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# Select steroid hormone glucuronide metabolites can cause toll-like receptor 4 activation and enhanced pain

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ABSTRACT

We have recently shown that several classes of glucuronide metabolites, including the morphine metabolite morphine-3-glucuronide and the ethanol metabolite ethyl glucuronide, cause toll like receptor 4 (TLR4)-dependent signaling *in vitro* and enhanced pain *in vivo*. Steroid hormones, including estrogens and corticosterone, are also metabolized through glucuronidation. Here we demonstrate that *in silico* docking predicts that corticosterone, corticosterone-21-glucuronide, estradiol, estradiol-3-glucuronide and estradiol-17-glucuronide all dock with the MD-2 component of the TLR4 receptor complex. In addition to each docking with MD-2, the docking of each was altered by pre-docking with (+)-naloxone, a TLR4 signaling inhibitor. As agonist versus antagonist activity cannot be determined from these *in silico* interactions, an *in vitro* study was undertaken to clarify which of these compounds can act in an agonist fashion. Studies using a cell line transfected with TLR4, necessary co-signaling molecules, and a reporter gene revealed that only estradiol-3-glucuronide and estradiol-17-glucuronide increased reporter gene product, indicative of TLR4 agonism. Finally, in *in vivo* studies, each of the 5 drugs was injected intrathecally at equimolar doses. In keeping with the *in vitro* results, only estradiol-3-glucuronide and estradiol-17-glucuronide caused enhanced pain. For both compounds, pain enhancement was blocked by the TLR4 antagonist lipopolysaccharide from *Rhodobacter sphaeroides*, evidence for the involvement in TLR4 in the resultant pain enhancement. These findings have implications for several chronic pain conditions, including migraine and temporomandibular joint disorder, in which pain episodes are more likely in cycling females when estradiol is decreasing and estradiol metabolites are at their highest.



## 1. Introduction

Recent evidence suggests that certain glucuronide metabolites have neuroinflammatory actions. Specifically, morphine-3-glucuronide, ethyl-β-D-glucuronide and glucuronic acid have been shown to activate the innate immune toll-like receptor 4 (TLR4) and cause TLR4-dependent enhanced pain when administered intrathecally (Lewis et al., 2010, 2013) or systemically (Due et al., 2012). Another major class of molecules with glucuronidated metabolites is the steroid hormones. Androgens (Belanger et al., 1994), estrogens (Guillemette et al., 2004), and corticosteroids (Ikegawa et al., 2009) are all glucuronidated as part of metabolism. However, the potential for TLR4 activation and/or enhanced nociception by these glucuronidated metabolites has not been studied.

Several of the steroid hormones also have associations with enhanced pain, including corticosterone and estradiol. Acute stress and acute stress-associated corticosterone release in rats has, by and large, been shown to inhibit nociception, while chronic stress has been linked to hyperalgesia (Imbe et al., 2006), although analgesic effects have also been repeatedly reported (reviewed in Miguez et al., 2014). Clinically, chronic stress has also been associated with chronic pain conditions such as fibromyalgia, chronic headache, inflammatory bowel disease and temporomandibular joint pain (McEwen and Kalia, 2010). Corticosterone, intriguingly, has both anti-inflammatory and pro-inflammatory effects on inflammation caused by the classic TLR4 ligand lipopolysaccharide (LPS). Corticosterone inhibits proinflammation by LPS when LPS

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precedes corticosterone, whereas corticosterone potentiates proinflammation by LPS when LPS is given 2 or 24 h following corticosterone (Frank et al., 2010). One potential explanation for this finding is that the anti-inflammatory effect of corticosterone was modulated by a proinflammatory effect of its glucuronidated metabolite. Corticosterone is metabolized by extensive degradation or conjugation with a glucuronide group to form corticosterone-21-glucuronide (CortG). CortG has been found in mouse brain homogenates, indicating that it has the potential to directly affect the central nervous system (Kallonen et al., 2009; Maeda et al., 2013). In humans, the major stress hormone cortisol and several of its metabolites are also glucuronidated, with over 90% of cortisol metabolites in the urine being glucuronide conjugated (Kornel and Saito, 1975).

Large epidemiology studies, in different countries and cultures, show women are more likely to report chronic pain (Blyth et al., 2001; Johannes et al., 2010) and chronic pain that limits daily activities (Blyth et al., 2001). Estrogens, including estradiol, are the primary female sex hormones and have reports of both pro-inflammatory and pro-nociceptive activity as well as antinociceptive effects (for review, Craft, 2007). Notably, the drop from peak estradiol levels during pre-menses in women is associated with increased incidence of migraine (Martin and Behbehani, 2006; Somerville, 1972) and temporomandibular joint pain (LeResche et al., 2003). This period of declining estradiol coincides with peak to consistent levels of glucuronidated estradiol metabolites (Stanczyk et al., 1980), as glucuronidated metabolites typically have longer half-lives than parent molecules. The declining estradiol and steady glucuronidated metabolites in this time period increases the relative effect of the glucuronidated metabolites. Estradiol may also affect the LPS response. Chronic estradiol administration in ovariectomized females and male rats increased the LPS-stimulated inflammatory response in hippocampal microglia *ex vivo* and increased proinflammatory cytokine transcription *in vivo* (Loram et al., 2012). Estradiol is metabolized into estradiol-3-glucuronide ($E_2$-3-G) and estradiol-17-glucuronide ($E_2$-17-G), and both of these metabolites have also been found in brain tissue homogenates, indicating that they have access to the central nervous system (Kallonen et al., 2009). Neither metabolite is believed to have activity at the estrogen receptors (Guillemette et al., 2004).

The first step to determine if glucuronidated corticosterone and estradiol metabolites could contribute to the pain-enhancing effects of the parent hormones is to determine if the metabolites have the ability to potentiate pain. Given the TLR4-dependent allodynia caused by other glucuronidated metabolites, we hypothesize that CortG, $E_2$-3-G and $E_2$-17-G will cause an increase in TLR4 signaling as well as TLR4-dependent enhanced pain.

## 2. Materials and methods

### 2.1. Drugs

Corticosterone, estradiol, $E_2$-3-G, and $E_2$-17-G were purchased from Sigma (St. Louis, MO). CortG was synthesized by the authors (MMF, TS) from D-(+)-glucurono-6,3-lactone. The lactone was converted to the protected trichloroacetimidate by the procedure of Nakajima et al. (2005), coupled with corticosterone, then deprotected according to the procedure of Ciuffreda et al. (2009). The identity of the product was confirmed by comparison of $^1$H and $^{13}$C NMR data with that reported by Ciuffreda et al. (2009). The competitive TLR4 antagonist lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS) was purchased from Invivogen (Thousand Oaks, CA) and (+)-naloxone was obtained from the National Institute on Drug Abuse, synthesized by an author (KR). CortG, $E_2$-3-G, LPS-RS and (+)-naloxone were dissolved in endotoxin-free sterile water (Hospira, Lake Forest, IL) for *in vitro* studies (Experiment 2) and endotoxin-free, sterile 0.9% saline (Hospira, Lake Forest, IL) for *in vivo* studies (Experiment 3). $E_2$-17-G was dissolved in 10% DMSO (Sigma, St. Louis, MO) and sterile water for *in vitro* studies (Experiment 2) and 1% DMSO and sterile saline for *in vivo* studies (Experiment 3). Corticosterone and estradiol were dissolved in 100% DMSO for both *in vitro* and *in vivo* studies. Corticosterone, CortG, estradiol, $E_2$-3-G, and $E_2$-17-G, (+)-naloxone, saline and water were all confirmed to be endotoxin-free by the limulus amebocyte lysate (LAL) assay (Lonza, Walkersville, MD). Where appropriate, doses are reported as a free base concentration.

### 2.2. In silico docking simulations

*In silico* docking simulation methods were similar to those previously described in detail (Hutchinson et al., 2012, 2010b). These were employed to examine the docking of corticosterone, CortG, estradiol, $E_2$-3-G, and $E_2$-17-G to the TLR4/MD-2 complex. The *in silico* docking analyses were conducted in Experiment 1 using the recently published high-resolution crystalline structure of the dimer of human TLR4 and MD-2 (Park et al., 2009) and the *in silico* docking software vina, PyRx and the AutoDock Tools package. Briefly, the complexed human TLR4 and MD-2 pdb file was obtained from RCSB Protein Data Bank database (PDBID: 3fxi). Docking was conducted using Vina (version 1.1.2; (Trott and Olson, 2010) within PyRx (version 0.8; (Wolf, 2009)). An exhaustiveness factor of 8 was used for all simulations, with the Vina search space dimensions and center defined using the auto-maximize function. Structures were gathered using PubChem isomeric SMILES then converted to .pdb using a structure file generator (http://cactus.nci.nih.gov/services/translate/).

### 2.3. In vitro assay for TLR4 signaling

A human embryonic kidney-293 (HEK 293) cell line was used in Experiment 2. This cell line was stably transfected by Invivogen (San Deigo, CA) to over-express human TLR4 and co-receptor molecules (MD-2, CD14) (293-htlr4a-md2cd14; referred to here as HEK-TLR4). In addition, these cells stably express an optimized alkaline phosphatase reporter gene under the control of a promoter inducible by transcription factors, such as NFκB and AP-1, activated as part of the TLR4 signaling cascade. Secreted alkaline phosphatase (SEAP) protein is produced as a consequence of TLR4 activation.

HEK-TLR4 cells were grown at 37 °C (5% $CO_2$; VWR incubator model 2300) in 10 cm dishes (Greiner Bio-One, CellStar 632171; Monroe, NC, USA) in normal supplement selection media (DMEM media [Invitrogen, Carlsbad, CA, USA] supplemented with 10% fetal bovine serum [Hyclone; Logan, UT, USA], HEK-TLR4 selection [Invivogen]; Penicillin 10,000 U/ml [Invitrogen]; streptomycin 10 mg/ml [Invitrogen], Normocin [Invivogen], and 200 nM L-glutamine [Invitrogen]). The cells were then plated for 48 h in 96 well plates (Microtest 96 well plate, flat bottom, Becton Dickinson; $5 \times 10^3$ cells/well) with the same media. After 48 h, supernatants were removed and replaced with fresh media. Drugs tested were added in concentrations indicated and incubated for 24 h. Supernatants (15 µl) were then collected from each well for immediate assay.

SEAP levels in the supernatants were assayed using the Phospha-Light System (Applied Biosystems) according to the manufacturer's instructions. This chemiluminescent assay incorporates Tropix CSPD chemiluminescent substrate. The 15 µl test samples were diluted in 45 µl of 1 × dilution buffer, transferred to 96-well plates (Thermo, Waltham, MA, USA), and heated at 65 °C in a water bath (Model 210, Fisher Scientific, Pittsburgh, PA, USA) for 30 min,