

Increased Serum Corticosterone and Glucose in Offspring of Chromium(III)-Treated Male Mice

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Preconceptional carcinogenesis occurs in animals and is suspected for humans—for example, after occupational metals exposure. Several characteristics in animal models, including high frequency and non-Mendelian inheritance patterns, have suggested an epigenetic mechanism, possibly involving hormone changes in offspring. To test this hypothesis, we treated male mice with chromium(III) chloride, a preconceptional carcinogen, 2 weeks before mating, in two separate experiments. Their 10-week-old offspring showed highly significant increases in average serum corticosterone and glucose, compared with control offspring. Average serum levels of insulin-like growth factor 1 (IGF1) showed more modest possible increases. A previous microarray experiment identified hepatic insulin-like growth factor binding protein 1 (IGF BP1) gene expression as consistently changed in correlation with serum corticosterone levels. In the present study, hepatic IGF BP1 mRNA correlated with serum IGF1 in male offspring of chromium-treated fathers, but not in controls; serum glucose correlated positively with hepatic IGF BP1 in chromium-group offspring but negatively in controls. These results support the hypothesis that preconceptional exposure effects may alter hormones, metabolism, and control of tissue gene expression, probably through epigenetic mechanisms. Risk of neoplasia may be influenced by these changes. **Key words:** chromium(III), corticosterone, insulin-like growth factor 1, insulin-like growth factor binding protein 1, offspring, paternal, preconceptional, stress, transgenerational. *Environ Health Perspect* 110:801–804 (2002). [Online 28 June 2002] <http://ehpnet1.niehs.nih.gov/docs/2002/110p801-804cheng/abstract.html>

Causative factors for childhood cancers, some of which are apparently increasing in the United States, remain largely elusive (1). Furthermore, evidence is increasing for influence of perinatal circumstances on risk of adult cancers (2–4). Risk of childhood cancer has been associated with preconceptional parental exposures in numerous epidemiologic studies; paternal occupational exposure to metals has been repeatedly implicated (1,5–7). Preconceptional carcinogenesis by chemicals and radiation is well established in animal models (1,7,8). Characteristics of preconceptional carcinogenesis in animal models, including non-Mendelian inheritance patterns (9), rates that are much higher than expected for point mutation (10), and absence of mutations in cancer genes (11,12), have led us to propose that the mechanism is epigenetic (13,14). The operation of a novel mechanism is also suggested by reports of high-frequency preconceptional effects on nontumor end points, some of them adaptive rather than toxicologic, for mammals, invertebrates, and plants (15,16).

In a test in mice of preconceptional carcinogenicity of metals present in welding fumes, we observed a significant effect with chromium(III) chloride (CrCl₃) (13). A more extensive study confirmed this finding, revealing that endocrine and secretory tissues were prominently targeted in the offspring (14). This led to the hypothesis that alterations in hormones in the offspring could be

part of the mechanism of preconceptional carcinogenesis.

To test this hypothesis, we treated male mice with chromium(III) chloride and mated them 2 weeks later, following the preconceptional carcinogenesis protocol. We assessed the offspring at 10 weeks of age for serum levels of corticosterone, glucose, and insulin-like growth factor 1 (IGF1), under closely controlled conditions. We observed highly significant increases in corticosterone and glucose in the sera of offspring of chromium(III)-treated fathers.

Materials and Methods

We injected NIH Swiss NCR male mice, 8 weeks old, from the Frederick Cancer Research Facility Animal Production Area, intraperitoneally with 1 mmol/kg CrCl₃•6H₂O (Aldrich Chemical Co., Milwaukee, WI) in 0.9% NaCl. We adjusted the original CrCl₃ solutions, which were highly acidic, to pH 4.0 by adding NaOH. Controls received saline that had been adjusted to pH 4.0 by addition of HCl. We injected the male mice in the afternoon in experiment 1 and in the morning in experiment 2. We maintained mice in a facility accredited by the American Association for Accreditation of Laboratory Animal Sciences. We bred each male to an 8-week-old female of the same strain, 2 weeks after treatment, as in previous studies (13,14).

We developed a euthanasia method to minimize increases in serum corticosterone

due to the procedure. We acclimatized the mice to the laboratory and handled them for several minutes before exsanguination via cervical incision. A separate study showed that mice euthanized in this way had lower and more uniform serum corticosterone compared with those used directly from the animal room (data not shown). In experiment 1, we euthanized the fathers after breeding was complete, in the morning, an average of 26.2 ± 0.8 days after treatment for chromium-treated mice and 25.2 ± 0.8 days for control mice. The offspring were euthanized at 68–72 days old, between 0900 and 1100 hr, with treated-group and control mice included in each set.

In preparation for experiment 2, we determined the time in the morning during which serum corticosterone levels were constant. For untreated male mice (10 per group), corticosterone values were 29.4 ± 3.2 (± SE) ng/mL at 1000 hr and 30.1 ± 2.6 ng/mL at 1100 hr, so in experiment 2, male offspring were euthanized between 1000 and 1100 hr. Females showed 248 ± 30 ng/mL corticosterone at 1100 hr and 236 ± 38 ng/mL at 1300 hr, and so were euthanized between 1100 and 1200 hr.

We followed the fathers for experiment 2 for weight changes and euthanized them 10 weeks after treatment; at this time point, we observed no significant differences between groups regarding serum corticosterone, glucose, or IGF1 (data not shown).

AniLytics, Inc. (Rockville, MD, USA) analyzed serum glucose, corticosterone, and IGF1 with kits for glucose/hexokinase (Boehringer Mannheim, Indianapolis, IN), ¹²⁵I-corticosterone (ICN Pharmaceuticals, Costa Mesa, CA), and rat IGF1 (Diagnostic Systems Laboratories, Houston, TX), respectively. Statistical analysis of the data for these parameters used simple data descriptive techniques, mixed-models analysis of variance (ANOVA), analysis of covariance, regression analysis, and post hoc tests. In particular, we considered chromium(III)-treated and control father mice as random factors in mixed,

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hierarchically nested designs. We transformed analyses of corticosterone measures to their common logarithms to satisfy homogeneity of variance requirements in the ANOVAs. We performed analyses of glucose measures on the raw data. We made inferences regarding significant differences among corticosterone, glucose, and other parameters in male and female offspring as a result of analyses taking the fathers as the treated experimental unit.

We evaluated hepatic mRNA for insulin-like growth factor binding protein 1 (IGF BP1) from selected mice by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR). The average serum values for control and chromium groups, respectively, were, for corticosterone, 44 ± 9 and 102 ± 17 ng/mL; for glucose, 217 ± 6 and 238 ± 6 mg/dL; and for IGF1, 282 ± 13 and 323 ± 26 ng/mL. Thus, these mice were representative of the larger population of the study. The primer pair for IGF BP1 PCR was CTCTGCATGCCCTCACC (forward) and CCAGCAGCTGTTCTCT (reverse) (17). Linear amplification occurred over cycles 15–27; we used 21 cycles. We quantified the IGF BP1 expression in two RT-PCR assays and pooled the results by normalizing each value to the lowest value in that assay. For statistical evaluation of the relationships between IGF BP1 expression and serum IGF1 and glucose, we performed regression analyses to determine the significance of differences of line slopes from zero. In addition, we performed comparative regression analyses for offspring of control and chromium(III)-treated fathers. We carried out analyses of covariance to determine whether regression lines in each group were homogeneous and to evaluate the significance of differences in slopes and intercepts.

Results

In the first experiment, 1 mmol/kg chromium(III) chloride proved significantly toxic, as indicated by failure of some treated males to father offspring (11/20 bred vs. 18/20 vehicle-treated controls, $p = 0.031$) and by significantly increased serum corticosterone and decreased serum glucose at 25–26 days after treatment (Figure 1A,B). We assessed the offspring of the males that succeeded in breeding at 10 weeks old for serum corticosterone and glucose. All mice were euthanized in the morning and both treatment and control group mice were included each day.

The female offspring of the chromium (III)-treated fathers had markedly higher average serum corticosterone compared with offspring of vehicle-treated fathers (Figure 1C). Average serum glucose also showed a highly significant increase (Figure 1D).

These differences remained significant after statistical consideration of litter membership, day of euthanasia, and exact age. Thus, we observed a shift toward higher serum corticosterone over most or all of the female offspring from the treated fathers. We noted no significant differences in the male offspring.

Chromium(III) chloride hexahydrate does not always exhibit this toxicity when administered to male mice (13,14). We found that chromium(III) toxicity related in part to the time lapsing between preparation and injection of the solution and could involve decreases in pH. In a second experiment, we injected solution within 30 min of preparation, in the morning, rather than after about 4 hr and in the afternoon, as in the first study. In addition, we added a second dose level, 0.1 mmol/kg. We observed no significant effects on breeding, indicating less toxicity. One male in each group of 10 failed to father offspring. In addition, one male in the high-dose chromium group and two males in the low-dose chromium group fathered litters that did not survive to weaning. Nevertheless, some residual toxic stress was evident, as indicated by the significantly lower body weights in the high-dose group, for example, at 3 days after treatment, 30.2 ± 0.7 g for the controls versus 27.3 ± 0.5 g for the high-dose chromium-treated fathers ($p = 0.0027$).

Experiment 2 offspring were euthanized within a 1-hr time span in the morning, when corticosterone levels had been determined in a separate study to be at a steady minimum (see “Materials and Methods”). The results for the offspring of this experiment confirmed those for the female offspring of the first trial, and in addition, we saw similar effects in the male offspring. We found a highly significant, 2-fold increase in average corticosterone in the offspring of both sexes after the higher dose (Figure 2A). The male offspring showed an apparent corticosterone increase after the lower dose as well (Figure 2A), although this fell short of statistical significance. We found a significant increase in serum glucose in the male offspring of the fathers treated with the higher chromium dose (Figure 2B) and a small increase in serum glucose in the females, although this fell short of significance. The statistical methods used, involving mixed-model ANOVAs, confirmed that the increases in serum corticosterone and glucose were not due to large changes in a few offspring.

In another study (18), we found that chromium high-dose treatment of fathers resulted in significantly increased IGF1 in offspring, and microarray analysis of gene expression in the offspring livers revealed a

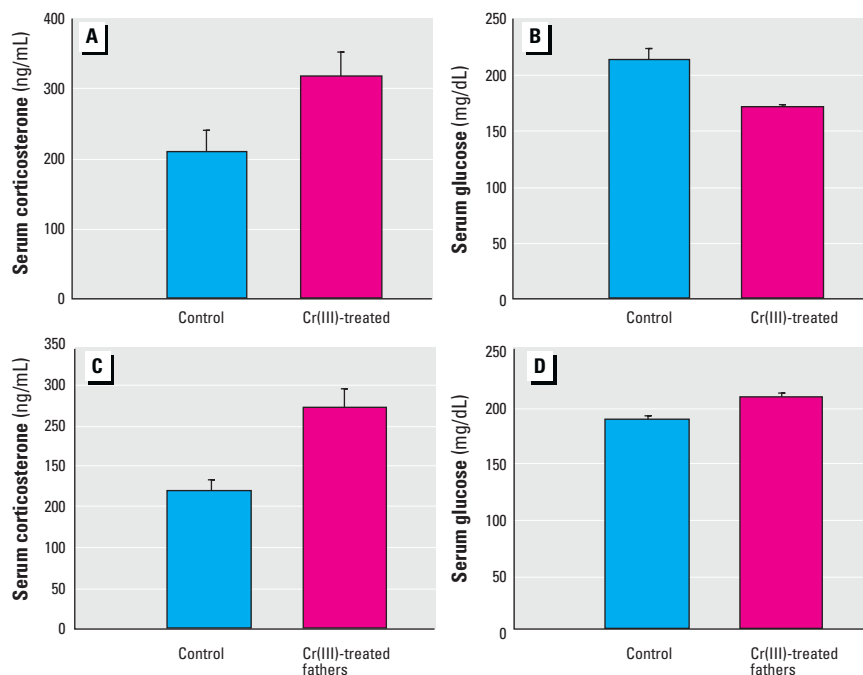


Figure 1. Twenty Swiss male mice, assessed ~25 days after intraperitoneal treatment with 1 mmol/kg Cr(III)/kg body weight, showed a significant increase ($p = 0.0098$; Student's *t*-test) in average serum corticosterone (A) and a significant decrease ($p = 0.0004$; *t*-test with Welch correction) in serum glucose (B) compared with 20 mice treated with acidified saline vehicle (control). The 41 female offspring of the Cr(III)-treated fathers showed significant increases in average serum corticosterone ($p = 0.021$) (C) and glucose ($p = 0.002$) (D) compared with the 45 controls. We obtained *p*-values for significance for offspring values by ANOVA using mixed models with random nested factors; the fathers were the unit of treatment. We performed corticosterone analyses on log-transformed data to maximize homogeneity but display results in direct format.

decreased expression of IGF BP1 compared with controls. IGF1 is a negative regulator of IGF BP1 expression (19). In experiment 2 of the present study, IGF1 was moderately elevated in the offspring after the high-dose chromium compared with controls, 309 ± 15 ng/mL versus 274 ± 14 ng/mL for females ($p = 0.090$) and 285 ± 12 ng/mL versus 265 ± 6 ng/mL for males ($p = 0.14$). We further analyzed livers of representative offspring from experiment 2 for IGF BP1 mRNA by semi-quantitative RT-PCR. We found no marked differences in female offspring. In the male offspring, normalized IGF BP1 expression was again somewhat lower in the chromium high-dose group compared with controls, 3.0 ± 0.3 relative fluorescence units versus 4.6 ± 1.0 ($p = 0.069$, one-tailed test). The normalized relative IGF BP1 hepatic expression levels showed significant negative correlation with serum IGF1 in the chromium group but not in the controls (Figure 3B). Most striking, serum glucose was positively associated with IGF BP1 in the chromium group but negatively associated in the controls (Figure 3C).

Discussion

The findings of the two independent experiments reported here confirm the hypothesis that preconceptional effects can involve alterations in hormone levels in the offspring. The increase in serum corticosterone in the offspring of the chromium(III)-treated fathers was marked and highly significant in both experiments. A more pronounced preconceptional effect occurred in the second experiment. This may have related to the different times of day used for paternal treatment and/or to the fact that most of the treated fathers from experiment 2 produced surviving offspring. Significant alterations in serum glucose, well known to be regulated by glucocorticoids, confirmed the biologic significance of the difference in corticosterone levels.

The measurements of IGF BP1 expression in liver provided additional evidence for the persistence of effects of paternal exposure in the tissues of offspring. Consistent with downregulation of IGF BP1 expression by IGF1, we observed a significant negative

relationship between serum IGF1 and hepatic IGF BP1 mRNA in selected male offspring of the chromium-treated fathers. However, this relationship was not found in the control male offspring. The differences in slopes and intercepts for the regression lines for the two groups were highly significant. We observed even more striking differences for the relationship between serum glucose and relative hepatic expression of IGF BP1, which was strongly positive for the offspring of the chromium-treated fathers and strongly negative for control offspring.

A possible interpretation of these complex interrelationships derives from the known counterregulatory role of IGF BP1 in glucose homeostasis (20). The 2-fold increase in average serum corticosterone in the male offspring of chromium-treated fathers may have tended to increase IGF BP1 synthesis; correlation between corticosterone and IGF BP1 mRNA levels was close to significance for these animals ($p = 0.19$; data not shown). IGF BP1 gene expression is known to be regulated by glucocorticoids (19). Sequestration of IGF1 by the IGF BP1 might then have increased serum glucose due to decreased tissue uptake, and hence the strong positive correlation between IGF BP1 and serum glucose, as shown in Figure 3C. Countering these effects could have been negative feedback regulation of IGF BP1 expression by the elevated IGF1, as shown in Figure 3B, resulting in the small net decrease in IGF BP1 expression. In the control animals with lower corticosterone and IGF1, by contrast, the known negative control of IGF BP1 by glucose predominated (20), and feedback regulation by IGF1 was not dominant. More experiments are required to test these ideas. In any event, our data indicate that the complex relationships among IGF BP1, glucose, IGF1, and possibly corticosterone can be influenced by paternal exposures.

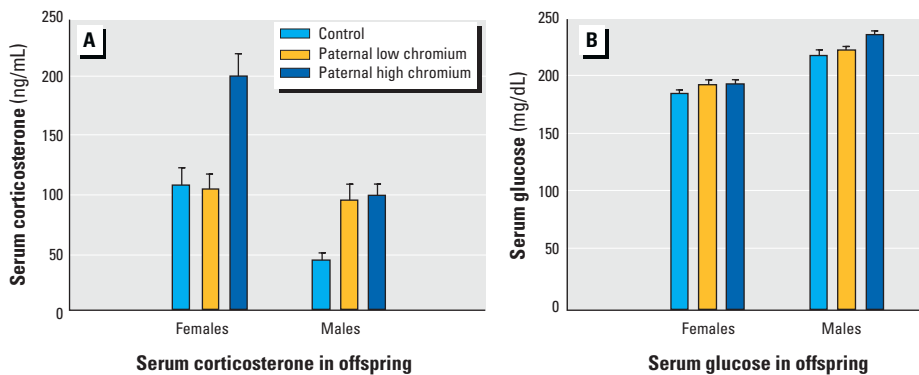


Figure 2. In a second experiment, we treated fathers with freshly prepared CrCl_3 at 0.1 mmol/kg (low) or 1 mmol/kg (high) or acidified saline vehicle (control). We mated them after 2 weeks and assessed the offspring at 10 weeks of age. Numbers of offspring were 36 control, 37 low-dose, and 30 high-dose females and 38 control, 26 low-dose, and 40 high-dose males. p -Values indicate the significance of differences between vehicle controls and the high chromium dose: (A) Females, $p = 0.0001$; males, $p = 0.0023$. (B) Both females and males, $p = 0.0018$.

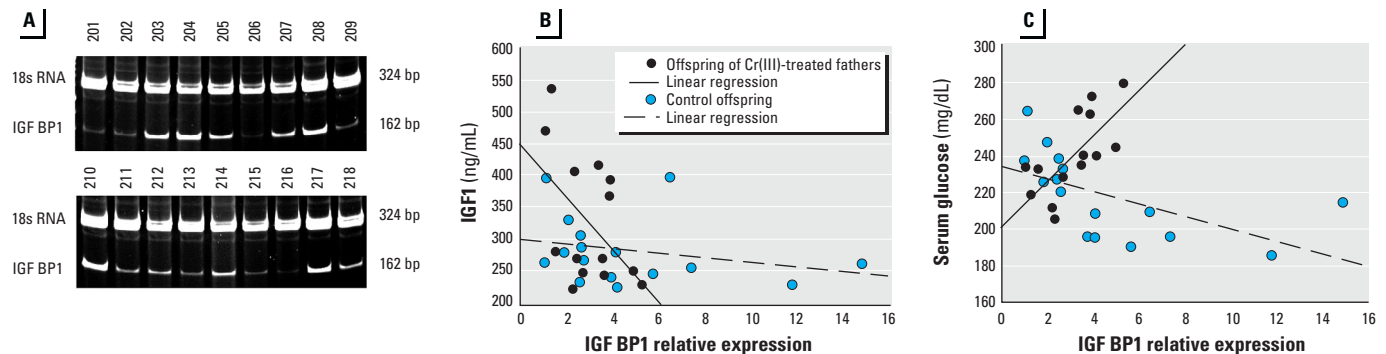


Figure 3. Effect of paternal exposure to 1 mmol/kg CrCl_3 in experiment 2 on relationship between hepatic IGF BP1 mRNA and serum IGF1 and glucose in selected male offspring. We made measurements on 16 control offspring from 9 fathers and 15 chromium-group offspring from 7 fathers. (A) Representative RT-PCR for IGF BP1 mRNA in offspring livers. Chromium-group offspring were numbers 201, 204, 206, 208, 210, 211, 215, 216, 217, and 218. Control offspring were numbers 202, 203, 205, 207, 209, 212, 213, and 214. (B) Correlation of serum IGF1 with hepatic IGF BP1 mRNA. The p -value, $p = 0.050$, is the significance of the difference of the negative slope for the chromium-group offspring versus zero. The regression lines for the two groups differed significantly regarding their intercepts ($p = 0.017$) and slopes ($p = 0.018$). (C) Correlation of serum glucose with hepatic IGF BP1 mRNA. The p -values, $p = 0.0051$ (solid line) and $p = 0.019$ (dashed line), are the significances of the differences of the slopes from zero. The regression lines had significantly different intercepts ($p = 0.033$) and, importantly, significantly different slopes in opposite directions ($p = 0.0006$).

Whether the observed differences in serum corticosterone, glucose, and IGF1 are sufficient to account for known preconceptional effects on neoplasms and other phenomena requires further study. The findings are consistent with this interpretation. High-caloric diets increase tumor incidence in rodents and are suspected to have a similar role regarding cancer and other diseases in humans, and increases in serum glucose and IGF1 are correlated (21–24). IGF1 and IGF BP1 have been repeatedly implicated in human cancer risk (25–28). Corticosteroids have a complex, wide range of positive and negative effects, depending on concentration, duration, physiologic context, and tissue type (29). Both stimulatory and suppressive effects of corticosteroids on the immune system, metabolism, and other hormones have been described (29). Correspondingly, corticosteroids may have either positive or negative association with tumor development or behavior, depending on the cancer type (30,31). This is consistent with the fact that previously observed preconceptional effects have included both increases and decreases in incidences of specific neoplasms in the offspring (14,32).

Beyond neoplasia, transgenerational effects have been observed on reproduction, metabolism, immunity, and behavior (15), all likely targets of neuroendocrine disruption. Thus, transgenerational phenomena might influence risk of not only cancer but also other conditions such as obesity, diabetes, and asthma. We propose a further, broader hypothesis, that what we have uncovered is not primarily a toxicologic phenomenon but an adaptive one: Stressed fathers prepare their offspring for a stressful environment by somehow preprogramming them to upregulate serum corticosterone. The wide-ranging types of transgenerational effects that have been described across phyla (15,16) suggest that this adaptive communication between the physiology of parent and offspring may be a general biologic phenomenon.

The mechanism of the transgenerational effect on serum corticosterone, glucose, and IGF1 is unknown at present. The fact that most or all of the offspring were affected argues against a mutational effect. Furthermore, under the conditions that we employed, we did not detect chromium(III), which enters cells poorly, in the sperm even with highly sensitive techniques, although we found low levels in interstitial tissue of the testis (33,34). An indirect, epigenetic effect seems likely. It is well

known that controls may be imposed on gene expression during gametogenesis, in the parent-specific imprinting phenomenon (35), in which change in gene methylation seems to be part of the mechanism. Further study of this novel phenomenon will be of considerable basic interest as well as relevant to public health concerns.

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