Acetaminophen Increases Aldosterone Secretion While Suppressing Cortisol and Androgens: A Possible Link to Increased Risk of Hypertension

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BACKGROUND
Acetaminophen (paracetamol) is a widely used analgesic and antipyretic drug. Potential side effects are of public health concern, and liver toxicity from acute overdose is well known. More recently, a regular use of acetaminophen has been associated with an increased risk of hypertension.

METHODS
We investigated effects of acetaminophen on steroidogenesis as a possible mechanism for the hypertensive action by using the human adrenocortical cell line, H295R. Cells were treated with 0.1, 0.5, and 1 mM of acetaminophen for 24 hours, and secretion of steroids and gene expression of key steps in the steroidogenesis were investigated.

RESULTS
Progesterone and aldosterone secretion were increased dose dependently, while secretion of 17α-OH-progesterone and cortisol as well as dehydroepiandrosterone and androstenedione was decreased. CYP17α-hydroxylase activity, assessed by the ratio 17α-OH-progesterone/progesterone, and CYP17-l-lyase activity, assessed by the ratio androstenedione/17α-OH-progesterone, were both dose-dependently decreased by acetaminophen. No effects were revealed on cell viability. Treatment of cells with 0.5 mM of acetaminophen did not cause any effects on the expression of 10 genes in the steroidogenic pathways.

CONCLUSIONS
The pattern of steroid secretion caused by acetaminophen can be explained by inhibition of CYP17A1 enzyme activity. A decreased secretion of glucocorticoids and androgens, as demonstrated by acetaminophen, would, in an in vivo situation, induce adrenocorticotropic hormone release via negative feedback in the hypothalamic–pituitary–adrenal axis and result in an upregulation of aldosterone secretion. Our results suggest a novel possible mechanism for acetaminophen-induced hypertension, which needs to be further elucidated in clinical investigations.

Keywords: blood pressure; acetaminophen; paracetamol; hypertension; CYP17; aldosterone; steroidogenesis; H295R.

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Acetaminophen (paracetamol, n-(4-hydroxyphenyl)ethanamide, n-acetyl-p-aminophenol) is a widely used analgesic and antipyretic drug. More than 20% of the women in a US study consumed acetaminophen at least 1 d/wk and 5.5% of older women consumed acetaminophen more than 6 d/wk.1 Acetaminophen is generally regarded as safe, although the window of therapeutic concentration is close to the toxic concentration. The most frequently reported toxic effect is acute liver failure, but more recently, regular use of acetaminophen has been associated with an increased risk of hypertension in several epidemiological studies.2–6 However, the mechanism for a hypertensive action of acetaminophen is not known. The increase in blood pressure may explain the increased risk of cardiovascular events (myocardial infarction and stroke), which has been reported after high-frequency use of acetaminophen.7 Other studies have not reported an increased risk of myocardial infarction and stroke associated with the use of acetaminophen in hypertensive populations.8,9

Recent reports have indicated that acetaminophen may have an effect on steroidogenesis and the secretion of steroid hormones.10,11 An association between the intake of acetaminophen and increased risk of cryptorchidism in newborn boys has been reported and explained by reduced testosterone secretion after acetaminophen exposure.12–16 In addition to sex hormones, steroidogenesis also involves secretion of mineralocorticoids and glucocorticoids from the adrenal cortex. Effects on adrenal steroidogenesis and secretion of aldosterone and glucocorticoids have not been explored as a possible mechanism for hypertension induced by acetaminophen.

The steroid hormones are synthesized from cholesterol by steroidogenic enzymes via hormone intermediates and a number of chemicals have been shown to act via inhibition of these steroidogenic pathways in the adrenal cortex.17,18
Steroidogenesis (Figure 1) starts with cholesterol, which is transported by the steroidogenic acute regulatory protein (StAR) into the inner mitochondrial membrane, where the side-chain cleavage enzyme cytochrome P450 (CYP) 11A1 (CYP11A1) converts it to pregnenolone. Pregnenolone is converted to aldosterone by 3β-hydroxysteroid dehydrogenase type II (HSD3B2), CYP21A2, CYP11B1, and CYP11B2. CYP17A1 catalyzes 2 distinct enzymatic steps in the steroidogenesis. The 17α-hydroxylase catalyzes 17-hydroxylation of pregnenolone and progesterone, and the 17,20-lyase catalyzes the reaction of the 17-hydroxylated products to dehydroepiandrosterone (DHEA) and androstenedione (Figure 1). Cortisol is produced by HSD3B2, CYP21A2, and CYP11B1 from the 17-hydroxylated intermediates.

We and others have used the human adrenocortical cell line H295R as an in vitro model to study effects and mechanisms of drugs and other chemicals on steroidogenesis and hormone secretion. This cell line expresses all the enzymes and transport proteins required for secretion of adrenal steroids. The H295R cell line was used in the present study for investigating the effects of acetaminophen on steroidogenesis and hormone secretion as a possible mechanism for the hypertensive action.

**METHODS**

**Test chemicals**

Acetaminophen was purchased from Sigma–Aldrich (StLouis, MO) and dissolved in dimethyl sulfoxide (Sigma–Aldrich).

**Cell culture**

NCI-H295R cells were purchased from ATCC (Manassas, VA) and cultured as a monolayer in DMEM:F-12 medium with GlutaMAX (Invitrogen, Carlsbad, CA), supplemented with 1% ITS + Premix (BD Biosciences, Bedford, MA), 2% Ultroser SF (Soprachem, France), and 1% penicillin-streptomycin (Lonza, Basel, Switzerland). Cells were cultured in a humidified environment at 37 °C containing 95% air and 5% CO₂. Medium was changed 2–3 times a week, and TrypLE Express (Invitrogen) was used for subculturing of the cells.

**Cell viability assay**

H295R cells of passage 4–24 were seeded in 96-well plates, 1.7 × 10⁴ cells/well, in a volume of 100 µl Ultroser SF-supplemented medium. After 72 hours, the medium was replaced with Ultroser SF-free medium containing acetaminophen dissolved in dimethyl sulfoxide. Dimethyl sulfoxide concentration did not exceed 0.1% in medium. Staurosporin (Sigma–Aldrich) was used as a positive control for cytotoxicity. Following 24 hours of exposure, 20 µl CellTiter 96 AQone Solution Reagent (Promega, Madison, WI) was added to each well. Absorbance at 490 nm was measured after 1-hour incubation at 37 °C using a Wallac Victor3 1420 microplate reader (PerkinElmer, Waltham, MA). Relative effects on cell viability were determined from the mean absorbance value based on 3 replicates.

**Treatment of H295R cells and analysis of steroid secretion**

H295R cells of passage 4–24 were seeded in 2.5 ml Ultroser SF-supplemented medium at a concentration of 5 × 10⁵ cells/well in 6-well plates. Medium was withdrawn after 72 hours and replaced with Ultroser SF-free medium supplemented with acetaminophen at 0, 0.1, 0.5, and 1.0 mM. After 24 hours of incubation at 37 °C, the cell culture medium was
withdrawn and stored at −20 °C for subsequent hormone analysis. Relative effects on steroid secretion were determined in 3 independent experiments with n = 3 for controls and treated cells in each experiment.

Cortisol level in culture medium was measured by enzyme-linked immunosorbent assay (ELISA) (#402710, NeoGen Corporation, Lansing, MI) according to manufacturer’s instructions. Absorbance was measured at 405 nm in a Labsystems Multiskan MS (Labsystems, Finland), and cortisol concentrations were calculated based on a standard curve. The cortisol ELISA had an assay range of 0.04–10 ng cortisol/ml, and the samples were diluted in the provided kit buffer.

Levels of progesterone, 17α-OH-progesterone, DHEA, androstenedione, and aldosterone were measured by ELISA in culture medium from cells treated with acetaminophen (ELISA assays: #DE1561, #DE1292, #DE3415, #DE3265, and #DE4128, respectively; Demeditec Diagnostics GmbH, Kiel-Welselfe, Germany). Absorbance was measured with a 450-nm filter. The absorbance values of the samples were converted into percent of maximal binding, and the concentration was given by the standard curve. The assay ranges for the kits were as follows: 0.3–40 ng progesterone/ml, 0.15–20 ng 17α-OH-progesterone/ml, 0.37–30 ng DHEA/ml, 0.1–10 ng androstenedione/ml, and 0.015–1 ng aldosterone/ml. Medium samples for analysis with the aldosterone kit were treated as urine samples, according to manufacturer’s recommendations, and diluted in urine dilution buffer. If needed, samples for analysis of progesterone, 17α-OH-progesterone, and androstenedione were diluted with Standard 0 solution, as recommended by the manufacturer.

RNA isolation, cDNA synthesis, and RT-qPCR

Total RNA was isolated from the cells using Nucleospin RNA II Kit (Clontech, Mountain View, CA) following manufacturer’s instructions. Quant-iT RiboGreen RNA Reagent (Invitrogen) was used to determine the concentration of the RNA samples according to manufacturer’s high-range protocol. Verso cDNA Kit (ABgene House, UK) was used to synthesize cDNA from purified total RNA. RNA (1 µg) was heated to 70 °C for 5 minutes and mixed with 5× cDNA synthesis buffer, dNTP mix (0.5 mM final concentration), and Verso Enzyme Mix. Finally, random hexamers (400 ng/µl) were added. Tubes were incubated at 42 °C for 30 minutes, followed by 95 °C for 2 minutes. cDNA samples were diluted to a final volume of 100 µl with nuclease-free water.

Quantitative real-time reverse transcriptase PCR (RT-qPCR) reactions were set up using ABgenes Absolute QPCR Mix (ABgene House) according to manufacturer’s instructions. In short, Absolute QPCR Mix, gene-specific forward (0.4 µM) and reverse (0.4 µM) primers, dual-labeled probes (0.1 µM) labeled with 5’-FAM and 3’-TAMRA, and nuclease-free water were mixed in a final volume of 20 µl. Sequences for primers and probes used in the reactions have previously been described (Oskarsson et al.21). cDNA was added to the mixture at a volume of 5 µl cDNA, corresponding to 50 ng cDNA/reaction. RT-qPCR was performed using a Rotor-Gene 3000 (Corbett Life Science, Sydney, New South Wales, Australia). Amplification of cDNA was done by 15-minute enzyme activation at 95 °C, followed by 40 cycles of 15-second denaturation at 95 °C and 1 minute of annealing/extension at 60 °C. The RT-qPCR program generated a standard curve based on 5 concentrations of the genes, ranging from 10^2 to 10^5 copies. This standard curve was used to calculate the copy number for each gene in the samples. Results are presented as mean relative gene expression levels compared to the vehicle control.

Statistical analysis

Nonparametric methods were used for statistical analysis. The results were analyzed by Kruskal–Wallis to detect differences between the experimental groups, followed by Mann–Whitney U for verifying differences between pair of groups. Statistical testing was performed with StatView 5.0.1 (SAS Institute, Cary, NC). Statistical significance was set as P < 0.05.

RESULTS

Effect on cell viability and steroid secretion

As shown in Figure 2, the concentrations used in the studies of acetaminophen did not influence cell viability, thus excluding nonspecific cytotoxic effects in the studies on steroidogenesis.

H295R cells were treated with acetaminophen for 24 hours, and the effects on hormone secretion were analyzed by ELISA. A dose-dependent increase of progesterone and androstenedione secretion was demonstrated, which was 2- and 2.5-fold, respectively, at 1 mM, while the secretion of 17α-OH-progesterone and cortisol were reduced relative to vehicle control (Figure 3). Levels of aldosterone and cortisol in the vehicle control samples were 0.40–0.53 and 21–23 ng/ml, respectively. Levels of progesterone and 17α-OH-progesterone in medium from vehicle control cells were 1.7–1.9 and 10.1–10.6 ng/µl, respectively. Levels of progesterone and 17α-OH-progesterone in medium from acetaminophen treated cells in each experiment.

Acetaminophen caused a reduced secretion of DHEA and androstenedione (Figure 3). The suppression was statistically significant compared to the vehicle control.

![Figure 2](https://example.com/figure2.png)
Acetaminophen Increases Aldosterone Secretion

Acetaminophen is one of the most widely used medical drugs in the world, and potential side effects are of significant public health concern. Liver toxicity of acetaminophen from acute overdose or continuous use at high doses is well known, and actions are taken to reduce the risk, such as a limit of acetaminophen per dosage unit and a warning label on the product. However, more recent epidemiological data point to an increased risk of hypertension in patients taking acetaminophen even in low doses.1–6

The underlying mechanism for a hypertensive action of acetaminophen is not known. One suggested mechanism is inhibition of cyclooxygenase-2, leading to a decrease in endogenous prostaglandins. However, in contrast to the traditional nonsteroidal anti-inflammatory drugs, acetaminophen is not considered as primarily a cyclooxygenase-2 inhibitor but suggested to act by activation of cannabinoid (CB1) receptors.24 Excess aldosterone due to effects on adrenal steroidogenesis has not been explored as a possible mechanism for hypertension induced by acetaminophen.

In the present investigation we found a dose-dependent increase in the secretion of aldosterone and progesterone, while glucocorticoids and androgens were suppressed by acetaminophen. Decreased secretion of glucocorticoids and androgens in connection with increased secretion of progesterone and mineralocorticoids is indicative of inhibition 17/20-lyase activity being more efficiently inhibited than 17α-hydroxylase activity.

DISCUSSION

Acetaminophen is one of the most widely used medical drugs in the world, and potential side effects are of significant public health concern. Liver toxicity of acetaminophen from acute overdose or continuous use at high doses is well known, and actions are taken to reduce the risk, such as a limit of acetaminophen per dosage unit and a warning label on the product. However, more recent epidemiological data point to an increased risk of hypertension in patients taking acetaminophen even in low doses.1–6

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**Effect of acetaminophen on the expression of steroidogenic genes**

To examine whether the specific effects on steroid secretion could be explained by changes in the expression of steroidogenic genes, gene expression levels were analyzed by RT-qPCR following treatment with 0.5 mM acetaminophen. No statistically significant effects of acetaminophen treatment were observed on expression of 10 steroidogenic genes (Figure 4).

**Effect of acetaminophen on steroidogenic enzyme activity**

The effect of acetaminophen on 17α-hydroxylase enzymatic activity was assessed as the ratio between the product 17α-OH-progesterone and the substrate progesterone. Similarly, the 17/20-lyase activity of CYP17A1 was assessed as the ratio between androstenedione and 17α-OH-progesterone and the total CYP17A1 activity as the ratio between androstenedione and progesterone. As illustrated in Figure 5, acetaminophen treatment of H295R cells dose-dependently inhibited CYP17A1 activity with 17/20-lyase activity being more efficiently inhibited than 17α-hydroxylase activity.
The product-to-precursor ratio 17α-OH-progesterone/progesterone, which reflects the CYP17α-hydroxylase activity, was dose-dependently decreased by acetaminophen. This may explain the accumulation of progesterone and aldosterone and reduced secretion of 17α-OH-progesterone. In addition, the ratio of androstenedione/17α-OH-progesterone was decreased by acetaminophen in a dose-dependent manner, indicating inhibition of the CYP17/20-lyase activity, which explains the strong downregulation of androstenedione and more modest downregulation of 17α-OH-progesterone and cortisol. Gene expression analysis did not reveal any effects of acetaminophen on CYP17A1 or on any other of the steroidogenic genes. Thus, the effects of acetaminophen on CYP17A1 activity cannot be explained by effects on gene expression, implying posttranscriptional effects, probably direct effects on enzyme activity.

Aldosterone is synthesized and secreted by zona glomerulosa in the adrenal cortex under the regulation of the renin–angiotensin system. A number of physiological pathways and mechanisms, including renin and potassium levels, may affect aldosterone secretion in vivo. In this study, however,
we show that acetaminophen has a direct effect on adrenal steroidogenesis and aldosterone secretion. Aldosterone regulates extracellular fluid, salt balance, and arterial blood pressure, and excessive aldosterone secretion results in hypertension. The main stimuli for aldosterone secretion are angiotensin II and extracellular potassium levels. In addition, aldosterone synthesis is induced by adrenocorticotropic hormone (ACTH), while lacking negative feedback to ACTH secretion. The decreased cortisol and androgen secretion by acetaminophen, as demonstrated in the present investigation, would in an in vivo situation induce ACTH release via negative feedback followed by upregulation of aldosterone synthesis. Our results on CYP17A1 inhibition and upregulation of aldosterone secretion suggest a novel mechanism for acetaminophen-induced hypertension. This mechanism is supported by data from a study in rats, treated with acetaminophen, showing increased aldosterone secretion and elevated arterial blood pressure. Furthermore, plasma aldosterone concentrations were increased in patients with fulminant hepatic failure due to acetaminophen overdose.

Increased aldosterone secretion and hypertension as a result of CYP17A1 inhibition has been demonstrated as a side effect in the treatment of prostate cancer patients with abiraterone, a drug aimed at suppressing testosterone synthesis via CYP17A1 inhibition. Similar to what we have demonstrated for acetaminophen in the present study, abiraterone also inhibits cortisol and androgen secretion via CYP17A1 inhibition in H295R cells. The suppressed cortisol secretion induces release of ACTH and increased aldosterone secretion. The side effects of abiraterone due to hyperaldosteronism can be reduced by treatment with corticosteroids, such as hydrocortisone or prednisone, preventing the release of ACTH.

Furthermore, the impact of CYP17A1 activity on mineralocorticoid synthesis and association with hypertension is known from certain diseases. CYP17A1 deficiency, caused by mutations in the CYP17A1 gene, is a rare form of congenital adrenal hyperplasia, associated with hypertension and hypokalemia as a result of accumulation of mineralocorticoids. The CYP17A1 deficiency leads to reduced production of cortisol and sex steroids and thus an increase in ACTH and synthesis of mineralocorticoids. Low apparent CYP17A1 activity was associated with elevated ambulatory blood pressure when salt intake was high in a family-based population study. Primary aldosteronism is a cause of secondary hypertension, in most cases due to either a bilateral (nodular) adrenal hyperplasia or an aldosterone-producing adenoma. Cui et al. reported downregulated gene and protein expressions of CYP17 in adrenals of patients with aldosterone-producing adenoma and nodular hyperplasia compared to normal adrenals.

The effects of acetaminophen in vivo are dependent on the concentrations at the target sites. To our knowledge, no data on concentrations of acetaminophen in the adrenal gland are available. Plasma acetaminophen levels of 10–20 µg/ml, corresponding to 0.066–0.13 mM, are considered within accepted therapeutic range, and levels of 120 µg/ml, corresponding to 0.8 mM, are considered as toxic. The concentrations of acetaminophen used in the present investigation was nontoxic to the cells and compared to clinical plasma levels ranged from therapeutic to toxic. Although quantitative extrapolation from in vitro to in vivo dose-response relationships is doubtful, the concentrations used in the present study causing significant effects on steroidogenesis may be of relevance for human exposure to acetaminophen.

There is evidence from epidemiological studies that the use of the nonsteroidal anti-inflammatory drugs aspirin and ibuprofen, similar to acetaminophen, is associated with increased blood pressure. The proposed mechanism is cyclooxygenase-2 inhibition and reduced synthesis of prostanoids. Considering the prevalent use of analgesics, a hypertensive effect of the drugs is of high importance for public health. Due to the negative cardiovascular effects of nonsteroidal anti-inflammatory drugs, acetaminophen has been suggested as a safer alternative in patients with osteoarthritis and cardiovascular disorders. Our data suggest a novel mechanism for acetaminophen-induced hypertension, which needs to be further elucidated in clinical investigations.

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DISCLOSURE

The authors declared no conflict of interest.

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