511	639	668	713
Poly-3 test (by dose)	P=0.2362	P=0.0792	P=0.1373	P=0.1722
Dietary-Controlled				
Overall rate	9/48 (19%)	7/48 (15%)	10/48 (21%)	10/48 (21%)
Adjusted rate	19.1%	15.2%	21.2%	21.8%
Terminal rate	9/45 (20%)	7/44 (16%)	10/47 (21%)	9/41 (22%)
First incidence (days)	757 (T)	757 (T)	757 (T)	625
Poly-3 test (by dose)	P=0.3381	P=0.4111N	P=0.5013	P=0.4753
Poly-3 test (comparison)	P=0.3238	P=0.0046	P=0.0624	P=0.0951
Hepatocellular Carcinoma				
Ad Libitum-Fed				
Overall rate	4/48 (8%)	10/48 (21%)	10/47 (21%)	7/48 (15%)
Adjusted rate	8.5%	21.4%	22.0%	14.7%
Terminal rate	2/41 (5%)	5/37 (14%)	5/36 (14%)	4/44 (9%)
First incidence (days)	689	666	668	629
Poly-3 test (by dose)	P=0.3737	P=0.0716	P=0.0631	P=0.2713
Dietary-Controlled				
Overall rate	2/48 (4%)	5/48 (10%)	4/48 (8%)	8/48 (17%)
Adjusted rate	4.2%	10.9%	8.5%	17.3%
Terminal rate	2/45 (4%)	5/44 (11%)	4/47 (9%)	4/41 (10%)
First incidence (days)	757 (T)	757 (T)	757 (T)	486
Poly-3 test (by dose)	P=0.0371	P=0.2078	P=0.3382	P=0.0422
Poly-3 test (comparison)	P=0.3356	P=0.1364	P=0.0617	P=0.4740N
Hepatocellular Adenoma or Carcinoma				
Ad Libitum-Fed				
Overall rate	16/48 (33%)	25/48 (52%)	23/47 (49%)	22/48 (46%)
Adjusted rate	33.4%	52.6%	50.6%	46.2%
Terminal rate	11/41 (27%)	16/37 (43%)	17/36 (47%)	19/44 (43%)
First incidence (days)	511	639	668	629
Poly-3 test (by dose)	P=0.2154	P=0.0437	P=0.0684	P=0.1430
Dietary-Controlled				
Overall rate	11/48 (23%)	11/48 (23%)	14/48 (29%)	18/48 (38%)
Adjusted rate	23.4%	23.9%	29.7%	38.6%
Terminal rate	11/45 (24%)	11/44 (25%)	14/47 (30%)	13/41 (32%)
First incidence (days)	757 (T)	757 (T)	757 (T)	486
Poly-3 test (by dose)	P=0.0450	P=0.5728	P=0.3231	P=0.0844
Poly-3 test (comparison)	P=0.1976	P=0.0030	P=0.0309	P=0.2975

(T) =Terminal sacrifice; Overall rate = Number of neoplasm-bearing animals/number of animals with tissue examined microscopically; Adjusted rate = Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality; Terminal rate = Observed incidence at terminal kill. Beneath the dietary-controlled group incidence are the P values corresponding to pairwise comparisons between the *ad libitum*-fed group and the corresponding dietary-controlled group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A lower incidence in the *ad libitum*-fed group is indicated by N.

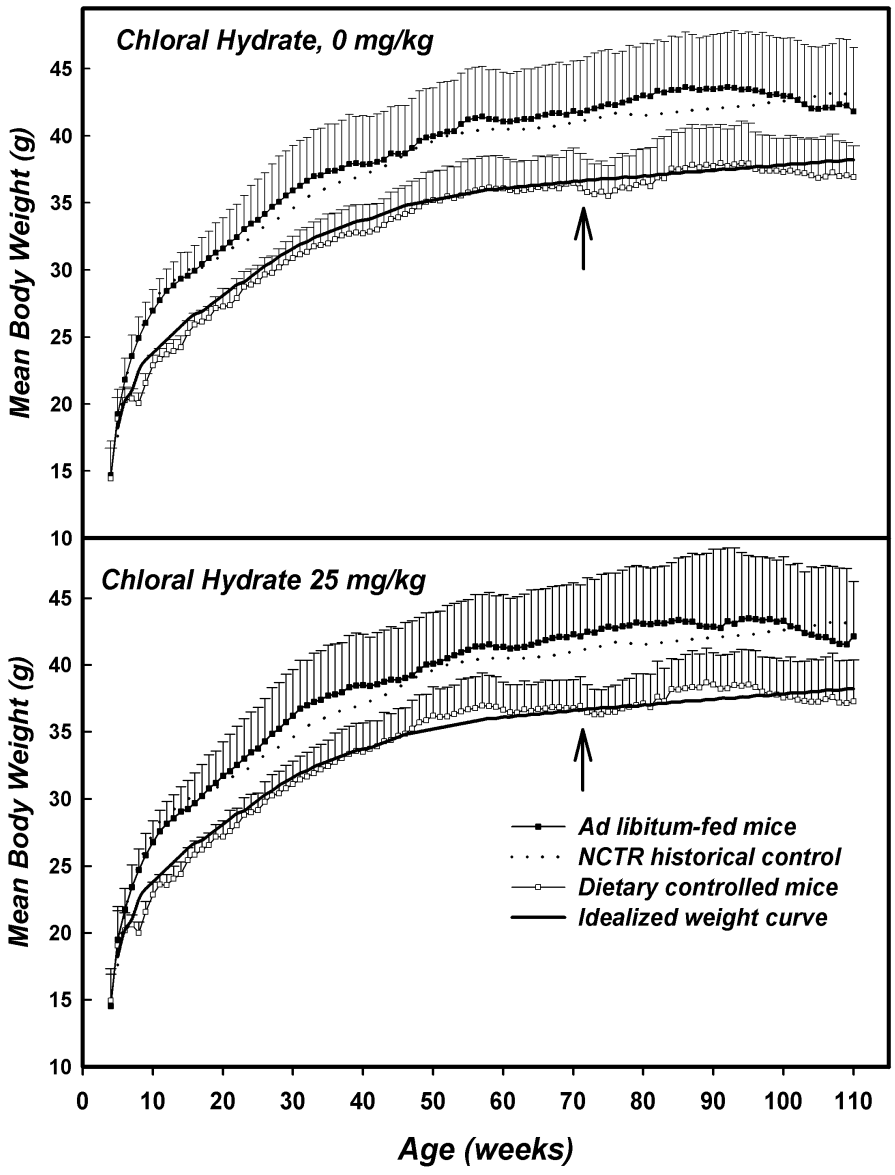

Figure 9. Growth Curves for *Ad Libitum*-Fed and Dietary-Controlled from the Chloral Hydrate Study - Low Dose and Vehicle Control Groups

The graphs show the standard deviation of each weekly mean body weight values for the control and 25 mg/kg chloral hydrate dose groups respectively. The idealized weight curve and the NCTR historical growth curve for male B6C3F$_1$ mice are shown on each graph for reference. The arrow marks the time point at which 12 mice were removed for the interim evaluation.

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tween 5% and 75% (238). This increased variability is partly due to altered housing conditions, but other factors such as genetic drift may also be responsible (238,239). However, differences in mean body weights between treatment groups within individual studies pose a greater problem since they may result in artifactual assumptions about the carcinogenicity of certain test chemicals (228,229).

Such differences usually arise when toxic responses to the test chemical reduce body weight gain, and a 10% reduction in body weight gain has been used as a criteria for achieving a maximum tolerated dose (240). Chemically induced body weight reductions can arise for a number of reasons, including decreased food consumption due to palatability problems in feed studies, anorexia due to toxic stress, disrupted intestinal absorption, or toxic wasting syndromes due to disruption of metabolism or endocrine systems. In most cases, such body weight gain decreases would be expected to be associated with hypercorticism, which would result from either nutrient or classic, CRF-mediated toxic stress. Nutrient stress resulting from reduced food consumption would be expected to decrease the inflammatory response in a similar manner to glucocorticoid administration or caloric restriction, whereas stress due to chemical toxicity would be expected to enhance the inflammatory response due to increased CRF and interleukin levels (Figure 6). In addition, excessive body weight gain in rodents may also involve an altered inflammatory response. Such animals could exhibit reduced efficiency in leptin expression or function, analogous to the *ob/ob* mouse, and this would result in hypothermia, hypercorticism, hyperglycemia, but a generally reduced inflammatory response due to elevated corticosterone (37,241,242). Conversely, they could exhibit excessive food consumption, which would result in low corticosterone levels, excessive production of arachidonic acid and an increased inflammatory response (243). Excessive inflammation exacerbates toxic responses to chemicals (244-246), directly promotes neoplasia in certain systems such as mouse skin (134) and can result in degenerative conditions such as renal inflammatory disease (243). Therefore, whether alterations in weight gain in

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bioassay rodents are accompanied by changes in inflammatory response may influence the relationship between body weight and terminal tumor incidence as illustrated in Figure 6. Such effects are not only relevant to two-year cancer bioassays, but also to the ancillary studies associated with these bioassays. As noted in the previous section, diet and body weight can influence the toxicokinetics of many chemicals. Dietary restriction has also been shown to influence rates of tumor progression in transgenic mouse models that are currently being introduced for rapid carcinogenesis screens (247,248). This should be considered during the interpretation of cancer bioassay data.

Dietary restriction has been suggested as a possible means for eliminating background tumors from the bioassay control populations (14,15). However, as stated above, dietary restriction inhibits chemically induced carcinogenesis in rodents (230,238,249). Moreover, dietary restriction is generally implemented by limiting food consumption to a set percentage of *ad libitum* food consumption, and this may vary between rodent populations in different laboratories (230,238).

An alternative approach involves using dietary control to manipulate the body weights and growth rates of rodents used in bioassays so that they conform to strain-specific standardized weight curves. Such standardized or idealized weight curves have been created for male and female B6C3F$_1$ mice and could potentially be used throughout industry and the regulatory community to standardize background neoplasm incidences between laboratories (3). The body weights of mice used for both control and treatment groups in future bioassays could be manipulated to fit these growth curves by moderate feed restriction or dietary supplementation.

Testing Dietary Control

The concept of using idealized weight curves has recently been tested as part of a standard NTP bioassay of chloral hydrate in B6C3F$_1$ mice that was conducted at the National Center for Toxicological Research [NCTR] (219,231,250-252). Data from mice used in NTP and NCTR chronic bioassays and aging studies were used to construct idealized weight curves for male and female B6C3F$_1$ mice that predicted a liver neoplasm incidence of 15% to 20% at 26 months of age. A 15% to 20% liver neoplasm incidence is sufficiently high to guarantee that the sensitivity of the mouse to chemical carcinogenesis has not been com-

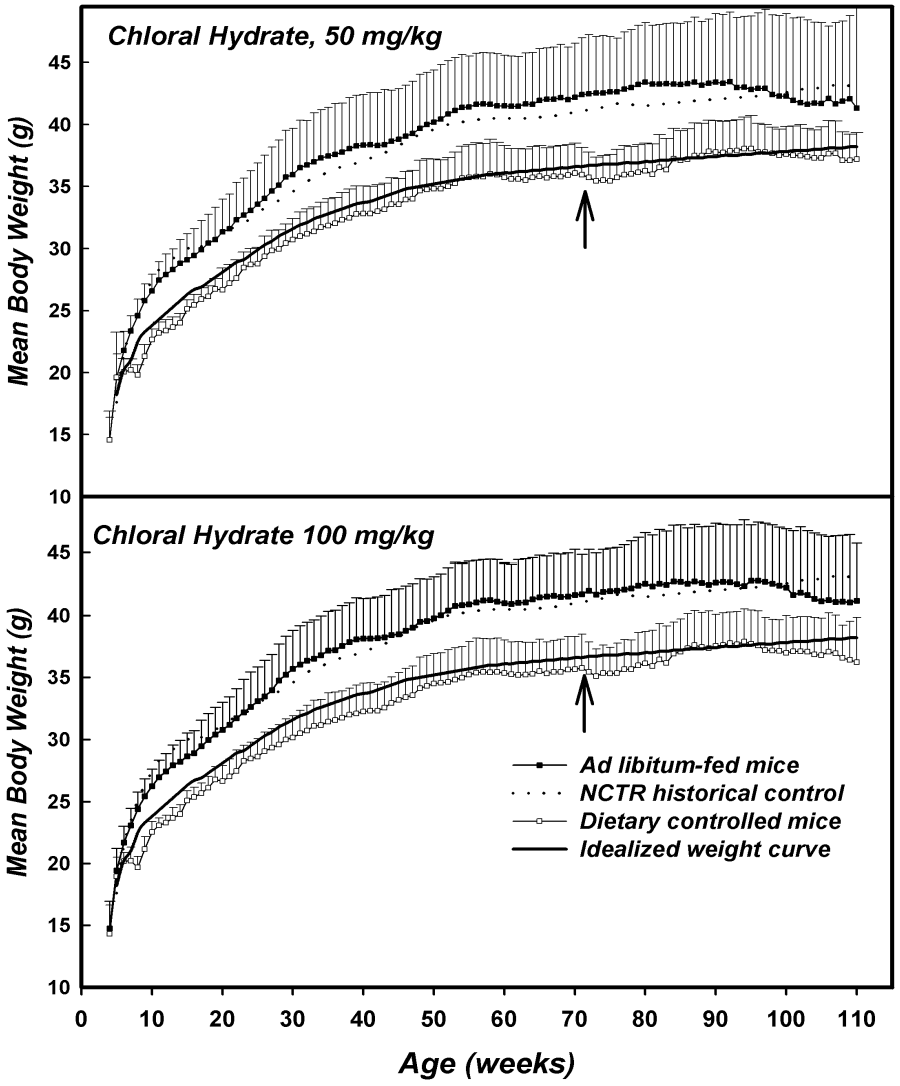

Figure 10. Growth Curves for *Ad Libitum*-Fed and Dietary-Controlled from the Chloral Hydrate Study - Medium and High Dose Groups

The graphs show the standard deviation of each weekly mean body weight values for the 50 and 100 mg/kg chloral hydrate dose groups respectively. Other details are given in Figure 9.

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promised, and it is low enough to ensure that the spontaneous neoplasms will not obscure any chemically induced liver tumors, and that sufficient mice will survive to the end of a two-year study. Initially the relationship between body weight and liver tumor incidence was calculated for historical control populations of male and female *ad libitum*-fed mice (approx. 2,750 and 2,300 animals respectively). However, it was determined that male B6C3F$_1$ mice, which had been subjected to forced body weight reduction due to either dietary restriction or exposure to non-carcinogenic test chemicals, differed from *ad libitum*-fed mice in their relationship between body weight and tumor incidence. A second *weight-reduced* historical control population (approx. 1,600 animals) was therefore used to construct the idealized weight curve for male mice (231). These curves are shown in Figure 7.

Weight-reduced mice exhibited a more linear relationship between body weight and liver tumor incidence in the low weight range than did *ad libitum*-fed mice, which exhibited a J-curve profile (3,229,231). These differences did not occur in females and were less apparent in sexually senescent males older than 60 weeks (Figure 8) and result in a larger sex-difference in liver tumor incidence in light mice than in heavy mice (231).

It is probable that stress due to dietary restriction or chemical exposure reduces not only the body weight-related liver tumors in male mice, but also the sex-dependent liver tumors which occur independently of body weight in small male B6C3F$_1$ mice and cause the J-curve profile in the tumor risk curve. Castration studies with the parent strains of B6C3F$_1$ mice, which also show sex differences in liver neoplasm risk, suggest that this increased incidence of liver neoplasms in the small male mice is partly due to testicular androgens (253-255). As discussed above, short-term caloric restriction has been reported to reduce the testosterone/estradiol ratios and impair male reproductive function in rodents, and restraint stress or food depression suppresses LH secretion in male mice (256).

The NCTR bioassay of chloral hydrate compared dietary-controlled mice with *ad libitum*-fed mice. Groups of 120 male mice received chloral hydrate in distilled water by gavage at doses of 0, 25, 50, or 100 mg/kg, 5 days per week for 104 to 105 weeks; vehicle controls received distilled water only. Each dose group was divided into two dietary groups of 60 mice. The *ad libitum*-fed mice had feed (NIH-31 autoclaved pelleted diet, Purina Mills, Richmond, IN) available *ad libitum,* and the dietary-controlled mice received the same feed in measured daily amounts calculated to maintain body weight on a previously computed idealized body weight curve. Twelve mice from each diet/dose group were evaluated at 15 months. Weekly feed allocation values required to control body weight in mice to conform to the idealized body weight curve were calculated as grams of NIH-31 pellets per day from food consumption and body weight data from previous NCTR studies using B6C3F$_1$ mice. This is described in detail elsewhere (231). It was anticipated that individual mice would exhibit body weights that

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Table 3. Liver-Weight-to-Body-Weight Ratios in Male Mice Evaluated at 15 Months in the Two-Year Study of Chloral Hydrate

	Ad Libitum-Fed				Dietary-Controlled			
	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
n	12	12	12	12	12	12	12	12
Mean[a]	47.08	46.96	40.87	51.11	35.63	37.46	38.31	39.55
SD[b]	17.59	17.40	4.13	19.58	1.02	1.37	2.09	2.29
SEM[c]	5.08	5.02	1.19	5.65	0.30	0.39	0.60	0.66
Tukey's test[d]	A	A	A	A	A	AB	BC	C
Dunnett's test[e]					0.0001	0.0394	0.0017	0.0000

[a] Ratios are given as mg liver per g body weight.
[b] Standard deviation
[c] Standard error of the mean
[d] Each diet group was treated on a separate ANOVA, and diet/dose groups not sharing the same letter are significantly different from each other (P<0.05).
[e] Beneath the vehicle control group is the P value associated with the trend analysis. Beneath the dosed groups are the P values relative to the vehicle control group.

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differed significantly from the idealized body weight curve at certain times during their growth. These mice were identified on a weekly basis and their food allocation adjusted in either 1.0 or 1.5 g increments to manipulate the body weight back onto the idealized body curve.

While chloral hydrate was less potent than expected, it did produce a weak, but statistically significant, hepatocarcinogenic response in both the *ad libitum*-fed and the dietary-controlled mice (Table 2). In the *ad libitum*-fed mice, this consisted of a significant increase in combined hepatocellular adenoma and carcinoma incidence in the 25-mg/kg-dose group with no further increase at higher doses. In the dietary-controlled mice, the combined hepatocellular adenoma and carcinoma incidence increased from 23.4% in the control group to 38.6% in the 100 mg/kg dose group, with a statistically significant dose trend; this increase was due to a statistically significant increase in hepatocellular carcinomas in the high dose group. Observed numbers of liver tumors were less in all the dietary-controlled dose groups than in the corresponding *ad libitum*-fed dose groups. Dietary control also significantly increased survival in the control, 25 and 50 mg/kg dose groups and decreased body weight variability in all groups. Dietary control reduced individual body weight variation in all four-dose groups (Figures 9 & 10). This was associated with smaller variation in related parameters. For example, a significant dose-response in liver per body weight values was observed in dietary-controlled mice used for an interim evaluation in the study (252), whereas a significant dose-response was not observed in the *ad libitum*-fed mice, which exhibited much greater individual variation (Table 3).

The dietary control procedures were relatively easy to run in this study and did not generate a large amount of extra labor once the feed allocation software had been devel-

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Table 4. Body Weight Derived Predictions of Liver Tumor Incidence in Dietary-Controlled and *Ad Libitum*-Fed Male B6C3F$_1$ Mice Administered Chloral Hydrate

Dose	*0 mg/kg*	*25 mg/kg*	*50 mg/kg*	*100 mg/kg*
Observed Rates - Dietary Control				
Overall rate	22.9 %	22.9 %	29.2 %	37.5 %
Poly 3 Adjusted rate	23.4 %	23.9 %	29.7 %	38.6 %
Number with tumors	11/48	11/48	14/48	18/48
Predicted Rates - sorted for < 5% ad libitum				
Poly 3 - Overall rate	22.4 ± 2.3 %	22.3 ± 3.0 %	23.1 ± 2.2 %	21.5 ± 2.3 %
Adjusted rate	22.9 ± 2.3 %	23.3 ± 3.0 %	23.1 ± 2.2 %	22.8 ± 2.0 %
Number with tumors	11/48	11/48	11/48	10/48
Z_h Statistic	0.229	0.226	2.98	8.030
Significance *P* =	0.4094	0.4145	0.0014	< 0.00001
Predicted Rates - sorted by body weight decrease				
Poly 3 - Overall rate	23.2 ± 3.2 %	24.0 ± 3.2 %	22.7 ± 3.3 %	21.6 ± 3.7 %
Adjusted rate	23.6 ± 3.2 %	25.1 ± 3.1 %	23.1 ± 3.3 %	23.0 ± 3.5 %
Number with tumors	11/48	12/48	11/48	10/48
Z_{tr} Statistic	0.073	0.376	2.001	4.493
Significance *P* =	0.4710	0.3534	0.0227	< 0.00001
Observed Rates - Ad Libitum-fed				
Overall rate	33.3 %	52.1 %	48.9 %	45.8 %
Poly 3 Adjusted rate	33.4 %	52.6 %	50.6 %	46.2 %
Number with tumors	16/48	25/48	23/47	22/48
Predicted Rates - sorted by body weight decrease				
Poly 3 - Overall rate	33.8 ± 8.9 %	33.7 ± 9.5 %	33.6 ± 9.6 %	33.1 ± 7.8 %
Adjusted rate	34.5 ± 8.9 %	35.8 ± 9.6 %	35.9 ± 9.6 %	34.1 ± 7.9 %
Number with tumors	16/48	16/48	16/48	16/48
Z_{tr} Statistic	0.176	1.751	1.524	1.542
Significance *P* =	0.4303	0.0400	0.0637	0.0616

Tumor risk was assigned to each mouse for each week of evaluation by specific sort criteria as described in (231). The Z_{tr} statistics describe comparisons between the predicted survival adjusted background tumor rate and the observed survival adjusted rate. The predicated rates refer here to background liver tumor incidence predicted by the body weight profiles of the individual mice in each dose group. Thus, significant differences between predicted and observed rates in the groups receiving chloral hydrate imply a carcinogenic effect due to the chemical.

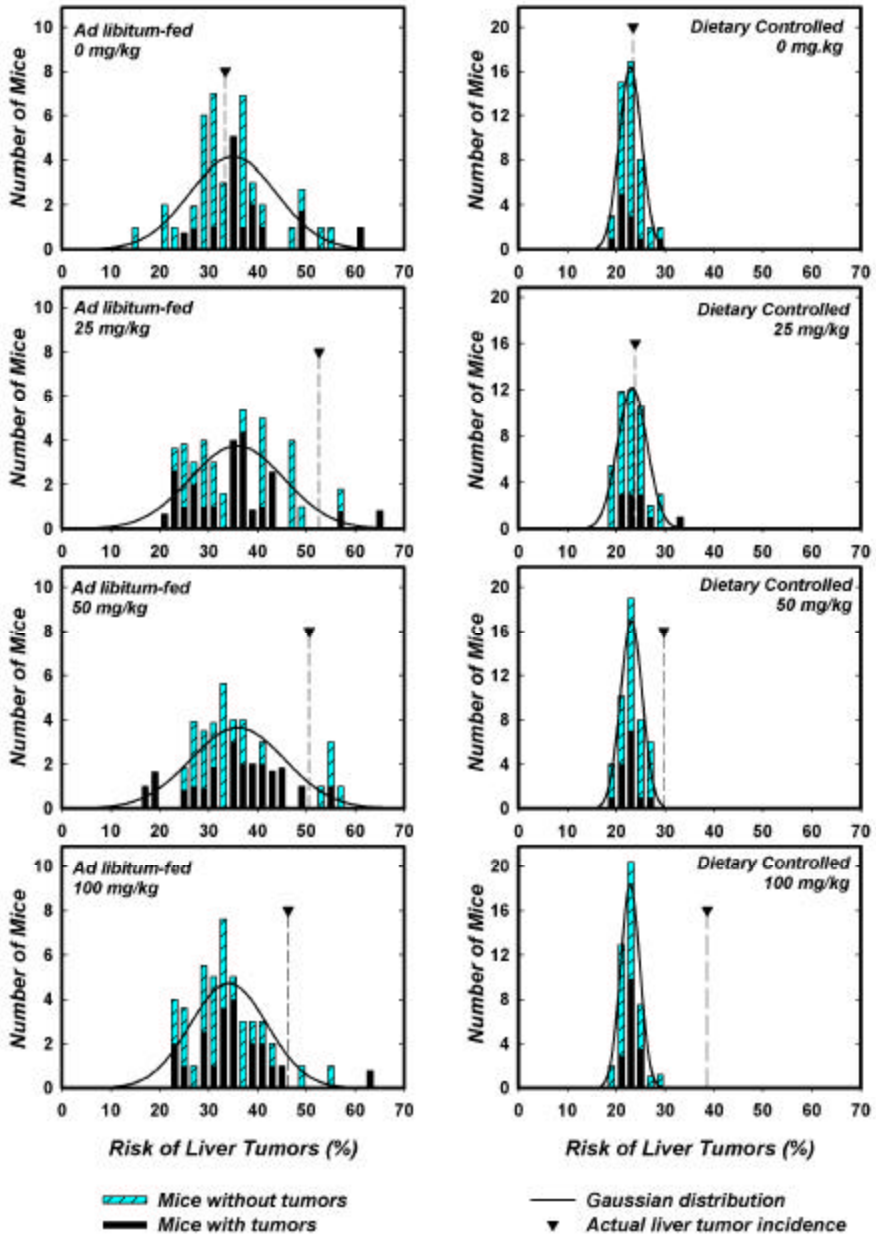

Figure 11. Cumulative Tumor Risk Plots for the Chloral Hydrate Study

The mice from each experimental group were sorted by tumor risk into sequential 2% incremental groups. Tumor risk was calculated for the *ad libitum*-fed mice by sorting by body weight decrease and for the dietary controlled mice by sorting by "<5% *ad libitum"* (see Table 4). Each mouse is represented in the incremental group by its a value to adjust for intercurrent mortality. The Gaussian distributions for each experimental group are calculated from the means and standard deviations of the adjusted tumor risk values given in Table 4. Taken from Leakey *et al* (231), full details are given in this reference.

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oped. Access to a feed pellet sorter and prior experience with caloric restriction studies also facilitated diet preparation. Since the study used gavage dosing, weekly weights were readily available. The procedure would be potentially more expensive and complicated for studies, which dose via the feed because these animals are generally not weighed every week, and the variable amounts of feed required for dietary control would result in variable dose levels. However, dose variation occurs in all feed studies since individual animals consume different amounts of feed. Dietary control could in fact standardize dosing to a more defined level if the required level of dietary restriction is relatively high and each animal consumes its entire daily feed allowance (231, 250).

During the course of this study, a procedure was developed to use the historical control data from *ad libitum*-fed and *weight-reduced* mice to calculate predicted background liver tumor rates for individual mice based on their body weight values between 21 and 68-weeks of age. Full details of this procedure are given elsewhere (231). Using this procedure, it was possible to predict background liver tumor rates for each experimental group in the chloral hydrate study. As shown in Table 4 and Figure 11, this procedure was able to accurately predict the background tumor rates in both the dietary-controlled and the *ad libitum*-fed dose-control groups. As illustrated in Figure 11, the variation in predicted background liver tumor risk of individual mice in each dose group was much less for the dietary-controlled mice than for the *ad libitum*-fed mice. Furthermore, the technique showed the observed liver tumor incidence in the dietary-controlled 50 mg/kg and 100 mg/kg dose groups were significantly greater than predicted background tumor incidence even though these groups did not show a statistically significant increase on the Poly-3 test (Table 2). This is because the Z_{tr} statistic used in Table 4 is an estimate of the probability that the observed tumor rate is an acceptable background tumor rate for the body weight-adjusted historical control population and is dependent on the variance of calculated tumor risk of the mice in each group

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rather than assuming a fixed binomial variance (231). As such it can give valuable supportive evidence on the relevance of test chemically induced increases in tumor incidence, but it assumes that no other factors significantly influence tumor incidence between studies other than body weight, survival and the test chemical.

Dietary control, therefore, can potentially improve both the sensitivity and reproducibility of cancer bioassays in mice. However, mouse liver neoplasms are frequently induced epigenetically by chemicals, which appear to not be carcinogenic for humans (257-259). Moreover, although incidence of liver cancer is increasing in the U.S. and other Western countries, the primary risk factor appears to be chronic inflammation resulting from hepatitis C or B infection rather than linked to the ongoing rise in obesity or exposure to chemical carcinogens (260-262). It could therefore be argued that a more sensitive mouse bioassay would merely compound the problem of accumulating misleading or false positive animal data that are irrelevant to human risk. Thus, it might not be useful to the regulatory community.

There are two main answers to this. First, it must be remembered that most other neoplastic lesions are also reduced by caloric restriction and related stress responses. For example, in $B6C3F_1$ mice positive correlations have been reported between body weight and incidence of tumors of the pituitary gland, lung and Harderian gland and of hemangiomas/hemangiosarcomas in addition to liver tumors (229,232). Caloric restriction has also been shown to delay or inhibit the development of these tumor types in $B6C3F_1$ mice (263). Reducing variability and body weight artifacts will therefore increase sensitivity to detect a wide range of neoplastic responses in addition to liver tumors.

Second, many potent genotoxic chemicals also cause liver tumors in $B6C3F_1$ mice, and several are hepatocarcinogenic in humans (264,265). Evidence as to whether a positive tumor response has relevance to humans and whether safe exposure levels can be determined depends on mechanistic data ancillary to bioassay tumor data. The emerging revolution in *"-omics* " technology holds promise that such mechanistic data will become more comprehensive and informative, but it is dependent on the quality and reproducibility of available tissue samples. Microarray techniques are especially vulnerable to sample variation, because of the large number of interactive endpoints that have to be measured simultaneously. Dietary control offers an approach to greatly reduce both variability within studies and between studies.

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Glossary

Apoptosis: Apoptosis is a process of programmed cell death triggered by either external hormonal signals or internal damage, which is characterized by ordered degradation of chromatin and the rapid phagocytosis of cellular debris without the release of inflammatory mediators. Thus, apoptosis differs from necrotic cell death in that there is no associated inflammation or compensatory mitogenesis.

Body weight reduction: In general, body weight reductions resulting from dietary restriction are reductions in body weight gain relative to corresponding *ad libitum*-fed control animals. That is the restricted animals are gaining weight but at a slower rate. However, individual animals, particularly older rodents on long-term dietary restriction studies, may actually lose weight on a short-term basis, but generally not on a long-term basis unless the weight-loss is associated with morbidity.

Dietary restriction: The term is used loosely here to incorporate both food restriction and caloric restriction. Caloric restriction is defined as the balanced and moderate reduction of the carbohydrate, protein and fat content of a diet without a reduction in vitamin or micronutrient content. Caloric restriction by 10 - 40% of *ad libitum* food consumption has typically been used in aging studies. Food restriction, where total food intake is reduced, is simpler to perform and has been used in several toxicity studies. It runs the risk of causing vitamin deficiencies when used in excess.

Dietary control: As used here, dietary control involves the manipulation of food allocations to maintain experimental animals on a pre-determined body weight growth curve. It can involve either dietary restriction of dietary supplementation depending on the individual animal and on the required growth curve.

Glucocorticoid: A steroid ligand of the glucocorticoid receptor, which evokes the glucocorticoid effect of raising blood glucose levels. Includes pharmaceuticals such as dexamethasone in addition to endogenous cortisol and corticosterone.

Hypercorticism: Elevated secretion and blood concentrations of adrenal corticosteroids; predominantly corticosterone in rodents and cortisol in primates.

Nutrient stress: Hypoglycemia-induced stress resulting from reduced caloric intake, which can result from dietary restriction, short-term fasting or starvation.

Restraint stress: Experimental technique of producing a classic stress response in rodents by restricting their movements.

Weight-reduced mice: Mice which had lower body weight values and lower rates of growth due to either dietary restriction or exposure to a non-carcinogenic test chemical. See reference 231 for more details.

See also legends of Figures 1 and 2 for full names of hormones and signal transduction proteins .

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