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# Correlation between thyroid hormone status and hepatic hyperplasia and hypertrophy caused by the peroxisome proliferator-activated receptor alpha agonist Wy-14,643

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## Abstract

**Background:** The metabolic inhibitor rotenone inhibits hepatocellular proliferation and the incidence of liver cancer resulting from exposure to the PPARα agonist Wy-14,643, *via* unknown mechanisms. Since the absence of thyroid hormones diminishes hepatomegaly, an early biomarker for the hepatocarcinogenicity induced by PPARα agonists, this study was undertaken to investigate whether rotenone might interference with the ability of Wy-14,643 to alter the animal thyroid status.

**Methods:** Male B6C3F1 mice were given Wy-14,643 (100 ppm), rotenone (600 ppm) or a mixture of both, in the feed for 7 days. Bromodeoxyuridine (BrDU), marker of cell replication, was delivered through subcutaneously implanted osmotic mini-pumps. At the end of the experiment, sera were collected and corticosterone and thyroid hormone levels were measured by solid-phase radioimmunoassay kits. In addition, liver tissue samples were stained immunohistochemically for BrDU to determine percentages of labeled cells. Further, cell surface area was determined from images generated by a Zeiss Axioplan microscope equipped with a plan Neofluar ×40 0.75 na objective. Tracings of individual hepatocyte perimeters were then analyzed and cell-surface areas were calculated using MicroMeasure FL-4000.

**Results:** Wy-14,643 caused a significant increase in liver weights, hepatocyte BrDU labeling index (LI), and hepatocyte surface area. In animals which received both Wy-14,643 and rotenone simultaneously, all of these effects were significantly less pronounced compared with mice that received Wy-14,643 alone. Rotenone alone decreased liver weights, LI and surface area. The Free Thyroid Index (FTI), which provides an accurate reflection of the animal's thyroid status, was 5.0 ± 0.3 in control mice. In animals exposed to rotenone, these values decreased to 2.0 ± 0.9, but in animals which received Wy-14,643, levels increased significantly to 7.7 ± 0.9. FTI values decreased to 3.4 ± 0.8 in mice receiving both rotenone and Wy-14,643.

**Conclusion:** A strong correlation was observed between the animal thyroid status and both, hepatocyte proliferation ($r^2 = 0.62$), and hepatocyte surface area ($r^2 = 0.83$). These results support the hypothesis that the thyroid status of the animal plays a role in PPARα-induced hepatocellular

proliferation and liver cell enlargement. Both these events are known to contribute to the expression of liver cancer in response to the activation of PPARα.

## Background

Although hepatocellular neoplasms occurred in wild-type, but not in PPARα-null mice upon exposure to PPARα agonists [1,2], molecular mechanisms involved in this effect remained unknown. However, important factors involved in this hepatocarcinogenic effect are thought to include: (**i**) enhanced hepatic oxidative stress due to elevated peroxisomal and nonperoxisomal oxidative metabolism, (**ii**) inhibition of apoptosis in livers of exposed animals, and/or (**iii**) increased hepatocellular proliferation [3,4].

Thyroid hormones have been shown by our laboratory [5] as well as by others [6] to alter hepatic responses to PPARα agonists, including hepatomegaly, an early biomarker for the hepatocarcinogenicity induced by these compounds [7,8]. Hepatomegaly was blunted in thyroidectomized animals treated with PPAR agonists, compared with intact animals [5,6]; however, the contribution of hyperplasia and hypertrophy to this effect is not known. Furthermore, agonists of PPARα impart a thyromimetic effect in exposed animals [9]. This effect is significantly more pronounced in intact animals compared with thyroidectomized counterparts [5]. These findings suggest that thyroid hormones may play a role in the effects attributed to the activation of PPARα, including hepatocellular cancer.

Previously, our laboratory [10], as well as others [11] have reported that the pesticide rotenone inhibited hepatocellular proliferation induced by the PPARα agonist Wy-14,643, and also reduced the incidence of liver cancer resulting from exposure to Wy-14,643 [11]. Consequently, this study was undertaken to investigate whether rotenone blocks the hepatic effects of the PPARα agonist Wy-14,643 by potentially interfering with its ability to modulate the animal thyroid status. The results demonstrate that rotenone and Wy-14,643 produced opposing effects on thyroid hormone levels. They also show a strong correlation between the animal thyroid status and both hepatocyte proliferation ($r^2 = 0.62$), and hepatocyte surface area ($r^2 = 0.83$). In conclusion, the results suggest that thyroid hormones play a major role in the events known to contribute to the expression of liver cancer in response to the activation of PPARα. These events include hepatocellular proliferation and hepatocyte enlargement.

## Methods

### *Animal treatment*

Male B6C3FI mice (Charles River, Potage, Michigan) weighing 25 ± 2 gram were maintained on a daily cycle of alternating 12 h periods of light and darkness, with room temperature set at 22 ± 2°C and on a standard Purina diet for seven days prior to experiment. Mice were then fed Wy-14,643 (100 ppm), rotenone (600 ppm) or a mixture of both blended in the feed for 7 days. Water was supplied *ad labitum*. Our experimental protocol was approved by the Institutional Animal Care and use Committee, and experiments were performed in accordance with established guidelines for care and use of animals.

### *Measurement of cell proliferation*

Osmotic minipumps (Alza Cooperation, palo Alto, CA, model 2002) were implanted subcutaneously into the backs of the mice to deliver bromodeoxyuridine (30 mg/ml) which is incorporated into DNA of replicating cells. Seven days later, animals were euthanized by $CO_2$ inhalation. Following determination of liver weights, a mid-lobe radial section of the right anterior lobe was fixed in neutral buffered formalin for 24 h. A cross section of small intestine was also fixed as a positive control for the proper operation of the mini-pump and the staining technique because these cells are constantly in S phase. Tissues were embedded in paraffin and serial sections were mounted onto poly-l-lysine-coated slides. After deparafination and rehydration, slides were stained immunohistochemically for BrDU incorporation [12]. Random areas of the slides were chosen for counting stained and unstained hepatocyte nuclei (>1000 hepatocytes/animal).

### *Determination of cell surface area*

Mice were fed control diet or a diet containing rotenone (600 ppm), Wy-14,643 (100 ppm) or a mixture of both for 7 days prior to these experiments. Using a Zeiss Axioplan microscope equipped with a plan Neofluar ×40 0.75 na objective (Carl Zeiss, Inc., Thornwood, NJ), individual hepatocyte perimeters were traced by a mouse-driven pointer. Generated images were analyzed and cell-surface areas were calculated using a commercial software (Micro-Measure FL-4000, Georgia Instruments, Inc., Rosswell, GA), as described previously [13,14].

### *Serum corticosterone and thyroid hormone levels*

Hormones were quantified in sera. Corticosterone levels were measured using a solid-phase radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). In this assay, $^{125}$I-labeled rat corticosterone was allowed to

compete with serum 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. Both, T3 and T4, as well as T3 value uptake were detected using a solid-phase chemiluminescent enzyme immunoassay kits (Diagnostic Products Corporation, Los Angeles, CA). The Free Thyroxine Index of Clark and Horn, which provides a more accurate picture of thyroid status [15], was calculated according to the manufacturer's instructions [(T3% Uptake/100) × T4 μg/dl].

### *Statistical Analysis*

Data were analyzed by ANOVA (Stat Work™), and statistical significance was defined as $p < 0.05$. All data reported are means ± SEM of 4–7 mice per group.

## Results

### *Liver/body weight ratios, hepatocyte proliferation and surface area in treated mice*

Liver/body weight ratios in control mice were 5.3 ± 1.02% (Fig. 1A). These ratios did not change in animals treated for seven days with rotenone (Fig. 1A). However, as expected in mice treated with Wy-14,643, ratios increased significantly to 8.8 ± 0.17% (Fig 1A). In animals treated with a mixture of Wy-14,643 and rotenone, the ratio was increased to only 7.5 ± 0.9%, which is significantly lower than those obtained with Wy-14,643 alone (Fig. 1A).

The BrDU labeling index in control mice was 6.7 ± 1.4% (Fig. 1B). Treatment with Wy-14,643 increased labeling indices significantly to 27.2 ± 4.30% (Fig. 1B). However, animals treated with a mixture of rotenone and Wy-14,643 had a BrDU labeling index which was significantly lower compared to those which received Wy-14,643 alone, nonetheless they were higher than control values (Fig. 1B). Rotenone alone decreased the BrDU labeling index by 50% (Fig. 1B).

Hepatocyte surface area in control mice was 318 ± 18 $\mu m^2$ (Fig. 1C). Seven days following treatment with Wy-14,643, cell size increased significantly to 765 ± 36 $\mu tm^2$, while feeding mice a diet containing a mixture of Wy-14,643 and rotenone resulted in a cell size of 387 ± 17 $\mu m^2$ which is approximately that of the control mice and significantly less than values observed in mice treated with Wy-14, 643 alone (Fig. 1C). Animals treated with rotenone alone showed a 24% reduction in cell surface area, compared to control animals (Fig. 1C),

### *Perturbation of serum hormone levels by rotenone*

Feeding mice diets containing rotenone, Wy-14,643, or a combination of both compounds did not alter serum T3 levels. These levels ranged from 42.4 ± 1.7 U% to 49.5 ± 1.2 U% among all tested groups (Fig 2A). However, when given separately, rotenone and Wy-14,643 produced opposing effects on serum T4 levels. While rotenone decreased these levels significantly by 60% (from 10.5 ± 0.65 μg/dl to 4.2 ± 0.5 μg/dl), Wy-14,643 almost doubled serum T4 to 18.0 ± 1.6 μg/dl (Fig 2B). Mice which simultaneously received rotenone and Wy-14,643 showed serum T4 levels of 6.1 ± 1.2 mg/dl (Fig 2B). The Free Thyroid Index (FTI), which accurately reflects the animal thyroid status [15], closely followed changes in serum T4 levels. FTI was 5.0 ± 0.3 in control mice (Fig 2C). These values decreased following exposure to rotenone to only 2.0 ± 0.9, but increased significantly to 7.7 ± 0.9 in animals which received Wy-14,643 alone (Fig 2C). Similar to T4, FTI values were lower in mice receiving rotenone and Wy-14,643 simultaneously (3.4 ± 0.8), compared with control mice (Fig 2C).

Mice which received drug-free diet had serum corticosterone levels of 159 ± 33 ng/ml (Fig 3). These levels were increased by 3 fold in mice fed rotenone-containing diet (Fig 3). However, Wy-14,643 failed to alter serum corticosterone levels significantly, where levels remained at 181 ± 3 ng/ml (Fig 3). The diet containing both rotenone and Wy-14,643 resulted in animal serum corticosterone levels which were 27% lower than those in mice exposed to rotenone alone, yet 2-fold higher than detected in sera of control mice (Fig 3).

### *Correlation between serum hormone levels and hepatic changes*

There was a strong correlation between the animal thyroid status and both, hepatocyte proliferation ($r^2 = 0.62$, Fig 4A), and hepatocyte surface area ($r^2 = 0.83$, Fig 4B). While diet containing Wy-14,643 significantly elevated the Free Thyroid Index (Fig 2C), and increased hepatocyte BrU labeling indices (Fig 1B), and hepatocyte surface area (Fig 1C), co-administration of rotenone with Wy-14,643 reduced all parameters toward control values (Figs 1&4). Rotenone alone decreased all parameters to levels below control values (Figs 1&4).

Conversely, there was a poor correlation ($r^2 = 0.26$) between serum corticosterone levels and hepatocyte BrU labeling indices (Fig 4C). For example, while diet containing Wy-14,643 did not significantly alter serum corticosterone levels (Fig 3), labeling indices in animals receiving this diet were 4 folds higher compared with control mice (Fig. 2B).

Similarly, serum corticosterone levels did not correlate well ($r^2 = 0.35$) with changes in hepatocyte surface area (Fig 4D).

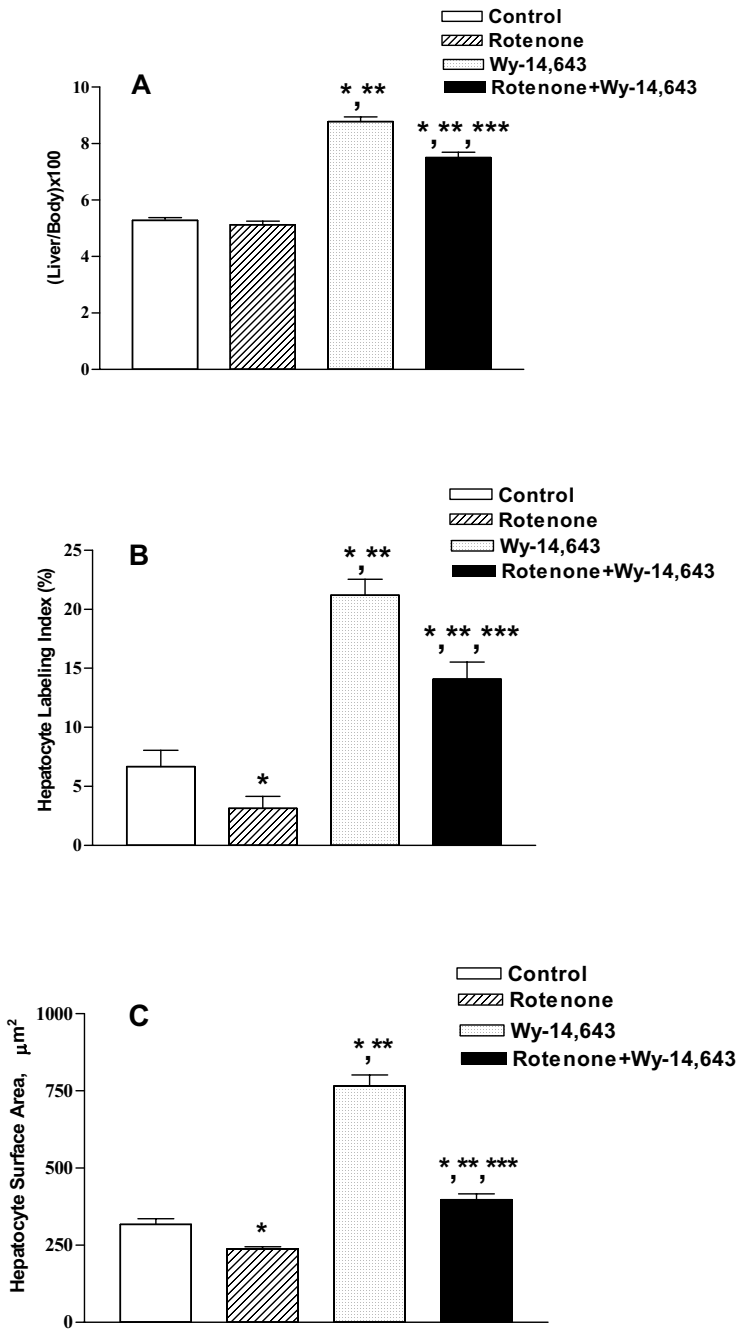


**Figure 1**

**Liver/body weight ratios, hepatocellualr proliferation and surface area.** Mice were fed control diet or a diet containing Wy-14,643 (100 ppm), rotenone (600 ppm), or a mixture of both for seven days. Animal body and liver weights were recorded, and ratios were calculated (**A**). Bromodeoxyuridine incorporation into hepatocyte DNA (**B**), and hepatocyte surface areas (**C**) were determined as detailed under "Materials and Methods". Data are means ± SEM from 4–6 mice per group. * P < 0.05 compared to control values. **p < 0.05 compared to rotenone values, and ***p < 0.05 compared to Wy-14,643 values.

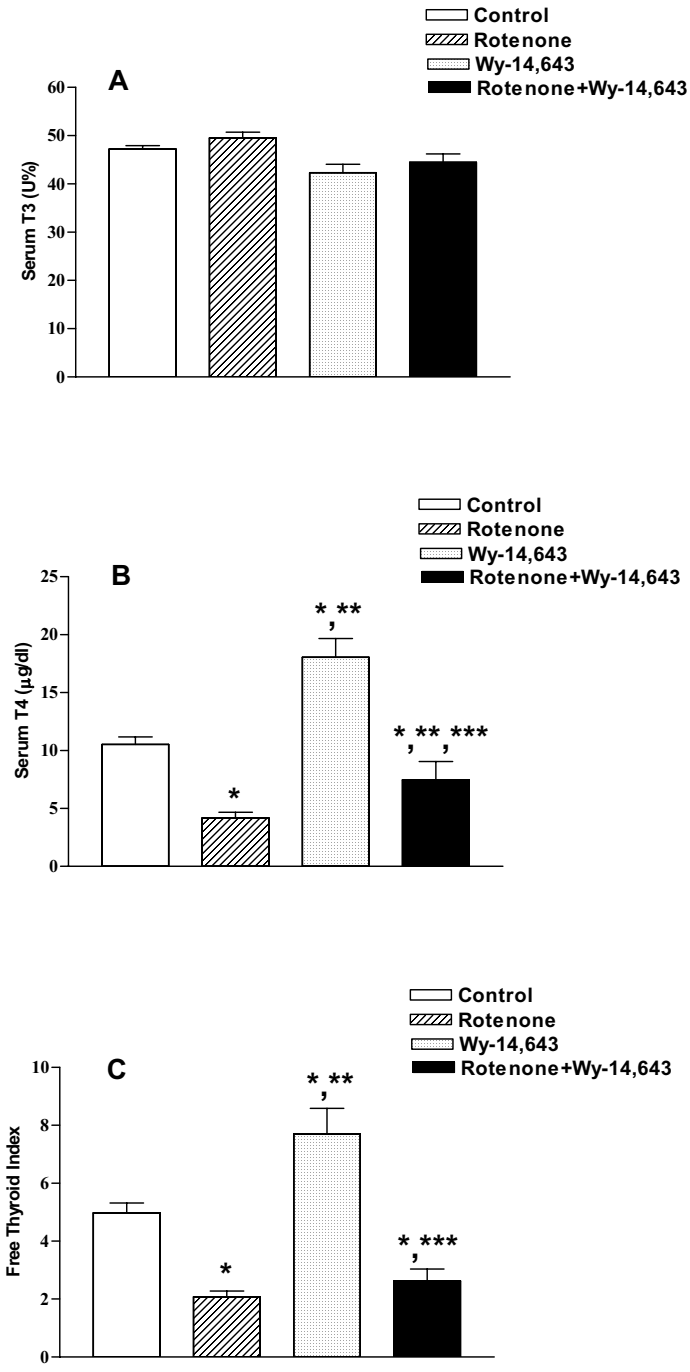


**Figure 2**

**Perturbations of serum thyroid levels by rotenone and Wy-14,643.** Animals were treated, and sera were collected, and analyzed, as detailed under "Materials and Methods", for T3 (**A**), and T4 (**B**). Free Thyroxine Index (**C**) was calculated [(T3% Uptake/100) × T4 μg/dl]. Data are means ± SEM from 4–6 mice per group. * P < 0.05 compared to control values. **p < 0.05 compared to rotenone values, and ***p < 0.05 compared to Wy-14,643 values.

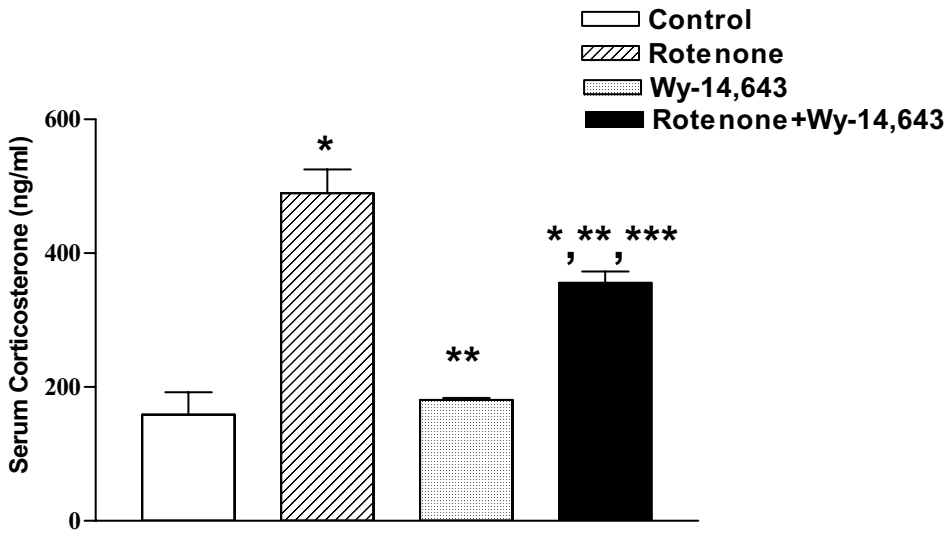


**Figure 3**
**Increase in serum corticosterone levels by rotenone.** Corticosterone was quantified in sera from various animal groups as described under "Materials and Methods". Data are means ± SEM from 4–6 mice per group. * P < 0.05 compared to control values. **p < 0.05 compared to rotenone values, and ***p < 0.05 compared to Wy-14,643 values.

## Discussion

The non-genotoxic hepatocarcinogens, PPARα agonists, elicit marked liver enlargement; encompassing an increase in both, cell number (hyperplasia) as well as cell size (hypertrophy). Hyperplasia results from the stimulation of DNA synthesis and subsequent cell division [16]. A probable causal link between the hyperplasia and the subsequent development of liver tumors in rodents has been suggested [17-19]. The potential role of hypertrophy in the hepatocarcinogenic effect of PPAR agonists is not clear, and differential mechanisms controlling these two phenomena are not understood.

Rotenone inhibits hepatocellular proliferation and reduces the incidence of liver cancer resulting from exposure to the PPARα agonist Wy-14,643 [10,11]. Conversely, thyroid hormones are known to mediate hepatic responses to PPARα agonists [5,6]. Hepatomegaly, which is considered an early biomarker for the hepatocarcinogenicity induced by these nongenotoxic hepatocarcinogens [7,8], is blunted in thyroidectomized animals [5,6], and PPAR agonists impart a thyromimetic-like effect in exposed animals [9]. Accordingly, we sought to investigate whether rotenone alters the animal thyroid status, in a manner that may explain its antagonistic effects of hepatocellular responses to the PPARα agonist Wy-14,543 [10,11].

In this study, the hepatocarcinogen Wy-14,643 and the antihepatocarcingen rotenone produced opposing effects on hepatocellular proliferation (Fig 1B), hepatocyte surface area (Fig 1C), and Free Thyroid Index (FTI; Fig 2C). While rotenone decreased all three parameters significantly, Wy-14,643 caused remarkable increases in these parameters. These values were lower in mice receiving rotenone and Wy-14,643 simultaneously, compared with mice that received Wy-14,643 alone. There was a strong positive correlation between FTI and both BrDU labeling ($r^2 = 0.62$; Fig 4A), as well as with hepatocyte surface area ($r^2 = 0.83$; Fig 4B).

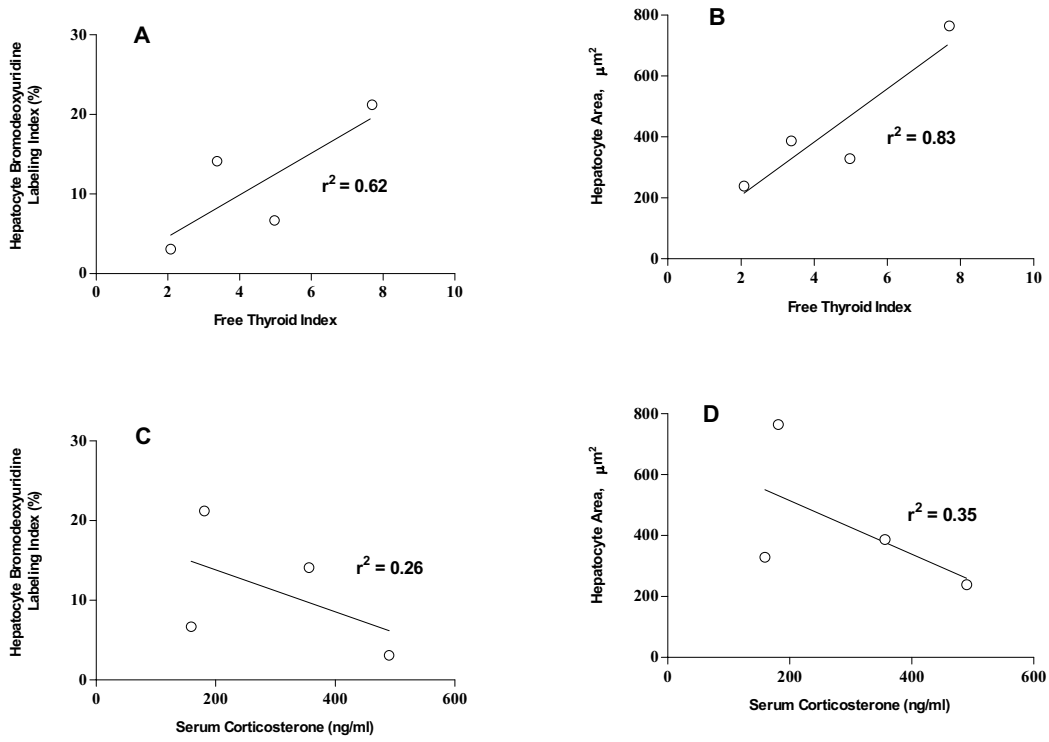


**Figure 4**

**Correlation between serum hormone levels and hepatocellular changes.** Relationship between serum thyroid hormone and corticosterone levels and either hepatocye BrDU labeling (**A,C**), or hepatocyte surface area (**B,D**) were evaluated. Regression coefficient analyses were performed using the Prism Software (GraphPad Software, Inc., San Diego, CA).

In contrast to thyroid hormone levels, and in agreement with a recent study from our laboratories [20], rotenone increased serum corticosterone levels (Fig 3). Wy-14,643, on the other hand, failed to alter these levels (Fig 3). Based on these findings, we previously suggested that increasing serum glucocorticoid levels by rotenone may, at least in part, explain the anticarcinogenic effect of this compound [20]. Results of the current study support the hypothesis that decreasing serum thyroid hormone levels may play a more prominent role, compared with that of glucocorticoids, in the mechanism of antihepatocarcingenicity attributed to rotenone. Animals receiving diet containing both rotenone and Wy-14,643 showed serum corticosterone levels which were 27% lower than those caused by rotenone alone, yet 2-fold higher than detected in sera of control mice (Fig 3). Yet, poor correlation was found between serum corticosterone levels and either BrDu ($r^2$ = 0.26; Fig 4C), or hepatocyte surface areas ($r^2$ = 0.35; Fig 4D). In previous studies [21,22], it was shown that while corticosterone mediated hepatomegaly and liver hypertrophy due to the organochlorine mirex, thyroid hormones exclusively mediated liver hyperplasia due to this compound. The current studies, however, show that increasing serum corticosterone levels by rotenone was accompanied by a decrease in hepatomegaly as well as diminished hypertrophy in response to Wy-14,643 (Fig

1). Furthermore, exposure to rotenone alone, which increased serum glucocorticoid levels remarkably (Fig 3), diminished hepatic hypertrophy (Fig 1C). It is possible that the simultaneous effect of rotenone on thyroid hormone levels, in addition to corticosterone, is responsible for this observation.

In conclusion, thyroid status appears to regulate PPARα-controlled increases in hepatocellular proliferation and liver cell enlargement; events known to contribute to the expression of liver cancer. Further, diminishing Wy-14,643's induction of serum thyroid hormone levels may represent an important aspect of the mechanism by which rotenone reduces the formation of hepatocellular cancer in response to Wy-14,643.

## Authors' Contributions

CW conducted hepatocyte surface area measurements. JY performed cell proliferation experiments. MLC measured serum hormone levels, and participated in the hepatocyte BrDU labeling experiments. MB conceived, designed and coordinated the study. JY, MC, and MB participated in writing the manuscript.

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