Glucocorticoid-like effects of antihepatocarcinogen Rotenone are mediated via enhanced serum corticosterone levels: Molecular Fitting and Receptor Activation Studies

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Abstract

Background: Recent studies suggest that rotenone alters cell signal transduction pathways in a manner similar to glucocorticoids. Histological and biochemical markers of glucocorticoid effects in vivo, evaluated in our laboratories, provide further evidence for similarities in the activity of glucocorticoids and rotenone. The purpose of this study was to investigate the mechanism by which rotenone produces glucocorticoid-like effects.

Methods: Male B6C3F1 mice were treated for 7 days with rotenone (600 ppm in diet), the glucocorticoid antagonist RU486 (2 mg/kg/day, ip), corticosterone (2 mg/kg/day, ip), or both rotenone and RU 486. Control mice received drug-free diet and the vehicle (corn oil, ip). Following preservation in 10% neutral buffered formalin, tissues were embedded in paraffin. Sections were stained with hematoxylin, eosin, and were examined by light microscopy. Tissue sections were processed for in situ enzymatic end labeling of 3’-hydroxy-DNA strand breaks, a measure of apoptosis. Corticosterone was quantified in sera, using a solid phase radioimmunoassay kit. Cells (cell line 1470.2 derived from C127 mouse mammary adenocarcinoma cells) were transiently transfected with 5 µg of pLTRLuc and 1 µg of β-Galactosidase expression vectors using a BTX square-wave pulser at 155 V, 4 pulses (40 ms each). Cells were then treated with dexamethasone, rotenone, or a mixture of both for 6 hr, harvested and assayed for luciferase and β-Galactosidase activity. Using Root Mean Square (RMS) fit analysis (Alchemy™, Tripose, Inc., St Louis, MO), we assessed possible structural similarities between rotenone and corticosterone, dehydrocorticosterone, glucocorticoid antagonists ZK 98.299, and RU 486. RMS fit was calculated by selecting three atoms in each of the molecules, followed by calculating the distance between these atoms. An RMS value of zero between two molecules indicates identical molecular characteristics. A positive value suggests diminished similarity with a value of 1 or higher excluding any such similarities.
Results: Although the stimulatory effect exerted by rotenone on hepatocellular apoptosis was in the opposite direction of that produced by the glucocorticoid antagonist RU 486, data suggested that rotenone does not directly activate the glucocorticoid receptor. Molecular fitting of rotenone to glucocorticoid receptor agonists and antagonists as well as examination of the transcriptional activation of a glucocorticoid-responsive reporter gene (Mouse MammaryTumorVirus) in response to rotenone indicated that it is highly unlikely that rotenone interacts directly with the glucocorticoid receptor. However, feeding male B6C3F1 mice a diet containing rotenone (600 ppm for 7 days) resulted in a 3-fold increase in serum levels of corticosterone relative to control animals. Corticosterone is the major glucocorticoid in rodents.

Conclusion: Rotenone does not interact directly with the glucocorticoid receptor. Elevation of serum corticosterone levels in response to rotenone may explain the glucocorticoid-like effects of this compound, and may play a role in its anti-hepatocarcinogenic effect.

Background
Previously, we [1] demonstrated that rotenone, a pesticide which specifically inhibits complex I of the mitochondrial respiratory chain [2], altered hepatocellular signal transduction pathways in a manner consistent with its anticarcinogenic activity in the liver [3]. Treatment of male B6C3F1 mice with rotenone enhanced hepatic apoptosis, inhibited cell proliferation and altered the expression of oncogenes and tumor suppressor genes [1]. Since rotenone is structurally similar to steroids and since the effects of rotenone and glucocorticoids appear similar [4,5], we tested the hypothesis that rotenone may act as a glucocorticoid receptor agonist. The impact of rotenone on several glucocorticoid-responsive organs (thymus, adrenal gland and liver), was evaluated in male B6C3F1 mice, and was compared with effects produced by corticosterone as well as by the glucocorticoid antagonist RU 486. Furthermore, we used molecular fitting techniques to examine the potential structural similarities between glucocorticoids and rotenone, and tested the ability of rotenone to activate the transcription of a glucocorticoid receptor responsive reporter gene.

The results of our study demonstrate that despite the fact that rotenone produces histological and biochemical effects similar to those produced by corticosterone, glucocorticoids and rotenone do not share structural molecular similarities. Moreover the transcriptional activation profile of glucocorticoids and rotenone were different. Taken collectively, data suggest that rotenone does not directly activate the glucocorticoid receptor. Interestingly, dietary rotenone significantly increased serum corticosterone levels in treated mice. This finding strongly suggests that glucocorticoid-like-effects of rotenone may be a consequence of increasing serum corticosterone levels caused as a result of exposure to rotenone.

Methods
Animal Treatments
Male B6C3F1 mice (Charles River, Portage, Michigan) weighing 20–25 g were maintained on a daily cycle of alternating 12 hours periods of light and darkness. Mice were randomly divided into five groups. In the first group, mice received rotenone (600 ppm in diet). In the second group, mice received glucocorticoid antagonist RU486 (2 mg/kg/day, ip), and in the third group, mice received corticosterone (2 mg/kg/day, ip). Another group of mice received both rotenone and RU 486. Control mice received drug-free diet and the vehicle (corn oil, ip). All animals were treated for 7 days, at which time mice were anesthetized, and tissues were isolated, blotted and weighed immediately. Care and handling of animals were in accordance with the guidelines of the USDA and Animal Welfare Act Guide for the Care and Use of Laboratory Animals, Department of Health and Human Services Publication No 85–23.

Histological Studies
Following preservation in 10% neutral buffered formalin, tissues were embedded in paraffin, and then sectioned (4 µm thickness). Sections were stained with hematoxylin, eosin, and were examined by light microscopy. Magnification of micrographs was 200×.

Hepatocellular Apoptosis
Tissue sections were processed for in situ enzymatic end labeling of 3’-hydroxy-DNA strand breaks, a measure of apoptosis, as previously described [1].

Transfections
Cell line 1470.2 derived from C127 mouse mammary adenocarcinoma cells was used to determine whether rotenone could affect glucocorticoid-induced transcription of the Mouse Mammary Tumor Virus (MMTV) promoter [6]. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% charcoal-stripped fetal calf serum. Subconfluent cells were transiently transfected with 5 µg
of pLTRLuc and 1 µg of β-Galactosidase expression vectors using a BTX square-wave pulser at 155 V, 4 pulses (40 ms each). PLTRLuc consists of a full-length MMTV Long Terminal Repeat (LTR) driving a luciferase reporter gene [6]. After 14 h transfection, cells were treated with indicated concentrations of dexamethasone, rotenone, or a mixture of both for 6 hr. Cells were harvested and assayed for luciferase and β-Galactosidase activity as previously described [6]. All transfections were done in triplicates and all experiments were repeated at least four times.

Molecular Fitting
Using Root Mean Square (RMS) fit analysis (Alchemety™, Tripose, Inc., St Louis, MO), we assessed possible structural similarities between rotenone and corticosterone, dehydrocorticosterone, glucocorticoid antagonists ZK 98.299, and RU 486. RMS fit was calculated by selecting three atoms in each of the molecules, followed by calculating the distance between these atoms. An RMS value of zero between two molecules indicates identical molecular characteristics. A positive value suggests diminished similarities with a value of 1 or higher excluding any such similarities.

Quantification of Serum Corticosterone Levels
Corticosterone was quantified in sera, using a solid phase radioimmunnoassay kit (Diagnostic Products Corporation, Los Angeles, CA). In this assay, 125I-labeled rat corticosterone was allowed to compete with corticosterone for antibody sites in the sample. Since corticosterone levels follow diurnal variations, assays were done at the same time periods for the different treatment groups.

Statistical Analysis
Means of various treatment groups were compared with respective controls and were analyzed by ANOVA (Stat Work™). Statistical significance was defined as p < 0.05. All data reported are means ± SEM of 4–7 mice per group.

Results
Effect of Rotenone on Thymus and Adrenal Glands
Since the thymus and the adrenal gland are sensitive indicators of changes in the animal glucocorticoid status, we investigated the effect of rotenone on these two organs. In control animals, thymus/body weight ratios were 0.26 ± 0.02 (Fig 1). Treatment of mice with exogenous corticosterone resulted in a significant decline of 42% in thymus/body weight ratios down to 0.15 ± 0.03 (Fig 1). Rotenone also decreased thymus/body weight ratios to 0.17 ± 0.02 (Fig 1), values that were similar to those observed in response to corticosterone (Fig 1). Next, we examined the effect of rotenone treatment on the histology of the adrenal gland. The adrenal cortex of control animals showed the expected well-demarcated division among z. glomerulosa, z. fasciculata, and z. reticularis. While cells of z. glomerulosa were arranged in the normal, irregular ovoid clumps, separated by a delicate trabecular system and a fine distribution of capillaries, those of z. fasciculata consisted of narrow cords of secretory cells with a large mildly stained cytoplasm, presenting a foamy appearance (Fig 2). Also, cells of z. reticularis were smaller and more intensively stained than those of z. fasciculata, and were arranged in an irregular network of glandular cells separated by numerous wide diameter capillaries (Fig 2). Rotenone treatment diminished the adrenal cortical width. Cells in z. fasciculata and z. reticularis were compact, with an area difficult to assign to either zone (Fig 2). Furthermore, nuclei, particularly in z. fasciculata, were rather small, intensively stained, with quite a few pyknotic nuclei present (Fig 2). The effects of rotenone were similar to those observed in adrenals of animals treated with corticosterone (not shown). Conversely, however, RU 486 treatment resulted in a rather wide adrenal cortex, with a well-demarcated z. glomerulosa, but unclear demarcation between z. reticularis and z. fasciculate. Both of these two areas were very compact and exhibited intensively stained cells (Fig 2). The nuclei in z. reticularis and z. fasciculata in RU 486-treated mice were small and hyperchromatic, with minimal evidence of pyknotic nuclei (Fig 2). Simultaneous administration of the glucocorticoid antagonist RU 486 with rotenone partially abrogated the effect of the later on the adrenal gland (Fig 2). Treatment with rotenone and RU 486 resulted in an increased cortical width, with the restoration of a very well demarcated z. glomerulosa. However, the effect of RU 486 was more evident on cells of z. fasciculata and z. reticularis which remained very compact with intensively stained eosinophilic cytoplasm (Fig 2). These two areas were also less defined and contained many pyknotic nuclei, compared with adrenals from control animals (Fig 2).
Opposite Modulation of Hepatic Apoptosis by Rotenone and RU 486

Rotenone has been demonstrated to increase hepatic apoptosis [1,7]. To evaluate whether this effect was mediated via glucocorticoid-responsive pathways, we compared the effect of rotenone with that of the glucocorticoid antagonist, RU 486. In livers of control mice, only about 3–4% of hepatocytes are undergoing self-imposed destruction (Fig 3). As expected, rotenone accelerated this process resulting in a two-fold increase in the number of hepatocytes undergoing apoptosis (Fig 3). Conversely, treatment with the glucocorticoid antagonist RU 486 resulted in more than a 60% decrease in this process (Fig 3).

Figure 2
Effect of Rotenone on Adrenal Histology. Shown are representative sections of adrenal glands from control, rotenone-treated, and RU 486-treated mice. Also shown is a representative section of adrenal gland from animals treated with the combination of rotenone and RU 486. Tissues were handled and examined as described under “Materials and Methods”. Micrographs were taken at 200× magnification.
To probe whether the in vivo glucocorticoid-like action of rotenone is mediated at the transcription level, mouse mammary adenocarcinoma cells (1470.2) were transfected with pLTRLuc and treated with dexamethasone and/or rotenone. Treatment of cells with 10 nM dexamethasone for 6 hours, activated the reporter gene transcription approximately 25-fold (Fig. 4). Moreover, 50 nM dexamethasone treatment for 6 h further activated the reporter gene transcription to 100-fold, suggesting that the effect of dexamethasone on transactivation is dose-dependent (data not shown). While dexamethasone increased reporter gene activity (Fig 4), rotenone alone neither changed the reporter gene activity at concentrations as high as 100 nM, nor did alter the dexamethasone-induced transactivation when it was added simultaneously with dexamethasone (Fig 4). These results suggest that rotenone does not activate transcription of glucocorticoid-inducible Mouse Mammary Tumor Virus promoter.

**Molecular Fitting Studies**

RMS fit was performed between rotenone and two glucocorticoid receptor agonists (corticosterone and dehydrocorticosterone; Fig 5) as well as between rotenone and two glucocorticoid receptor antagonists (ZK 98.299 and RU 486; Fig 5). In comparing rotenone with all of these compounds, RMS values ranging from 0.87 to 1.4 were obtained, suggesting minimum degree of similarities between rotenone and any of these compounds, if any (Table 1).

**Serum Corticosterone Levels**

In mice receiving drug-free diet, serum corticosterone levels were 168 ± 28 ng/ml (Fig 6). When animals were given a diet containing 600 ppm rotenone for 7 days, serum corticosterone levels climbed significantly to 490 ± 33 ng/ml, a 3-fold increase in these levels (Fig 6).
Figure 5
Chemical Structures of Rotenone, Glucocorticoid Agonists and Antagonists.
Glucocorticoid-Like Effects of Rotenone

Increases in hepatic apoptosis were observed in the livers of rotenone-treated mice [1,7]. Since apoptosis can act as a safeguard mechanism that allows an organ to purge itself of already transformed cells [1], increased apoptosis may be a crucial event in explaining the anticarcinogenic effect of rotenone [1,7]. However, how rotenone modulates hepatocellular apoptosis remains unclear. In search of an answer to this question, it is noteworthy that like rotenone, glucocorticoids have hepatic anticarcinogenic effects [8]. These hormones modulate the expression of oncogenes and apoptosis in a fashion similar to that observed in animals treated with rotenone [1,9–11]. In agreement with our previous studies [1,12], we observed that while rotenone enhanced hepatic apoptosis, the glucocorticoid antagonist RU 486 diminished this activity (Fig 3). In order to further characterize the relationship between rotenone and glucocorticoids, we compared their effects on the thymus and adrenal glands; organs which are also sensitive to glucocorticoids [13,14]. In this study, exposure of mice to exogenous corticosterone significantly reduced thymus/body weight ratios; this effect was mimicked by rotenone (Fig 1). Furthermore, in rotenone-treated mice the decrease in the ratio of adrenal-to-body weight was completely eliminated by the simultaneous administration of the glucocorticoid antagonist RU 486 with rotenone (not shown). Histological changes in adrenal glands in response to rotenone and RU 486 co-treatment (Fig 2) resembled those known to occur in response to exogenous corticosterone [14]. Based on these findings, we initially hypothesized that rotenone may act as a glucocorticoid agonist.

Hormonal Disruption as Basis for Rotenone-Induced Antihepatocarcinogenic Effect

In spite of advances made in the field of cancer research, events leading to cancer and mechanisms of action by anticancer agents remain elusive [15]. We [1,3] as well others [7] have reported that the antihepatocarcinogenic activity of rotenone may be produced by diminishing liver cell proliferation and/or by enhancing hepatocellular apoptosis. We have also shown that rotenone alters hepatocellular transduction pathways in a manner consistent with a pro-apoptotic effect [1]. Nonetheless, molecular mechanisms involved remained unclear. In this study, we present evidence showing that the antihepatocarcinogenic effect of rotenone may be mediated, at least in part, through elevated serum glucocorticoid levels, resulting from exposure to this compound. Despite the fact that endocrine disruptors have been linked to numerous pathological conditions [16], discovery of such compounds may in contrast, lead to development of novel cancer preventive agents.

Conclusion

The data demonstrate that despite the fact that rotenone produces histological and biochemical effects similar to those produced by corticosterone, rotenone does not directly activate the glucocorticoid receptor. Glucocorticoid-like-effects of rotenone may be a consequence of increasing serum corticosterone levels caused as a result of exposure to rotenone. This novel finding leads to the conclusion that rotenone is a unique endocrine disruptor of glucocorticoids, and warrants further investigation of its effects on homeostasis of other hormones. Although
mechanisms by which rotenone elevates serum corticosterone levels remain unclear, the possibility that rotenone acts via a receptor-mediated mechanism to stimulate the adrenal gland deserves further evaluation. Furthermore, compounds structurally related to rotenone should be screened to evaluate their potential as disruptors of glucocorticoid homeostasis.

**Author's Contributions**

JY performed the apoptosis assay. CE and BW carried out the glucocorticoid receptor transfection and transactivation assay. RN and DY performed the molecular fitting studies. AM performed the tissue histological evaluations. MC analyzed serum corticosterone levels. MB conceived, designed and coordinated the study. All authors participated in writing the manuscript.

**Acknowledgements**

This work is supported, in part, by NIH AG 18479 to MB. The authors are grateful to Ms Hongmei Meng for technical assistance.

**References**

1. Wang C, Youssef J, Saran B, Rotthberg P, Cunningham ML, Molteni A and Badr M. Diminished energy metabolism and enhanced apoptosis in livers of B6C3F1 mice treated with the antihepatocarcinogen rotenone. *Mol Cell Biochem* 1999, 201:25-32
2. Oberg K. Site of action of rotenone in the respiratory chain. *Exp Cell Res* 1961, 24:163-164
3. Cunningham ML, Soliman MS, Badr M and Matthews HB. Rotenone, an anticarcinogen, inhibits cellular proliferation but not peroxisome proliferation in mouse liver. *Cancer Lett* 1995, 95:93-97
4. King KL and Cidlowski JA. Cell cycle regulation and apoptosis. *Annu Rev Physiol* 1998, 60:601-617
5. Telford WG, King LE and Fraker PJ. Evaluation of glucocorticoid-induced DNA fragmentation in mouse thymocytes by flow cytometry. *Cell Prolif* 1991, 24:447-459
6. Fryer C, Kinyamu HK, Rogatsky I, Garabedian MJ and Archer TK. Selective activation of the glucocorticoid receptor by steroid antagonists in human breast cancer and osteosarcoma cells. *J Biol Chem* 2000, 275:17771-17777
7. Isenberg J, Kolaja K, Ayoubi S, Watkins JB and Klaunig JE. Inhibition of Wy-14,643 induced hepatic lesion growth by rotenone. *Carcinogenesis* 1997, 18:1511-1519
8. Henderson IC, Fischel RE and Loeb JN. Suppression of DNA synthesis by cortisone. *Endocrinol* 1971, 88:1471-1474
9. Henderson IC and Loeb JN. Hormone-induced changes in liver DNA synthesis: effects of glucocorticoids and growth hormones: growth and DNA polymerase activity. *Endocrinol* 1974, 93:1637-1643
10. Denis G, Humbelt M, Varlaet J, Boniver J and Defresne MP. p53, Bax and Bcl-2 in vivo expression in the murine thymus after apoptotic treatments. *Anticancer Res* 1998, 18:3315-3321
11. Medh RD, Saeed MF, Johnson BH and Thompson EB. Resistance of human leukemic CEM-C1 cells is overcome by synergism between glucocorticoid and protein kinase A pathways: correlation with c-myc suppression. *Cancer Res* 1998, 58:3684-3693
12. Youssef J and Badr M. Hepatocarcinogenic potential of the glucocorticoid antagonist RU 486 in B6C3F1 mice: effect on apoptosis, expression of oncogene and the tumor suppressor gene p53. *Mol Can* 2003, 2:3
13. Selye E and Hall C. Pathological changes induced in various species by overdosage with desoxycorticosterone. *Arch Pathol* 1943, 36:19-28
14. Skelton FR. Production and inhibition of hypertensive disease in rat by corticosterone. *Endocrinol* 1958, 62:364-369
15. Gopala K, Shiff S, Telang N, Das K, Kohgo Y, Narayan S and Li H. Carcinogenesis: The more we seek to know the more we need to know-challenges in the post genomic era. *J Carcinogenesis* 2003, 2:1
16. Birnbaum L and Cummings A. Dioxins and Endometriosis: A Plausible hypothesis. *Environ Health Perspect* 2002, 110:15-21