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Tumor Necrosis Factor Alpha May Act as an Intra-Ovarian Mediator of Luteinizing Hormone-Induced Oocyte Maturation in Trout

Keywords: GVBD, Maturation-inducing steroid (MIS), Cytokine, Ovary, Teleost

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Abstract

In fish, like in other vertebrates, luteinizing hormone (Lh) is an essential hormone for the completion of oocyte maturation. In salmonid fish (i.e., salmon and trout), oocyte maturation is induced by Lh through its stimulation of the production of the maturation-inducing steroid, 17alpha,20beta-dihydroxy-4-pregnen-3-one (17,20beta-P). In mammals, several factors, including ovarian cytokines and growth factors, have been reported to contribute to the regulation of oocyte maturation. In fish, there is growing evidence suggesting that tumor necrosis factor alpha (hereafter referred to as Tnf) could play multiple physiological roles in the control of ovarian function. In the present study, we have investigated the possible involvement of Tnf in the regulation of oocyte maturation in brown trout (*Salmo trutta*). Our results show that in vitro treatment of brown trout preovulatory follicles with coho salmon Lh (sLh) significantly increased oocyte maturation, as assessed by germinal vesicle breakdown (GVBD), and this effect was blocked by TAPI-1 (an inhibitor of Tnf converting enzyme or Tace/Adam17). Furthermore, treatment of preovulatory follicles with sLh increased the expression of *tnf* and *tace/adam17* as well as the secretion of the Tnf protein. Importantly, recombinant trout Tnf (rtTnf) significantly increased GVBD in vitro. Our results also show that the stimulatory effects of rtTnf on oocyte maturation may be the result of the direct involvement of rtTnf in stimulating the production of the maturation-inducing steroid as evidenced, first, by the stimulatory effects of rtTnf on 17,20beta-P production in vitro and on the expression of cholesterol side-chain cleavage P450 cytochrome (*p450scc*) and 20beta-hydroxysteroid dehydrogenase/carbonyl reductase 1 (*cbr1*), the enzyme responsible for the production of 17,20beta-P, and, second, by the ability of TAPI-1 to block the stimulatory effects of sLh on 17,20beta-P production and *cbr1* expression. Furthermore, sLh and rtTnf increased the expression of the Lh receptor (*lhr*) and decreased the expression of aromatase (*cyp19a1*) and TAPI-1 completely blocked the effects of sLh. These results strongly suggest that Tnf may contribute to the regulation of oocyte maturation by Lh in trout.

Introduction

In teleost fish, it is well established that following the follicular growth phase, oocyte maturation (the resumption of meiosis) is under the control of luteinizing hormone (Lh) [1]. Oocyte maturation consists in a multistep process in which Lh is involved through its stimulation of the production of the maturation-inducing steroid (MIS) by follicle cells that, in turn, induces the breakdown of the germinal vesicle (GVBD) of the oocyte [2, 3].The capacity to synthesize MIS by follicle cells must also be accompanied by the acquisition of sensitivity by the oocyte to respond to MIS (i.e. maturational competence). In salmonid fish (trout and salmon), the MIS is the progestin 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) [4]. The ability to produce 17,20β-P in response to Lh is associated with the increase of mRNA expression and activity of 20β-hydroxysteroid dehydrogenase/carbonyl reductase 1 (Cbr1) [5-7], the enzyme responsible for the conversion of 17α-hydroxyprogesterone (17-OHP) to 17,20β-P.

In addition to the cascade of events induced by Lh and steroids during oocyte maturation, several lines of evidence suggest a possible role of members of the tumor necrosis factor (Tnf) family and corresponding receptors during the periovulatory ovary. In mammals, cytokines such as TNFα, hereafter referred to as TNF, have been studied as a possible mediators of LH on ovulation rate and steroid production [8, 9]. Furthermore, the presence of TNF in human follicular fluid has been related to a more optimal follicular and oocyte developmental progression and maturation [10]. There is also evidence suggesting that several growth factors are implicated in the functioning of the mammalian ovary during maturation [11-13]. In fish, the nature of the regulatory factors that control oocyte maturation and the possible involvement of cytokines as mediators in this process are not well documented. Members of Tnf family have been identified in the trout ovary by molecular studies [14, 15]. In a previous study, we have reported that Tnf could have an important role in the preparatory events leading to ovulation in the trout ovary by stimulating granulosa cell apoptosis and follicle contraction [16]. In fact, treatment with recombinant trout Tnf (rtTnf) also stimulated the mRNA abundance of cathepsins, cysteine proteases known to be expressed during oocyte maturation in different fish species [17-19], and potentiated Lh-induced testosterone production by trout follicles. In addition to this, Lh has been previously identified as the determining factor regulating the production of MIS and the induction of meiosis resumption in salmonids [20]. In view of these results, we hypothesize that Tnf could be an important physiological mediator of Lh action in the trout ovary probably through its stimulation of the production of key steroids during oocyte maturation.

The purpose of this study was to investigate the *in vitro* effects of purified coho salmon (*Oncorhynchus kisutch*) Lh (sLh) and the possible mediatory role of Tnf in preovulatory brown trout (*Salmo trutta*) follicles at various levels: (1) stimulation of oocyte maturation (assessed by GVBD), (2) steroid production (17-OHP and 17,20β-P) and (3) changes in the expression of genes involved in steroid production and action (*cbr1*, *p450scc, star, cyp19a1* and *pgmrc1*), luteinizing hormone receptor (*lhr*) and Tnf-related genes (*tnf* and *tace/adam17*).

Materials and methods

Animals

Reproductively mature female brown trout (*Salmo trutta*) from a cultured stock at the Piscifactoria de Bagà (Generalitat de Catalunya, Bagà, Spain) were kept under natural conditions of temperature and photoperiod. *In vitro* experiments were conducted using intact ovarian

follicles prior to GVBD (preGVBD). The stage of maturation was determined using a clearing solution as previously described [21]. Fish were anesthesized in 3-aminobenzoic acid ethyl ester (0.1 g/l; Sigma, Alcobendas, Spain) dissolved in fresh water, and sacrificed by concussion prior to the collection of the ovaries. The dissected ovaries were immediately used either for the *in vitro* induction of oocyte maturation and steroid production or flash frozen in liquid nitrogen and stored at -80ºC until RNA isolation. The experimental protocols used for trout in this study have been reviewed and approved by the Ethics and Animal Welfare Committee of the University of Barcelona, Spain.

Hormones and reagents

Purified coho salmon (*Oncorhynchus kisutch*) luteinizing hormone (sLh) [22] was a kind gift from Dr. Penny Swanson (National Marine Fisheries Service, Seattle, WA, USA) and was dissolved directly in incubation medium. The mature form of recombinant trout Tnf (rtTnf) from rainbow trout (*Oncorhynchus mykiss*) was subcloned into an expression vector, produced in *E. coli*, refolded, concentrated and shown to be biologically active using a fish cell line [23] and in trout ovarian and muscle tissue [16, 24]. The TNFprocessing inhibitor-1 (TAPI-1) was purchased from Calbiochem (San Diego, CA, USA) and dissolved in dimethyl sulfoxide (DMSO) before being diluted to the appropriate concentration in incubation medium. Control incubations containing DMSO at the appropriate concentration had no influence on any of the parameters measured.

Ovarian follicle incubations

After dissection, brown trout ovaries were placed in Hank´s balanced salt solution (HBSS) and individual ovarian follicles were manually separated with forceps from each ovary on ice, as previously described [25]. For the *in vitro* induction of GVBD, brown trout follicles at the preGVBD stage were incubated in HBSS containing 0.2% BSA (fraction V, Sigma; HBSS-BSA). Ten follicles were placed in each well of a 6-well culture plate containing 4 ml of HBSS-BSA in the absence or presence of the test compounds for 48 h at 15°C with shaking. At the termination of the incubation period the culture medium was removed and stored at -20ºC to determine the *in vitro* production of 17-OHP and 17,20β-P by brown trout ovarian follicles. The levels 17-OHP and 17,20β-P were analyzed directly in the incubation medium using a commercial radioimmunoassay (RIA; Schering-CIS, Madrid, Spain) and a custom enzyme immunoassay (EIA; Mañanós et al., unpublished data), respectively.

To collect ovarian tissue for RNA extraction, preGVBD follicles from each of a total of three females were incubated (20 follicles/5 ml in triplicate) in HBSS-BSA in the absence or presence of test compounds, at 15ºC for 48 h with shaking. At the end of the incubation period, the ovarian follicles were removed. Also, theca and granulosa layers were manually dissected from ovarian follicles after treatment with the test compounds, as previously described [20]. Subsequently, ovarian tissues were flash frozen in liquid nitrogen and stored at -80ºC until assayed.

Western blot analysis of supernatants

Brown trout ovarian follicles were incubated with sLh (25 ng/ml) in the presence or absence of TAPI-1 (50 μM) during 16 h at 15ºC under shaking conditions. After the incubation period, the culture medium was removed and centrifuged at 12000g for 5 min at 4ºC. Supernatants were

boiled for 5 min and separated on 15% SDS-PAGE. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF; Millipore, Madrid, Spain) membrane using a Miniprotean III system (Bio-Rad, Alcobendas, Spain). The membrane was blocked with blocking buffer (TBST with 5% non-fat dry milk) for 12 h at 4ºC under continuous shaking. For Tnf detection, membranes were incubated with a rabbit polyclonal antibody against rtTnf, as previously described [23], diluted to 1:500 in blocking buffer for 12 h at 4ºC and, after three washes with TBST, the membrane was incubated with HRP-antibody (HRP-conjugated goat anti rabbit IgG; Jackson Immunoresearch, Barcelona, Spain) diluted to 1:5000 in blocking buffer for 1.5 h at 20-22ºC. The membranes were developed with the enhanced chemiluminescence method using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Madrid, Spain). Immunoreactive bands were visualized (Fujifilm LAS-3000) and quantified with an image analyzer (ImageJ; http://rsb.info.nih.gov/ij/).

RNA isolation and cDNA synthesis

Total RNA from ovarian tissue was isolated using TRIzol Reagent (Invitrogen, Barcelona, Spain), following the manufacturer's instructions and quantification was carried out with a NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA synthesis was performed with 2 µg DNase (RQ1 DNase, Promega, Barcelona, Spain)-treated total RNA using SuperScript III Transcriptase (Invitrogen), oligo(dT) primer and random hexamer primers (Promega), according to the manufacturer's protocols. RNA and cDNA were stored at -80 and -20°C respectively until use.

Quantitative real-time PCR (qPCR)

In order to quantify mRNA expression of individual genes, quantitative real-time PCR (qPCR) was carried out with ovarian tissue from each of three separate females. cDNA was diluted 1:25 for target mRNA and 1:1000 for *18s*, used as the reference gene. The reactions (20 µl of final volume) contained 10 µl of SYBR GreenER qPCR SuperMix (Invitrogen), 500 nM of forward and reverse primers and 5 µl of cDNA. Reactions were run in a MyiQ Real-Time PCR Detection System (Bio-Rad) under the following protocol: 2 min at 50°C, 8 min at 95ºC, followed by 40 cycles of 15 sec denaturation at 95°C and 30 sec at 60°C, and a final melting curve of 81 cycles from 55°C to 95°C (0.5ºC increments every 10 sec). Primer sequences, GenBank accession numbers of the target genes and PCR product sizes are presented in Table 1. Primers for *tace/adam17* were designed using an annotated (DY696186) salmonid expressed sequence tag (EST) that has not yet been published. All samples were run in triplicate and fluorescence was measured at the end of every extension step. Fluorescence readings were used to estimate the values for the threshold cycles (*C*t). Values for each sample were expressed as fold change, calculated relative to control group and normalized for each gene against those obtained for *18s* [26]. Expression of *18s* was not affected by any of the treatments (data not shown). For all primer pairs, a dilution curve obtained from a serially diluted ovarian cDNA pool was used to ensure that PCR efficiency was higher than 90%.

Statistical analysis

Statistical differences were calculated by the non parametric Kruskal-Wallis test followed by Mann-Whitney U test or one-way analysis of variance (ANOVA) followed by the Fisher protected least significant different test for the determination of differences among groups, using

StatView 5.0 (SAS Institute, Cary, NC, USA). Results are expressed as mean ± SEM and differences between groups were considered to be significant if $p < 0.05$.

Results

Effects of Tnf on basal and Lh-stimulated oocyte maturation and steroid production

First, in order to demonstrate that purified piscine Lh stimulates oocyte maturation in the brown trout, as has been shown in other salmonid species (i.e. brook trout) [20], brown trout ovarian follicles were incubated in the presence of purified salmon Lh (sLh). Brown trout ovarian follicles significantly ($p < 0.05$) increased their ability to undergo GVBD in response to sLh (25 ng/ml) (Fig. 1A). Second, in order to investigate whether Tnf could be involved in mediating the stimulatory effects of Lh on oocyte maturation, we incubated trout ovarian follicles with sLh in the presence of TAPI-1, an inhibitor of Tnf converting enzyme (Tace), also known as a disintegrin and metalloproteinase (Adam17), that has recently been shown to inhibit Tnf secretion from trout macrophages [23]. Interestingly, TAPI-1 (50 μM) completely ($p < 0.05$) blocked the effect of sLh on GVBD, whereas TAPI-1 alone had no effect on GVBD (Fig. 1A). In view of these results, we directly investigated whether rtTnf can affect oocyte maturation *in vitro*. Our results showed that incubation of trout ovarian follicles with rtTnf (1, 10 and 50 ng/ml) stimulated GVBD (significantly at 50 ng/ml; $p < 0.05$) (Fig. 1B), clearly indicating that Tnf can stimulate oocyte maturation in trout, although with less potency than sLh.

Since it is well known that in salmonid fish Lh stimulates oocyte maturation indirectly by stimulating the production of 17,20β-P [20], we hypothesized that rtTnf might exert its stimulatory effects on oocyte maturation by regulating the production of 17,20β-P in trout ovarian follicles. First, we investigated the effects of TAPI-1 on sLh-induced production of 17-OHP, the steroid precursor of the maturation-inducing steroid 17,20β-P, by trout ovarian follicles. TAPI-1 (50 μM) completely ($p < 0.05$) blocked the stimulatory effects of sLh (25 ng/ml) on 17-OHP (Fig. 2A) and 17,20β-P production (Fig. 2B). More importantly, rtTnf (50 ng/ml) significantly ($p < 0.05$) stimulated the production of 17-OHP (Fig. 2A) and 17,20β-P (Fig. 2B) by trout ovarian follicles at levels comparable to those induced by sLh. Therefore, these results suggest that rtTnf directly stimulates oocyte maturation in trout follicles by stimulating the production of the maturation-inducing hormone. Furthermore, the observation that the Tnf secretion inhibitor TAPI-1 was able to completely block the stimulatory effects of sLh on oocyte maturation and 17,20β-P production raised the question whether Tnf could be produced by the trout ovarian follicle in response to sLh. In order to address this question, we performed Western blot analyses of media samples from trout ovarian follicles incubated in the absence or presence of sLh and with sLh and TAPI-1 to determine the amount of Tnf protein secreted into the media. As shown in Fig. 3, sLh (25 ng/ml) strongly stimulated the secretion of Tnf (3.96 ± 0.68 fold) after 16 h of incubation. However, when the Tace inhibitor (TAPI-1 at 50 μM) was added, the effect of sLh to induce the secretion of Tnf to the medium was completely abrogated, even below control levels (Fig. 3). Interestingly, Tnf protein was also detectable in media incubates from control ovarian follicles indicating that, despite the much lower band intensity when compared to sLh treatment (Fig. 3), low basal levels of Tnf are actually being secreted by trout ovarian follicles. This observation supports the notion that Tnf may be playing a normal physiological function in the trout ovary.

Effects of Tnf on ovarian gene expression

In order to further investigate the possible involvement of Tnf in mediating the effects of Lh on oocyte maturation we analyzed changes in gene expression by qPCR in brown trout follicles. We specifically investigated if Lh can exert at least some of its effects through Tnf by inducing the expression of *tnf* and of *tace/adam17*, the gene coding for the enzyme that cleaves the immature form of Tnf, leading to the secretion of the soluble protein, and that is the target of TAPI-1. In addition, we investigated if Tnf can stimulate the expression of genes involved in the steroidogenic action of Lh leading to the production of 17,20β-P: steroidogenic acute regulatory protein (*star*), cholesterol side-chain cleavage P450 cytochrome (*p450scc*) and, most importantly, *cbr1*. Finally, we investigated whether Tnf and Lh can regulate the expression of P450 aromatase (*cyp19a1*) and a membrane progesterone receptor (*pgmrc1*). Gene expression analyses were performed in whole follicles in order to determine the overall effects of sLh and rtTnf in brown trout follicles as well as in isolated theca and granulosa layers in order to determine the site of action of sLh and rtTnf within the follicle wall.

We first observed in whole follicles that sLh (25 ng/ml) significantly ($p < 0.05$) stimulated the expression of *tnfh* (Fig. 4A). TAPI-1 significantly blocked the stimulatory effect of sLh on *tnf* expression and rtTnf (50 ng/ml) did not have any significant effect (Fig. 4A). In addition, we observed a significant increase in the mRNA expression of *tace/adam17* after sLh and rtTnf treatments (Fig. 4B) and TAPI-1 decreased, although not significantly, the stimulatory effect of sLh on *tace/adam17* expression (Fig. 4B). Importantly, the expression of *cbr1* was significantly ($p < 0.05$) stimulated by sLh and this effect was significantly blocked by TAPI-1 (Fig. 4C). Furthermore, rtTnf strongly stimulated (over 20-fold) *cbr1* expression in whole follicles (Fig. 4C). These results clearly suggest that rtTnf can mediate, at least in part, the Lh-induced expression of *cbr1*, the enzyme responsible for 17,20β-P production. In addition, the expression of *p450scc* and *star* was significantly stimulated ($p < 0.05$) by sLh (Figs. 4D and E) and TAPI-1 had little effect, only partially blocking the sLh effects on *star* expression as suggested by the loss of the stimulatory effects of sLh on *star* expression in the presence of TAPI-1 (Fig. 4E). rtTnf increased the expression of *p450scc* (Fig. 4D) but not of *star* (Fig. 4E). Interestingly, sLh and rtTnf increased the expression of the Lh receptor (*lhr*) and TAPI-1 completely blocked the sLh-induced effects (Fig. 4F), suggesting a possible role for Tnf in the responsiveness to Lh by the brown trout follicle.

In isolated theca layers, similarly to whole follicles, treatment with sLh significantly ($p < 0.05$) increased the expression of *tnf* and *tace/adam17* (Figs. 5A and B). Moreover, TAPI-1 significantly blocked the sLh-induced *tace/adam17* expression (Fig. 5B) and, although it did not significantly block the sLh-induced *tnf* expression, TAPI-1 caused sLh to lose its stimulatory effects on *tnf* expression (Fig. 5A). rtTnf induced the expression of *tace/adam17* but not of *tnf* (Figs. 5A and B). Furthermore, sLh and rtTnf significantly stimulated the expression of *cbr1* and TAPI-1 decreased, although not significantly, the stimulatory effect of sLh (Fig. 5C). As in whole follicles, rtTnf stimulated the expression of *p450scc* (Fig. 5D) but not of *star* (Fig. 5E). TAPI-1 blocked the stimulatory effects of sLh on *p450scc* expression (Fig. 5D) and decreased, although not significantly, the sLh effect on *star* expression (Fig. 5E). The expression of *lhr* was significantly stimulated by sLh and rtTnf and TAPI-1 did not significantly alter the sLh effects (Fig. 5F).

In contrast with the observed expression of *tnf* in whole follicles and theca layers, its expression was not detectable in granulosa layers (Fig. 6A). The expression of *tace/adam17* was stimulated by sLh in granulosa layers and this effect was completely blocked by TAPI-1 (Fig. 6B). However, rtTnf did not affect *tace/adam17* expression (Fig. 6B). As expected, the expression of *cbr1* was stimulated by sLh and rtTnf in granulosa layers; however, TAPI-1 did not significantly alter the sLh-induced effects (Fig. 6C). As in whole follicle and theca layers, sLh treatment stimulated the expression of *p450scc* (Fig. 6D) and *star* (Fig. 6E) in granulosa layers, whereas rtTnf only stimulated *p450scc* expression (Fig. 6D). However, TAPI-1 did not alter the stimulatory effects of sLh on *p450scc* and *star* expression (Figs. 6D and E). The expression of *lhr* was stimulated by sLh but not by rtTnf and TAPI-1 in the presence of sLh decreased, although not significantly with respect to sLh alone, the stimulatory effects of sLh (Fig. 6F).

Given that oocyte maturation in teleosts is inhibited by estrogens [27-29] and is associated with a decrease in P450 aromatase expression and activity [3], we investigated if Lh and Tnf can regulate the expression of *cyp19a1*, the enzyme responsible for the production of 17β-estradiol, in the brown trout ovary. In whole follicles, sLh and rtTnf significantly inhibited *cyp19a1* expression and incubation with TAPI-1 significantly reversed the inhibitory effects of sLh (Fig. 7A). Very similar results on the regulation of *cyp19a1* by rtTnf and sLh were found in granulosa layers (Fig. 7C) but not in theca layers (Fig. 7E), were no changes were observed under any of the treatments. These results suggest that Lh inhibits *cyp19a1* expression primarily in granulosa layers and that Tnf is a possible mediator of the inhibitory effects of Lh on *cyp19a1* expression. On the other hand, the expression of the membrane progesterone receptor *pgmrc1*, a non-classical single transmembrane progesterone receptor [30] whose expression was shown to be localized to the oocyte in rainbow trout vitellogenic follicles [31], was significantly induced by sLh in brown trout whole follicles, as well as in theca and granulosa layers (Figs. 7B, D and F). Interestingly, *pgmrc1* expression was not affected by rtTnf in whole follicles, granulosa or theca layers and TAPI-1 did not modulate the stimulatory effects of sLh (Figs. 7D and 7F), suggesting that Lh stimulates *pgmrc1* expression without the involvement of Tnf.

Discussion

In fish, like in other vertebrates, Lh is the hormone responsible for final oocyte maturation and for the acquisition of oocyte competence and ovulation [1, 3]. However, studies in mammals point to the possible involvement of ovarian cytokines such as IL1B and TNF on oocyte maturation [32-34]. An ovarian role for TNF is supported by the reported expression of TNF and its receptors at the mRNA and protein levels [35] and the presence of TNF receptor superfamily members [36, 37] in mature mammalian follicles. Since Tnf and Tnf receptors have been detected in the trout ovary [14, 15] and given that trout preovulatory follicles respond to trout Tnf of recombinant origin by regulating follicle contraction, steroid production, granulosa cell apoptosis and gene expression [16], we hypothesized that Tnf could also be involved in the regulation of oocyte maturation in the trout ovary. Specifically, the purpose of this study was to investigate the direct effects of Tnf and its possible mediatory role on Lh action on oocyte maturation in brown trout ovarian follicles. The results from the present study show that rtTnf is able to directly stimulate GVBD *in vitro* in brown trout follicles. To the best of our knowledge, these results provide the first demonstration for the involvement of Tnf in the control of oocyte maturation in teleosts, as has been shown in some studies in mammals [10, 38]. The induction of oocyte maturation by Tnf in brown trout follicles is supported by our results on the ability of

rtTnf to increase the *in vitro* production of 17,20β-P, the MIS for this species, and the expression of *cbr1*, the enzyme responsible for its production. Moreover, the inhibition of P450 aromatase (*cyp19a1*) expression by rtTnf is consistent with the maturational effects of this cytokine. In addition, as suggested in a previous study [16], Tnf may also stimulate earlier steroidogenic events in brown trout ovarian follicles as evidenced by the stimulation of the production of 17-OHP, the precursor of 17,20β-P, and the expression of *p450scc* by rtTnf. In mammals, the role of TNF in steroidogenesis has been studied in several species [9, 37, 39]. In rat preovulatory follicles, TNF increases progesterone production [40, 41]. Furthermore, TNF stimulates progesterone, estradiol and androstenedione production in incubated preovulatory follicles of the adult cycling rat [42]. In contrast, other studies revealed an inhibitory effect of TNF on progesterone production in porcine follicles [43] and bovine granulosa cells [44], suggesting that TNF may have stimulatory or inhibitory actions depending on the type of ovarian cells and the species studied. In view of the results from the present study, we can reasonably deduce that Tnf may induce oocyte maturation in brown trout follicles by stimulating the production of the MIS (Fig. 8).

Interestingly, very similar effects to those of rtTnf on GVBD, steroid production and follicle gene expression were also observed when brown trout follicles were incubated with sLh, supporting the results of previous studies from our laboratory in this and other salmonid species [16, 20, 25]. The possibility of the existence of a causal relationship between the effects of sLh and rtTnf on oocyte maturation in the brown trout is suggested by two lines of evidence. First, sLh stimulates the expression of *tnf* in brown trout follicles. Analyses of *tnf* expression in isolated brown trout theca and granulosa layers show that *tnf* is expressed in theca layers and not in granulosa layers, suggesting that the site of Tnf production in the brown trout follicle is the theca layer. However, the specific cell type responsible for the production of Tnf within the trout ovary is not known yet. Moreover, sLh stimulates the expression of *tace/adam17* in both theca and granulosa layers, an enzyme that sheds membrane-anchored Tnf thus releasing the mature, soluble form of Tnf, as shown in trout macrophages [23]. Importantly, we show here that sLh stimulates the secretion of Tnf by brown trout follicles. Therefore, we propose that sLh induces the secretion of Tnf from theca layers due to its stimulation of the expression of *tnf* in theca layers as well as of *tace/adam17* in theca and granulosa layers (Fig. 8). Interestingly, rtTnf itself stimulates the expression of *tace/adam17* in theca, but not granulosa layers, suggesting that Tnf could be involved in stimulating its own secretion. Second, incubation of brown trout follicles with sLh in the presence of TAPI-1, a known inhibitor of Tace/Adam17 that has recently been shown to effectively inhibit Tace-like activity and Tnf secretion in trout macrophages [23], results in the abrogation of the stimulatory effects of sLh on Tnf secretion and oocyte maturation. Specifically, inhibition of Tnf secretion by TAPI-1 completely blocks sLh-induced GVBD, 17,20β-P production and whole follicle *cbr1* expression, as well as sLh-inhibited *cyp19a1* expression, therefore suggesting that Tnf secretion may be necessary for Lh to exert its maturational actions in the brown trout follicle. Therefore, these results combined lead us to propose that Tnf may represent an intra-ovarian mediator of the effects of Lh on oocyte maturation in brown trout follicles (Fig. 8). To the best of our knowledge, these results constitute the first direct demonstration in any vertebrate species that Lh stimulates the intra-ovarian production of Tnf leading to the stimulation of oocyte maturation.

Although we have shown that inhibition of Tace/Adam17 by TAPI-1 results in the complete inhibition of sLh-induced secretion of Tnf and of most of the effects of sLh that are independently stimulated by Tnf, thereby suggesting that TAPI-1 is inhibiting the shedding of Tnf in brown trout ovarian follicles, it must be recognized that pro-Tnf is not the only known substrate of Tace/Adam17. Most notably, TAPI-1 can also shed ligands of the epidermal growth factor (EGF) family such as the EGF-like growth factors pro-amphiregulin and pro-epiregulin [45], known to be involved in the stimulation of oocyte maturation by LH in the mammalian ovary [46]. Despite recent evidence for estrogens inhibiting oocyte maturation in zebrafish by activating EGF receptor signaling through binding to a membrane estrogen receptor (Gper) [47], the possible role of EGF-like ligands in the regulation of oocyte maturation in teleosts is virtually unexplored. Consequently, we cannot rule out the possibility that part of the Lh maturational activity that is blocked by TAPI-1 could be also the result of the inhibition of shedding of EGF-like ligand(s). In fact, we have data showing that sLh stimulates the expression of pro-epiregulin in brown trout follicles (Crespo and Planas, unpublished observations) independently of Tnf, supporting a possible stimulatory role of EGF-like ligands on oocyte maturation in fish. Overall, these data support the notion that the Tnf as well as other signaling pathways (e.g. EGF receptor, inhibin, insulin-like growth factors, transforming growth factor α, etc), some of which have been demonstrated to operate in zebrafish [48], could converge to induce oocyte maturation in teleosts, as has been suggested in mammals.

The two major differences between the effects of sLh and rtTnf on gene expression reside on the ability of sLh, but not rtTnf, to stimulate the expression of *star*, a cholesterol-transport molecule that constitutes one of the key limiting steps in the production of steroids [49], and *pgmrc1*, a membrane progesterone receptor believed to be involved in mediating the anti-apoptotic effects of progesterone in the ovary [30]. While the stimulation of *star*, *p450scc* and *cbr1* expression by sLh agree perfectly with the idea posed by Nakamura et. al. [5] that the increased production of 17,20β-P in post-vitellogenic trout follicles is dependent on increased Star activity, that does not appear to be the case in the rtTnf-induced 17,20β-P production. Since rtTnf stimulates *p450scc* and *cbr1* expression in brown trout follicles, it is possible that Tnf may act in concert with Lh to modify cholesterol that has entered the mitochondria as a result of Lh-induced Star activity. On the other hand, the observed stimulation of the expression of *pgmrc1* by sLh is interesting in the light of the maturational effects of Lh shown in the present study. Although membrane progesterone receptor α (mPgr) is believed to be the main receptor mediating the stimulation of oocyte maturation by the MIS [30], there is no evidence to date linking *pgmrc1* and Lh- and/or MIS-induced oocyte maturation. In the absence of known sequences for mPgr in salmonid fish, further studies will be needed, first, to identify mPgr in trout and investigate the regulation of its expression and, second, to establish if Lh-induced *pgmrc1* expression is necessary for the induction of oocyte maturation by MIS.

The maturational effects of Lh in the salmonid ovary are believed to take place through the binding of Lh to specific Lh receptors that appear at the time of oocyte maturation [50, 51]. At the molecular level, few studies have focused on the expression of *lhr* in the salmonid ovary. In female amago salmon (*Oncorhynchus rhodurus*) [52], rainbow trout (*Oncorhynchus mykiss*) [17, 53] and Atlantic salmon (*Salmo salar*) [54], *lhr* has been shown to expressed in the ovary during sexual maturation. Recently, *lhr* expression was shown to be induced by follicle-stimulating hormone (FSH) in coho salmon (*Oncorhynchus kisutch*) previtellogenic follicles [55]. However,

no information is available to date regarding the hormonal regulation of *lhr* in the salmonid ovary during final oocyte maturation, when plasma Lh levels are elevated [56-60]. In the present study, we provide evidence for the stimulation of the *in vitro* expression of *lhr* by Lh in both theca and granulosa layers. Although receptors for Lh have been only identified in granulosa layers from coho salmon preovulatory follicles by radiolabeled ligand binding studies [50, 51], our results support recent observations in the Atlantic salmon ovary [54] that expression of *lhr*, as assessed by *in situ* hybridization, is found in both theca and granulosa layers. However, since the manual separation of the follicular layers may yield theca layers with approximately a 10% contamination of granulosa cells [20], we cannot rule out the possibility that at least part of the expression of *lhr* in theca layers may have resulted from contaminating granulosa cells. Importantly, the results from the present study represent the first demonstration that Lh stimulates the ovarian expression of its own receptor during the period of sexual maturation in teleosts. Moreover, our results suggest that Tnf may mediate the stimulatory effects of Lh on *lhr* expression in the trout ovary, as shown by the ability of rtTnf to increase *lhr* expression (specifically in theca layers) and by the ability of TAPI-1 to block sLh-induced *lhr* expression in whole follicles. Therefore, we believe that Lh, possibly through the production of Tnf, could be participating in the control of oocyte maturation by sensitizing the ovarian follicles to its own biological activity. It would also be interesting to determine whether Lh stimulates the expression and the number of ovarian receptors for Tnf in trout. However, these studies must await a precise characterization of the specific receptors mediating the biological action of Tnf in trout since the classical Tnf receptors (Tnfr1 and Tnfr2) have not yet been identified in any salmonid species.

A two-cell type model involving theca and granulosa cells has been proposed in the salmonid ovary to account for the production of 17,20β-P [2, 61]. According to this model, during final oocyte maturation the theca cell layer, in response to Lh, produces 17-OHP that diffuses through the basal lamina and is converted to 17,20β-P in the granulosa cell layer as a result of the stimulation of Cbr1 activity by Lh. However, here we show that *cbr1* is expressed in brown trout theca and granulosa layers and that *cbr1* expression in theca layers is induced by sLh (5-fold increase), albeit not as strongly as in granulosa layers (25-fold increase). Two closely related *cbr1* cDNAs (98.7% homology at the protein level) were previously identified in rainbow trout ovarian follicles but only one had Cbr1 activity [62]. Given that the primers used in the present study to amplify *cbr1* in brown trout follicles detect both forms of Cbr1, with the available data it is not possible to determine if *cbr1* expressed in brown trout theca layers has Cbr1 activity. Further studies should examine if *cbr1* expression in theca layers correlates with the ability of theca layers to convert 17-OHP to 17,20β-P. As indicated above for the expression of *lhr* in theca layers, it is also possible that the detection of Lh-induced *cbr1* expression in theca layers could be the result, at least in part, of contamination with granulosa cells. Studies on the localization of *cbr1* expression in the trout ovary by *in situ* hybridization should help resolve this issue. In contrast to *cbr1* expression, *p450scc* and *star,* two genes involved in the *de novo* synthesis of steroids (and expected to reside only in thecal cells), were expressed in granulosa layers and were induced by Lh at slightly higher levels than in theca layers. Although we cannot rule out the possibility that these observations were caused by contamination of granulosa layers by thecal cells, further studies on the localization of these genes in the trout ovary should be performed to shed light onto the cellular origin of steroidogenic enzyme activity in the trout ovary.

In summary, the results of the present study clearly indicate that Tnf can directly stimulate oocyte maturation through the stimulation of the production of 17,20β-P in brown trout follicles. Due to the stimulatory effects of Lh on Tnf secretion and the follicular expression of *tnf* and *tace/adam17*, on one hand, and to the abrogation of the maturational effects of Lh by an inhibitor of Tnf secretion, on the other, we can conclude that Lh may control oocyte maturation in brown trout follicles through the production of Tnf (Fig. 8). Therefore, Tnf may represent an intra-ovarian mediator of the stimulatory effects of Lh on oocyte maturation in the brown trout ovary. Overall, our results further highlight the complex orchestration of signaling events triggered by Lh in its control of oocyte maturation in vertebrates.

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Figure legends

FIG. 1. Regulation of oocyte maturation by Lh and Tnf in brown trout ovarian follicles. In A, the *in vitro* effects of salmon Lh (sLh; 25 ng/ml) in the absence or presence of TAPI-1 (50 μM), an inhibitor of Tnf secretion, are shown. In B, the *in vitro* effects of recombinant trout Tnf (rtTnf; 1, 10 and 50 ng/ml) are shown. Oocyte maturation, as indicated by germinal vesicle breakdown (GVBD), was determined after 48 h at 15ºC. Each bar represents the mean ± SEM of four independent experiments, each with ovarian tissue from a separate female and assayed in triplicate. Statistically significant ($p < 0.05$) differences with respect to the control group are indicated by an asterisk (*) and between the sLh and sLh+TAPI-1 groups by a pound symbol (#).

FIG. 2. Regulation of steroid production by Lh and Tnf in brown trout ovarian follicles. Brown trout ovarian follicles were incubated with salmon Lh (sLh; 25 ng/ml) in the absence or presence of TAPI-1 (50 μM), an inhibitor of Tnf secretion, or with recombinant trout Tnf (rtTnf; 50 ng/ml) for 48 h at 15°C. At the termination of the incubation period, 17α-hydroxyprogesterone (17-OHP) (A) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) (B) were measured in the incubation medium. 17-OHP and 17,20β-P levels were analyzed using a commercial radioimmunoassay (RIA) and enzyme immunoassay (EIA), respectively. Each bar represents the mean ± SEM of 4 independent experiments, each performed with ovarian tissue from a separate female and assayed in triplicate. Values are expressed as fold change with respect to the control, which has been set at 1 (basal levels of 17-OHP and 17,20β-P are 129.5 ± 28.9 and 988.8 ± 199.4 pg/ml, respectively). Statistically significant ($p < 0.05$) differences with respect to the control group are indicated by an asterisk (*) and between the sLh and sLh+TAPI-1 groups by a pound symbol (#).

FIG. 3. Ovarian Tnf secretion in brown trout follicle incubates. Preovulatory trout follicles were incubated with sLh (25 ng/ml) in the absence or presence of TAPI-1 (50 μM) for 16 h at 15°C. Tnf secretion in the medium was analyzed by Western blot at the end of the incubation period. Each bar represents the mean ± SEM of three independent experiments, each with ovarian tissue from a separate female. The results are expressed as mean of fold change with respect to the control. * indicates significantly ($p < 0.05$) different from control group. # indicates significantly ($p < 0.05$) different from the sLh group. A representative Western blot is shown in the graph.

FIG. 4. Effects of Lhand Tnf on gene expression in brown trout ovarian follicles. Brown trout ovarian follicles were incubated with salmon Lh (sLh; 25 ng/ml) in the absence or presence of TAPI-1 (50 μM), an inhibitor of Tnf secretion, or with recombinant trout Tnf (rtTnf; 50 ng/ml) for 48 h at 15°C. The relative expression of *tnf*\ (A), *tace/adam17* (B), *cbr1* (C), *p450scc* (D), *star* (E) and *lhr* (F) was determined by real-time PCR (qPCR) and normalized to the abundance of *18s*. The results from ovarian tissue from 3 separate brown trout females are expressed as mean of fold change ± SEM ($n = 3$) with respect to the control, which has been set at 1. Statistically significant ($p < 0.05$) differences with respect to the control group are indicated by an asterisk (*) and between the sLh and sLh+TAPI-1 groups by a pound symbol (#).

FIG. 5. Effects of Lh and Tnf on gene expression in theca layers from brown trout ovarian follicles. Brown trout ovarian follicles were incubated with salmon Lh (sLh; 25 ng/ml) in the absence or presence of TAPI-1 (50 μM), an inhibitor of Tnf secretion, or with recombinant trout Tnf (rtTnf; 50 ng/ml) for 48 h at 15ºC and, subsequently, theca layers were manually separated. The relative expression of *tnf* (A), *tace/adam17* (B), *cbr1* (C), *p450scc* (D), *star* (E) and *lhr* (F) in isolated theca layers was determined by real-time PCR (qPCR) and normalized to the abundance of *18s*. The results from ovarian tissue from 3 separate brown trout females are expressed as mean of fold change ± SEM (n = 3) with respect to the control, which has been set at 1. Statistically significant ($p < 0.05$) differences with respect to the control group are indicated by an asterisk (*) and between the sLh and sLh+TAPI-1 groups by a pound symbol (#).

FIG. 6. Effects of Lh and Tnf on gene expression in granulosa layers from brown trout ovarian follicles. Brown trout ovarian follicles were incubated with salmon Lh (sLh; 25 ng/ml) in the absence or presence of TAPI-1 (50 μM), an inhibitor of Tnf secretion, or with recombinant trout Tnf (rtTnf; 50 ng/ml) for 48 h at 15ºC and, subsequently, granulosa layers were manually separated. The relative expression of *tnf* (A), *tace/adam17* (B), *cbr1* (C), *p450scc* (D), *star* (E) and *lhr* (F) in isolated theca layers was determined by real-time PCR (qPCR) and normalized to the abundance of *18s*. The results from ovarian tissue from 3 separate brown trout females are expressed as mean of fold change ± SEM (n = 3) with respect to the control, which has been set at 1. Statistically significant ($p < 0.05$) differences with respect to the control group are indicated by an asterisk (*) and between the sLh and sLh+TAPI-1 groups by a pound symbol (#). ND indicates no detectable expression.

FIG. 7. Effects of Lh and Tnf on the expression of aromatase and a membrane progesterone receptor in ovarian follicles and in theca and granulosa layers from brown trout. Brown trout ovarian follicles were incubated with salmon Lh (sLh; 25 ng/ml) in the absence or presence of TAPI-1 (50 μM), an inhibitor of Tnf secretion, or with recombinant trout Tnf (rtTnf; 50 ng/ml) for 48 h at 15ºC and, subsequently, theca and granulosa layers were manually separated, The relative expression of *cyp19a1* and *pgmrc1* in whole follicles (A and B), isolated theca (C and D) and granulosa layers (E and F) was determined by real-time PCR (qPCR) and normalized to the abundance of *18s*. The results from ovarian tissue from 3 separate females are expressed as mean of fold change ± SEM (n = 3) with respect to the control, which has been set at 1. The expression of *cyp19a1* in granulosa layers (E) was detectable in only one female (n = 1). Statistically significant ($p < 0.05$) differences with respect to the control group are indicated by an asterisk (*) and between the sLh and sLh+TAPI-1 groups by a pound symbol (#).

FIG. 8. Proposed model for a possible mediatory role of Tnf on Lh-induced oocyte maturation in the trout ovarian follicle. The results from the present study suggest that Lh stimulates oocyte maturation (GVBD) in trout follicles, at least in part, through the production of Tnf. Lh increases the expression of *tnf* in theca layers and of *tace/adam17* in theca and granulosa layers and also the secretion of the Tnf protein. Tnf in turn stimulates GVBD by stimulating the production of 17α-hydroxyprogesterone (17-OHP) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), the maturation-inducing steroid for this species, as well as increasing the expression levels of *p450scc* and *cbr1* and decreasing the expression levels of *cyp19a1*. Lh, in a process possibly mediated by Tnf, also increases the expression of *lhr* in theca and granulosa layers. GV, germinal vesicle.

Tables

TABLE 1. Nucleotide sequence of real-time PCR (qPCR) primers used in gene expression analyses, amplicon size (base pairs, bp) and associated accession numbers from GenBank.

GenBank accession	Gene name	Primer sequence (5´-3´)	Amplicon size (bp)
AJ278085	Tumor necrosis factor α (*tnf*)	(F) AGCATGGAAGACCGTCAA (R) TTCGTTTACAGCCAGGCT	271
DY696186	TNFα converting enzyme (*tace/adam17*)	(F) TGGTGGTCTTCTCGTTGGTCTTC (R) TCCACACAGTGAACCAGGATGC	58
AF100931	20β-hydroxysteroid dehydrogenase (*cbr1*)	(F) CTACAAGCCAAGTTCCGTGATAC (R) TGTGCCATAGGCTGTGTTTGGC	129
S57305	Cholesterol side-chain cleavage cytochrome P450 (*p450scc*)	(F) AACGCTGAGGCTTCATCCAGTT (R) ACCAGAGTCCCACAAGGTATGT	96
AB047032	Steroidogenic acute regulatory protein (*star*)	(F) ACATGACAGGATTGAGGAAG (R) CATGGCTGATCCAGCTGCTA	93
AF439404	Luteinizing hormone receptor (*lhr*)	(F) GGACAATCTGAAATGGCACCCTAT (R) TCCCATCTGAACAATAACCTCCC	185
AY427786	P450 aromatase (*cyp19a1*)	(F) GTGCCGAAGGGGACAAACATC (R) CGAAAGGCTGGAAGAAACGATTAG	130
AY069921	Progesterone membrane receptor component 1 (*pgmrc1*)	(F) CCCAACCAAGCAGAGAGAAA (R) AGAGGCCAAGCAGACTCAAA	116
AF308735	18S ribosomal RNA (*18s*)	(F) CGGAGGTTCGAAGACGATCA (R) TCGCTAGTTGGCATCGTTTAT	62

F: forward, R: reverse.

Figure 1

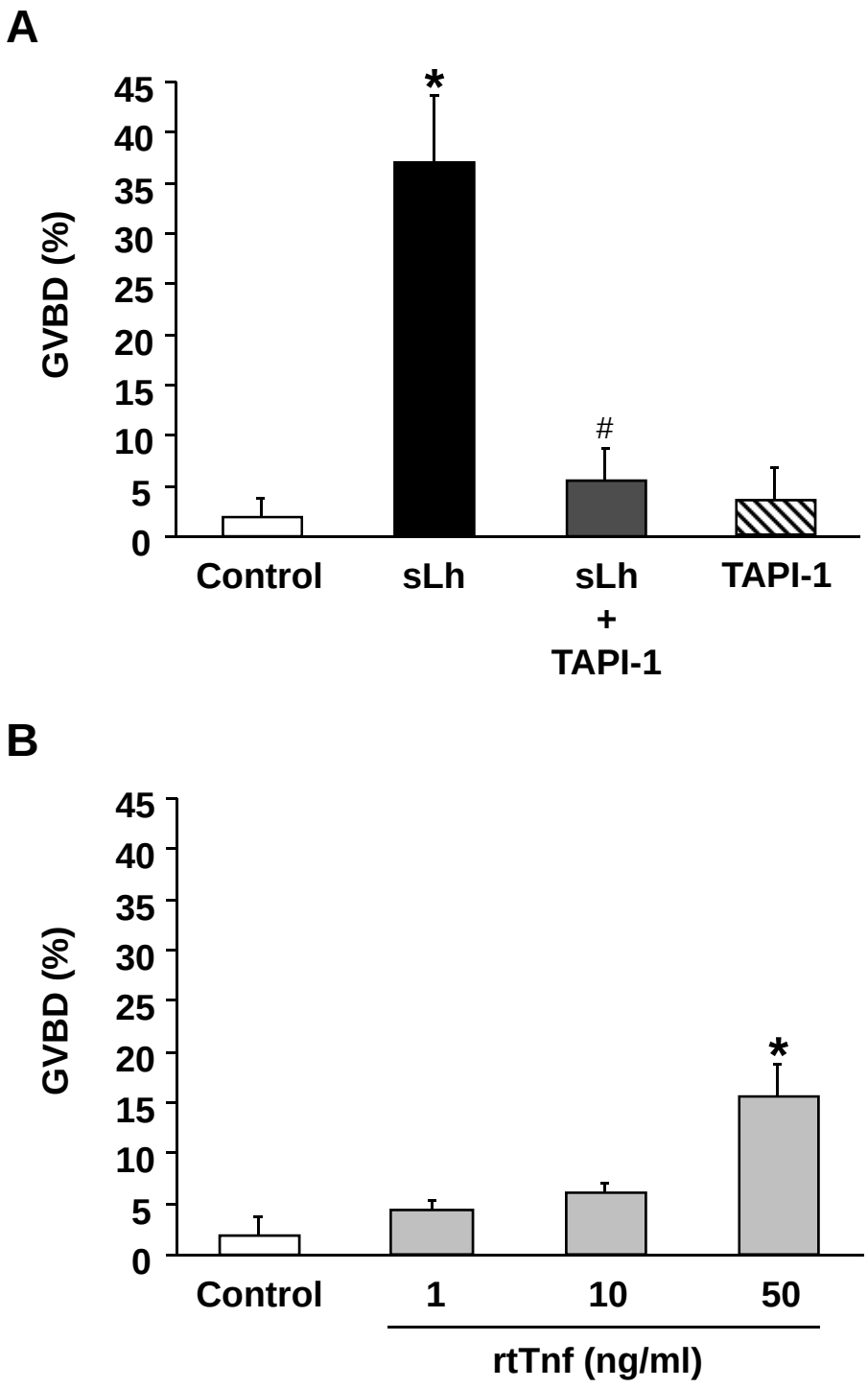

A
GVBD (%)
45
40
35
30
25
20
15
10
5
0
*
#
Control
sLh
sLh
+
TAPI-1
TAPI-1
B
GVBD (%)
45
40
35
30
25
20
15
10
5
0
*
Control
1
10
50
rtTnf (ng/ml)

Figure 2

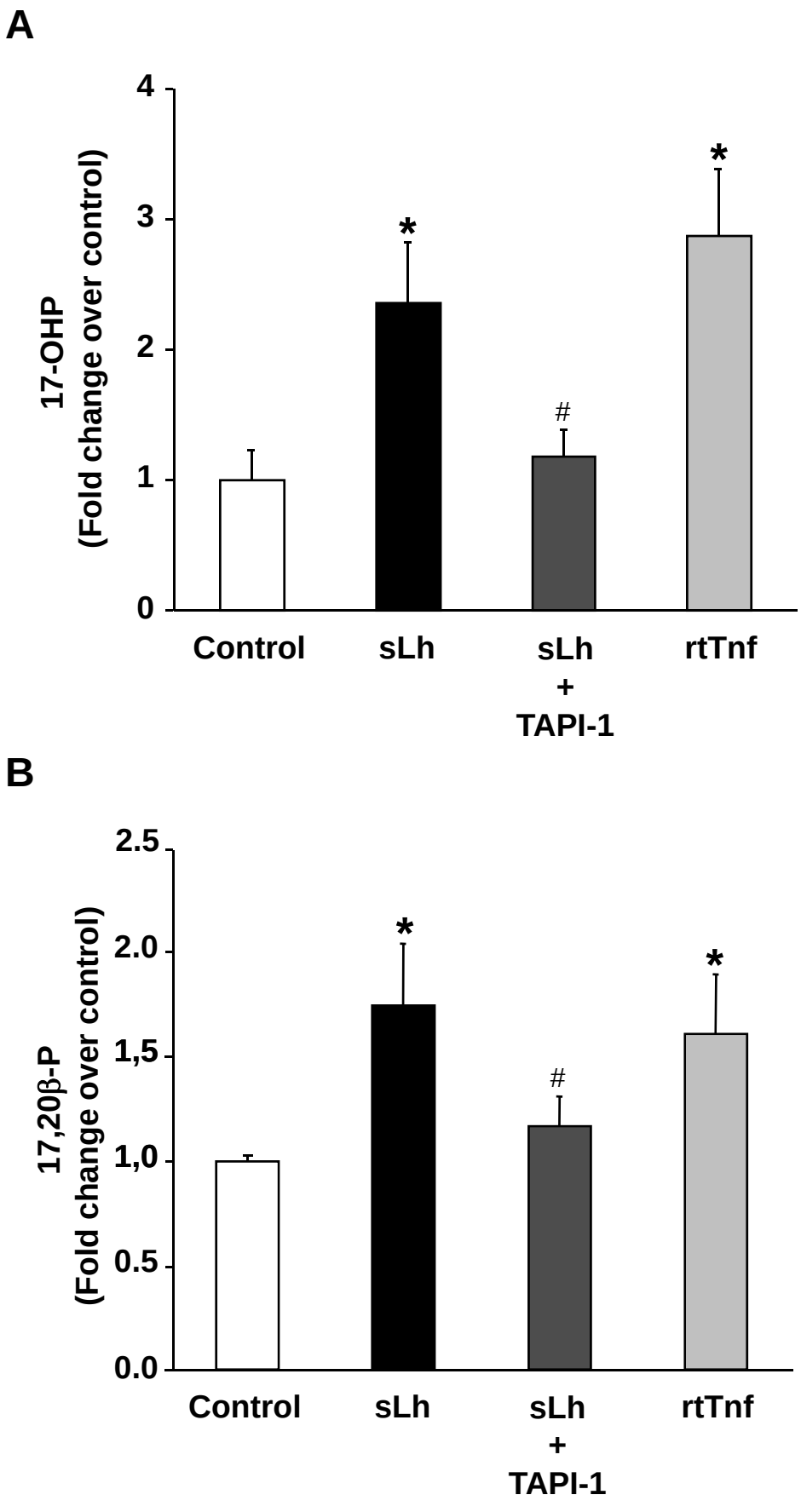

A
17-OHP
(Fold change over control)
4
3
2
1
0
*
*
#
Control
sLh
sLh
+
TAPI-1
rtTnf
B
17,20β-P
(Fold change over control)
2.5
2.0
1,5
1,0
0.5
0.0
*
*
#
Control
sLh
sLh
+
TAPI-1
rtTnf

Figure 3

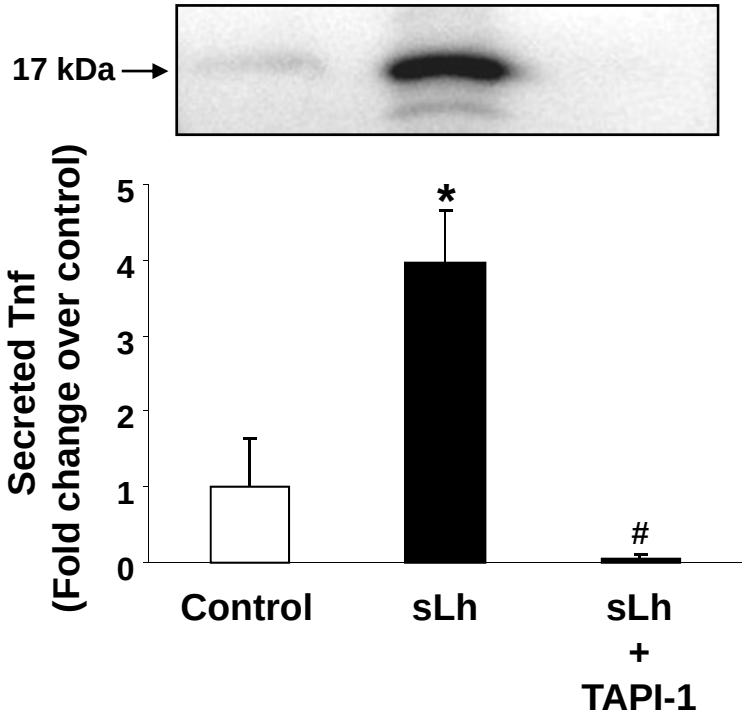

17 kDa
Secreted Tnf
(Fold change over control)
5
4
3
2
1
0
*
#
Control
sLh
sLh
+
TAPI-1

Figure 4

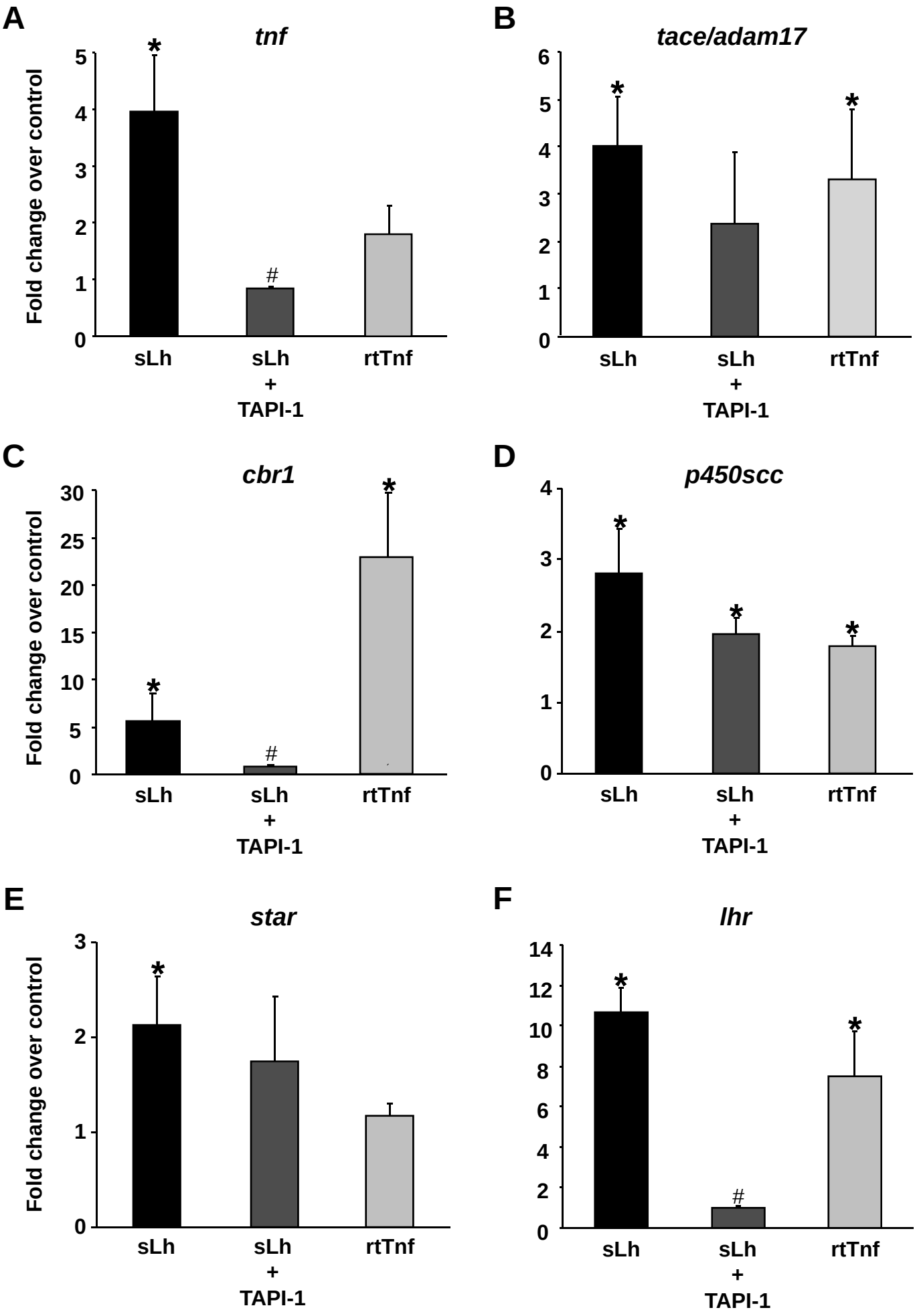

A
tnf
Fold change over control
0
1
2
3
4
5
*
#
sLh
sLh + TAPI-1
rtTnf
B
tace/adam17
0
1
2
3
4
5
6
*
*
sLh
sLh + TAPI-1
rtTnf
C
cbr1
Fold change over control
0
5
10
15
20
25
30
*
#
*
sLh
sLh + TAPI-1
rtTnf
D
p450scc
0
1
2
3
4
*
*
*
sLh
sLh + TAPI-1
rtTnf
E
star
Fold change over control
0
1
2
3
*
sLh
sLh + TAPI-1
rtTnf
F
lhr
0
2
4
6
8
10
12
14
*
#
*
sLh
sLh + TAPI-1
rtTnf

Figure 5

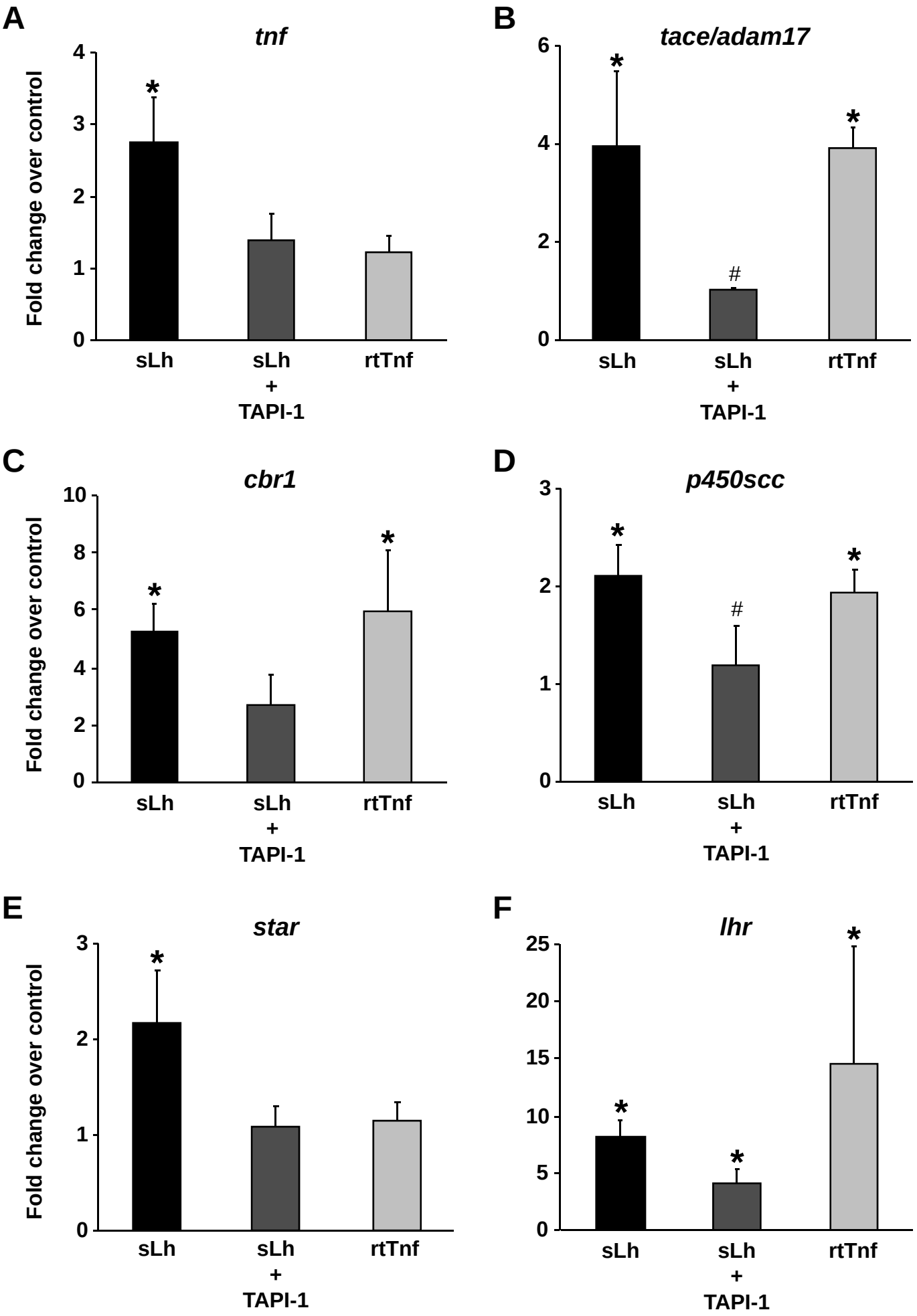

A
tnf
Fold change over control
0
1
2
3
4
*
sLh
sLh + TAPI-1
rtTnf
B
tace/adam17
0
2
4
6
*
#
*
sLh
sLh + TAPI-1
rtTnf
C
cbr1
Fold change over control
0
2
4
6
8
10
*
*
sLh
sLh + TAPI-1
rtTnf
D
p450scc
0
1
2
3
*
#
*
sLh
sLh + TAPI-1
rtTnf
E
star
Fold change over control
0
1
2
3
*
sLh
sLh + TAPI-1
rtTnf
F
lhr
0
5
10
15
20
25
*
*
*
sLh
sLh + TAPI-1
rtTnf

Figure 6

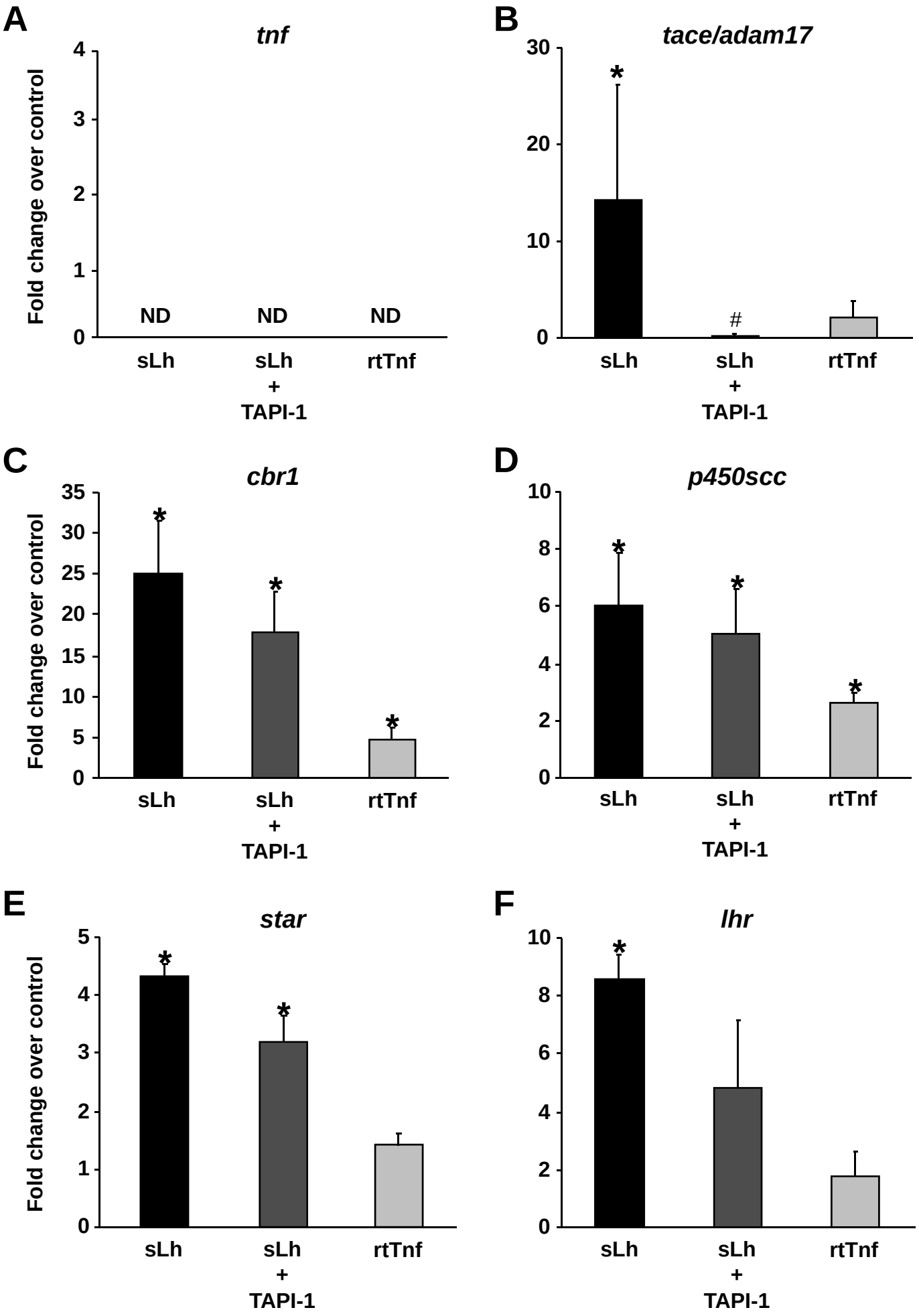

A
tnf
Fold change over control
4
3
2
1
0
ND
ND
ND
sLh
sLh + TAPI-1
rtTnf
B
tace/adam17
30
20
10
0
*
#
sLh
sLh + TAPI-1
rtTnf
C
cbr1
Fold change over control
35
30
25
20
15
10
5
0
*
*
*
sLh
sLh + TAPI-1
rtTnf
D
p450scc
10
8
6
4
2
0
*
*
*
sLh
sLh + TAPI-1
rtTnf
E
star
Fold change over control
5
4
3
2
1
0
*
*
sLh
sLh + TAPI-1
rtTnf
F
lhr
10
8
6
4
2
0
*
sLh
sLh + TAPI-1
rtTnf

Figure 7

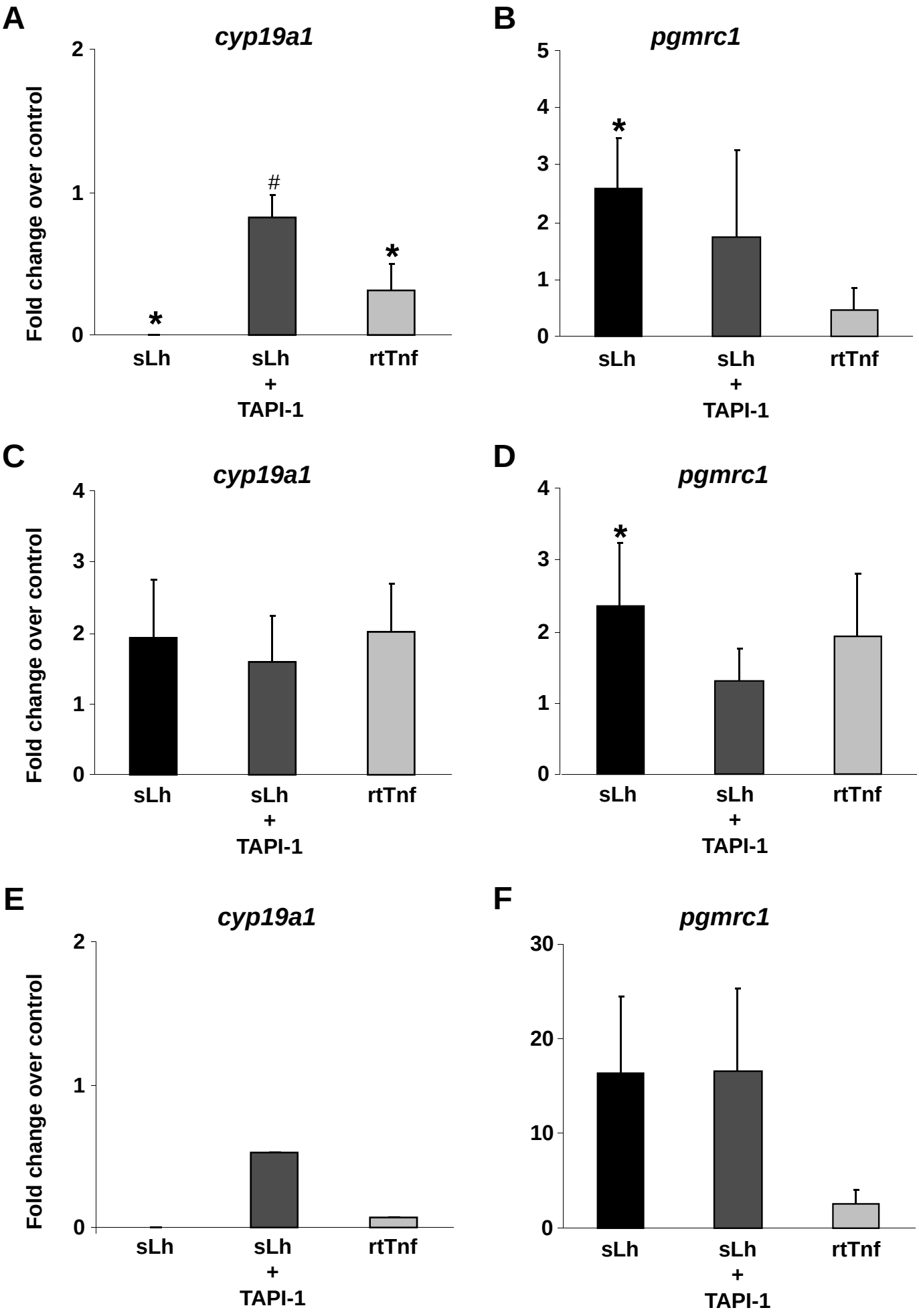
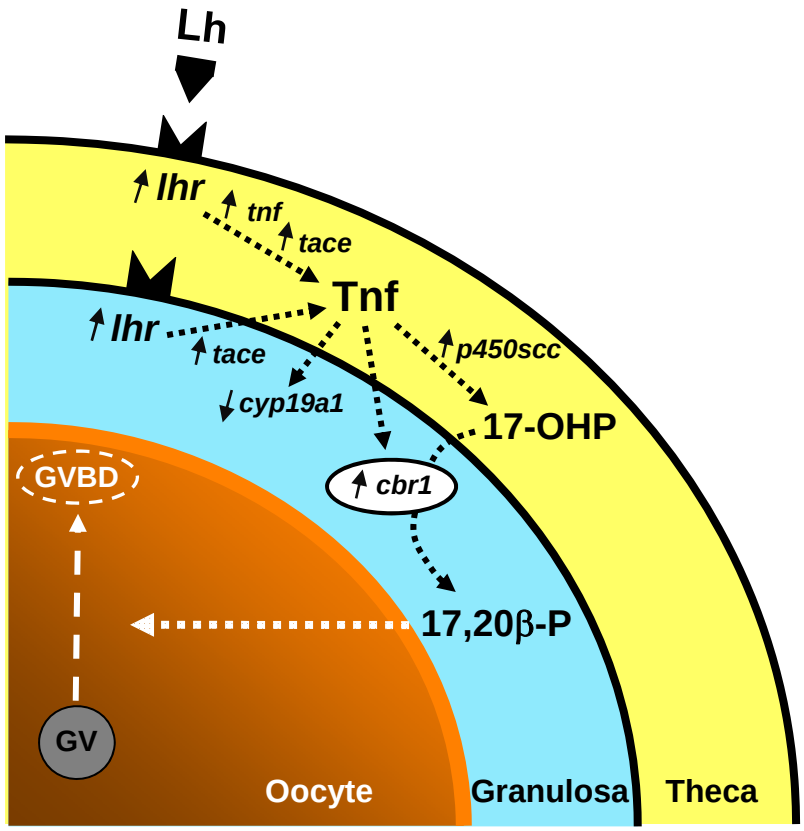

A
cyp19a1
Fold change over control
2
1
0
#
*
*
sLh
sLh + TAPI-1
rtTnf
B
pgmrc1
5
4
3
2
1
0
*
sLh
sLh + TAPI-1
rtTnf
C
cyp19a1
Fold change over control
4
3
2
1
0
sLh
sLh + TAPI-1
rtTnf
D
pgmrc1
4
3
2
1
0
*
sLh
sLh + TAPI-1
rtTnf
E
cyp19a1
Fold change over control
2
1
0
sLh
sLh + TAPI-1
rtTnf
F
pgmrc1
30
20
10
0
sLh
sLh + TAPI-1
rtTnf

Figure 8


Lh
lhr
tnf
tace
Tnf
lhr
tace
p450scc
cyp19a1
17-OHP
GVBD
cbr1
17,20β-P
GV
Oocyte
Granulosa
Theca