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17β-estradiol modulates the expression of hormonal receptors on THP-1 *T. gondii*-infected macrophages and monocytes in an AKT and ERK-dependent manner

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A R T I C L E I N F O

A B S T R A C T

Toxoplasma gondii (*T. gondii*) is a parasite common in pregnancy. Monocytes and macrophages are a significant immunologic barrier against *T. gondii* by boosting up inflammation. This outcome is highly regulated by signaling pathways such as MAPK (ERK1/2) and PI3K (AKT), necessary in cell growth and proliferation. It may be associated with the hormonal receptors' modulation by *T. gondii* (Estrogen Receptor (ER)-α, ERβ, G Protein-coupled ER (GPER), and Prolactin Receptor (PRLR)), as previously reported by our research group. 17β-estradiol also activates MAPK and PI3K; however, its combined effect in THP-1 monocytes and macrophages, infected with *T. gondii*, has not yet been evaluated.

This study aimed to evaluate the combined effect of 17β-estradiol in the activation of signaling pathways using a model of THP-1 monocytes and macrophages infected with *T. gondii*.

THP-1 monocytes were cultured and differentiated into macrophages. Inhibition of AKT and ERK1/2 was performed with specific inhibitors. Stimuli were performed with 17β-estradiol (10 nM), *T. gondii* (20,000 tachyzoites), and both conditions for 48 h. Proteins were extracted and quantified, and Western Blot assays were performed.

17β-estradiol performed activation of ERK1/2 and AKT in *T. gondii*-infected macrophages. 17β-estradiol modulated the expression of hormonal receptors in infected cells: increases the PRLR and PrgR in *T. gondii*-infected macrophages and decreases the PRLR and ERα in *T. gondii*-infected monocytes. As for GPER, its expression is abolished by *T. gondii*, and 17β-estradiol cannot restore it. Finally, the blockage of ERK and AKT pathways modified the expression of hormonal receptors. In conclusion, 17β-estradiol modifies the receptors of *T. gondii*-infected THP1 macrophages and monocytes in an ERK/AKT dependent manner.

1. Introduction

Toxoplasmosis is a disease caused by the infection with *Toxoplasma gondii* (*T. gondii*), an intracellular protozoan Apicomplexa able to infect humans [1]. Discovered more than 100 years ago by Nicole and Manceaux [2], toxoplasmosis is still a global health problem, having a high prevalence in the worldwide population. It has been reported to be remarkably higher in pregnant women, especially in the second and third trimesters, when estrogen levels increase [3]. High levels of pregnancy-related hormones are associated with susceptibility to *T. gondii* infection, in particular, 17β-estradiol due to its main actions: cell growth, proliferation, and calcium mobilization [4].

17β-estradiol exerts its actions through its receptors (ER, ER, and GPER) and induces the activation of signaling pathways, such as MAPK and PI3K. These pathways are well-known for activating proliferation and anti-apoptotic actions through their effector molecules ERK1/2 and AKT, respectively [5], supporting cell proliferation. High levels of 17β-estradiol protect regulatory T cells against the apoptosis induced by *T. gondii* infection and induce PD1 expression, which can be a supporting mechanism for the parasite [6]. Since *T. gondii* is an obligate intracellular parasite, cell proliferation and immune regulation are extremely important for its survival. However, the effects of 17β-estradiol over *T. gondii*-infected cells and the related molecular mechanisms are yet to be defined.

T. gondii can activate these signaling pathways in many ways, which may contribute to the effects 17β-estradiol carries on the infection [7]. Proinflammatory cytokines produced by *T. gondii* infection, IL-12 and IL-23 in particular, are made by activating of MAPK and PI3K in THP-1 monocytes [8]. It is known that the parasite can activate Jnk MAPK, and therefore, the inflammatory response, by releasing exosomes [9]. Finally, some proteins of *T. gondii*, like GRA24, can modulate the immune response by activating MAPK [10].

Monocytes and macrophages are immune cells that mediate the host response against *T. gondii*, and their role in infection, particularly on toxoplasmosis, has been widely documented to be important in mucosal immunity against *T. gondii*. Their main actions are phagocytosis, antigenic presentation, and proinflammatory cytokine production [11–14]. These actions could be strongly mediated by the activation of signaling pathways, such as MAPK and PI3K, extensively described to participate in the processes mentioned above.

Our research group reported that 17β-estradiol increases *T. gondii*`s ability to infect monocytes and also modulates the expression of hormonal receptors in THP-1 monocytes. The modulation of hormonal receptors' expression induces a decrease in the expression of PRLR and an increase of ERα and ERβ, which in turn may enhance 17β-estradiol's effects, leading to an anti-inflammatory environment, therefore, increasing susceptibility to infection [15]. However, the exact mechanism used by the parasite and the 17β-estradiol to conduct these effects in monocytes/macrophages is still unknown.

Given the importance of these cells in controlling the primary infection with *T. gondii*, and these results, we are further interested in unraveling the possible mechanisms carried by the hormone-parasite interaction. This study aimed to evaluate the activation of the main signaling pathways activated by 17β-estradiol and *T. gondii* on monocytes and macrophages to identify whether a cooperative effect is carried and to approach the elucidation of the mechanism through which estrogen performs these effects.

2. Materials and methods

2.1. Reagents

17β-estradiol (Sigma Aldrich), Polyvinylidene difluoride (PVDF) membranes, enhanced chemiluminescence (ECL), and Western blotting detection kit (WBKLS0500) were purchased from Merck Millipore® (EMD Millipore Corporation Billerica, MA, USA). Charcoal Stripped Fetal Bovine Serum (FBS) and antibiotic/antimycotic were purchased from Gibco®, Life technologies (Carlsbad, CA). Anti-pERK 1/2 (Thr 202) sc-101760, anti-pAKT 1/2/3 (ser 473) sc-101629, anti-AKT 1/2/3 (H-136) sc-8312 as well as the monoclonal antibodies anti-ERK2 (H-9) sc-271451, Phospho-p38 (Thr 180/Tyr 182) (D3F9) and #4511 and p38 MAPK #9212 were purchased from Cell Signaling Technology®. A polyclonal antibody against ERα (MC-20; cat. no. sc-542) and monoclonal antibodies against PRL (E-9; cat. no. sc-48383) and ERβ (B-3; cat. no. sc-373853) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibody against GPER was purchased from Abcam (Cat. 39742, Cambridge, UK). ERK inhibitor (PD098059) and Akt inhibitor V Triciribine were purchased from Sigma Aldrich and Calbiochem, respectively.

2.2. Parasites

Six-week-old female Swiss mice, weighing approximately 20–25 g were intraperitoneally injected with 1×10^5 *T. gondii* tachyzoites (strain RH) and sacrificed after seven days; the infection was maintained by injecting new mice every three days. The mice were sacrificed to harvest the *T. gondii* used to infect THP-1 cells.

2.3. Cell culture

THP-1 cells (ATCC® TIB-202™) were grown in RPMI 1640 medium, supplemented with 10 % Charcoal stripped FBS (1 % of penicillin G, streptomycin, and amphotericin B) until reaching a confluence of approximately 70 %. The cells were stored in a jacket-water incubator at 37 °C with an atmosphere of 5 % CO_2. They were checked daily, taking care of not exceeding a density of 500 000 cells/mL. The medium was changed on a 2-day basis to maintain its integrity.

2.4. Macrophage differentiation

THP-1 monocytes were treated with PMA (200 nM) for 72 h and kept at 37 °C and 5 % CO_2. After this time, cells were observed by an inverted microscope (Zeiss ID 02) to verify that macrophages have adhered to the plate. Three washes with PBS buffer were performed to eliminate non-adherent cells (death cells). Finally, the attached cells were maintained with RPMI supplemented media to carry the stimuli.

2.5. Hormonal and parasitic stimuli

2 000 000 THP-1 cells (monocytes/macrophages) were taken for every stimulus; they were seeded in 6-well plates and kept at 37 °C and 5 % of CO_2. Hormonal stimuli consisted of 17β-estradiol (40 nM). Infection was performed with 20 000 tachyzoites of *T. gondii* (Ratio 1:100 tachyzoites:cells). Mixed stimuli consisted of the concomitant *T. gondii* and 17β-estradiol. Once infected and stimulated, cells were incubated at 37 °C and 5 % CO_2 for 30 min (for signaling pathway evaluation) or 48 h (for hormonal receptors evaluation). Afterward, protein extraction was conducted.

2.6. Western blotting

Forty micrograms of total protein extract were mixed with loading buffer and denatured at 95 °C for 5 min. Afterward, the samples were loaded onto 10 % SDS polyacrylamide gels to be resolved. Electrotransference was performed in polyvinylidene difluoride membranes (BioRad Laboratories, Hercules, CA), previously activated with methanol. Blocking solution was prepared with 5 % of Blotting Grade Blocker (BioRad Laboratories, Hercules, CA), dissolved in PBST, and membranes were incubated in this solution for 24 h. Solutions with primary antibodies were prepared at a dilution of 1:500 and 1:10,000 (in the case of β-actin) using PBST, and membranes were kept overnight with it. Afterward, anti-mouse or anti-rabbit secondary antibody solutions (diluted

1:5000) were added for one and a half hours at room temperature. The reveal process was developed with a chemiluminescence system (Immobilon, Merck Millipore®). Microchemi 6.0 was used to reveal membranes, and GelQuant v13.2 software was used to measure the optical density of the images.

2.7. Statistical analysis

Data obtained from western blotting optical densities were analyzed using GraphPad Prism software (GraphPad version 6.01). ANOVA analysis were performed to evaluate significant effects. p-values <0.05 were considered statistically significant.

3. Results

To understand more about the possible mechanisms related to the interaction between 17β-estradiol and *T. gondii* on monocytes and macrophages, critical molecules of signaling pathways involved in *T. gondii* pathogenesis were evaluated, and the results are the following:

3.1. p38 is not modulated by 17β-estradiol in infected THP-1 cells

The activation of p38 was evaluated in infected macrophages and monocytes with and without 17β-estradiol stimulus for 30 min.

In concordance with other reports, the basal expression of p-p38 in macrophages is deficient in comparison to that in monocytes [16–18]. Compared to it, *T. gondii* increased p-p38 phosphorylation in both macrophages and monocytes ($p < 0.0001$); however, when both stimuli were added simultaneously, no significant differences were observed when compared to the *T. gondii* effect (Fig. 1A).

3.2. β-estradiol regulates differently the activation of ERK1/2 in macrophages and monocytes infected with T. gondii

17β-estradiol stimuli were performed for 30 min on THP-1 macrophages and monocytes infected with *T. gondii,* and the phosphorylation of ERK was measured. An opposite behavior is observed in the ERK phosphorylation after 17β-estradiol is added to infected macrophages and monocytes.

T. gondii infection significantly increased ERK phosphorylation in

monocytes ($p < 0.0001$); however, this outcome was considerably reduced by 17β-estradiol ($p < 0.0001$). Even though macrophages did not show a drastic increase in ERK phosphorylation after *T. gondii* infection, the stimulus with 17β-estradiol augmented p-ERK expression in these cells ($p < 0.0001$) (Fig. 1B).

3.3. β-estradiol activates AKT in T. gondii-infected macrophages but not in monocytes

AKT phosphorylation was increased in macrophages and monocytes after being infected with *T. gondii*; however, two different outcomes were observed when adding 17β-estradiol.

For *T. gondii*-infected macrophages, the 17β-estradiol stimulus for 30 min led to increased levels of p-AKT ($p < 0.0001$), while the opposite effect was observed in infected monocytes (Fig. 2), where a significative reduction ($p < 0.0001$) was observed.

Moreover, total AKT expression in macrophages and monocytes was also increased after every *T. gondii* stimulus was present, regardless of the presence or absence of the hormone (data not shown).

3.4. β-estradiol modulates the hormonal receptors differentially in T. gondii-infected macrophages and monocytes

T. gondii can deregulate the prolactin receptor (PRLR) when infecting monocytes, as shown in a previous report [15], besides it enhances the expression of ERα and ERβ. Collectively, 17β-estradiol increased the parasite's viability on THP-1 monocytes and modified the inflammatory response induced by *T. gondii*, increasing regulator cytokines and decreasing the proinflammatory profile. Therefore, an interplay parasite-hormone is stated; thus, we decided to evaluate the effect of the 17β-estradiol on the expression of hormonal receptors expressed in *T. gondii*-infected macrophages and monocytes treated with 17β-estradiol.

First, the expression of ERα and ERβ was highly expressed after *T. gondii* infection was performed in macrophages and monocytes; however, when the infected macrophages were also stimulated with 17β-estradiol, the expression of ERβ significantly decreased ($p < 0.0001$). On the other hand, after the mixed stimulus, monocytes reduced the expression of ERα ($p < 0.0001$) and increased that of ERβ ($p < 0.05$).

For GPER, the behavior of its expression was very similar in
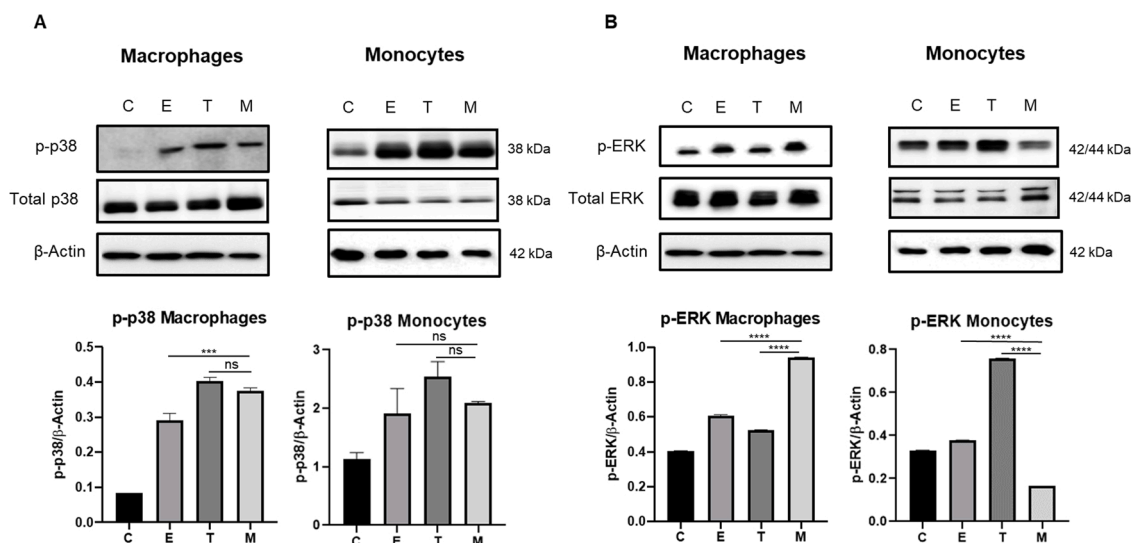
Fig. 1. **Activation of MAPK on macrophages and monocytes infected with *T. gondii* *after* the 17β-estradiol stimulus** Macrophages and monocytes were grown in supplemented RPMI medium and treated with 17β-estradiol (10 nM), *T. gondii* (20 000 tachyzoites), or a combination of both stimuli for 30 min. Western blot assays were performed to evaluate the presence of A) p-p38 and B) p-ERK1/2. (upper panel). Densitometric analysis was conducted for phosphorylated molecule's expression, and normalized to the constitutive protein, β-actin; this is shown in the graphics (lower panel). C = Control, E = 17β-estradiol, T= *T. gondii,* M = Mixed, *** = p<0.001, **** = p < 0.0001, ns = no significant. Data are presented as the mean ± standard deviation of three independent assays.
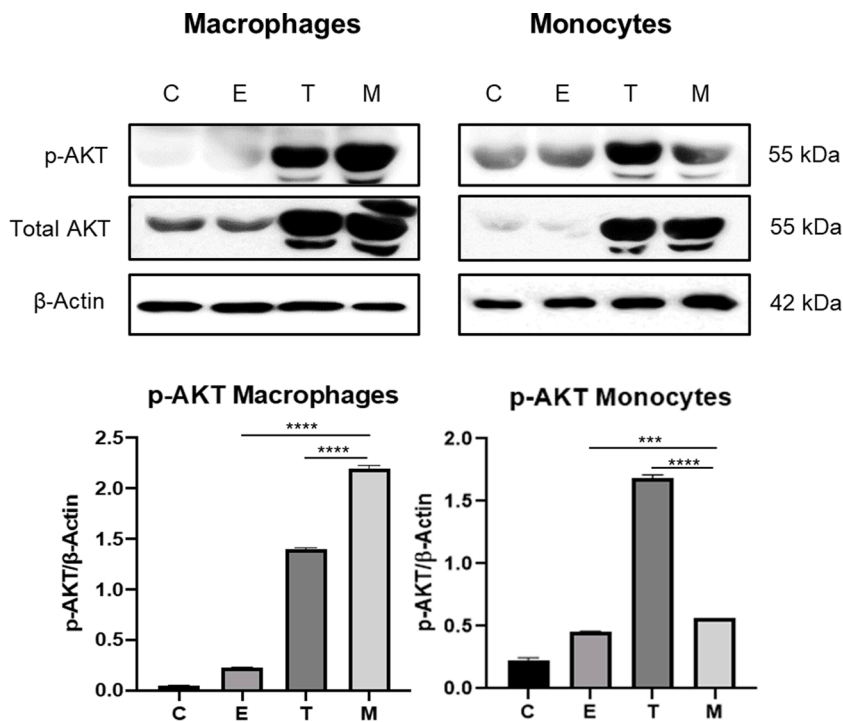
Fig. 2. Activation of AKT on macrophages and monocytes infected with *T. gondii* after the 17β-estradiol stimulus Macrophages and monocytes were grown in supplemented RPMI medium and treated with 17β-estradiol (10 nM), *T. gondii* (20 000 tachyzoites), or a combination of both stimuli for 30 min. Western blot assays were performed to evaluate the presence of p-AKT. (upper panel). Densitometric analysis was conducted for phosphorylated molecule's expression, and normalized to the constitutive protein, β-actin; this is shown in the graphics (lower panel). C = Control, E = 17β-estradiol, T= *T. gondii,* M = Mixed, *** = p<0.001, **** = p < 0.0001. Data are presented as the mean ± standard deviation of three independent assays.

macrophages and monocytes. *T. gondii* entirely abolished the expression of GPER, and no further regulation was observed by the addition of 17β-estradiol to the infected cells, showing no expression of this receptor after the mixed stimulus.

The behavior observed in macrophages regarding PRLR expression was the opposite to that of monocytes. Macrophages overexpressed long isoforms of PRLR after being infected with *T. gondii*, and the stimulus with 17β-estradiol resulted in a higher expression of it (p < 0.0001). Conversely, the expression of this receptor in monocytes was reduced after infection, and when comparing *T. gondii* infection with and without 17β-estradiol stimulation, an even lower expression was observed; however, this decrease was not statistically significant; thus, only a tendency to decrease was observed.

Finally, the expression of progesterone receptor (PrgR) was measured. As expected, its expression increased after the infection of *T. gondii* in both types of cells; furthermore, the addition of 17β-estradiol to infected cells turned into a higher PrgR expression in macrophages and monocytes (p < 0.05 for both cell types).

3.5. Blockage of ERK and AKT pathways turned into different hormonal receptors expression

With the results regarding the signaling pathways modulated by 17β-estradiol in *T. gondii*-infected cells and the modulation of the hormonal receptor, we decided to block p-ERK and p-AKT because they were highly modulated by the hormone, to test if the expression of the receptors was related to those signaling pathways.

First, we performed an assay to validate the inhibition of the phosphorylation of ERK and AKT. Specific inhibitors (ERK inhibitor: PD098059 and AKT inhibitor: V Triciribine) were used for 30 and 60 min to avoid the phosphorylation of these molecules. As can be seen in Fig. 4A, after 60 min, the phosphorylation was utterly abolished. Therefore, we used this condition for further assays.

Fig. 4B shows the expression of hormonal receptors in macrophages and monocytes infected with *T. gondii* with and without blockage of phosphorylation of ERK and AKT.

As seen, in some cases, the level of expression is reverted after blocking some pathways. For example, ERα expression increased after

T. gondii infection in both macrophages and monocytes; however, when ERK or AKT phosphorylation was blocked, its expression was considerably diminished. The opposite behavior is observed for GPER expression; the infection abolished its expression. Surprisingly, the blockade of any, p-ERK, or p-AKT resulted in a high expression, even higher than that observed in the basal condition in most cases.

Finally, stimuli with *T. gondii* and 17β-estradiol were performed on monocytes and macrophages with the same signaling pathway blockage and compared to those without it.

Fig. 4C shows that, all cases seem to be significant for monocytes when comparing every hormonal receptor pattern with p-ERK or p-AKT blocked versus that on monocytes without the blockage.

ERα and ERβ showed similar behavior; mixed stimuli increased their expression on monocytes; however, blocking signaling pathways activation decreased its expression significatively (p < 0.0001 to p-ERK and p-AKT inhibition). Monocytes reduced the expression of PRLR and GPER when the mixed stimulus was performed, but when any of the pathways was blocked, the expression was higher than control cells.

Macrophages only showed this change on GPER expression, whose behavior was very similar to that observed in monocytes, a decrease in its expression after the stimulus, and a high increase when pathways were blocked.

4. Discussion

Toxoplasmosis is a worldwide infection with a higher prevalence in pregnant women. Hormones have been previously involved in modifying *T. gondii* infective capacity. Particularly 17β-estradiol has shown to increase susceptibility to *T. gondii* infection by modulating hormonal receptors and creating an anti-inflammatory environment.

In this work, we analyzed the activation of signaling pathways involved in cell proliferation, and we observed the following:

p38 is activated in macrophages and monocytes after *T. gondii* infection, which is in agreement with the literature. GRA24 is a dense granule protein from *T. gondii* that modulates the immune response against infection with this parasite at an early stage by activating p38 MAPK [10]. p38 is essential for proinflammatory cytokines to be secreted in THP-1 cells as described by Quan et al. [8]; however, no

significant differences were shown when 17β-estradiol was present, which may indicate that 17β-estradiol is not related to p38 phosphorylation in infected macrophages nor monocytes.

For ERK1/2, an opposite effect is observed: macrophages increased p-ERK1/2 after mixed stimulus while that in monocytes was considerably reduced. As it has been very well documented, different cell types present distinct outcomes and respond differently to the same stimulus [19]. In this regard, the increase in the expression of p-ERK1/2 in *macrophages* after *T. gondii* infection has already been reported [20,21]; and the secreted parasite kinases, through MAPK modulation, may be responsible for it. The latter has been described in a published paper focused on an integrative approach [22].

Monocytes' ERK phosphorylation increased in response to *T. gondii* than macrophages, which is consistent with previous studies [23]; however, the presence of 17β-estradiol abolished this effect. In monocytes, as in dendritic cells, high ERK phosphorylation is necessary for parasite invasion, and TgMIF protein from the parasite has a direct effect on increasing ERK and modulating the host immunity [21,24]; therefore, 17β-estradiol might be supporting the "trojan horse" effect by decreasing the parasite's motility and invasion capacity.

The anti-inflammatory effects exerted by 17β-estradiol have been widely documented [25]; however, little has it been explored about its involvement in resolving of the inflammatory processes. Villa et al. published that 17β-estradiol can activate ERα to decrease the time of macrophages in the inflammatory status, making it clear the 17β-estradiol's ability to resolve the inflammation [26]. In our results, *T. gondii* significantly increased the expression of ERα in macrophages, which could represent an advantage for *T. gondii* itself by decreasing the inflammation through the mechanism mentioned before.

Taking the results together, the explanation for this opposite effect observed in monocytes and macrophages could be related to these cell's actions. While monocytes are circulating cells, macrophages are usually resident in tissues; this difference is important because *T. gondii* can take advantage of infecting monocytes to move through the body, leading the parasite to infect distant sites such as the brain, for example; to achieve this, 17β-estradiol decreases the phosphorylation of ERK and Akt in monocytes, which correlates with a decreased proliferation, reduced adhesion, and an anti-inflammatory status [27–29]. On the other hand, macrophages' proliferation makes it more accessible for the parasite to have target cells to infect but also, through the induction of ERα, controlling the inflammation, to evade the immune system responses.

p-AKT shows similar behavior to that observed with ERK1/2. An enhanced phosphorylation level is observed in macrophages stimulated with both (parasite and hormone), but p-AKT diminished in monocytes after the same conditions. As discussed before, for ERK results, macrophages and monocytes play different roles in the infection course, which is why their regulation is different in response to 17β-estradiol. The immunomodulatory effect of the hormone has been widely reviewed, and the cell type is an essential factor for the outcome observed; previous reports show that *T. gondii*-infected astrocytes increased the parasite replication in response to the hormone [19], while in T regulatory cells, an immune modulatory and antiapoptotic effect is observed [6]. Both effects support the cooperation of the hormone to *T. gondii* infection on distinct actions observed in different cell types.

In this study, besides increasing its phosphorylation, total AKT was highly expressed after *T. gondii* stimuli. AKT has been reported to be increased in *T. gondii* infection, which not only favors cell proliferation but also may support the dysregulation of tight junctions, which would make the migration of the parasite easier [30]. Proteomic approaches have been conducted to evaluate other important effectors regarding tight junction profiles. STAT and NF-kB signaling pathways are also modulated by infection [31].

Previous reports of our group have documented the interplay between 17β-estradiol, *T. gondii,* and the expression of hormonal receptors [15]. In this regard, the expression of hormonal receptors was measured in monocytes and macrophages.

The expression of ERα and ERβ is increased after *T.gondii* infection. However, when 17β-estradiol is added, a slight decrease of ERβ in macrophages and ERα in monocytes is observed. Unlike ERβ, ERα is vital for AKT and ERK1/2 activation; in our results, the activation of AKT and ERK1/2 seems to be mostly related to the expression of ERα after the *T. gondii* infection and also after the stimulus with 17β-estradiol. In turn, AKT might modulate the expression and actions of ERα [32,33]; however, in our results, we do not observe a direct regulation on the expression of ERα expression by AKT. This could be due to the model or the time of stimulation performed. ERβ increases in monocytes and decreases in macrophages after the 17β-estradiol stimulus. For this receptor, antiproliferative and proapoptotic functions have been reported in breast cancer cells [34]. Together with p-ERK downregulation (also carried out by 17β-estradiol in monocytes), this ability could support the "trojan horse" effect.

Interestingly, GPER expression was abolished entirely in monocytes and macrophages after *T. gondii* infection, even in the mixed stimulus. This finding is important because this receptor is involved in calcium release, which might modulate several processes related to toxoplasmosis, such as motility and invasion through Calcium-dependent protein kinases (CDPK) [35–37]. Conversely, other intracellular pathogens have been reported to increase GPER expression, like *Cryptococcus gattii*; Costa et al. reported that GPER is involved in the modulation exerted by 17β-estradiol, improving the control against infection by this parasite [38]. More research focused on GPER modulation on *T. gondii* infection must be conducted to identify possible mechanisms governing these effects.

One of the functions of GPER is activating calcium mobilization. Some calcium-dependent kinases have been reported to have an essential role in *T. gondii's* invasion and motility [39]. Nevertheless, this result could depend on the activation of ERK1/2 and AKT because when each of those molecules where inhibited, GPER expression was re-established.

In line with previous reports of our group of research, after *T. gondii* infection, the expression of long isoforms of PRLR was reduced in monocytes [15], as seen in Fig. 3B. Decreasing long isoforms of PRLR may represent an evasion mechanism of *T. gondii*, given the significant regulating functions of this receptor in intracellular infections [40]; thus, lower inflammation may be achieved by downregulating these isoforms.

Nevertheless, it has been reported that monocytes infected with *T. gondii* undergo conversion into TipDC, a subtype of dendritic cells able to produce TNFα and inducible nitric oxide synthase [41]; whether hormonal receptors mediate this differentiation is still unknown; however, more experiments must be conducted to state the regulation in this regard. As for 17β-estradiol, its anti-inflammatory effect has been widely reported [42,43]. Estrogens are known to decrease the production of key inflammatory mediators such as TNFα, IL1β, and IL-6, and increase the production of anti-inflammatory molecules, such as TGF-β [43]. This modulation is context-dependent, which means the microenvironment plays a significant role in the outcome. In this context, the parasite might modify other molecules' expression, which might interfere with the effects exerted by estradiol. Performing an RNA sequence analysis would clarify this issue, and prospectively we will perform it. ERα and ERβ play a role in the modulation of inflammation. ERα presents several isoforms, but one, in particular, ERα36, has been shown to interact with GPER to carry anti-inflammatory actions [44]. ERβ has an anti-inflammatory role in a murine model deregulating $P2 \times 7R$ in macrophages [45]. It has also been reported that estradiol might have differential effects in the presence or absence of ERβ, reflecting the vital modulating role of this receptor in inflammation [46]. Interaction between estrogen receptors might modify the effect ERβ also interacts with isoforms of Era to modulate the genic expression regulating effects such as proliferation and apoptosis [34]. Once again, further analysis are to be conducted to describe if a modulation of the gene expression is conducted in this model.

On the other hand, infected macrophages responded to 17β-estradiol
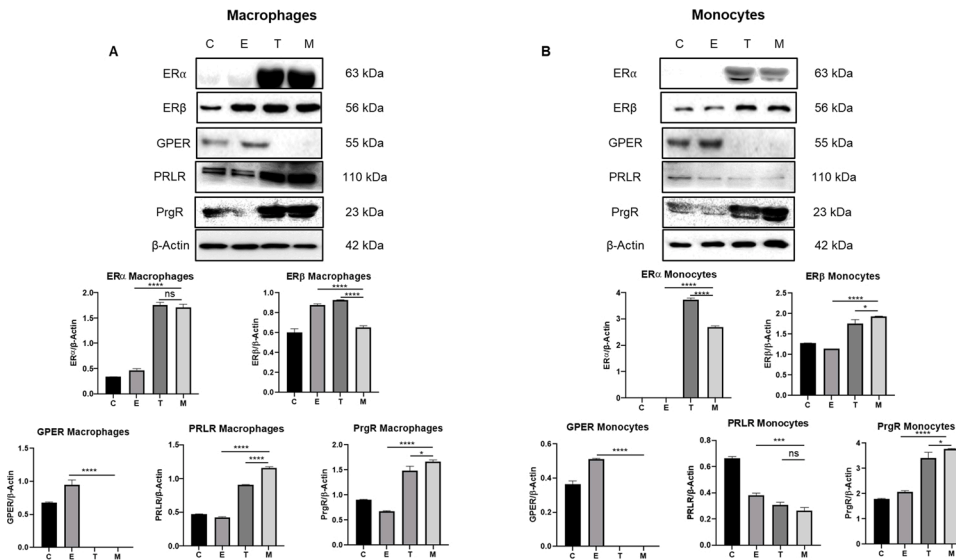
Fig. 3. Expression of hormonal receptors in macrophages and monocytes infected with *T. gondii* after the 17β-estradiol stimulus THP-1 cells were grown in supplemented RPMI medium and treated with 17β-estradiol (10 nM), *T. gondii* (20 000 tachyzoites), or a combination of both stimuli. Western blot assays were performed to evaluate the expression of ERα, ERβ, GPER, PRLR, and PrgR (upper panel) in A) Macrophages and B) Monocytes. Densitometric analysis was conducted for the hormonal receptor's expression and normalized to the constitutive protein, β-actin; this is shown in the graphics (lower panel). C = Control, E = 17β-estradiol, T = *T. gondii*, M = Mixed, * = p < 0.05, *** = p<0.001, **** = p < 0.0001, ns = no significant. Data are presented as the mean ± standard deviation of three independent assays.
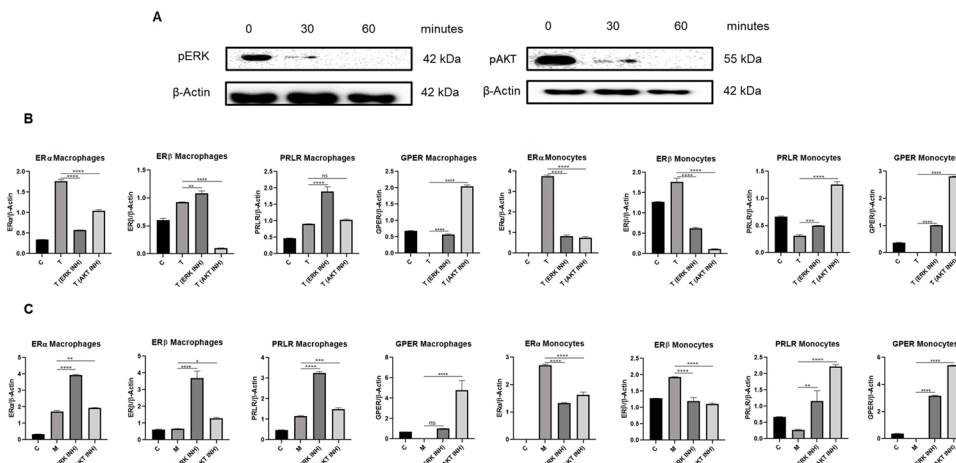
Fig. 4. Expression of hormonal receptors in macrophages and monocytes with ERK and AKT blocked, after infection with *T. gondii* and the 17β-estradiol stimulus A) THP-1 cells were grown in supplemented RPMI medium and treated ERK and AKT inhibitors during 30 and 60 min. Western blot analysis was performed to observe the expression of p-ERK (left panel) and p-AKT (right panel). B and C) After the signaling pathways inhibition *T. gondii* (20 000 tachyzoites) (B) and mixed stimuli (C) were conducted. Western blot assays were performed to evaluate the expression of ERα, ERβ, GPER, and PRLR in macrophages and monocytes. Densitometric analysis was conducted for the hormonal receptor's expression and normalized to the constitutive protein, β-actin; this is shown in the graphics. C = Control, T= *T. gondii,* M = Mixed, ERK INH = ERK inhibited, AKT INH = AKT inhibited, * = p < 0.05, ** = p<0.01, *** = p<0.001, **** = p < 0.0001, ns = no significant. Data are presented as the mean ± standard deviation of three independent assays.

by increasing PRLR expression. *T. gondii* infection can be conducted in two ways: either by active invasion or being passively phagocytosed. Nevertheless, the proinflammatory cytokine response is only established after the second mechanism [35]. After phagocytosis, macrophages undergo a series of events reviewed by Legorreta-Herrera, including the increase of the PRLR expression, production of proinflammatory cytokines, ROS, and NO [47].

The expression of PrgR was increased in response to 17β-estradiol in monocytes and macrophages. Some evidence supports an interplay between progesterone, estrogen, and their receptors to generate an anti-inflammatory microenvironment. Stimulus with progesterone increases the expression of ERα and ERβ on THP-1 monocytes infected with *T. gondii* [15]. Zhang et al. reported that progesterone reduces *T. gondii's* ability to exit the cell by decreasing MIC2 protein secretion; therefore, it reduces its pathogenicity [48]. This outcome is regulated by calcium-flux induction, which is closely related to GPER's actions, and it supports the results observed on GPER expression.

Finally, ERK and AKT have been widely documented to play an essential role in toxoplasmosis [8]. In this article, we show the importance of the activation of these signaling pathways to modulate the expression of hormonal receptors. Blocking ERK and AKT in infected monocytes and macrophages resulted in a different expression of hormonal receptors.

In monocytes, the result of the inhibition of both pathways (ERK / AKT) turned out in similar patterns for all the receptors in *T. gondii*-infected cells and those with the mixed stimulus; the ERα and ERβ's expression decreased significantly (p < 0.0001) after the blockade assays, while PRLR and GPER's increased (Fig. 4 B). On the other hand, macrophages' behavior was more heterogeneous. ERα decreased its expression after the blockade of both signaling pathways in infected cells, while augmented in those infected cells with 17β-estradiol. ERβ decreased in monocytes after inhibition in infected cells with or without the hormone; however, a significant increase was observed in macrophages with the blockage of ERK after stimulation with the parasite or the parasite-hormone stimulus. Finally, PRLR and GPER increased in all cases when ERK or AKT were blocked. It is crucial to remind that each cell type responds differently to stimuli. Further research will clarify the results here presented; however, it is clear that ERK and AKT are essential to modulate the expression of the hormonal receptors in monocyte and macrophages during *T. gondii* infection.

At the immunological level, MAPK and PI3K modulate the secretion of proinflammatory cytokines [8]. PRLR, through its long isoforms, modulates the inflammatory response in other intracellular infection models [40]. Additionally, these signaling pathways are also crucial for oxygen radicals formation [49].

Because MAPK and PI3K signaling pathways are essential in promoting cell proliferation, and 17β-estradiol highly modulated them in *T. gondii* infected monocytes and macrophages, more studies about the role they play in toxoplasmosis infection should be conducted.

In conclusion, a modulation by 17β-estradiol was observed in *T. gondii*-infected cells. The fact that ERK and AKT are essential in the modulation of hormonal receptors in infected THP-1 cells treated with 17β-estradiol opens a new field that remains unexplored so far, raising some questions like what are the differential expressed genes under these stimuli? What is the role of these genes in the *T. gondii* infection? And how do they interact with ERK and AKT? Could they be taken into account so new therapeutical approaches are developed? More research on this regard must be conducted to answer these questions and to widen the current landscape of 17β-estradiol modulation on toxoplasmosis. The answer to these interrogators could lead to a better comprehension of the pathology in particular in the context of pregnancy, which could lead to a new focus on therapies directed to these patients. This descriptive analysis states the importance of the interplay between 17β-estradiol and *T. gondii* and opens the landscape for further projects focused on the evaluation of the consequences of this signaling regulation at the functional level.

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Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code Availability

Not applicable.

Author's contributions

Conception and design of experiments: Adrian Ramirez de Arellano and Ana Laura Pereira Suarez; Performance of the experiments: Christian David Hernandez Silva, Inocencia Guadalupe Ramirez Lopez, Grecia Viridiana Morales Amaya, and Luis David Lopez Cabrera; Analysis of results: Edgar Ivan Lopez Pulido and Jose Francisco Muñoz Valle; Parasite collection and quantification: Maria de la Luz Galvan Ramirez and Laura Rocio Rodriguez Perez; Supervision: Adrian Ramirez de Arellano, Ana Laura Pereira Suarez, and Jose Francisco Muñoz Valle; Writing, reviewing and editing: Adrian Ramirez de Arellano, Ana Laura Pereira Suarez, Jose Francisco Muñoz Valle, Edgar Ivan Lopez Pulido, and Maria de la Luz Galvan Ramirez.

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Molecular and Biochemical Parasitology Journal.

Declaration of Competing Interest

The authors declare they do not have any competing interest.

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