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Regulation of VEGF-A, VEGFR-I, Thrombospondin-1, -2, and -3 Expression in a Human Pituitary Cell Line (HP75) by TGFβ1, bFGF, and EGF

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Pituitary tumors are highly vascular neoplasms, which suggest an important role of angiogenesis in pituitary tumor growth. We used the human pituitary cell line (HP75) to examine the effects of the growth factors TGFβ1, bFGF, and EGF on cell growth, and on the regulation of the pro-angiogenic growth factor VEGF-A and the VEGFR-I and the anti-angiogenic molecules thrombospondin (TSP) TSP-1 and TSP-2 along with TSP-3. Real-time RT-PCR was used to measure mRNA levels, and Western blot was used to analyze TSP-1 and TSP-2 protein levels.

TGFβ1 treatment (1×10^{-9} *M*) increased VEGF-A mRNA levels significantly ($p < 0.05$) after 4 and 24 h of treatment. TGF β1 treatment decreased VEGF-R mRNA levels after 96 h of treatment ($p < 0.05$). After 96 h of treatment, TSP-1 and TSP-2 mRNA levels were significantly increased ($p < 0.05$) by TGFβ1 treatment, which also inhibited HP75 cell growth. Basic FGF also increased TSP-1 mRNA levels after 96 h of treatment, but did not regulate growth of the pituitary tumor cells. Basic FGF and EGF did not modulate changes in VEGF-A mRNA levels after 4 and 24 h of treatment, but EGF increased VEGF-A significantly ($p < 0.05$) after 96 h of treatment.

These results indicate that TGFβ1 treatment may regulate angiogenesis in pituitary cells by initially increasing levels of pro-angiogenic VEGF-A and then stimulating the anti-angiogenic molecules TSP-1 and TSP-2 levels.

Key Words: Pituitary; VEGF; thrombospondin;TGFβ; HP75 cells.

Introduction

Angiogenesis has an important role in the development of neoplasms including tumors of the anterior pituitary gland *(1–3)*. The complexity of angiogenesis is reflected in the many stimulatory and inhibitory proteins regulating new vessel formation *(4)*. Some of the widely studied proteins include VEGF and its receptor *(5,6)*, TGFβ *(7,8)*, FGF-2 or basic FGF (bFGF) *(9,10)*, EGF *(11,12)*, and thrombospondin-1 and -2 *(13–16)*. The role of VEGF and its receptors in pituitary tumor angiogenesis has been studied extensively *(2–5,17–21)*. However, thrombospondins (TSP) have not been examined extensively in pituitary cells or tumors. The TSPs are a family of five secreted proteins, which are widely distributed in the extracellular matrix of tissues *(13)*. TSP-1 and TSP-2 have been shown to have anti-angiogenic activity, and their expression is mediated by hypoxia and oncogenes. They are secreted from a variety of epithelial and mesenchymal cells *(13)*. A recent study analyzing the distribution of TSP-2 and TSP-3 in various tissues showed that the pituitary had relatively low levels of TSP-2 but high levels of TSP-3 as measured by RNA expression *(22)*. TSP-1 has been studied extensively because of its critical role in platelet aggregation, in coagulation, and in angiogenesis *(23,24)*. Because TSP-1 and TSP-2 have similar structural organizations, these molecules are often studied together *(13)*. However, the function of TSP-3 in the pituitary is relatively unknown, and because this molecule is present in relatively high levels in the pituitary tissues, we were interested in studying the role of TSP-3 in the pituitary.

TSP-1 is a potent inhibitor of in vivo neovascularization and tumorigenesis. Recent studies have suggested that TSP-1 is an important mediator of the angiogenic switch in which disruption of the normal ratio of angiogenic stimulators and inhibitors in a tumor mass allows for autonomous vascularization *(25)*. More recent studies with transformed embryonic kidney cells and human mammary epithelial cells have shown that, in these cells, the angiogenic switch is dependent on the level of expression of oncogenic RAS and that TSP-1 was the mediator of the angiogenic switch in these tumors. There is very little information about the expression of TSPs in pituitary cells and tumors. In order to examine the role of pro- and anti-angiogenesis factors in the pituitary, we used a human pituitary cell line HP75 *(26–28)* to examine the regulation of VEGF, VEGFR-I, TSP-1, TSP-2, and TSP-3 by various growth factors in vitro. Our results indicate that TGFβ1 has a significant role in regulating the anti-angiogenesis proteins TSP-I and TSP-II.

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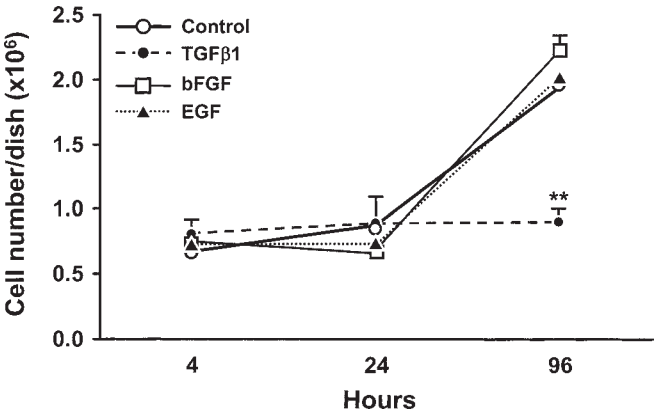

Fig. 1. Analysis of HP75 cell growth. Cells were treated with TGFβ1 (1×10^{-9} *M*), basic FGF (10 ng/mL), or EGF (1 μg/mL) for 4 h, 24 h, and 96 h, and cell counts were performed with a hemocytometer. The results are the mean ± standard error of five separate experiments. There was significant inhibition by TGFβ1 on cell growth after 96 h of treatment (**$p < 0.01$).

Results

Cell Growth Treatment

TGFβ1 treatment (1×10^{-9} *M*) produced a significant inhibition of cell growth after 96 h ($p < 0.01$) (Fig. 1). There was no difference in cell growth after treatment with bFGF and EGF.

Treatment with Growth Factors

After 4 h of treatment, there was a significant ($p < 0.05$) increase in VEGF-A by TGFβ1 treatment. TSP-1 showed a 3.6-fold increase, although this change was not statistically significant (Fig. 2). Treatment with bFGF and EGF did not lead to changes in mRNA expression for the VEGF-A, VEGFR-I, TSP-1, TSP-2, and TSP-3 (Fig. 2A).

After 24 h of treatment with TGFβ1, there was a significant ($p < 0.05$) increase in VEGF-A. There was a 3.4-fold increase in TSP-1 after TGFβ1 treatment, but this difference was not statistically significant (Fig. 2B).

After 96 h of treatment with various growth factors, there was a significant ($p < 0.05$) increase in TSP-1 mRNA by TGFβ1 and bFGF above control levels (Fig. 2C). TSP-2 was significantly ($p < 0.05$) increased by 2.4-fold after TGFβ1 treatment. There were no changes in TSP-3 with any treatment (Fig. 2C). There was a significant decrease in VEGFR mRNA levels by TGFβ1 after 96 h of treatment (Fig. 2C). EGF treatment led to a significant ($p < 0.05$) increase in VEGF-A.

Western Blot Analysis

Changes in the levels of TSP-1 protein as measured by Western blotting with densitometric analysis after 96 h of treatment were similar to those seen with mRNAs. There was a significant ($p < 0.05$) increase in TSP-1 protein expression after treatment with TGFβ1, while basic FGF and EGF treatments did not cause significant changes in TSP-1 proteins (Figs. 3A,B). Protein levels of TSP-2 were increased after TGFβ1, bFGF, and EGF treatments, but these changes were not statistically significant (Figs. 3C,D).

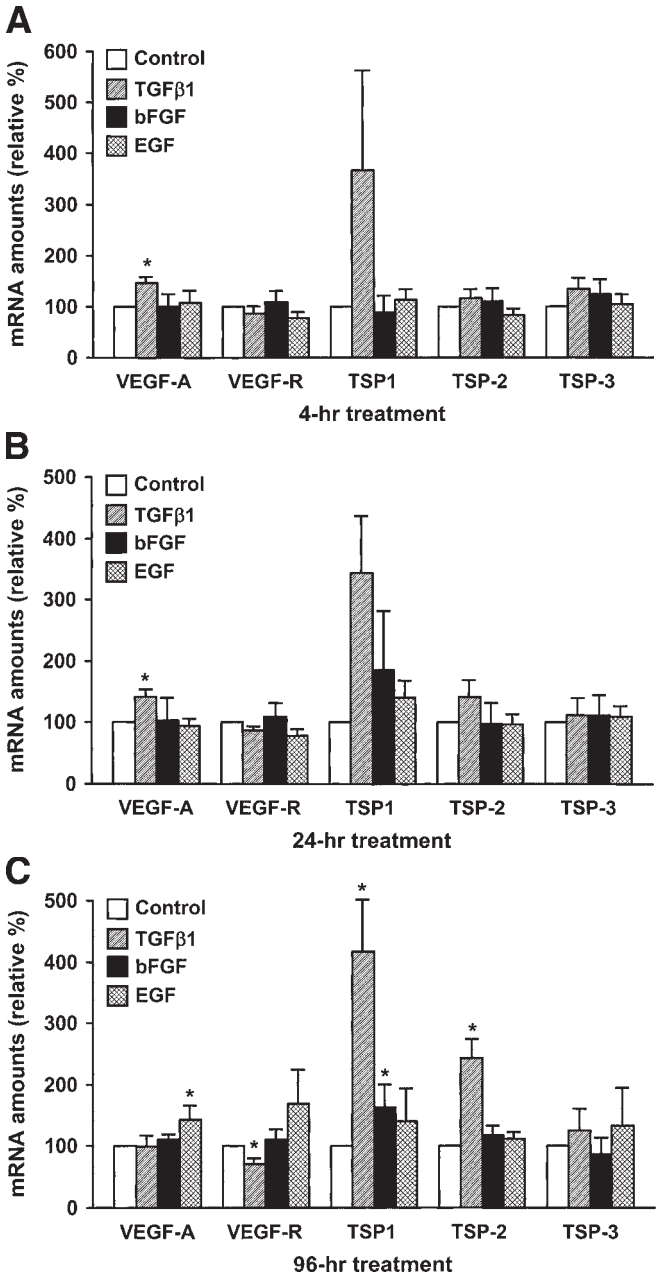

Fig. 2. Effects of growth factor treatment on mRNA expression for VEGF-A, VEGFR1, TSP-1, TSP-2, and TSP-3. Cells were treated with TGFβ1, basic FGF, and EGF for 4 h (**A**), 24 h (**B**), and for 96 h (**C**). RNA was extracted and measured by real-time RT-PCR as described in Materials and Methods. Results are expressed as fold change relative to the untreated control cells after normalizing to GAPDH and represent the mean ± SEM of five separate experiments (*$p < 0.05$).

Discussion

Analysis of the regulation of VEGF-A, VEGFR-1, TSP-1, TSP-2, and TSP-3 mRNAs by real time RT-PCR showed

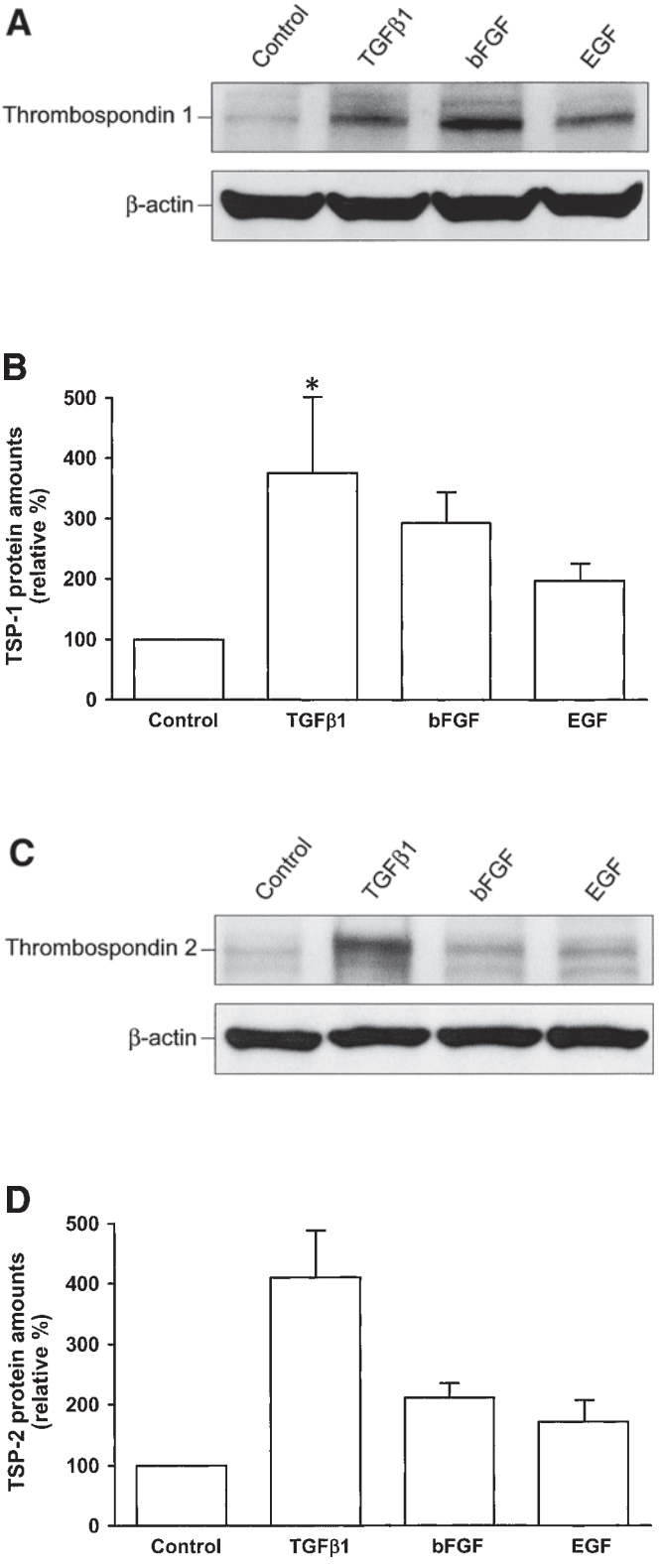

Fig. 3. Effects of growth factor treatment and protein expression for TSP-1 (**A** and **B**) and TSP-2 (**C** and **D**) on HP75 cells after 96 h of treatment with TGFβ1 (1×10^{-9} *M*), basic FGF (10 ng/mL), and EGF (1 μg/mL). Protein was extracted and used for Western blotting as described in Materials and Methods. Results are expressed as fold change relative to the untreated control cells and represent the mean ± SEM for three separate experiments (*$p < 0.05$).

that TGFβ1 treatment upregulated VEGF-A after 4 h of treatment. After 96 h of TGFβ1 treatment, there was a significant increase in TSP-1 and TSP-2 mRNA levels and a significant decrease in VEGF-A mRNA levels in the human pituitary HP75 cell line. EGF also increased VEGF-1 mRNA levels after 96 h of treatment. Analysis of TSP-1 after TGFβ1 treatment for 96 h showed increased levels of protein expression.

VEGF proteins and mRNAs expression has previously been examined in normal and in neoplastic pituitary tissues *(17–20)*. Lloyd et al., found that VEGF proteins were more highly expressed in normal human pituitary cells and in pituitary carcinomas compared to adenomas *(17)*. In the present study, treatment with TGFβ1, which inhibits proliferation of anterior pituitary cells, led to an increase in VEGF-A mRNA after 4 h, although this effect was not seen with longer treatment suggesting a refractory effect of TGFβ1 on VEGF-A levels with chronic exposure. The inhibitory effect of TGFβ1 on HP75 cell proliferation was similar to our previous studies in which ^{3}H-thyrimidase incorporation was used to show this inhibition *(26)*.

Thrombospondin is an extracellular matrix protein and belongs to a family of five proteins *(22)*. TSP-1 and TSP-2 have been shown to be involved with angiogenesis in various tumor types *(13–16)*. Although TSP-2 and TSP-3 have been described in pituitary tissues *(23)*, the function of these TSPs in pituitary tissues has not been previously examined. Our study shows for the first time that TSP-1 and TSP-2 are regulated by the growth factor TGFβ1 with a marked upregulation of both TSP-1 and TSP-2mRNAs and proteins after chronic treatment for 4 d. Although TSP-1 and TSP-2 are considered to be predominantly anti-angiogenic *(22)*, some studies have suggested a pro-angiogenic role for TSP-1 in vitro *(29)*. TSP-1 and TSP-2 have been shown to be regulated by hypoxia. The regulation of TSP-1 and TSP-2 expression by hypoxia is dependent on tissue type, cell transformation, and experimental conditions *(22, 30)*. In various human tumors, such as gliomas, colon, adenocarcinomas, and adrenocortical carcinomas, TSP-1 levels were lower in malignant tumors compared to benign ones *(31–33)*. Interactions between TSP-1 and TGFβ have previously been observed in some cell types *(34–36)*. TSP-1 appears to bind to the latent precursor of TGFβ-1, which favors release of TGFβ-1 directly or indirectly. TGFβ is known to stimulate apoptosis in different tumor types and may contribute to tumor growth by stabilizing the tumor vasculature *(37)*. The overall effects of TSP-I on tumor cells probably reflects a balance between anti-angiogenic forces and conditions leading to tumor invasion and progression *(14,42)*.

Although relatively high levels of TSP-3 have been reported in pituitary tissues compared to other tissues *(23)*, the function of this molecule in the pituitary remains unknown. The tissue distribution of the various TSPs may partly reflect their functions. TSP-1 is the only member of the family expressed by platelets, while TSP-2 is highly expressed

in bone. TSP-3 is present in lung and has relatively high levels in the pituitary. In the current studies, TSP-3 was not up-regulated by TGFβ1 or the other growth factors. This may imply a different role for TSP-3 compared to the anti-angiogenic roles of the other two TSPs studied.

Although only TGFβ1 inhibited HP75 cell growth, bFGF also upregulated TSP-1 mRNA expression in cultured pituitary cells in our study. A striking difference of the effects of TGFβ1 compared to bFGF and EGF was that TGFβ1 inhibited cell growth, and this effect was not observed with bFGF or EGF in the HP75 pituitary cell line. Thus, the anti-proliferative effect of TGFβ1 on cultured pituitary cells may be related to its anti-angiogenic effect. Recently, Watnik et al., showed that the oncogenes *RAS* and *MYC* genes, which influenced cell growth had regulatory effects on angiogenesis *(25)*. *RAS* was shown to up-regulate the upstream pathway of angiogenesis, while *MYC* controlled TSP-I expression directly as a final modulator. This led to an angiogenic switch between VEGF and TSP-1, which was regulated by *RAS* and *MYC (25)*. In our study, under the influence of TGFβ1, there was initial early up-regulation of VEGF-A and later with chronic treatment (96 h), both TSP-1 and TSP-2 mRNA levels were significantly up-regulated in the TGFβ1-treated cells with down-regulation of VEGF-R suggesting a switch from a pro-angiogenic regulatory role to a predominantly anti-angiogenic role in pituitary tumor cells under the influence of this growth factor.

In summary, our study shows that TGFβ1 increased VEGF-A mRNAs after 4 and 24 h of treatment, while TGFβ1 treatment for longer periods (96 h) led to an upregulation of both TSP-1 and TSP-2 mRNA and TSP-1 protein levels in the cultured pituitary cells suggesting a regulatory role of TGFβ1 in angiogenesis in pituitary cells.

Materials and Methods

Cell Lines and Reagents

The HP75 cell line was developed in our laboratory as previously reported *(26–28)* and is available from American type tissue culture collection (Manassas, VA). Cells were maintained in Dulbecco's minimum essential medium (DMEM) with phenol red and supplemented with 2.5% fetal bovine serum (FBS) and 15% horse serum and 1% antibiotics/antimycotics (Invitrogen, Carlsbad, CA).

Chemicals

TGFβ1 was from R and D Systems (Minneapolis, MN), and was used as 10^{-9} *M* as previously described *(26)*. bFGF was from Promega (Madison, WI), and was used at 10 ng/mL. EGF was from Promega and was used at 1 μg/mL. The optimal concentration of bFGF and EGF for the experiments was determined by preliminary titration.

Cell Culture Treatment

Approximately 1×10^6 HP75 cells were cultured in DMEM with 2% FBS. The medium was changed every other day. Cells were treated for 4 h, 24 h, and 96 h for each experiment. The number of cells at the end of each experiment was counted with the aid of a hemocytometer.

RNA Extraction

Total RNA was extracted from the harvested cells using Trizol reagent (Invitrogen). The RNA was precipitated by isopropanol, washed in 70% ethanol, then dissolved in RNAase-free water. RNA concentrations were calculated using optical density measurements taken with a spectrophotometer.

Synthesis of cDNA for Standard Curve on Real-Time PCR

One microgram of total RNA from a pituitary gonadotroph adenoma was converted to cDNA using the ProStar™ First Strand RT-PCR kit (Stratagene, La Jolla, CA). PCR products to be used as standards for VEGF-A, VEGFR-I, TSP-1, TSP-2, and TSP-3, and GAPDH were made by conventional PCR. The PCR primers used are listed in Table 1. Each PCR product was inserted into the pGEMT easy vector plasmid (Promega) and grown on (100 μg/mL ampicillin) at 37°C overnight. Single colonies were selected and grown overnight in LB broth (100 μg/mL ampicillin) at 37°C with shaking. The insert-containing plasmid DNA was then extracted from the bacterial cells using the Qiagen midi-prep kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The plasmid DNA was digested with *Eco*R1 restriction enzyme (Promega) at 37°C for 4 h and run on a 1% agarose gel and briefly stained with ethidium bromide. The band containing the insert DNA was cut out from the agarose gel and extracted with an ultra-free DA centrifugal filter device (Millipore Corp, Bedford, MA) and concentrated using a Microcon YM-10 and/or 100 (Millipore). The concentration of the purified DNA was measured using a spectrophotometer. The gene sequence of each product was verified by automated sequencing using M-13 primers (Mayo Molecular Biology Core Facility).

The copy number was calculated using the following formula: Copy number (copies/microliter) = $6 \times 0.023 \times 10^{23}$ molecules/mole × (OD_{260} × 5 μg/mL/OD × 10^{-9} g/μL)/(662 × molecular weight/bp × *Eco*RI fragment bp). The standard solutions were adjusted to a concentration of 0.5×10^7 copies/μL, aliquotted and stored at –70° C Just before using in the PCR reaction, samples were diluted 1 to 10 with sterile water and used to make standard curves.

Real-Time PCR

The primer and annealing temperatures used are shown in Table 1 *(38–43)*. Real-time RT-PCR was performed using the LightCycler Instrument (Roche) as previously reported *(26)*. SYBR Green-1 kit (Roche) was used for detection. Each PCR reaction contained primers (0.5 m*M* each), $MgCl_2$ (4 m*M*), 2 μL of LightCycler FastStart DNA master SYBR Green-I, and 2 μL of template in a volume of 20 μL. The standard curve PCR reactions were included in the

Table 1
Sequence of Primers and Annealing Temperature for PCR

Gene	Sequence[a]	Annealing temperature (°C)	Basepair of product	Ref.
VEGF	SS 5'-AGGAGGAGGGCAGAATCATCA-3'			
	AS 5'-CAGGGATTTTCTTGTCTTG-3'	56	336	*38*
VEGFR-1	SS 5'-GCAAGATTCAGGGACTATG-3'			
	AS 5'-ACCAAGTGATCTGAGGCTCG-3'	56	200	*39*
TSP-1	SS 5'-CACCAACCGCATTCCAGAG-3'			
	AS 5'-TCAGGGATGCCAGAAGGAG-3'	57	233	*40*
TSP-2	SS 5'-GCAACATCAACCGCAAGAC-3'			
	AS 5'-AAGCAAACCCCTGAAGTGACT-3'	59	541	*41*
TSP-3	SS 5'-GTCCTCTTTGGCCTCTATTCT-3'			
	AS 5'-CATCCTCAATACGCCTTC-3'	56	330	*42*
GAPDH	SS 5'-AAGGTGAAGGTCGGAGTCAACG-3'			
	AS 5'-GTTGTCATGGATGAACCTTGGCC-3'	56	495	*43*

[a]SS, sense primer; AS, antisense primer.

same run as the experimental samples per capillary of standard DNA was used for VEGF-A, VEGFR-I, TSP-1, -2, and -3. For GAPDH 10^5 to 2×10^{10} copies of standard cDNA was used in each capillary. PCR was performed using the following conditions: (1) preincubation: 95°C × 10 min; (2) amplification (45 cycles): 95°C for 10 s, annealing temperature 56–59°C for 5 s, and extension at 72°C for 8–22 s; (3) melting curve analysis at 95°C at a transition rate of 0.1°C/s.

To ensure that the correct product was amplified, all products were separated in a 2% Agarose gel and stained with ethidium bromide. A water blank and a no-RT reaction were used as negative controls.

Quantification

To normalize the sample, each copy number calculated from the standard curve was divided by the copy number of the housekeeping gene *GAPDH*. The ratio (target gene copy number/*GAPDH* copy number) × 10^4 was used as a relative ratio. A total of five experiments for real-time RT-PCR and three experiments for Western blot analysis were performed. Results were expressed as the mean ± SEM. Student's *t* test was used for comparison of the treated and control groups. A *p* value less than 0.05 was considered statistically significant.

Western Blotting

Western blot analysis was performed as previously reported *(28)*. The anti-TSP-1 and anti-TSP-2 antibodies were used at the 1:250 dilution each (Becon-Dickinson Company, Mountain View, CA). Beta-actin (Sigma, St. Louis, MO) was used to correct for gel loading, and quantitation was done by densitometry with Chemi Doc System and Quantity One Software (Biorad Labs). Bands were visualized by chemiluminescence (Amersham, Arlington, IL).

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