Prostaglandin Synthases Influence Thyroid Follicular Cell Proliferation But Not Carcinogenesis in Rats Initiated With N-Bis(2-hydroxypropyl)nitrosamine

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To clarify roles of prostaglandin synthases in rat thyroid follicular carcinogenesis, effects of an antithyroid agent, sulfadimethoxine (SDM), and two prostaglandin H synthase (COX) inhibitors, indomethacin and nimesulide, on prostaglandin synthase expression, follicular cell proliferation, and tumor induction in thyroids of rats with or without N-bis(2-hydroxypropyl)nitrosamine (DHPN) initiation were examined. In experiment 1, F344 male rats were allowed free access to drinking water containing SDM (0.1%), SDM + indomethacin (0.0025% in diet), or SDM + nimesulide (0.04% in diet) for 4 weeks. Both COX inhibitors suppressed goitrogenic activity of SDM, but they did not significantly affect microsomal prostaglandin E synthase-2 (mPGES-2) expression levels enhanced by SDM. In experiment 2, all rats received an injection of DHPN (2800 mg/kg body weight), and starting 1 week later, they were treated as in experiment 1 for 4 or 10 weeks. Cell proliferation was suppressed or showed a tendency for suppression by the COX inhibitors in the follicular preneoplastic/neoplastic lesions and surrounding parenchyma, and this was obviously thyroid stimulating hormone independent at least at week 4. However, neither of the COX inhibitors altered the incidence or multiplicity of preneoplastic/neoplastic lesions. Immunohistochemistry revealed significant reduction and elevation of COX-2 and mPGES-2 expression, respectively, in the lesions, but these were also not changed by the COX inhibitors. These results suggest that COX-2 and PGES, and in turn PGE2, might play important roles in follicular cell proliferation but do not affect tumor induction in this rat thyroid carcinogenesis model. Further studies are needed to clarify the significance of the reduction of COX-2 expression in preneoplastic/neoplastic lesions.

Key Words: sulfadimethoxine; indomethacin; nimesulide; thyroid follicular carcinoma; F344 rats.

Arachidonic acid (AA) and its derivatives, the prostaglandins (PGs) and thromboxane (TX), are important in many physiological processes (Smith et al., 1996). Cyclooxygenase (COX), also known as prostaglandin H synthase (PGHS) or prostaglandin endoperoxide synthase (E.C.1.14.99.1), was first identified as the key enzyme in the oxidative conversion of AA to PGG2 and PGH2 (Hamberg et al., 1974; Smith and Lands, 1972). PGH2 is then converted to various bioactive PGs (PGD2, PGE2, PGF2, and PGI2) and TX by the respective terminal prostanoid synthases, which have diverse structures and exhibit cell- and tissue-specific distributions (Kudo and Murakami, 2005). Three isoforms of COXs have been identified (Sobolewski et al., 2010). COX-1 is recognized as a constitutively, almost ubiquitously expressed form found in most organs and tissues, whereas COX-2 is a mitogen-inducible form, upregulated in inflammatory states and cancers (Smith et al., 1996). Although COX-3 has been shown to be present mainly in brain and spinal cord (Kis et al., 2003), its functions remain to be elucidated (Sobolewski et al., 2010).

COX-2 inhibitors have been shown to inhibit cell growth in a number of tumors such as colon, skin epidermal, gall bladder, esophageal, and pancreatic cancers (Grossman et al., 2000; Higashi et al., 2000; Molina et al., 1999; Sheng et al., 2000; Souza et al., 2000) with possible suppression of their development (Castelao et al., 2003; Garcia-Rodrı´guez and Huerta-Alvarez, 2001).

We previously reported that COX-2 is constitutively expressed in nonneoplastic thyroid follicular cells, but COX-2 reactivity was significantly reduced or negative in the neoplastic/neoplastic lesions induced by N-bis(2-hydroxypropyl)nitrosamine (DHPN) followed by an antithyroidal agent, sulfadimethoxine (SDM), or propylthiouracil treatment in rats (Imai et al., 2005). On the other hand, in human thyroid carcinomas, contradictory results have been documented, with COX-2 found elevated in papillary carcinomas (Krawczyk-Rusiecka et al., 2011; Omi et al., 2009; Scarpino et al., 2009), but less frequent or lacking in follicular carcinomas and/or
undifferentiated carcinoma (Cornetta et al., 2002; Ito et al., 2003; Ozolins et al., 2010). The peroxisome proliferator–activated receptor (PPAR) γ, whose activation can be regulated by an endogenous ligand PGJ2, is thought to be related to thyroid tumor progression (Kroll et al., 2000). In addition, PPAR γ ligands inhibit cell proliferation by inducing apoptosis through upregulation of Bax protein without any change in Bcl-2 protein in human anaplastic thyroid carcinoma cell lines (Hayashi et al., 2004). Therefore, we hypothesized that the COX-2-15d-PGJ2-PPAR γ axis might be associated with thyroid carcinogenesis not only in human anaplastic carcinoma cases but also in DHPN-induced rat follicular lesions. In addition, COX inhibitors have been shown to exert antitumor and chemopreventive effects via reduction of PGE2 synthesis in several different forms of cancer (Cui et al., 2005; Li et al., 2008; Moore et al., 2009; Shalk et al., 2004). Therefore, the present study was performed to clarify the roles of COX-2 in thyroid follicular cells and thyroid carcinogenesis in rats. For this purpose, the effects of indomethacin and nimesulide, a COX dual inhibitor and a COX-2 inhibitor, respectively, on goitrogenic activity of SDM in rats were first examined. Subsequently, effects on development of DHPN-initiated follicular preneoplastic/preneoplastic lesions and cell proliferation activities in these and surrounding parenchyma were assessed in the DHPN-SDM model. In addition, we analyzed the expression of microsomal prostaglandin E synthase type-2 (mPGES-2) in rat thyroids. PGES metabolize COX-derived PGH₂ to PGE₂ (Kudo and Murakami, 2005), reported to be expressed in thyroid follicular cells (Han and Smith, 2002).

MATERIALS AND METHODS

Chemicals

DHPN was purchased from Nacalai Tesque (Kyoto, Japan). SDM and nimesulide were from Sigma Chemical (St Louis, MO), and indomethacin was from Wako Pure Chemical Industries (Osaka, Japan). DHPN was dissolved in physiological saline immediately prior to the treatment, and SDM was dissolved in deionized water. Drinking water was prepared at least once a week. Indomethacin and nimesulide were mixed in commercial basal diet (CE-2; CLEA Japan, Tokyo, Japan for experiment 1 or CRF-1, Oriental Yeast, Tokyo, Japan for experiment 2).

Experimental Animals

Six-week-old male F344 rats were purchased from Charles River Japan (Kanagawa, Japan) and used after a 1-week acclimatization period. The animals were housed at a maximum of five to a polycarbonate cage, with white chip bedding in an air-conditioned room. Animals were allowed access to a basal diet and deionized water ad libitum.

Study Designs

**Experiment 1.** Sixteen male F344 rats were divided into four groups, three, three, three, and seven rats for groups 1, 2, 3, and 4, respectively, and drinking water containing 0.1% SDM was provided to groups 1, 2, and 3 for 4 weeks. Group 2 and 3 animals received 25 ppm indomethacin and 400 ppm nimesulide, respectively, in the diet, the dose levels being determined based on the previous literature (Imai et al., 2006; Kitamura et al., 2004; Okajima et al., 1998). Group 4 animals were given deionized water and basal diet. Body weights were recorded once a week. At week 4, after commencement of the SDM treatment, all animals were killed under isoflurane anesthesia by exsanguination. The bilateral thyroid lobes were excised, weighed, and cut in half, and one half was fixed in phosphate-buffered 10% formalin, routinely processed for embedding in paraffin, and serially sectioned at the largest cut surface of each thyroid lobe for staining with hematoxylin-eosin and immunohistochemistry. The other halves of thyroid tissues were stored in a −80°C freezer for Western blot analysis.

**Experiment 2.** One hundred and five male F344 rats were divided into six groups (n = 25 for groups 1–3; n = 10 for groups 4–6) and initiated with a single sc injection of DHPN, diluted to 560 mg/ml in saline, at 2800 mg/kg body weight. One week after the initiation, drinking water containing 0.1% SDM was provided ad libitum to groups 1, 2, and 3 for up to 10 weeks. Groups 2 and 5 animals were treated with 25 ppm indomethacin in the diet. Groups 3 and 6 animals were treated with 400 ppm nimesulide in the diet. Groups 4, 5, and 6 animals were given deionized water, whereas group 1 and 4 animals received basal diet. General conditions were checked daily, and body weights and food intake were recorded once a week. At weeks 4 and 10 from commencement of the SDM treatment, 10 or 15 rats from groups 1, 2, and 3 and 5 rats from groups 4, 5, and 6 were killed under deep ether anesthesia by exsanguination, after blood samples were collected from the abdominal aorta. The bilateral thyroid lobes were excised, weighed and fixed in phosphate-buffered 10% formalin, and routinely processed for embedding in paraffin, and serial sections of the largest cut surface of each thyroid lobe were prepared for staining with hematoxylin-eosin and immunohistochemistry. The experiments were carried out in accordance with the Guide for Animal Experimentation in the National Institute of Health Sciences and the Guideline for Animal Experiments of the National Cancer Center.

Histopathology and Immunohistochemistry

For histopathological evaluation, proliferative lesions of follicular epithelial cells were classified in hematoxylin-eosin–stained sections as diffuse hyperplasia, focal hyperplasia, adenomas, and adenocarcinomas, according to published criteria (Hardisty and Boorman, 1990). Adenocarcinomas were subclassified into invasive carcinomas, involving the thyroid capsule or extrathyroidal tissue, and intrathyroidal carcinomas. For distinguishing adenocarcinomas from focal hyperplasia/adenomas and other nonneoplastic lesions, cellular and structural atypia, characterized by large nuclei with a fine chromatin pattern and/or nuclear grooves, and irregular glandular structures that were not recognizable as follicles were fundamental (Imai et al., 2005). For immunohistochemistry, antigen retrieval was performed in an autoclave for 10 min at 121°C in 10mM citrate buffer (pH6.0). Anti-Ki-67 antigen monoclonal antibodies (clone MIB-5; DAKO, Glostrup, Denmark) were used for determination of cell proliferative activity. COX-2 monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY) and mPGES-2 rabbit polyclonal antibodies from Cayman Chemical (Ann Arbor, MI). The streptavidin-biotin-peroxidase complex method (StreptABComplex/HRP; DAKO) was employed to determine the expression and localization of each protein, and the sections were lightly counterstained with hematoxylin to facilitate microscopic examination. Ki-67-positive nuclei per up to 1000 cells of each type of randomly selected follicular lesion were counted. Expression of COX-2 in each proliferative lesion was divided into three levels (less than 20% of cells, 20–90%, and more than 90%) and assessed for each animal individually.

Western Blot Analysis

Thyroid samples of all rats of groups 1–4 in experiment 1 were homogenized in extraction buffer (50mM Tris-CHI pH7.4, 3mM EDTA, 100mM NaCl, 1% Tween-20, 10mM sodium orthovanadate, and 1mM PMSF) and centrifuged at 14,000 × g for 20 min. Equal amounts of protein samples (50 µg) from collected supernatants were subjected to SDS-polyacrylamide gel electrophoresis on 5–20% gradient acrylamide gels (ATTO, Tokyo, Japan), and the separated proteins were transferred to polyvinyliden difluoride membranes (Whatman, Sanford, ME). Immunoblotting was performed using rabbit
polyclonal antibodies against mPGES-2 (Cayman Chemical) or monoclonal antibodies against β-actin (clone AC-15; Sigma), followed by exposure to peroxidase-labeled anti-rabbit or mouse IgG goat antibodies (DAKO) and development of signals with 3,3′,5,5′ tetramethylbenzidine (ATTO).

**Hormone Determinations**

At necropsy, blood samples collected from the abdominal aorta of all animals in experiment 2 under ether anesthesia were assayed for serum thyroxine (T4), triiodothyronine (T3), and thyroid stimulating hormone (TSH) levels with radioimmunoassay kits, a GammaCoat Total T4 human kit (Dia Sorin, Saluggia, Italy), a RIABEAD Kit for human T3 (Dinabot, North Chicago, IL), and the Rat Thyroid Stimulating Hormone [125I] Biotrak Assay (Amersham Pharmacia Biotech, Hemel Hempstead, U.K.), respectively, at SRL (Tokyo, Japan).

**Statistical Analysis**

Statistical analyses to compare the body and thyroid weights, serum hormone levels, quantitative data for immunoblotting, and Ki-67-positive ratios, as well as the multiplicity of histopathological findings in experiment 1 and 2 were carried out using Tukey’s multiple comparison test. Ki-67-positive ratios in groups 1–3 in experiment 2 were additionally analyzed using the Student’s or Welch’s t-tests following the F-test. For serum TSH levels and Ki-67-positive ratios in experiment 1 were determined. Bars show mean and SD. (*p < 0.05, **p < 0.01 versus group 1; †p < 0.05, ††p < 0.01 versus group 2; and ‡‡p < 0.01 versus group 3) (Tukey’s multiple comparison test).

**RESULTS**

**Experiment 1**

No abnormalities in general condition were observed in any of the groups. Final body weights were significantly lowered by SDM treatment (groups 1–3), but no changes were observed with indomethacin or nimesulide treatment (Table 1). Thyroid absolute weights and relative to body weights were significantly increased by SDM (groups 1–3) and lowered by nimesulide treatment (group 3) (Table 1). Relative thyroid weights in SDM + nimesulide group 3 were lower than that in SDM + indomethacin group 2 (p < 0.05) (Table 1). Histopathologically, diffuse follicular cell hyperplasia and capsular thickening with inflammatory cells including macrophages in the thyroids were observed in rats of SDM-treated groups 1–3 as described earlier (Imai et al., 2004), but no obvious changes were found with indomethacin or nimesulide treatment (data not shown). Ki-67-positive ratios were significantly increased by SDM treatment (groups 1–3), and they were significantly lowered by indomethacin but not by nimesulide treatment (groups 2 and 3) (Table 1). Expression of mPGES-2 protein was trace in the control (group 4) and significantly enhanced or showed a tendency for increase with SDM treatment (groups 1–3). The SDM increase in mPGES-2 levels was not altered by indomethacin or nimesulide treatment (Fig. 1).

**FIG. 1.** Changes in levels of mPGES-2 protein in the thyroids of rats treated with SDM alone, or simultaneously with indomethacin or nimesulide, as compared with nontreated controls. Seven samples of control and three samples of the other each group were determined. Bars show mean and SD. *p < 0.05, **p < 0.01 (Tukey’s multiple comparison test) versus control.
Experiment 2

In-life parameters and thyroid weights. No abnormalities in general condition were observed in any of the groups. Final body weights were significantly lowered by SDM treatment (groups 1–3) at week 4 as compared with groups 4–6, and similar changes were observed also at week 10 (Table 2). Among the groups without SDM treatment, final body weights were significantly lowered by nimesulide (group 6) and appeared to be lowered by indomethacin treatment (group 5) as compared with the DHPN alone group 4 values at week 10, but relations between the body weights and chemical treatments were not clear because opposite effects or no changes were found at week 4. Although no differences in absolute and relative thyroid weights were found among the groups with and without SDM treatment at week 4, relative weights were significantly lowered by indomethacin or nimesulide treatment (groups 2 and 3) as compared with SDM-treated group 1 at week 10 (Table 2).

Serum hormone levels. At week 4, serum TSH levels were significantly elevated by indomethacin and showed a tendency for elevation by nimesulide treatment (groups 2 and 3) as compared with the SDM-treated group 1 (Table 3). The serum TSH levels in the groups with indomethacin or nimesulide treatment (groups 2, 3, 5, and 6) at week 4 were significantly decreased by week 10 (p < 0.01 or 0.05; 50.2, 46.8, 35.2, and 34.5% lowering, respectively) but not in the DHPN-SDM–treated group 1 (7.9% lowering) and the DHPN alone group 4 (16.2% lowering). At week 10, serum TSH levels showed a tendency for decrease in groups 2 and 3 as compared with group 1. Serum T3 levels was decreased at week 4 by indomethacin treatment in group 5, T4 and/or TSH levels showed a tendency for decrease at week 4 and were significantly decreased at week 10 by nimesulide treatment in group 6, as compared with the DHPN alone group 4.

Histopathology and immunohistochemistry. In SDM-treated groups 1–3, focal follicular cell hyperplasia and adenomas were observed at week 4 and 10, and carcinomas were additionally found at week 10. There were no significant differences in the incidences and multiplicities of the lesions among the groups (Table 4). No proliferative lesions of follicular epithelial cells were observed in groups 4–6 without SDM treatment at week 4 and 10 (Table 4). As a non-proliferative change, thickening of the thyroid capsule with inflammation was observed in the SDM-treated groups 1–3. Grading of the capsular lesions based on the spread of inflammation was carried out, but no significant variation was apparent among the groups (data not shown). Ki-67-positive ratios for focal hyperplasias and adenomas showed a tendency for decrease with indomethacin or nimesulide treatment (groups 2 and 3) as compared with the SDM-treated group 1 at weeks 4 and 10 (Table 5). In contrast, in surrounding follicular parenchyma, Ki-67-positive ratios were significantly decreased or showed a tendency for decrease in groups 2 and 3 as compared with group 1 at weeks 4 and 10 and in groups 5 and 6 at week 10 as compared with control group 4 without SDM treatment (Table 5). For comparison of Ki-67-positive ratios between weeks 4 and 10, those in both focal hyperplasias/adenomas and surrounding follicular parenchyma in groups 1–3 at week 4 were significantly declined by week 10 (p < 0.01). Immunohistochemistry revealed significant reduction and induction of COX-2 and PGES expression, respectively, in focal hyperplasias, adenomas, and carcinomas.

### TABLE 2
Final Body and Organ Weights in Rats Initiated With DHPN Then Receiving SDM/Indomethacin or Nimesulide Administration (Experiment 2)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHPN-SDM</td>
<td>DHPN-SDM + indomethacin</td>
<td>DHPN-SDM + nimesulide</td>
<td>DHPN alone</td>
<td>DHPN + indomethacin</td>
<td>DHPN + nimesulide</td>
</tr>
<tr>
<td>Week 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of animals</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>194.7 ± 13.9</td>
<td>189.7 ± 2.6</td>
<td>201.4 ± 11.5</td>
<td>237.0 ± 7.5</td>
<td>251.1 ± 10.5</td>
<td>237.6 ± 6.1</td>
</tr>
<tr>
<td>Absolute thyroid weight (mg)</td>
<td>95 ± 11</td>
<td>97 ± 10</td>
<td>94 ± 11</td>
<td>12 ± 3</td>
<td>12 ± 3</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Relative thyroid weight (mg/100g body weight)</td>
<td>49 ± 8</td>
<td>51 ± 5</td>
<td>47 ± 6</td>
<td>5 ± 1</td>
<td>5 ± 2</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Week 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of animals</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>232.9 ± 13.4</td>
<td>230.5 ± 13.2</td>
<td>236.6 ± 11.2</td>
<td>281.7 ± 28.1</td>
<td>269.0 ± 5.7</td>
<td>230.0 ± 12.9</td>
</tr>
<tr>
<td>Absolute thyroid weight (mg)</td>
<td>206 ± 31</td>
<td>179 ± 23</td>
<td>185 ± 33</td>
<td>13 ± 3</td>
<td>13 ± 1</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Relative thyroid weight (mg/100g body weight)</td>
<td>88 ± 9</td>
<td>77 ± 8</td>
<td>78 ± 12</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

Notes. Data are means ± SDs.

* *p < 0.05, **p < 0.01 versus group 1; ††p < 0.01 versus group 2; †*p < 0.01 versus group 3; §§p < 0.01 versus group 4; ‡‡p < 0.01 versus group 5 (Tukey’s multiple comparison test).
but they were not obviously changed by the COX inhibitors (Figs. 2 and 3).

**DISCUSSION**

To clarify the roles of constitutive expression of COX-2 in thyroid follicular cells and the significance of its reduction in thyroid carcinogenesis in rats, in experiment 1, the effects of COX inhibitors indomethacin and nimesulide on the goitrogenic actions of SDM were here evaluated in rats without DHPN initiation. The decreased body weight gain and significant increase in thyroid weights observed with SDM treatment under the present conditions were consistent with our previously reported data (Imai et al., 2004, 2005). The increased thyroid size, so called goiter, was histopathologically identified as due to diffuse follicular cell hyperplasia. One of the most important aspects in the present study was that goitrogenic actions of SDM were inhibited by the COX inhibitors, i.e., thyroid weights were lowered by nimesulide (but not by indomethacin) and Ki-67-positive ratios by indomethacin (but not by nimesulide).

### TABLE 3

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3 (ng/ml)</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.0</td>
<td>0.7 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>T4 (ng/ml)</td>
<td>35.4 ± 6.1</td>
<td>33.2 ± 2.5</td>
<td>37.7 ± 4.7</td>
<td>61.2 ± 5.0</td>
<td>71.8 ± 9.5</td>
<td>54.6 ± 7.1</td>
</tr>
<tr>
<td>TSH (ng/ml)</td>
<td>161.5 ± 59.2</td>
<td>220.2 ± 38.2</td>
<td>208.5 ± 36.6</td>
<td>9.9 ± 2.5</td>
<td>12.5 ± 2.8</td>
<td>8.7 ± 1.0</td>
</tr>
</tbody>
</table>

*Week 4*

| No. of animals | 10 | 10 | 10 | 5 | 4 | 5 |

Notes. Data are means ± SDs.

*p < 0.05, **p < 0.01 versus group 1; †p < 0.05, ††p < 0.01 versus group 2; **p < 0.01 versus group 3; ‡p < 0.05, ‡‡p < 0.01 versus group 4; ††p < 0.01 versus group 5 (Tukey's multiple comparison test).

### TABLE 4

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3 (ng/ml)</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>T4 (ng/ml)</td>
<td>35.5 ± 6.1</td>
<td>33.5 ± 4.8</td>
<td>38.5 ± 4.2</td>
<td>62.6 ± 6.0</td>
<td>60.2 ± 9.7</td>
<td>42.8 ± 4.1</td>
</tr>
<tr>
<td>TSH (ng/ml)</td>
<td>148.8 ± 65.3</td>
<td>109.7 ± 69.6</td>
<td>110.9 ± 51.9</td>
<td>8.3 ± 1.6</td>
<td>8.1 ± 1.4</td>
<td>5.7 ± 0.7</td>
</tr>
</tbody>
</table>

*Week 10*

| No. of animals | 15 | 15 | 15 | 5 | 5 | 5 |

Notes. Data are means ± SDs.

*p < 0.05, **p < 0.01 versus group 1; †p < 0.05, ††p < 0.01 versus group 2; **p < 0.01 versus group 3; ‡p < 0.05, ‡‡p < 0.01 versus group 4; ††p < 0.01 versus group 5 (Tukey's multiple comparison test).

*aIncidence.

*bMultiplicity (mean number of lesions per animal).
addition, an inhibitory action of the COX inhibitors on SDM-induced goiter was observed with reference to Ki-67-positive rates in surrounding follicular parenchyma at weeks 4 and 10. Several inconsistencies in the response to each COX inhibitor on thyroid weights and Ki-67-positive rates in experiments 1 and 2 were speculated to be caused by a gap in the reaction time of follicular cell proliferation and hypertrophy (each featured diffuse follicular cell hyperplasia) under different conditions. At the same time with the inhibitory action of the COX inhibitors on SDM-induced goiter, serum TSH levels were significantly increased by indomethacin and showed a tendency for increase by nimesulide as compared with DHPN-SDM–treated control at week 4. These results clearly indicated that constitutively expressed COX-2 in rat thyroid follicular cells is involved in their proliferation in a TSH-independent fashion. Western blot analysis in experiment 1 revealed enhanced mPGES-2 expression levels by SDM treatment. The results suggest that the COX-2-PGES-PGE2 axis might simultaneously stimulate follicular cell proliferation with TSH (Capen, 2008; Mitsumori et al., 1995) under conditions of antithyroidal action of SDM. The main regulators of thyroid growth and function are understood to be TSH and insulin, which is recognized by both insulin and insulin-like growth factor-I receptors (Dumont et al., 1992). TSH is known to activate thyroid epithelial cells via both G\textsubscript{s}-cyclic adenosine monophosphate/protein kinase A/extracellular signal-regulated kinases– and G\textsubscript{q/-Akt/protein kinase C–coupled signaling networks (Morshed et al., 2009). On the other hand, PGE\textsubscript{2} acts through different membrane receptors called EP receptors (EP1, EP2, EP3, and EP4). One of the EP1 signaling pathways is thought to be involve phospholipase C/inositol triphosphate signaling, leading to intracellular mobilization of calcium (Breyer et al., 2001). One of the important roles of PGE\textsubscript{2} is known to be stimulation of cell proliferation in several organs or tissues (Ansari et al., 2008; Harding and LaPointe, 2011; Hoggatt et al., 2009; Shao et al., 2006) and also in thyroid follicles cell proliferation might be controlled at least partly via PGE\textsubscript{2} signaling pathways, independent of conventional TSH. To our knowledge, this is the first report directly showing COX-2 and mPGES-2 involvement in thyroid follicular proliferation in rats.

However, neither COX inhibitor altered the incidence or multiplicity of preneoplastic/neoplastic lesions in experiment 2. Immunohistochemistry revealed induction of mPGES-2 expression, but COX-2 expression was significantly reduced in follicular preneoplastic/neoplastic lesions as also documented in our previous report (Imai et al., 2005). Although the significance and mechanisms of reduction of COX-2 in lesions have yet to be elucidated, Ki-67 analysis revealed that the inhibitory effects of COX inhibitors on SDM-induced cell proliferation in surrounding follicular parenchyma were more pronounced than those in preneoplastic/neoplastic lesions. In addition, reactively elevated serum TSH by COX inhibitors at week 4 appeared to paradoxically interfere with the protective effect of the COX inhibitors on DHPN-SDM–induced carcinogenesis in this rat model.

The elevated serum TSH levels in the groups with indomethacin or nimesulide treatment at week 4 were significantly decreased by week 10. Although the mechanisms are completely unclear, this is in line with the earlier finding of elevated serum TSH levels in early stages of SDM treatment gradually declining with continuous exposure (Mitsumori et al., 1995). Exhaustion of TSH-producing cells in the pituitary or acclimation of thyroid follicular cells to SDM may at least partly be involved. Actually, the elevated levels of serum TSH by indomethacin or nimesulide treatment at week 4 in DHPN-SDM–treated rats (208–220 ng/ml) were much higher than those of DHPN-SDM–treated controls not only in the present study (161 ng/ml) but also in a previously experiment (128 ng/ml) (Imai et al., 2009). On the contrary,

### Table 5

<table>
<thead>
<tr>
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<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHPN-SDM</td>
<td>3.3 ± 0.5</td>
<td>3.5 ± 0.6</td>
<td>3.2 ± 0.7</td>
<td>3.4 ± 0.6</td>
<td>3.6 ± 0.7</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>DHPN-SDM +</td>
<td>3.3 ± 0.5</td>
<td>3.5 ± 0.6</td>
<td>3.2 ± 0.7</td>
<td>3.4 ± 0.6</td>
<td>3.6 ± 0.7</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>indomethacin</td>
<td>3.3 ± 0.5</td>
<td>3.5 ± 0.6</td>
<td>3.2 ± 0.7</td>
<td>3.4 ± 0.6</td>
<td>3.6 ± 0.7</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>nimesulide</td>
<td>3.3 ± 0.5</td>
<td>3.5 ± 0.6</td>
<td>3.2 ± 0.7</td>
<td>3.4 ± 0.6</td>
<td>3.6 ± 0.7</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>DHPN alone</td>
<td>3.3 ± 0.5</td>
<td>3.5 ± 0.6</td>
<td>3.2 ± 0.7</td>
<td>3.4 ± 0.6</td>
<td>3.6 ± 0.7</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>indomethacin</td>
<td>3.3 ± 0.5</td>
<td>3.5 ± 0.6</td>
<td>3.2 ± 0.7</td>
<td>3.4 ± 0.6</td>
<td>3.6 ± 0.7</td>
<td>3.7 ± 0.8</td>
</tr>
</tbody>
</table>

**Notes.** Data are means ± SDs. $^a$ p < 0.01 versus group 1; $^b$ p < 0.05 versus group 2; **p < 0.01 versus group 3 (Tukey’s multiple comparison test); $^c$ p < 0.05 versus group 1 (Student’s t-test); $^d$ p < 0.05 versus group 1 (Welch’s t-test).
suppressive effects of the COX inhibitors on SDM-induced goiter were still observed at week 10, suggesting that COX inhibitory effects were maintained at the time. In the nimesulide-treated group without SDM, serum T4 and/or TSH levels showed a tendency for decrease at week 4 and were significantly decreased at week 10 as compared with the DHPN alone control group. Ki-67-positive ratios also showed a tendency for decrease in the nimesulide-treated group without SDM at week 10, and these data might suggest that COX-2 is related to cell proliferation and function in thyroid follicles in rats.

In conclusion, although COX-2 and PGES, and in turn PGE$_2$, might play important roles in thyroid follicular cell proliferation, they do not appear to affect tumor induction in the DHPN-SDM rat model. Further studies are needed to clarify the significance of reduction of COX-2 expression in preneoplastic/neoplastic lesions.

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**REFERENCES**


