Modern Pathology (2005) 18, 221–227

www.modernpathology.org

Role of COX-2, thromboxane A_2 synthase, and prostaglandin I_2 synthase in papillary thyroid carcinoma growth

Sabine Kajita, Katharina H Ruebel, Mary B Casey, Nobuki Nakamura and Ricardo V Lloyd

Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, MN, USA

The development of papillary thyroid carcinoma is influenced by many factors including genetic alterations, growth factors, and physical agents such as radiation. Arachidonic acid and its derivatives including prostaglandins (PG) and thromboxane along with the enzymes involved in their synthesis have been shown to influence the growth of various tumors. We analyzed the immunoreactivity for cyclooxygenase-2 (COX-2) and mRNA expression levels of the enzymes COX-2, thromboxane A_2 (TXA_2) synthase, and PGI_2 synthase by RT-PCR in papillary carcinomas and matching normal tissues to determine the role of these enzymes in the development of papillary thyroid carcinomas. A papillary thyroid carcinoma cell line TPC-1 was also studied *in vitro* to determine the role of the specific COX-2 inhibitor NS-398 on COX-2 and vascular endothelial growth factor-A, since COX-2 also has a role in regulating tumor angiogenesis. RT-PCR analysis showed significant increases in TXA_2 synthase mRNA levels in papillary thyroid carcinomas compared to normal thyroid tissues. Although COX-2 mRNA levels were generally increased in papillary carcinomas, the differences were not statistically significant. There were no significant differences in PGI_2 synthase mRNA levels. COX-2 protein expression was greater in papillary carcinoma compared to normal thyroid tissues; however, the levels were quite variable. *In vitro* studies with a COX-2 inhibitor, NS-398, showed inhibition of tumor growth along with increased levels of COX-2 and vascular endothelial growth factor-A mRNA expression. These results indicate that specific enzyme levels in the PG synthesis pathway such as TXA_2 synthase are increased in papillary thyroid carcinomas. COX-2 also has a role in papillary thyroid growth, since a specific inhibitor of COX-2 regulates papillary thyroid carcinoma cell proliferation. These results implicate several enzymes in the synthesis of prostanoids as regulators of thyroid papillary carcinoma proliferation and suggest that increased levels of expression of these enzymes may play a role in the pathogenesis of these tumors.
Modern Pathology (2005) **18,** 221–227, advance online publication, 8 October 2004; doi:10.1038/modpathol.3800285

Keywords: COX-2; thyroid; papillary carcinoma; thromboxane synthase

Arachidonic acid and its derivatives, the prostaglandins and thromboxane (Figure 1), are important in many physiological processes.[1] They are also mediators of tumor progression. There are two major cyclooxygenases (COX). COX-1 is the constitutive form of the enzyme, which catalyzes formation of prostaglandin from arachidonic acid, while COX-2 is expressed as an inducible isoform. COX-2 expression is increased by various substances including mitogens,[2] tumor promoters,[3,4] cytokines,[1,2,5,6] serum,[5] and free fatty acids.[7] Other studies have shown that COX-2 is also regulated by nonsteroidal anti-inflammatory drugs (NSAIDs) or by selective COX-2 inhibitors.[7–10]

COX-2 is expressed in many types of cancers including colon,[11,12] pancreas,[8,13,14] stomach,[15–17] lung,[12,18] breast,[12,19] prostate,[20] cervix,[6] head and neck,[21] esophagus,[22] bladder,[23] glioma,[24] and melanomas.[25] Recent studies have shown that COX-2 is expressed in thyroid lesions.[26–29] Thyroid tumors generally express higher levels of COX-2 than normal tissues and thyroid tissues from patients with thyroiditis.[26–29]

Thromboxane A2 (TXA_2) synthase and prostaglandin (PG) I_2 synthase are enzymes located downstream from COX-1 and COX-2, and catalyze synthesis of PG H, TXA_2 and PGI_2 (Figure 1). TXA_2 stimulates platelet aggregation, while PGI_2 inhibits aggregation of platelets. Some studies have suggested that TXA_2 synthase and PGI_2 synthase contribute to tumor metastasis.[30–32] TXA_2 synthase

Correspondence: Dr RV Lloyd, MD, PhD, Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First Street, SW, Rochester, MN 55905, USA.
E-mail: lloyd.ricardo@mayo.edu
Received 22 June 2004; revised 16 August 2004; accepted 17 August 2004; published online 8 October 2004

was shown to stimulate tumor angiogenesis, while PGI_2 synthase inhibited angiogenesis.[33–35] Our recent studies on COX-2 and TXA_2 synthase expression in thyroid tumors and other thyroid lesions using immunohistochemistry found higher levels of these proteins in papillary thyroid carcinomas compared to adenomatous nodules and benign thyroid tumors.[36] The present study examines COX-2, TXA_2 synthase, and PGI_2 synthase mRNA expression in normal thyroid tissue and papillary thyroid carcinomas using semiquantitative RT-PCR to analyze the role of these enzymes in thyroid tumor growth. We also examined the selective inhibition of COX-2 by NS-398 in the papillary thyroid carcinoma cell line TPC-1 to study the effects of this inhibitor on cell growth as well as COX-2 and VEGF-A mRNA expression.

Materials and methods

Tissue Samples

In all, 15 cases of papillary thyroid carcinomas and adjacent normal tissues were obtained at the time of surgery and stored at −70°C. Hematoxylin and eosin staining was performed for each case to determine the diagnosis and distribution of tumor and normal thyroid cells. There were 12 cases of classic variant and three cases of follicular variant of papillary carcinomas.

Immunostaining for COX-2 with a monoclonal antibody from Cayman Chemicals, Ann Arbor, MI at a 1/200 dilution was carried out using frozen tissues fixed in formalin.[36]

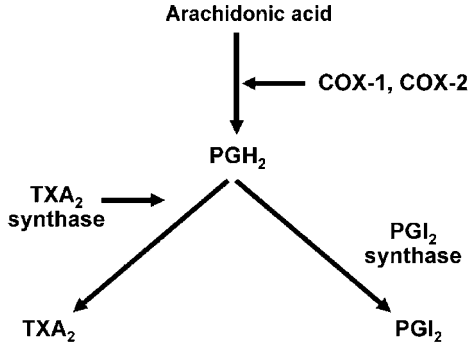

Figure 1 Schematic diagram showing the metabolic pathways of prostanoids. Arachidonic acid is converted to PGH_2 under the influence of COX-1 and COX-2. TXA_2 synthase influences synthesis of TXA_2 while PGI_2 synthase influences PGI_2 formation.

RNA Extraction and RT-PCR

Total RNA was extracted from frozen normal and tumor tissue samples and from cultured cells using the TRIzol reagent as previously reported.[37] A measure of 1 μg total RNA was converted to cDNA using the Prostar First Strand RT-PCR Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instruction. A measure of 5 μl cDNA was amplified by hot-start PCR in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Five specific primer sets with individual annealing temperatures were used (Table 1). Each PCR reaction was performed using 1 × PCR buffer, 2 mM $MgCl_2$, 1.25 U *Taq* polymerase (Promega, Madision, WI, USA), 0.2 μM each dNTP (Roche Diagnostics, Alameda, CA, USA) in a final volume of 50 μl. PCR product identity was verified by automated sequencing using the 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). In all, 20 μl of each PCR reaction as analyzed on a 1.5% agarose gel, stained briefly in ethidium bromide, and viewed under UV light.

Densitometric Analysis

Densitometric analysis was carried out with a Quantity One System (Bio-Rad Laboratories, Hercules, CA, USA). Relative densities of the samples were calculated and compared with the housekeeping gene HPRT expression level. Samples were normalized and expressed relative to HPRT.

Cell Culture

A human papillary thyroid carcinoma cell line, TPC1, was obtained from Dr Yuri Nikiforov (Uni-

Table 1 Sequence of primers used in experiments

		Sequence	*Cycle number*	*Product (bp)*	*Annealing temperature (°C)*	*References*
HPRT	Forward	5′-CTTGCTCGAGATGTGATGAAG-3′	28	290	58	[38]
	Reverse	5′-GTCTGCATTGTTTTGCCAGTG-3′				
COX-2	Forward	5′-GGTCTGGTGCCTGGTCTCATC-3′	30	724	56	[39]
	Reverse	5′-GTCCTTTCAAGGAGAATGGTGC-3′				
TXA_2 synthase	Forward	5′-CCTTCTCCTGGCTCATTTA-3′	32	270	56	[40]
	Reverse	5′-TCGTCTCGGTTCTTATTGG-3′				
PGI_2 synthase	Forward	5′-AGGAGAAGCACGGTGACATC-3′	35	387	65	[41]
	Reverse	5′-GCAGCGCCTCAATTCCGTAA-3′				
VEGF-A 165	Forward	5′-AGGAGGAGGGCAGAATCATCA-3′	30	336	56	[42]
	Reverse	5′-CAGGGATTTTCTTGTCTTG-3′				

versity of Cincinnati Medical Center). The cell line has a rearranged form of RET/PTC1.[43] Cells were cultured in DMEM, supplemented with 10% FBS, 1 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (InVitrogen Life Technologies, Carlsbad, CA, USA) using cell culture conditions as previously reported.[44]

Cell Treatments and Reagents

A stock solution of 40 mM NS398 (Cayman Chemical Company, Ann Arbor, MI, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma) and diluted in standard growth medium to a final concentration of 20–80 μM for the cell growth experiments. COX-2 expression experiments were performed with NS-398, which was diluted in serum-free medium and treated with 80 μM NS-398. The final concentration of DMSO was 0.2% in each experiment. The experiments were performed at least three times.

Growth Inhibition Experiments

Cells were seeded at a density of 0.2×10^6 per six-well plates. They were allowed to grow for 24 h, and then the medium was replaced with NS-398 and/or DMSO-containing control medium. Cells were counted with a hemocytometer after 72 h of NS-398 treatment.

COX-2 Induction Experiments

Cells were seeded at a density of 0.5×10^6 cells in a 75 cm^2 flask. After 72 h, the cells were treated with 80 μM NS-398 or DMSO in serum-free medium. At 24 h before treatment, the medium was replaced with serum-free media. Total RNA was extracted after 8, 24, and 32 h. The samples were subsequently analyzed by RT-PCR.

Statistical Analysis

Comparison between tumor and normal tissue were calculated using the Student's *t*-tests and by the Wilcoxon signed rank-sum test. NS-398 cell growth inhibition was analyzed by dose-response regression analysis. A *P*-value less than 0.05 was considered to be statistically significant.

Results

Immunohistochemistry

Immunoreactivity for COX-2 was stronger in the papillary carcinomas compared to the normal thyroid tissues (Table 2). However, immunostaining was variable among the tumors.

Table 2 Distribution of cases of papillary thyroid carcinomas

Case number	*Age/ sex*	*Tumor size (cm)*	*Extra-thyroidal extenasion*	*Lymph node metastasis*	*COX-2 immunoreactivity*	
					Normal	*Tumor*
1	68/M	3	+	+	0	2+
2	35/F	3	−	−	1+	3+
3	59/F	3.5	+	+	1+	2+
4	77/M	3.5	+	+	1+	1+
5	70/M	3.2	−	−	1+	3+
6	52/M	1.8	−	+	1+	1+
7	46/F	0.6	−	−	1+	1+
8	22/F	3.5	−	+	1+	2+
9	43/F	4	−	−	0	2+
10	41/M	3.5	−	−	0	2+
11	52/M	5	+	+	1+	1+
12	59/F	5.2	−	−	1+	3+
13	31/M	6	−	+	1+	1+
14	46/M	2.5	−	−	1+	1+
15	62/F	5.3	+	−	1+	1+

+, present; −, absent.
Immunoreactivity for COX-2 was graded as 0 negative; 1+ weakly positive; 2+ moderately positive; and 3+ strongly positive.

Expression of COX-2, TXA2 Synthase, and PGI-2 Synthase mRNA

Analysis of COX-2 mRNA by RT-PCR in 15 cases of papillary thyroid carcinoma (Table 2) (Figure 2) showed that COX-2 expression was upregulated more than 1.8-fold in four cases (Figures 3 and 4a). In four cases, the tumors were downregulated to less than 50% of normal tissues. The expression levels of TXA_2 synthase showed upregulation more than 1.8-fold in eight cases (Figures 3 and 4b). There were significant differences of TXA_2 synthase expression between carcinomas and normal tissues ($P = 0.008$). The expression of PGI_2 synthase was quite variable, and there were no significant differences between tumor and normal tissues (Figures 3 and 4c).

In Vitro Studies of the COX-2 Inhibitor NS-398

The effects of the specific COX-2 inhibitor NS-398 were examined in the TPC-1 cell line. A titration experiment with varying concentrations of NS-398 showed inhibition of cell growth which was statistically significant in a dose-dependent manner ($R^2 = 0.590$, $P = 0.0035$), and the maximum inhibition was at 80 μM of NS-398 (Figure 5).

When the TPC-1 cells were treated with NS-398 for varying periods under serum-free conditions, preliminary experiments showed no change over control after 1 and 2 h of treatment (data not shown). Treatment of cells with NS-398 for 8 and 24 h produced a significant increase in COX-2 and VEGF-A mRNAs ($P = 0.0025$ and 0.00189) (Figure 6a–c). After 32 h of treatment, there were no significant differences in the treated cells for COX-2 or VEGF mRNA levels (Figures 6a–c).

Discussion

COX are enzymes which convert arachidonic acid to PG-H. COX-2 has been reported to be upregulated in various cancers including thyroid tumors.[6,8,11–29] COX-2 is also induced in inflammatory conditions such as thyroiditis. COX-2 expression was also higher in the carcinomas compared to the normal thyroid tissue. However, as we recently reported,[36] there was variable expression in both normal thyroid tumors. Although COX-2 mRNA was elevated above levels in normal thyroids in several

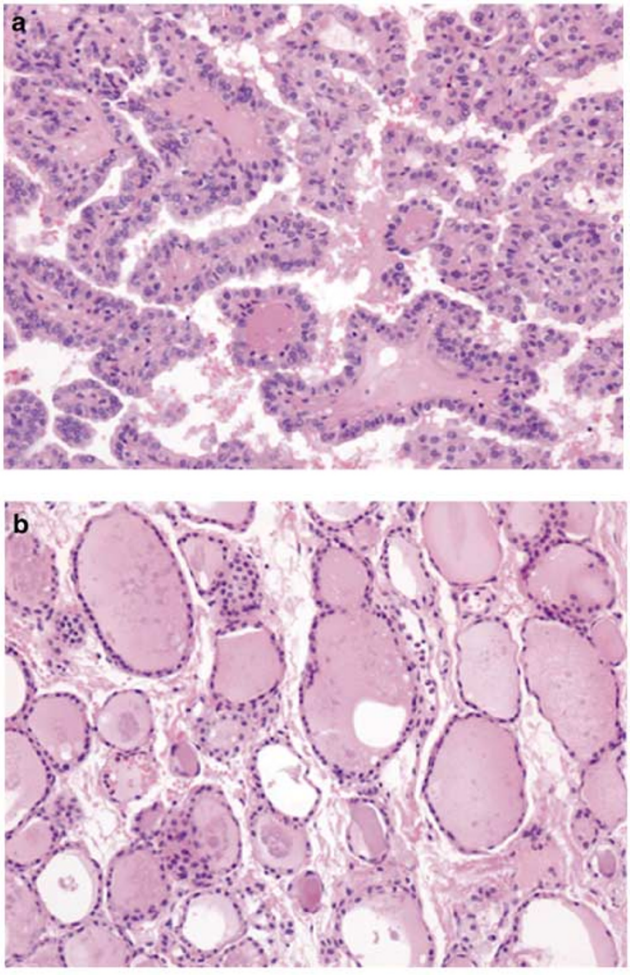

Figure 2 Hematoxylin and eosin section of (**a**) papillary thyroid carcinoma and (**b**) adjacent normal thyroid (Case 2).

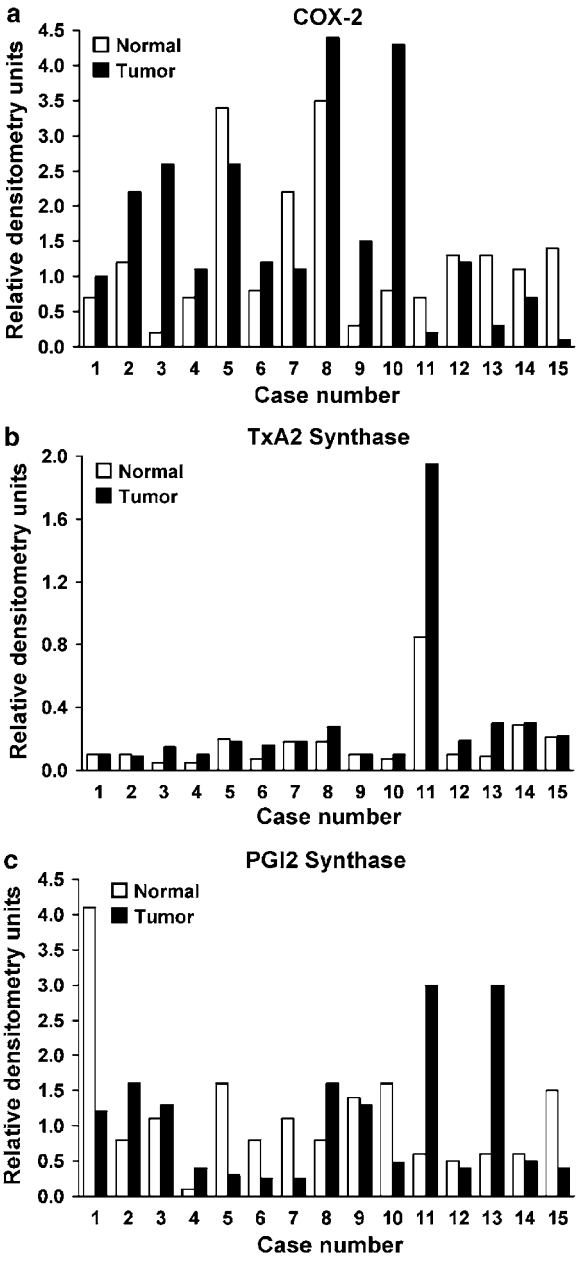

Figure 4 Densitometric analysis of mRNA for the three enzymes analyzed in the study. (**a**) COX-2 analysis in the 15 cases comparing normal and papillary carcinoma for each case. (**b**) Thromboxane synthase (TXA_2 synthase) analysis of normal and papillary carcinoma. (**c**) PGI_2 synthase analysis of normal and papillary carcinoma.

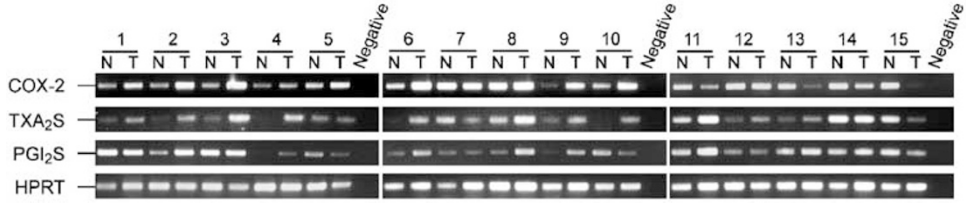

Figure 3 RT-PCR gel analysis of normal thyroid and tumors in the study. RT-PCR and gel analysis was performed as determined in Materials and methods. The negative lane was the no RT reaction.

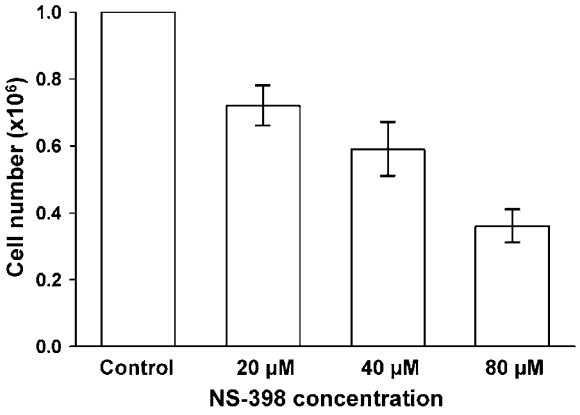

Figure 5 Effects of different concentration of NS-398 on the growth of TPC-1 cells in culture. The 80 μM concentration had the greatest inhibitory effect on tumor growth.

papillary carcinomas, our results did not show significant differences in COX-2 mRNA expression between normal thyroid and papillary carcinoma, because of the variation in mRNA levels. Other studies have also found variable levels of COX-2 proteins in papillary thyroid carcinomas.[29,36] Ito *et al*[29] found nine COX-2-negative cases (18.4%) among the 49 papillary carcinoma cases studied by immunohistochemistry. They showed that COX-2 levels varied with certain conditions and were significantly reduced in older patients, in patients with large tumors, and with advanced disease stages.[29] Three of the patients in our series were over 54 years of age (the cutoff age used by Ito *et al*.[30] Five cases had tumors larger than 4 cm which could contribute to the reduced expression levels of COX-2 in our study. Five patients had tumors showing extrathyroidal invasion. Seven cases had lymph node metastases. However, the influence of age on COX-2 expression remains controversial since another recent study found that COX-2 expression was increased with age in papillary thyroid carcinoma.[45]

Our studies found significant upregulation of TXA_2 synthase in papillary thyroid carcinomas which implicate TXA_2 synthase in the pathogenesis of papillary thyroid carcinoma. Pradono *et al*,[34] recently showed that TXA_2 synthase and PGI_2 synthase-transfected cancer cells had opposite stimulatory and inhibitory effects on their growth *in vivo*, and these effects were related to vascular density. PGI_2 synthase was not significantly different in normal thyroid or tumors in our study. TXA_2 has been associated with tumor proliferation and metastasis and is considered to be proangiogenic while PGI_2 is considered to be an anticancer prostanoid.[34] The number of cases of the nonclassical (follicular) variant of papillary carcinoma was too small ($n = 3$) to observe a relationship between tumor subtype and COX-2 or TXA_2 expression.

To examine directly the role of COX-2 on growth regulation of thyroid tumors, we performed *in vitro*

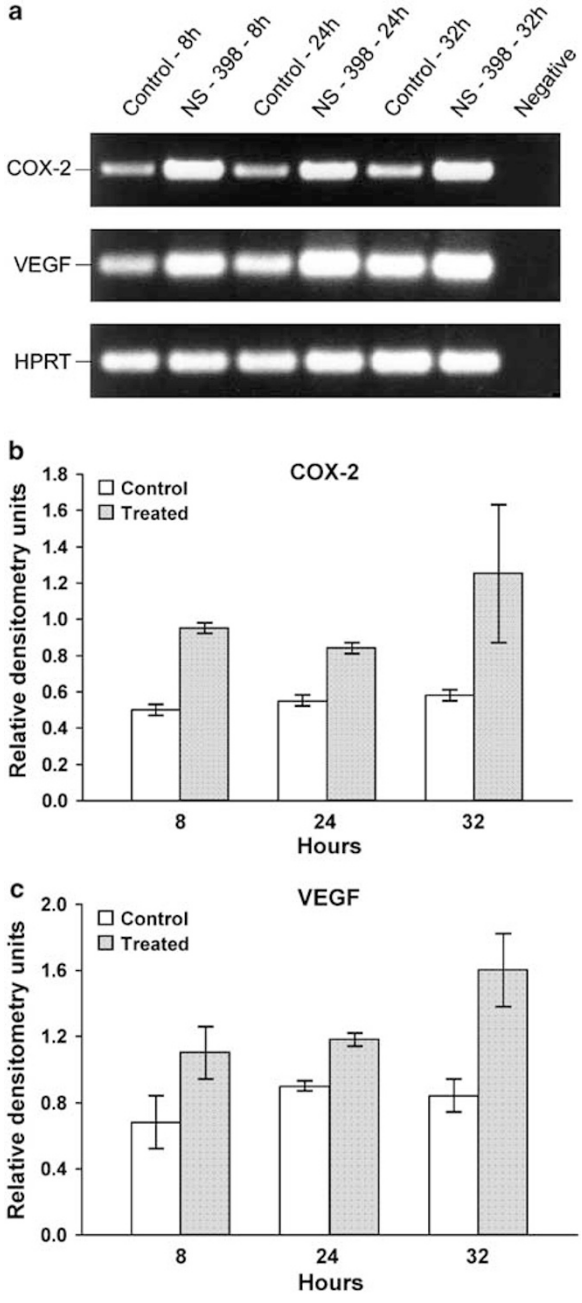

Figure 6 (**a**) RT-PCR analysis of COX-2 and VEGF-A levels after 8, 24, and 32 h of treatment with 80 μM NS-398 on PTG-1 cells. (**b**) Densitometric analysis of COX-2 mRNA levels after 8, 24, and 32 h of treatment with NS-398. There was a significant increase in COX-2 mRNA levels after 8 and 24 h of treatment. (**c**) Densitometric analysis of VEGF-A mRNA levels after 8, 24, and 32 h of treatment with NS-398. There was a significant increase in VEGF-A mRNA after 8 and 24 h of treatment.

studies with the TPC-1 thyroid papillary carcinoma cell line. Studies with NS-398, a COX-2 specific inhibitor of COX-2 enzymatic activity, inhibited growth of these tumor cells. Interestingly, with the inhibition of COX-2 enzymatic activity, there was an increase in COX-2 mRNA levels. These results indicate that COX-2 has a role in the growth of thyroid papillary cell lines. Previous studies have

shown that NSAIDs and COX-2 inhibitors have growth inhibitory effects on various tumors including the colon, pancreas,[8,37] stomach,[38] esophagus,[22] liver,[46] lung, and prostate,[48] carcinoma cell lines. NS-398 has been shown to inhibit cell growth, and induce apoptosis via activating caspase-3 *in vitro*.[47] This is the first report of the growth inhibitory effect of NS-398 on thyroid tumor cells with selective inhibition of COX-2. One earlier study examined NS-398 effects on COX-2 expression in a human thyroid epithelial cell line and found that NS-398 inhibited COX enzyme activity by proinflammatory cytokines.[49]

The TPC-1 cell line expressed COX-2, PGI_2 synthase, and VEGF. TPC-1 had very low expression levels of TXA_2 synthase. NS-398 upregulated COX-2 expression in TPC-1 cells. Our results are similar to those for other tumors such as colon,[10] pancreas,[8] and gliomas.[9] A previous study showed that in transfected colon carcinoma cells, COX-2 upregulated VEGF expression. This expression could be downregulated by NS-398.[50] Related drugs such as NSAIDs had similar effects in prostate,[48] transitional cell carcinoma[51] and pancreatic cells.[52] In our study, VEGF expression level was noticeably affected by NS-398 treatment. This would agree with previous studies, since we also observed an increase in COX-2 mRNA levels after NS-398 treatment. The importance of VEGF in thyroid tumor growth has been well documented.[53–55]

In summary, analysis of papillary thyroid carcinomas and normal thyroid tissue mRNAs by RT-PCR showed significantly increased expression of TXA_2 synthase in the tumors. Although there was an increase in COX-2 mRNA levels in some cases, the differences were not statistically significant. However, our *in vitro* studies showed that the COX-2-specific inhibitor NS-398 increased COX-2 messenger RNA expression, while inhibiting tumor growth implicating a role of COX-2 as well as TXA_2 synthase in papillary thyroid carcinoma proliferation.

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