Tumor Necrosis Factor α Inhibits Transcriptional Activity of the Porcine P45011A Insulin-like Growth Factor Response Element*

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We investigated the effects of tumor necrosis factor α (TNFα) on the transcriptional activity of the porcine P45011A (P450scc) insulin-like growth factor response element (IGFRE). TNFα inhibited insulin-like growth factor-I (IGF-I)-stimulated P450scc mRNA concentrations in cultures of porcine granulosa cells. Transient transfection experiments in granulosa cells with deletion P450scc/luciferase constructs showed that TNFα inhibited the transcriptional activity of the IGFRE. IGF-I binding and IGF-I receptor mRNA concentrations in porcine granulosa cells were not inhibited by TNFα. Electrophoretic mobility shift assay with nuclear extract protein from porcine granulosa cells treated with IGF-I and TNFα showed that Sp1 and a second transcription factor, P2, bound to the IGFRE. While IGF-I treatment increased the binding activity of both factors, TNFα specifically inhibited the IGF-I-stimulated binding activity of P2. Transient transfection studies done in mouse fibroblasts overexpressing the IGF-I receptor (NWTb3) with the porcine IGFRE (three repeats) in an SV40/luciferase construct also showed TNFα inhibited IGF-I-stimulated reporter gene expression. We conclude that TNFα inhibits the transcriptional activity of the porcine P450scc IGFRE by preventing IGF-I-stimulated binding of P2.

Tumor necrosis factor α (TNFα) is a cytokine that regulates steroidogenesis in the ovary. Studies indicate TNFα may be a key mediator in regression of the corpus luteum (CL) (1). In the porcine ovary, specific, high affinity TNFα receptors exist on granulosa and small luteal cells (2, 3). TNFα inhibits insulin-stimulated mRNA concentrations of P-450 cholesterol side-chain cleavage enzyme, P45011A (P450scc) in porcine granulosa cells (2).

Insulin-like growth factor I (IGF-I) is a growth factor that also regulates steroidogenesis in the ovary. In porcine granulosa cells, IGF-I increases P450scc mRNA concentrations (4). Isolation of the porcine P450scc gene and transient transfection studies in porcine granulosa cells identified a 30-base pair IGF-response region (IGFRE) in the gene (5). The porcine P450scc IGFRE is a GC-rich domain that binds Sp1 and another transcription factor, P2 (6).

This study determined that TNFα inhibits the function of the porcine P450scc IGFRE in porcine granulosa by preventing IGF-I-stimulated binding of P2. This finding presents a mechanism whereby TNFα can induce luteolysis by inhibiting IGF-I-supported steroidogenesis in the CL. Moreover, it establishes P2 as the transcription factor mediating the IGF-I response.

EXPERIMENTAL PROCEDURES

Materials—The 1-kilobase porcine P450scc cDNA clone was obtained from Drs. Tom Wise and George Mulheron, U. S. Department of Agriculture (Clay Center, NE) (7). All restriction enzymes were obtained from Life Technologies, Inc. Nitrocellulose filters were obtained from Micron Separations, Inc. (Westboro, MA). Multi-prime labeling kit, [α-32P]deoxycytidine-5′-triphosphate (dCTP), and [γ-32P]deoxyadenosine-5′-triphosphate (dATP) were obtained from Amersham. Pure human recombinant IGF-I and TNFα were obtained from Bachem Inc. (Torrance, CA). Mouse fibroblast cell lines (NIH-3T3), NWTb3 (overexpressing the IGF-I receptor; Ref. 8) and K1 (containing a mutation in the tyrosine kinase domain of the IGF-I receptor; Ref. 9) were obtained from Dr. Charles Roberts, Department of Pediatrics, University of Oregon.

Reporter Gene Constructs—The reporter gene constructs –100, −130, and −2320 P450scc/luc have been described (5). Briefly, these constructs contain 5′ deletions of the upstream region of porcine P450scc, the core porcine P450scc promoter, and the entire coding region of the firefly luciferase gene with a polyadenylation tract (10). rWT pSVPLUC contains three repeats of the porcine IGFRE cloned into pSVPLUC, a modified pGEM3 plasmid containing the luciferase gene described above, and the enhancerless SV40 early region promoter (10). The plasmids were obtained from Dr. Allan Brasier, University of Texas Medical Branch, Galveston, TX.

Porcine Granulosa Cell Culture, RNA Isolation, and P450scc cDNA Hybridization—Granulosa cells were isolated from 1–5-mm follicles of ovaries from immature swine (60–70 kg). The ovaries were collected from a local slaughterhouse. The granulosa cells were plated in Eagle’s minimum essential medium and 3% fetal calf serum for 12–16 h to facilitate granulosa cell attachment to the tissue culture plates as described previously (11). After granulosa cell attachment, all culture conditions were done in serum-free medium for experiments that measured mRNA concentrations.

At the time of cell harvesting, medium for measurement of progesterone concentrations and cells for DNA content were collected for each condition as described previously (11). Total cellular RNA was prepared by the method of Chirgwin et al. (12), and 15 μg was used to make Northern blots for hybridization to a P450scc cDNA clone. Membranes were hybridized with 50 ng of P450scc cDNA clone radioactively labeled by random priming with [α-32P]dCTP to a specific activity of 1 × 108 cpm/μg (11). After washing, filters were exposed to film as described previously (4).

Progesterone and DNA Assay—Progesterone concentrations in media were measured by radioimmunoassay after cleate microcolumn chromatography as described previously (11). Progesterone antisera used in the assay was rabbit-produced using progesterone-11-succinate/bovine serum albumin as described (13). All samples from each experiment were assayed in a single assay. Total cellular DNA was measured by fluorometric assay using Hoechst 33258 dye (14). Calf thymus DNA was used as standard. The assay has a sensitivity of 20 ng/tube and was linear to 400 ng/tube.

Densitometry—The 18 S ribosomal RNA band from photographs of ethidium-stained total RNA formaldehyde gels and hybridization bands

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from autoradiograms of corresponding membranes were measured for integrated optical density with a BVI 4,000 digital analysis system (Applied Imaging, Santa Clara, CA). The 18 S ribosomal band was used to correct for differences in RNA loading for Northern blots as described previously (11).

IGF-I Binding Assay and Receptor mRNA Concentrations—The IGF-I binding assay was done as described previously (11). Briefly, cells were cultured at a concentrations of 20–30 million cells/dish and received 0.3 ng/ml 125I-IGF to give 200,000 cpm/dish. Competition with unlabeled IGF-I was done at the following concentrations: 0.3, 1.0, 3.0, 10.0, and 30.0 ng/ml. Granulosa cells were maintained in serum-free medium (control) or treated with TNF a (30 ng/ml) for 48 h prior to binding assays. Type I IGF receptor mRNA concentrations were determined by Northern blot hybridization using a porcine riboprobe as described previously (11).

Transient Transfection in Porcine Granulosa Cells—Porcine granulosa cells were cultured in 60-mm culture dishes at a concentration of 3 × 10^7 cells/dish (5). At the time of transfection, cells received 30 μg of P450sc/cyclophilin construct (divided among three 60-mm culture plates) by the calcium phosphate precipitation technique (5, 15). After 24 h, the precipitate was removed and fresh medium was added with specified hormonal treatments. Cultures were maintained for designated treatment times, harvested, and measured for light production (5). Reporter gene activity for porcine transfection experiments was normalized by the measurement of protein concentrations from the supernatant of the samples using Bio-Rad Bradford protein assay kit.

Transient Transfection in Mouse Fibroblast Cells—The two variants (pSV2Apap, pSV2Apap) of mouse fibroblast (NIH-3T3) cells were cultured in Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum and 500 μg/ml Geneticin (Life Technologies, Inc.). Transient transfection was done by lipofection (Tfx-50 Reagent, Promega, Madison, WI). Transfection experiments were done on 60-mm plates as per the Promega protocol for Tfx-50 Reagent. A 3 to 1 ratio (1 μg of DNA:2.5 μl of Tfx-50) was used for each transfection. The control plasmid pSV2Apap (containing the SV40 early promoter and the human placental alkaline phosphatase gene; Ref. 16) was cotransfected with the chimeric construct of interest. After transfection, cells were maintained in 2.5% fetal bovine serum without Geneticin®. Cells were harvested and measured for alkaline phosphatase activity using p-nitrophenyl phosphate (Sigma) and measuring absorbance at 405 nm.

Electrophoretic Mobility Shift Assay (EMSA)—Oligonucleotides were 32P-labeled by polynucleotide kinase and γ-32PATP (4500 Ci/mmol). Nuclear extract protein from porcine granulosa (15 μg), 5 × gelshift binding solution (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5), 0.5 μg of poly(dI-dC)poly(dI-dC), and labeled oligonucleotide (50,000 cpm) were incubated at room temperature for 15 min for shift assays. For supershift assays, 2 μl of Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated at room temperature for 15 min with the nuclear extract protein, 5 × buffer, and poly(dI-dC)poly(dI-dC). Labeled oligonucleotide was then added for an additional 15 min at room temperature. The protein-DNA complexes were separated on a 4% polyacrylamide gel by electrophoresis in 0.5 TBE buffer (1M Tris-HCl, 100 mM boric acid, 20 mM EDTA) and visualized by autoradiography.

Statistical Methods—One-way analysis of variance (ANOVA) with Tukey multiple comparison test was used to determine differences in experiments. Probability values of p < 0.05 were considered statistically significant. Data are presented as mean ± S.E.

RESULTS

Effects of TNFa on IGF-I-stimulated P450sc mRNA Concentrations and Progesterone Production—TNFa inhibits insulin-stimulated P450sc mRNA concentrations and progesterone production in porcine granulosa cells (2), but the effects of TNFa on IGF-I-stimulated P450sc mRNA concentrations and progesterone production had not been tested. Porcine granulosa cells were treated for 48 h with IGF-I (20 ng/ml), TNFa (30 ng/ml), and IGF-I and TNFa. TNFa inhibited IGF-I-stimulated P450sc mRNA concentrations and progesterone production (Fig. 1).

TNFa Effects on the Porcine P450sc IGFRE in Porcine Granulosa Cells—Transient transfections of deletion constructs of the upstream region of the porcine P450sc gene were done in porcine granulosa cells during treatment with TNFa to determine whether TNFa inhibited the activity of the porcine IGFRE. Experiments were done with three deletion constructs of porcine P450sc as follows: 1) the sequenced upstream region including the IGFRE (−2320), 2) the IGFRE and porcine P450sc core promoter (−130), and 3) the core promoter only (−100). These constructs have been described (5). Transfected cells were treated with IGF-I, TNFa, or both for 48 h. TNFa inhibited the transcriptional activity of the porcine IGFRE, but had no effect on the core porcine P450sc promoter (Fig. 2).

Effects of TNFa on IGF-I Binding and Receptor mRNA Concentrations—TNFa could inhibit IGF-I-stimulated porcine P450sc mRNA concentrations by decreasing IGF-I binding or reducing IGF type I receptors. IGF-I binding assays performed in porcine granulosa cells after treatment with TNFa for 48 h found that TNFa had no effect on IGF-I binding (Fig. 3). Moreover, Northern blot hybridization with a riboprobe of the porcine P450sc IGF type I receptor (11) showed no decrease in receptor mRNA concentrations in porcine granulosa cells treated with TNFa for 48 h (Fig. 3).

Effects of TNFa on the Binding Activity of IGFRE Transcription Factors—EMSA was done with nuclear extract proteins from porcine granulosa cells treated with IGF-I or TNFa for 48 h to determine whether the binding affinity of the IGFRE proteins were changed with treatment. Supershift assay with an Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was also done to confirm that Sp1 bound to the IGFRE. As shown in Fig. 4, IGF-I increased the binding activity of both transcription factors to the IGFRE, but TNFa specifically prevented IGF-I-induced binding of P2. The binding activity of Sp2 was increased with IGF-I and TNFa treatment over IGF-I.
From experiments done in triplicate (representing 6 experimental conditions), the P450scc/luc constructs and treated with IGF-I (20 nM) or TNF-α. Porcine granulosa cells were transfected with porcine granulosa cells. The 6 construct contains only the IGFRE and the endogenous porcine P450scc promoter. The −2320 construct contains the sequenced upstream region of porcine P450sec including the IGFRE. Data are the mean ± S.E. from two experiments done in triplicate (representing 6 experimental data points). The asterisk denotes a statistically significant increase (p ≤ 0.05) as determined by ANOVA with Tukey multiple comparison test. 48 hr represents the time of treatment after transient transfection.

**FIG. 2.** TNF-α effects on the porcine P450sec IGFRE in porcine granulosa cells. Porcine granulosa cells were transfected with porcine P450sec/luc constructs and treated with IGF-I (20 nM) or TNF-α (30 ng/ml). The −100 construct does not contain the IGFRE. The −130 construct contains only the IGFRE and the endogenous porcine P450scc promoter. The −2320 construct contains the sequenced upstream region of porcine P450sec including the IGFRE. Data are the mean ± S.E. from two experiments done in triplicate (representing 6 experimental data points). The asterisk denotes a statistically significant increase (p ≤ 0.05) as determined by ANOVA with Tukey multiple comparison test. 48 hr represents the time of treatment after transient transfection.

**FIG. 3.** TNF-α effects on IGF-1 binding and mRNA receptor concentrations. Porcine granulosa cells were cultured in 60-mm dishes at a concentration of 20–30 million cells/dish for IGF-I binding experiments. All plates received 0.3 ng/ml [125I]-IGF to give 200,000 cpm/dish. Competition with unlabeled IGF-I was done at the following concentrations: 0.3, 1.0, 3.0, 10.0, and 30.0 ng/ml. Granulosa cells were maintained in serum-free medium (control) or treated with TNF-α (30 ng/ml) for 48 h. Data represent one experiment done in triplicate. Inset, type I IGF receptor mRNA concentrations in porcine granulosa cells treated with TNF-α were determined by Northern blot hybridization using a porcine ribonuclease (11). Data are the mean ± S.E. from three experiments. There was no statistical difference in IGF type I receptor mRNA concentrations between control and TNF-α treatment.

**FIG. 4.** EMSA of the porcine P40sec IGFRE and nuclear extract protein from granulosa cells treated with TNF-α. Nuclear extract proteins (10 μg) from porcine granulosa cells were used with radiolabeled porcine IGFRE oligonucleotide in EMSA. The nuclear extract proteins were harvested from cells treated with IGF-I (20 nM) or TNF-α (30 ng/ml) for 48 h. The first four lanes are control (C), IGF-I (I), TNF-α (T), and IGF-I + TNF-α (I+T). The fifth (S) lane represents IGF-I-treated cells supershifted with an Sp1 antibody (2 μg).

From the results of this study and the previous study in NIH-3T3 cells, we can develop the mechanism of how the porcine IGFRE is able to stimulate gene expression. The IGFRE requires the binding of Sp1 for basal function of the element. However, while necessary, this binding is not specific. This is evident from the EMSA results during treatment with TNF-α and IGF-I. Despite a reduction in P450sec gene expression (decreased mRNA concentrations), the binding of Sp1 to the IGFRE was increased over the binding that occurred with IGF-I treatment alone. It is the binding of P2 to the IGFRE that correlates with gene expression of the IGFRE. Therefore, P2 is
Cells were treated for 48 h after transfection with IGF-I (20 nM) and mouse fibroblast cell line overexpressing the IGF-I receptor (NWTb3). The data represent the mean ± S.E. from five replicates. The asterisk indicates a statistical significance increase, and the double asterisk indicates another significant decrease from control as determined by ANOVA.

Another significant finding in this study was the TNFα signaling pathway interacting with the IGF-I pathway in porcine granulosa cells. There is evidence in other cells of interactions between the pathways. TNFα inhibits IGF-I-stimulated proteoglycan synthesis in cartilage from hypophysectomize rats (18). In human obesity, TNFα expression from adipose tissue can be correlated with the level of hyperinsulinemia (19). Studies indicate that TNFα inhibits insulin receptor signaling by causing a modified form of IRS-1 that suppresses rather than enhances the signaling pathway (20). However, other studies have indicated that TNFα modulates insulin receptor signaling by protein-tyrosine phosphatase activation (21). Mutations to the IGF-I receptor show that the initial mechanisms of activation of the insulin and IGF-I receptor are almost identical (22). Therefore, understanding the mechanisms of TNFα inhibition of IGF-I-stimulated transcriptional activity of the porcine IGFRE may further our understanding of obesity and insulin resistance.

These findings in porcine granulosa cells must also be viewed from their physiologic significance regarding corpus luteum function. Corpus luteum that are regressing have an increase in resident ovarian macrophages and TNFα concentrations (23). The site of synthesis of TNFα in the CL has not been absolutely determined, but, in addition to resident macrophages, the endothelial cells of the CL have been implicated as a source of TNFα (24). In the bovine, an increase in intraluteal TNFα is related to luteolysis and the peak activity of luteal TNFα occurs before the decline in progesterone production (25). IGF-I is also important in the regulation of CL function. The porcine CL expresses mRNA concentrations for IGF-I and IGF-binding proteins (26). In women, elevated concentrations of insulin-like growth factor-binding protein-1 (IGFBP-1) occur in luteinizing follicles, further suggesting the importance of the IGF-I autocrine/paracrine system in regulation of CL function (27). This study presents a plausible mechanism for the interaction of TNFα and IGF-I on CL function. These experiments were done in granulosa cells, so these results must be extrapolated to the small luteal cell. Increasing concentrations of TNFα from either macrophages or epithelial cells in CL would inhibit IGF-I-supported progesterone production. This inhibition of steroidogenesis would be the initial step in the regression of the CL.

In summary, this study shows that TNFα inhibits the transcriptional activity of the porcine P450scc IGFRE in porcine granulosa and mouse fibroblasts cells. This finding supports a plausible mechanism for TNFα induction of CL luteolysis and further defines P2 as the transcription factor that mediates IGF-I-stimulated gene expression.
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