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Effects of 17β-Estradiol and Testosterone on *Nocardia brasiliensis* Gene Expression

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Abstract

Infection caused by *Nocardia brasiliensis*, known as actinomycetoma, occurs more frequently in men than in women. Several factors are involved in sex bias in infections between men and women. However, the molecular mechanisms underlying these differences are not completely understood. Sex steroid hormones play an important role in the development of infections because of their function in the host immune response and their direct effect on bacterial activity. Estrogen is generally understood to have a protective effect in women, whereas testosterone is associated with immunosuppression and predisposes men to many infections. This work aimed to study the *in vitro* effect of 17β-estradiol and testosterone on *N. brasiliensis* gene expression using a differential display assay, through RNA arbitrarily primed PCR. The results showed that both hormones affect the expression of genes encoding proteins involved in metabolism, transcriptional regulation, and other hypothetical proteins with unknown functions. Further studies are needed to better understand the exact role of these hormones in the *N. brasiliensis* virulence and pathogenesis of the infection, which may help in understanding the sex bias of this infection.

Keywords

Nocardia brasiliensis, Actinomycetoma, Sex Hormones, 17β-Estradiol, Testosterone, RAP-PCR

1. Introduction

Men are more susceptible to bacterial infections than women [1]. Actinomycetoma is a chronic infection of the skin and underlying tissue, mainly acquired by young

men who are fieldworkers, in a 3:1 ratio compared to women. This infection is caused by bacteria, including *Actinomadura pelletieri*, *A. madurae*, *Streptomyces somaliensis*, *Nocardia brasiliensis*, and *N. asteroides* complex [2]. This disease is endemic in tropical, subtropical, and temperate regions. While most cases occur in Africa, countries such as Mexico and Venezuela report the highest number of cases in the Americas, with Mexico having the highest reported number (3796 cases, 96.52% of actinomycetoma) [3]. Eighty-six percent of these cases are caused by *Nocardia*, with *N. brasiliensis* being the main causative agent (65.58%) of actinomycetoma in Mexico [3] [4].

Several factors are involved in the progression and outcome of infections in men and women, including genetics, behavior, exposure, immune response, and sex steroid hormones [5]. However, the predominance of actinomycetoma in men is substantial. One of the most common explanations is men's lifestyle and occupation related to work in the field, that most often involves traumatic inoculation of bacteria that live in the soil. Interestingly, women working in the field are not infected at the same rate as men, suggesting the presence of protective factors. *In vitro* studies have demonstrated that estrogen limits the development of actinomycetoma in mice. By contrast, hormones such as progesterone and testosterone induce the highest invasion rates in mice [6]. In our laboratory, we recently demonstrated that progesterone and dihydrotestosterone influence gene expression in *N. brasiliensis*. The identified genes were involved in metabolism, whereas others encoded hypothetical proteins with unknown functions [7]. However, to the best of our knowledge, no information is available on the effects of estrogen and testosterone on gene expression in this bacterium. Thus, the aim of this study was to analyze the *in vitro* effect of 17β-estradiol and testosterone on gene expression in *N. brasiliensis* using a differential display RNA arbitrarily primed (RAP)-PCR-based method described by Fislage *et al.* [7] [8], as an approach to help explain the susceptibility associated with sex through *N. brasiliensis* infection.

2. Materials and Methods

2.1. Strains and Culture Media

In this study, a strain of *Nocardia brasiliensis* FM825, identified by PCR-Sequencing, was obtained from a 54-year-old man from Puebla, Mexico, diagnosed with actinomycetoma [9]. Bacterial cultures were first grown on brain-heart infusion (BHI) agar for 7 days to obtain an isolated colony and continue the study. A growth curve was then generated. To study the effect of 17β-estradiol and testosterone on bacterial gene expression, *N. brasiliensis* was grown in BHI broth for 5 days, at which time the bacterium reached the exponential phase of growth. The inoculum was prepared as follows: bacteria were filtered, dried on sterile filter paper, and washed several times with sterile distilled water to eliminate residues in the culture medium. A bacterial suspension in sterile saline solution equivalent to 1.5×10^9 CFU/mL was prepared. Fifty microliters of this suspension were added to Erlenmeyer flasks containing BHI broth and were incubated at 28˚C for 5 days

under agitation. After this incubation period, 17β-estradiol or testosterone (Sigma®) was added at concentrations equivalent to the normal serum concentration in adult women's luteal phase (300 pg/mL) or the normal serum concentration in adult men (3 ng/mL), respectively, as well as concentrations ten times higher than these normal levels. As a control, bacteria were grown without hormones, but with the vehicle (ethanol to reconstitute the hormone; final concentration in the medium 0.54%). After the addition of hormones or vehicles to each flask, the cells were incubated for 2, 8, or 24 hours.

2.2. RNA Extraction

After incubation, RNA was extracted from *N. brasiliensis* grown in BHI medium containing 17β-estradiol, testosterone, or vehicle for 2, 8, or 24 h. The bacteria were filtered, washed, and dried on sterile filter paper. The bacterial mass was then transferred to a sterile mortar and macerated in liquid nitrogen. RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, MA, USA) following the manufacturer's instructions. The RNA obtained was treated with DNase I (Invitrogen, Thermo Fisher Scientific, MA, USA) to eliminate any possible genomic DNA residues. RNA quality was verified using a denatured 1% agarose gel stained with ethidium bromide. Finally, RNA was quantified using spectrophotometry.

2.3. RNA Arbitrarily Primed PCR (RAP-PCR)

RAP-PCR was performed using random arbitrary primers previously described and used in *N. brasiliensis* in our laboratory [7] [8]. First, a reverse transcription reaction was performed with 0.5 µg of RNA, SuperScript II™ reverse transcriptase (Invitrogen, Thermo Fisher Scientific, MA, USA), and random primers Ea3, Ea4, or Ea8, following the manufacturer's instructions. Second-strand cDNA synthesis was initiated by arbitrary priming using several combinations of primers (Ea3 + Es5, Ea4 + Es5, Ea4 + Es8, Ea8 + Es5, and Ea8 + Es7) under previously described conditions [7]. PCR products were separated on a 3.5% agarose gel stained with ethidium bromide and visualized using a UVP transilluminator.

2.4. Cloning of PCR Products

PCR products showing differential expression compared with that of the non-hormone-treated control were cut and purified using the Purelink Gel Extraction kit (Invitrogen, Thermo Fisher Scientific, MA, USA), following the manufacturer's instructions. The purified fragments were cloned using the PCR 2.1 TOPO vector from the TOPO™ TA Cloning™ Kit (Invitrogen, Thermo Fisher Scientific, MA, USA). Ligation, competent cell preparation, transformation, and positive clone selection were performed according to the manufacturer's instructions. For the transformation of competent cells, bacteria were grown on lysogeny agar containing ampicillin (100 µg/mL), X-Gal (40 mg/mL), and IPTG (100 mM). The plasmids were purified using the PureLink™ Quick Plasmid Miniprep kit (Invitrogen, Thermo Fisher Scientific, MA, USA) and stored at −20˚C.

2.5. Sequencing and Analysis

Purified plasmid DNAs were sequenced at the Molecular Biology Laboratory, Institute of Biology at UNAM. The sequences were analyzed using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to predict the encoded proteins.

3. Results

RAP-PCR using arbitrary primers was conducted to obtain bacterial fingerprints following the incubation of *N. brasiliensis* with two different concentrations of 17β-estradiol or testosterone (at physiological and 10-fold physiological concentrations). A total of 10 fragments exhibited distinct gene expression patterns solely after 2- and 8-h post-incubation with 17β-estradiol compared with those of the control (**Figure 1(a)**). No significant differences in gene expression were observed after 24 h (data not shown). Densitometric analysis revealed upregulation of six fragments and downregulation of four (**Table 1**). By contrast, 15 fragments displayed varying expression patterns following testosterone treatment at 2-, 8-, and 24-h intervals (**Table 1** and **Figure 1(b)** and **Figure 1(c)**), with the highest number of differential bands observed at 2 h. Subsequently, each PCR product was purified, and only 11 of the fragments were successfully cloned. The analysis of sequences from those cloned PCR products showed that 17β-estradiol affected the expression of two genes encoding proteins involved in transcriptional regulation: anti-sigma D factor RsdA and a putative Multiple Antibiotic Resistance Regulator (MarR) family transcriptional regulator; one gene encoding a pyruvate dehydrogenase subunit E1 protein; and two hypothetical proteins with unknown functions (**Table 2**). It is important to note that some of the cloned fragments corresponded to the same gene; therefore, the number of proteins identified and listed in **Table 2** is lower than the number of cloned fragments.

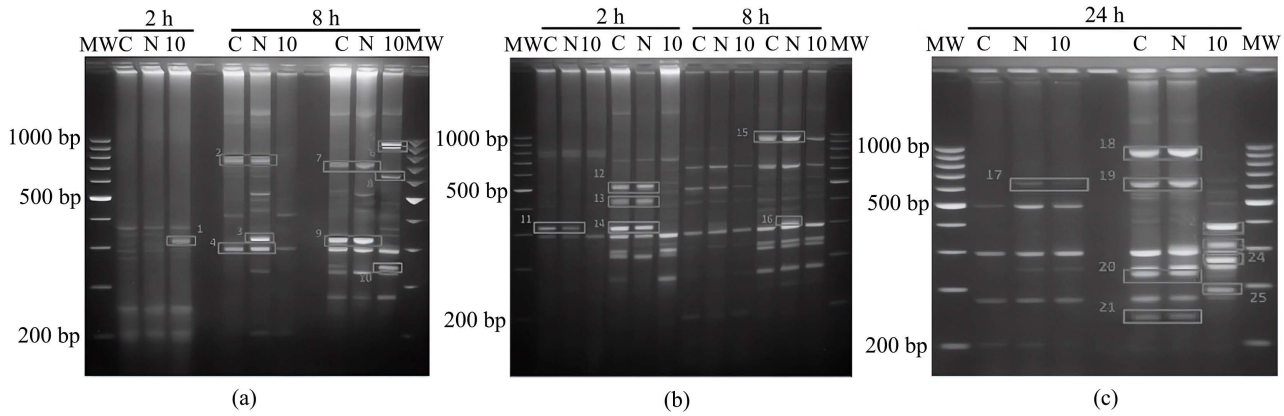

Figure 1. Differential display RNA arbitrarily primed PCR of *N. brasiliensis* with 17β-estradiol or testosterone. Total RNA of *N. brasiliensis* FM-825 without hormones (C for control), with (a) 17β-estradiol at the normal serum concentration in women during the luteal phase (N) or ten times that concentration (10), or with (b and c) testosterone at the normal serum concentration in men (N) or ten times that concentration (10), was reverse transcribed with an arbitrary primer and amplified using PCR. The cDNA products were resolved in a 3.5% agarose gel stained with ethidium bromide (10 mg/mL). Gray marks indicate differentially amplified and isolated products. 2, 8, and 24 h indicate the incubation time after the addition of hormones or the vehicle.

Table 1. Characteristics of fragments showing differential gene expression induced by 17β-estradiol and testosterone.

Hormone	Number of bands*	Molecular weight (bp)	Time of incubation (hours)**	Primers used	Hormone concentration***	Effect
17β-estradiol	1	325	2	Ea8 + Es5	10×	Increased
	2	790	8	Ea3 + Es5	10×	Decreased
	3	340	8	Ea3 + Es5	Normal	Increased
	4	300	8	Ea3 + Es5	10×	Decreased
	5	910	8	Ea4 + Es5	10×	Increased
	6	905	8	Ea4 + Es5	10×	Increased
	7	700	8	Ea4 + Es5	10×	Decreased
	8	620	8	Ea4 + Es5	10×	Increased
	9	320	8	Ea4 + Es5	10×	Decreased
	10	250	8	Ea4 + Es5	10×	Increased
Testosterone	11	315	2	Ea3 + Es5	10×	Decreased
	12	500	2	Ea4 + Es5	10×	Decreased
	13	410	2	Ea4 + Es5	10×	Decreased
	14	315	2	Ea4 + Es5	10×	Decreased
	15	915	8	Ea4 + Es5	10×	Decreased
	16	305	8	Ea4 + Es5	Normal	Increased
	17	615	24	Ea3 + Es5	Normal and 10×	Increased
	18	900	24	Ea4 + Es5	10×	Decreased
	19	600	24	Ea4 + Es5	10×	Decreased
	20	250	24	Ea4 + Es5	10×	Decreased
	21	150	24	Ea4 + Es5	10×	Decreased
	22	390	2	Ea4 + Es5	10×	Increased
	23	310	2	Ea4 + Es5	10×	Increased
	24	270	2	Ea4 + Es5	10×	Increased
	25	190	2	Ea4 + Es5	10×	Increased

4. Discussion

The direct effects of hormones on bacterial growth, virulence, and antibiotic susceptibility have previously been demonstrated. Some years ago, the concept of microbial endocrinology was introduced to define the interaction between the host and microbes via interkingdom signaling [10]. In this study, through a differential display technique using arbitrary primers, it was demonstrated that 17β-estradiol and testosterone also have effects on the gene expression of *N. brasiliensis*.

17β-estradiol positively affects the expression of two genes encoding proteins

involved in transcriptional regulation, including anti-sigma D factor RsdA and a putative MarR family of transcriptional regulators; one gene encoding a protein involved in metabolism, pyruvate dehydrogenase subunit E1 protein; and one gene encoding a hypothetical protein. By contrast, it negatively affected the expression of genes encoding hypothetical proteins with unknown functions (Table 2). Anti-sigma D factor RsdA has been characterized in bacteria related to *N. brasiliensis*, such as *Mycobacterium tuberculosis* and *Corynebacterium glutamicum* [11] [12]. Anti-sigma factors are small proteins that bind to sigma factors to prevent the binding of bacterial RNA polymerase. They undergo conformational change or proteolysis in response to environmental signals [13]. Conversely, sigma factors are critical for transcription initiation in bacteria, playing roles such as direct recognition of promoter elements to form the "closed" complex, stabilization of the "open" complex, and interaction with transcription activators [13]. RsdA binds to the sigma factor SigD. In *M. tuberculosis*, SigD regulates the expression of ribosomal RNA operons and maintains homeostasis during the late stationary growth phase [14]. In *C. glutamicum*, SigD regulates the synthesis and transfer of mycolic acids [11]. In both *M. tuberculosis* and *C. glutamicum*, SigD activity is controlled by RsdA [11] [12]. Although the function of SigD and RsdA in *Nocardia* has not been reported, it is possible to hypothesize that these proteins play roles similar to those described for related bacteria. Additionally, 17-β-estradiol could function as a stimulus for the anti-sigma RsdA in *N. brasiliensis* to regulate several responses induced by this hormone, which could play a protective role in the pathogenesis of *Nocardia* infection. However, this hypothesis warrants further investigation.

Table 2. Differential gene expression induced by 17β-estradiol and testosterone.

Identified protein	NCBI Accession Id	Hormone concentration	Incubation time (hours)	Effect
17β-estradiol				
Anti-sigma-D factor RsdA	AFT98974.1	Normal	8	Increased
Hypothetical protein O3I_028560	AFU03661.1	10×	8	Decreased
Hypothetical protein O3I_020245	AFU02008.1	10×	8	Increased
Putative MarR family transcriptional regulator	AFU04018.1	10×	8	Increased
Pyruvate dehydrogenase subunit E1	AFU00138.1	10×	8	Increased
Testosterone				
Hypothetical protein O3I_028560	AFU03661	Normal	8	Increased

Continued

Hypothetical protein O3I_005060.	AFT98974	Normal	8	Increased
rRNA adenine N-6-methyltransferase family protein	WP_042255886	Normal and 10×	24	Increased
Carbonic anhydrase	AFU03742.1	10×	24	Decreased
Hypothetical protein O3I_030545	AFU04058.1	10×	24	Decreased
Carboxylesterase	AFU00944.1/K0EV09	10×	24	Increased

The MarR family of transcriptional regulators controls the expression of genes involved in stress responses, virulence, degradation, and the export of organic compounds, antibiotics, and detergents [15]. The binding of DNA to MarR proteins is modified in the presence of specific ligands. MarR was first described in *Escherichia coli*, where it regulates an operon encoding an efflux pump [15]. Homologs have been described in many bacteria. For example, in *Mycobacterium abscessus*, an emerging non-tuberculosis mycobacterial pathogen, a MarR homolog (MAB_2648c) represses the expression of mmpSL5, which induces ethionamide resistance [16]. In *Streptomyces* spp., the MarR family of transcriptional regulators is involved in the positive control of the production of metabolic compounds widely used in human and veterinary medicine and pesticides, such as avermectin, antinorhodin, and daptomycin [17]-[19]. However, there is no information on the functions of this family of transcriptional regulators in *Nocardia*. It is possible to suggest that MarR senses the presence of 17β-estradiol within the culture media, playing a role in changing the expression of genes in response to the sex hormone, but this needs to be elucidated.

Expression of the gene encoding pyruvate dehydrogenase subunit E1 protein was also affected by 17β-estradiol at high concentrations (**Table 2**). This enzyme catalyzes the oxidative decarboxylation of pyruvate, an important intermediary in amino acid and fatty acid synthesis [20]. This result suggests that 17β-estradiol at higher concentrations could affect the energetic metabolism of *N. brasiliensis*; however, the exact role in pathogenesis in the presence of estrogens needs to be investigated.

Testosterone positively affected the expression of two genes encoding hypothetical proteins, whereas the others encoded an rRNA adenine N-6-methyltransferase family protein and carboxylesterase. By contrast, it negatively affected the expression of genes encoding hypothetical proteins and carbonic anhydrases (**Table 2**). rRNA adenine N-6-methyltransferase family proteins are involved in antibiotic resistance [21]. Carboxylesterases are major lipolytic enzymes that catalyze the cleavage of ester linkages and have been described in humans, animals, fungi, algae, and bacteria [22]. Particularly, carboxylesterases from *Pseudomonas* spp. have been widely studied and overproduced for industrial usage [23]. In *M. tu-*

berculosis, it has been suggested that carboxylesterases, such as Rv1288, modulate cell wall lipids (such as mycolic acids, the major and specific lipid components of the mycobacterial cell envelope) to favor the survival of bacteria under stress conditions [24], whereas Rv2223c may participate in the intracellular survival of bacteria such as *M. smegmatis* [25]. Carbonic anhydrase is involved in metabolic synthesis pathways and pH regulation and is important for bacterial survival in various niches [26]. Carbonic anhydrase has also been explored as a promising drug target because its activity influences microbial proliferation and pathogen persistence in the host. In *P. aeruginosa*, the carbonic anhydrase psCA1 contributes to adaptation to low CO_2 conditions and is involved in virulence by enabling calcium deposition [27]. In *Mycobacterium*, carbonic anhydrase is essential for growth and survival during starvation, and in nontuberculous mycobacteria, it is important for biofilm formation and the transport of extracellular DNA [28]. Neither the function of carboxylesterases nor carbonic anhydrases has been studied in *Nocardia* spp., suggesting that these enzymes have similar functions in *N. brasiliensis*. In this context, testosterone-induced modulation of such metabolic pathways could hypothetically enhance bacterial survival and virulence in male hosts by promoting stress resistance, optimizing nutrient utilization, and supporting persistence within host tissues.

In addition to the direct effects of estrogen and testosterone on *N. brasiliensis*, other studies have demonstrated their roles in bacterial functions, such as virulence, antimicrobial resistance, and metabolism. Recently, it was shown that estradiol reduces growth and biofilm formation and increases adhesion, invasion, and the minimum bactericidal concentration of gentamycin against *Staphylococcus aureus* [29]. Similarly, estradiol can alter the virulence of *Porphyromonas gingivalis* by increasing growth, biofilm formation, and release of gingipains, a trypsin-like cysteine protease important for adherence, colonization, nutrient acquisition, host immune evasion, inflammatory response, tissue destruction, invasion, and systemic dissemination of *P. gingivalis* [30]. Interestingly, the role of sex hormones has not only been described in bacteria and human pathogens but also in parasites, fungi, and bacterial pathogens of animals. A recent study showed that testosterone and estradiol induce the overexpression of proteins involved in adhesion to host cells, and estradiol limits the development of biofilms of *Actinobacillus seminis*, the causal agent of epididymitis and other clinical presentations in the reproductive tract of small ruminants and bovines [31]. In parasites, such as *Toxoplasma gondii*, an important human pathogen, estradiol can directly affect invasion *in vitro* and increase the parasitic load in mice [32]. Infections, such as vaginal candidiasis caused by the opportunistic fungus *Candida albicans*, have been recognized more frequently in pregnant women, young women using hormonal contraceptives, and postmenopausal women who have undergone hormonal replacement therapy. A study on the direct effect of estrogen on *C. albicans* showed that estrogen affects the expression of several genes and the secretion of several metabolites that help regulate C. albicans morphogenesis and virulence

[33]. In addition, estrogens have a direct effect on *Paracoccidiodes brasiliensis* by inhibiting the transition of mycelia or conidia (saprophytic form) to yeast (pathogenic form) through the modulation of gene expression involved in the heat-shock response, cell wall synthesis, energy metabolism, and cell signaling [34] [35]. Interestingly, a recent study showed that estradiol aggravates *Nocardia farcinica* respiratory infection in mice. However, the authors attributed the differences in these results compared with those of other studies to experimental participants and approaches, such as the *Nocardia* strains used and/or stimuli employed [36]. Some reports have also evidenced that in humans, hormonal changes, such as pregnancy and puberty, modify the *N. brasiliensis* actinomycetoma clinical evolution [37] [38].

Collectively, the results presented here show that 17β-estradiol and testosterone have a direct effect on gene expression in *N. brasiliensis*. Nonetheless, the exact functions of the proteins encoded by these genes are not well understood; therefore, further research is needed in this field. A limitation of this study is that gene expression was assessed using differential display PCR, which, although useful for identifying hormone-responsive transcripts, provides only a partial view of the transcriptional changes induced by sex steroid hormones. Whole-transcriptome sequencing approaches, such as RNA-seq, would allow for a more comprehensive and quantitative analysis of the global transcriptional response of *N. brasiliensis* to 17β-estradiol and testosterone. Incorporating such methods in future studies will strengthen our understanding of hormone-driven modulation of bacterial metabolism, virulence, and ultimately the observed sex bias in actinomycetoma.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Author Contribution

Francisca Hernández-Hernández: Conceptualization, Funding acquisition, Supervision, Writing—review & editing. Laura V. Jasso-Escutia: Investigation, Methodology, Formal analysis. Erika Córdova-Martínez: Methodology, Supervision. Patricia Manzano-Gayosso: Supervision, Writing—review & editing. Claudia C.

Paredes-Amaya: Formal analysis, Investigation, Writing—original draft, Writing—review & editing.

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