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Inhibitory effect of inducible nitric oxide synthase inhibitors on DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis

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Abstract

We investigated the effects of two NOS inhibitors (AG and L-NAME) on DMBA-induced hamster buccal–pouch carcinogenesis. Six hundred Syrian golden hamsters were split into two divisions (I and II); divisions split into three groups (experimental groups A and B, control group C); and each group into subgroups of 20 (A1–A6, B1–B6 and C1–C3). The pouches of animals in groups A1–A3 were painted first with AG of differing concentrations (10, 20, and 30 μmol/ml) and then 30 min later with DMBA (0.5%), thrice weekly for 9 weeks. Subgroups A4–A6 only received AG treatment. Groups B1 to B6 were similarly treated with L-NAME. Animals in division II were treated in the same manner for 13 weeks. Post-mortem analysis revealed that both inhibitors can suppress the development of epithelial dysplasias and squamous-cell carcinomas. An associated increase in the numbers of epithelial hyperplasias was paralleled by a decrease in iNOS protein expression. This animal model can be employed to evaluate the potential use of iNOS inhibitors as novel therapeutic tools for oral squamous-cell carcinogenesis.

Keywords: iNOS inhibitor; AG; L-NAME; Hamster; DMBA-carcinogenesis; Oral

Nitric oxide (NO), first described as an endothelium-derived relaxation factor in the 1980s, is a key signaling molecule that mediates many physiological processes including vasodilation and neurotransmission [1,2], as well being involved in a variety of pathological disorders including cancer [3,4]. NO is endogenously formed by a family of enzymes called NO synthases (NOSs) [5]. There are three isoforms, two of which are constitutively expressed and are Ca^{2+}-dependent (NOS1 [neuronal NOS (nNOS)] and NOS3 [endothelial NOS (eNOS)]), and one, the Ca^{2+}-independent isoform, (NOS2 [inducible NOS (iNOS)]) that usually requires induction [6,7].

Inducible NOS and NO have been associated with the development of human and animal cancers in vivo [8]. A number of activities mediated by NO may contribute to its tumor enhancing effects, including induction of DNA damage [9], increased angiogenesis [10], prevention of apoptotic cell death [11], and suppression of the immune system [12]. On the other hand, some reports indicate that NO is cytotoxic to tumor cells [13] and can decrease tumor growth and metastasis in vivo [14–16]. These contradictory roles of iNOS and NO in tumorigenesis can be explained as a concentration-dependent phenomenon, where relatively lower concentrations are beneficial to tumor cell proliferation, and relatively higher concentrations, from exogenous sources (NO donors), are inhibitory to tumor growth. With regard to oral carcinogenesis (both human and animal), most of the available data suggest that iNOS is present at levels that promotes tumor development [17]. The concentrations of NO released by oral dysplastic keratinocytes and cancer cells are insufficient to produce a tumorcidal effect, and are likely to facilitate angiogenesis and tumor dissemination [8,18].

Shang et al. [19] reported on the effects of exogenous NO on oral squamous-cell carcinomas. The authors

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found that high levels of exogenous NO caused death of tumor cells. This observation suggests that the manipulation of NO concentrations by providing exogenous NO is a possible avenue for future cancer therapy. However, such high doses of exogenous NO are obviously limited by their side effects-vasodilation, hypotension, and headache [20]. It is unlikely that the NO concentrations found by Shang et al. [19] to cause death of oral cancer cells in vitro would have a similar effect in vivo without causing marked systemic complications. There is, however, another potential area for possible future cancer therapy. Pharmacological inhibition of iNOS would reduce NO production resulting in significant reduction in angiogenesis in tumors [21,22].

All NOSs use free L-arginine and molecular oxygen as substrates to produce NO and L-citrulline. The L-citrulline produced is shunted into a metabolic pathway that regenerates free L-arginine [23]. Analogues of L-arginine modified at the terminal guanidine nitrogen and/or the carboxyl terminus are widely used to inhibit the production of NO. An example of one of these compounds is the methyl ester analogue N^G-nitro L-arginine methyl ester (L-NAME), which is a non-selective inhibitor that suppresses all types of NOSs [24]. Investigators have also developed some selective inhibitors such as aminoguanidine (AG), which has the same guanidine group as that of L-arginine. AG is approximately 10-fold more selective for iNOS than eNOS, and is minimally selective for nNOS [25].

The buccal–pouch mucosa of hamsters provides one of the most widely accepted experimental models for oral carcinogenesis [26]. Despite anatomical and histological differences between hamster pouch mucosa and human buccal tissue, experimental carcinogenesis protocols for the former induce premalignant changes and carcinomas that are similar to the development of premalignancy and malignancy in human oral mucosa [27]. In addition, metastasis to distant organs are extremely rare in this animal model [26,27]. To date, the effects of iNOS inhibitors on experimentally induced oral carcinogenesis are unknown. In this study, we investigate the effects of two NOS inhibitors (AG and L-NAME) on 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced hamster buccal–pouch squamous-cell carcinogenesis.

Table 1
Protocol of AG and L-NAME over a 9-week period on DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis

Division I	Treatment protocol
A	AG: aminoguanidine
A1	AG (10 μmol/ml)[a] DMBA–9 wk → killed
A2	AG (20 μmol/ml)[a] DMBA–9 wk → killed
A3	AG (30 μmol/ml)[a] DMBA–9 wk → killed
A4	AG (10 μmol/ml)–9 wk → killed
A5	AG (20 μmol/ml)–9 wk → killed
A6	AG (30 μmol/ml)–9 wk → killed
B	L-NAME:N^G-nitro L-arginine methyl ester
B1	L-NAME (10 μmol/ml)[a] DMBA–9 wk → killed
B2	L-NAME (20 μmol/ml)[a] DMBA–9 wk → killed
B3	L-NAME (30 μmol/ml)[a] DMBA–9 wk → killed
B4	L-NAME (10 μmol/ml)–9 wk → killed
B5	L-NAME (20 μmol/ml)–9 wk → killed
B6	L-NAME (30 μmol/ml)–9 wk → killed
C	Control
C1	DMBA–9 wk → killed
C2	Mineral oil–9 wk → killed
C3	No treatment–9 wk → killed

DMBA: 7,12-dimethyl benz[*a*]anthrance.
[a] AG/L-NAME: applied 30 min prior to DMBA painting; wk: week.

Table 2
Protocol of AG and L-NAME over a 13-week period on DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis

Division II	Treatment protocol
A	AG: aminoguanidine
A1	AG (10 μmol/ml)[a] DMBA–13 wk → killed
A2	AG (20 μmol/ml)[a] DMBA–13 wk → killed
A3	AG (30 μmol/ml)[a] DMBA–13 wk → killed
A4	AG (10 μmol/ml)–13 wk → killed
A5	AG (20 μmol/ml)–13 wk → killed
A6	AG (30 μmol/ml)–13 wk → killed
B	L-NAME:N^G-nitro L-arginine methyl ester
B1	L-NAME (10 μmol/ml)[a] DMBA–13 wk → killed
B2	L-NAME (20 μmol/ml)[a] DMBA–13 wk → killed
B3	L-NAME (30 μmol/ml)[a] DMBA–13 wk → killed
B4	L-NAME (10 μmol/ml)–13 wk → killed
B5	L-NAME (20 μmol/ml)–13 wk → killed
B6	L-NAME (30 μmol/ml)–13 wk → killed
C	Control
C1	DMBA–13 wk → killed
C2	Mineral oil–13 wk → killed
C3	No treatment–13 wk → killed

DMBA: 7,12-dimethyl benz[*a*]anthrance.
[a] AG/L-NAME: applied 30 min prior to DMBA painting; wk: week.

Materials and methods

Animals

Six hundred out-bred, young (6-weeks old), male, Syrian golden hamsters (*Mesocricatus auratus*; purchased from the National Science Council Animal Breeding Center, ROC), weighing about 100 g each at the commencement of the experiment, were randomly divided into two divisions, I (Table 1) and II (Table 2). The animals were housed under constant conditions (22 °C, 12-h light/dark cycle) and fed with tap water and standard Purina laboratory chow ad libitum. The animal-handling protocol ensured humane practices were observed throughout the experimental process.

The division I (Table 1) was divided into two experimental groups, A and B, as well as one control group, C. The experimental group A was further divided into subgroups A1-A6, each with 20 animals. Subsequent to 1 week of acclimatization to their new surroundings, on

Monday, Wednesday, and Friday of each subsequent week for 9 weeks, both pouches of each animal from the subgroups A1–A6 were painted with AG (Sigma, St. Louis, Missouri, USA) solution of various concentrations (A1: 10 μ mol/ml; A2: 20 μmol/ml; A3: 30 μmol/ml) and then 30 min later with a 0.5% DMBA solution (Sigma, purity: approx. 95%) which was dissolved in mineral oil (Sigma, purity: 100%), using a No. 4 sable-hair brush. Approximately 0.2 ml of the respective solution was applied topically to the medial walls of both pouches at each painting session. Bilateral pouches of subgroups A4–A6 were painted only with AG of various concentrations (A4: 10 μmol/ml; A5: 20 μmol/ml; A6: 30 μmol/ml). Bilateral pouches of each animal from subgroups B1 to B6, each with 20 animals, were similarly treated with a solution of L-NAME (Sigma, St. Louis, Missouri, USA) of different concentrations. Both pouches of each animal of one of the control groups (C1, 20 animals) were similarly treated with mineral oil while another control group (C2, 20 animals) was treated with 0.5% DMBA solution. The last control group (C3, 20 animals) remained untreated throughout the experiment. The treatment protocol for division II (Table 2) was similar to that of division I (Table 1) with the exception that the painting period was 13 weeks long.

At the end of 9 and 13 weeks of treatment respectively (three days following the final treatment), and in order to avoid the potential influence of diurnal variation [28], all of the animals from each division were simultaneously killed at 9 a.m. by inhaling a lethal dose of diethyl ether. The animals' pouches were exposed by dissection and examined grossly. Both pouches were then excised. The number of hamsters with tumors and the average number of tumors per hamster were counted. In addition, the average dimension of the tumors was determined. The tissues were subsequently fixed in 10% neutral-buffered formalin solution for about 24 h, dehydrated in a series of ascending-concentration alcohol solutions, cleared in xylene, and embedded in paraffin for immunohistochemistry studies.

Immunohistochemical staining

Paraffin-embedded, 4-μm-thick tissue sections were stained for the primary antibody for iNOS protein. Staining was performed using a standard avidin–biotin peroxidase-complex (ABC) method [29]. Polyclonal antibodies to mouse iNOS were generated in rabbits and obtained from Calbiochem-Novabiochem (Cat. No. 482728). This antibody recognizes iNOS protein in tissue derived from humans, rats, and mice and exhibits no cross-reactivity with eNOS and nNOS (information from manufacturer). The specificity of this antibody has been established in a previous study [30]. Subsequent to deparafinization in xylene and ethanol, the tissue sections were treated in 0.3% H_2O_2–methanol to block endogenous peroxidase activity. The antigenicity was subsequently unmasked by microwave heating for a period of 3 min in a 10 mM solution of sodium citrate. Following this, a 10% solution of normal goat serum was applied to reduce non-specific staining in all tissue sections stained for iNOS protein. All sections were subsequently incubated with the primary antibody (1:500) at room temperature for 30 min. Following rinsing with PBS, all sections were then incubated for 30 min at room temperature in the presence of biotin-conjugated goat anti-rabbit IgG (Vector, Burlingame, USA; 1:100), following which all the sections were again washed with PBS and then incubated with avidin–biotin complex conjugated to horseradish peroxidase (Dako, Santa Barbara, CA, USA) for a further 30 min. After a final PBS rinse, the sites of peroxidase binding were visualized as brown reaction products from a benzidine staining. The sections were then counterstained with hematoxylin. Each set of staining experiments included a human buccal squamous-cell carcinoma specimen known to express iNOS, which served as a positive control and ensured the reproducibility of the staining process. A negative control, in which the primary antibody step was omitted, was also included in each set of experiments. The quantitative determination of the iNOS immunoactivity has been counted according to our previous study [30], with the possible patterns of iNOS positivity, randomly distributed in a pouch mucosa, may vary from a single cell to a cluster of two or more cells without the interruption of unstained cells.

Table 3
Summary of the effects of AG and L-NAME over a 9-week period on DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis

Division I	Incidence of various histological findings	iNOS protein expression
A		
A1	Dysplasia[a] (17)**; Epithelial hyperplasia (3)	6.2* ± 2.4[b]
A2	Dysplasia (12)**; Epithelial hyperplasia (8)	5.3* ± 1.2
A3	Dysplasia (11)**; Epithelial hyperplasia (9)	5.1* ± 1.8
A4	Hyperorthokeratosis (20)	0 ± 0
A5	Hyperorthokeratosis (20)	0 ± 0
A6	Hyperorthokeratosis (20)	0 ± 0
B		
B1	Dysplasia (16)**; Epithelial hyperplasia (4)	6.8* ± 2.7
B2	Dysplasia (12)**; Epithelial hyperplasia (8)	5.7* ± 1.6
B3	Dysplasia (11)**; Epithelial hyperplasia (9)	5.4* ± 1.4
B4	Hyperorthokeratosis (20)	0 ± 0
B5	Hyperorthokeratosis (20)	0 ± 0
B6	Hyperorthokeratosis (20)	0 ± 0
C		
C1	Dysplasia (20)	8.6 ± 2.5
C2	Normal (20)	0 ± 0
C3	Normal (20)	0 ± 0

DMBA: 7,12-dimethyl benz[*a*]anthrance; [a]Dysplasia: epithelial dysplasia; iNOS: inducible nitric oxide synthase; number in bracket: number of animals; [b]mean ± standard deviation; *significantly difference from group C1 ($p < 0.001$); **significantly difference from group C1 ($p < 0.05$).

Statistical analysis

Quantitative differences between group values were statistically analyzed using ANOVA (analysis of variance) or χ^2 test.

Results

The effects of AG and L-NAME over a 9-week period on DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis are tabulated in Table 3 and some representative examples are shown (Figs. 1 and 2). The 9-week mineral oil-treated and 9-week untreated pouches revealed no obvious changes (subgroups C2 and C3, Table 3). However, a 100% incidence of epithelial dysplasia was noted for all of the 9-week DMBA-treated pouches (subgroup C1, Table 3). Those pouches treated with varying concentrations of AG and L-NAME alone for 9 weeks demonstrated hyperorthokeratosis (subgroups A4–A6; B4–B6, Table 3). It is noteworthy that a dose-dependent decrease in the incidence of dysplasia for those subgroups treated with different concentrations of AG (subgroups A1–A3, Table 3) prior to DMBA treatment for 9 weeks has been observed when compared with DMBA alone treated group (subgroup C1, Table 3) ($p < 0.05$), with a leveling effect at the 20 and 30 μmol/ml doses. L-NAME treatments in DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis over the 9-week experiment exhibited similar suppressive effects (subgroups B1–B3, Table 3). This decrease in dysplasia was associated with an increased incidence of epithelial hyperplasia and in parallel with a decrease in iNOS activity (Table 3) ($p < 0.001$), as demonstrated by the cytoplasmic/nuclear stainings (Fig. 2). Leveling of iNOS protein expression was noted for the concentrations of 20 and 30 μmol/ml of both AG and L-NAME (Table 3).

The chemopreventive effects of AG and L-NAME on DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis over a 13-week period are summarized in Table 4, with representative examples shown in Figs. 3–5. Squamous-cell carcinomas with a 100% tumor incidence were apparent for all of the 13-week DMBA-treated pouches (subgroup C1, Table 4). The 13-week mineral oil-treated and 13-week untreated pouches revealed no obvious changes (subgroups C2 and C3, Table 4). Those pouches treated with varying concentrations of AG and L-NAME alone for 13 weeks exhibited hyperorthokeratosis that was associated with the treatment (subgroups A4–A6; B4–B6, Table 4). There was a dose-dependent decrease in the incidence ($p < 0.05$),

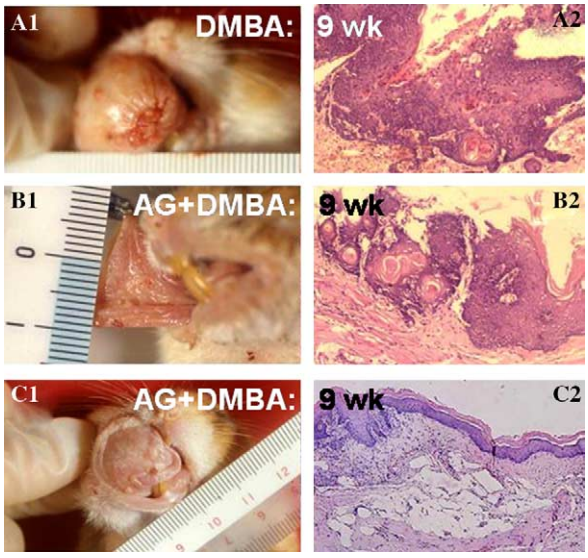

Fig. 1. Representative examples of effects of AG in 9-week DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis. Gross (A1) and histological pictures of epithelial dysplasia (A2, hematoxylin eosin stain, original magnification 100×) of DMBA-treated pouch mucosa. Gross (B1) and histological pictures of epithelial dysplasia (B2, hematoxylin eosin stain, original magnification 100×) of pouch mucosa treated with AG (30 μmol/ml) prior to DMBA. Gross (C1) and histological pictures of epithelial hyperplasia (C2, hematoxylin eosin stain, original magnification 40×) of pouch mucosa treated with AG (30 μmol/ml) prior to DMBA.

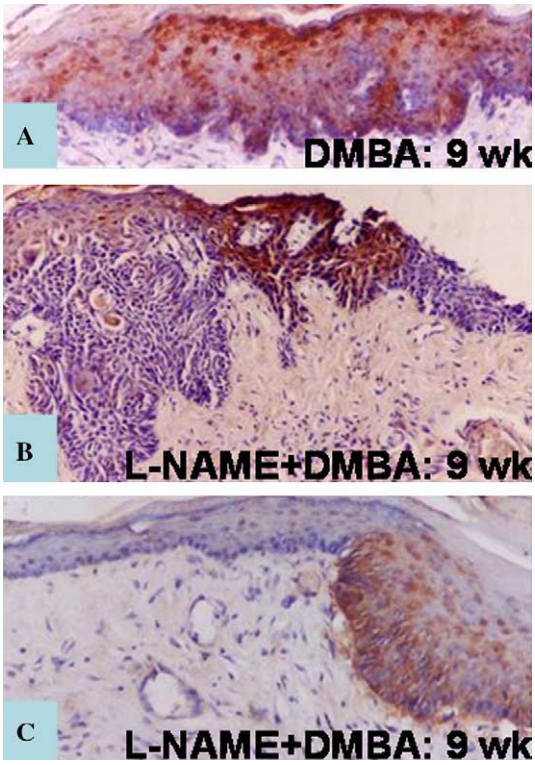

Fig. 2. Representative examples of iNOS immunoactivity in (A) epithelial dysplasia in hamster buccal–pouch treated with DMBA for 9-week (original magnification 40×); in (B) epithelial dysplasia in hamster buccal–pouch mucosa treated with L-NAME (30 μmol/ml) prior to DMBA for 13-week (original magnification 40×); in (C) epithelial hyperplasia in hamster buccal–pouch mucosa treated with L-NAME (30 μmol/ml) prior to DMBA for 13-week (original magnification 40×).

Table 4
Summary of the effects of AG and L-NAME over a 13-week period on DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis

Division II	Incidence of various histological findings	Multiplicity (Number of SCC/hamster)	Average dimension (mm)/SCC	iNOS protein expression
A				
A1	SCC (16)**; Dysplasia[a] (4), Epithelial hyperplasia (0)	2.14* ± 0.19[b]	2.73* ± 1.4	9.2 ± 4.2
A2	SCC (12)**; Dysplasia (6), Epithelial hyperplasia (2)	1.84* ± 0.35	2.27* ± 1.7	7.7* ± 2.3
A3	SCC (9)**; Dysplasia (6), Epithelial hyperplasia (5)	1.14* ± 0.29	1.72* ± 1.4	5.4* ± 1.4
A4	Hyperorthokeratosis (20)	0 ± 0	0 ± 0	0 ± 0
A5	Hyperorthokeratosis (20)	0 ± 0	0 ± 0	0 ± 0
A6	Hyperorthokeratosis (20)	0 ± 0	0 ± 0	0 ± 0
B				
B1	SCC (15)**; Dysplasia (4), Epithelial hyperplasia (1)	2.35* ± 0.35	2.57* ± 1.7	9.8* ± 3.8
B2	SCC (13)**; Dysplasia (3), Epithelial hyperplasia (4)	1.74* ± 0.57	1.89* ± 1.3	6.8* ± 2.8
B3	SCC (9)**; Dysplasia (4), Epithelial hyperplasia (7)	1.12* ± 0.25	1.46* ± 1.4	5.6* ± 1.3
B4	Hyperorthokeratosis (20)	0 ± 0	0 ± 0	0 ± 0
B5	Hyperorthokeratosis (20)	0 ± 0	0 ± 0	0 ± 0
B6	Hyperorthokeratosis (20)	0 ± 0	0 ± 0	0 ± 0
C				
C1	SCC (20)	4.22 ± 1.2	4.35 ± 1.3	11.2 ± 3.6
C2	Normal (20)	0 ± 0	0 ± 0	0 ± 0
C3	Normal (20)	0 ± 0	0 ± 0	0 ± 0

DMBA: 7,12-dimethyl benz[*a*]anthrance; [a]Dysplasia: epithelial dysplasia; iNOS: inducible nitric oxide synthase; SCC: squamous cell carcinoma; number in bracket: number of animal; [b]mean ± standard deviation; *significantly difference from group C1 ($p < 0.001$); **significantly difference from group C1 ($p < 0.05$).

multiplicity ($p < 0.001$) and average dimension ($p < 0.001$) of squamous-cell carcinomas for those subgroups treated with different concentrations of AG (subgroups A1–A3, Table 4) prior to DMBA treatment for 13 weeks when compared with those hamsters treated with DMBA alone for 13 weeks (subgroup C1, Table 4). L-NAME treatment for 13-week DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis showed similar preventive effects (subgroups B1–B3, Table 4). This reduction of squamous-cell carcinoma was associated with an increased incidence of epithelial hyperplasia, in parallel with a decrease in iNOS protein expression (Table 4) ($p < 0.001$).

No iNOS protein expression was detected in the untreated and mineral oil-treated (9- and 13-week) or in the AG- and L-NAME (9- and 13-week) treated pouch tissues.

Discussion

In recent years, overexpression of iNOS has been reported in both human oral carcinogenesis and chemically induced oral experimental carcinogenesis by our research group [30–37] and others [18,38–40]. Furthermore, it has been suggested that iNOS is expressed by cancer cells rather than by cancer-induced inflammatory cells in the surrounding stroma [41], implying that pharmacologic inhibition of iNOS is a possible method for oral cancer prevention. To date, few studies have examined the direct impact of iNOS inhibitors on carcinogenesis in animal models of human cancers of breast, colon, and liver [8,42] and no studies have yet been done on the animal model for human cancer of the oral cavity. Therefore, this study has been, to the best of our knowledge, the first to demonstrate the chemopreventive effect of iNOS inhibitors on DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis.

Our results indicate that iNOS inhibitors, AG and L-NAME, do not completely inhibit but can reduce the incidences of both epithelial dysplasias and squamous-cell carcinomas in a dose-dependent manner during DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis. This indicates that the development of squamous-cell carcinoma may have been multifactorial as the use of iNOS inhibitors alone has failed to induce complete suppression. Nevertheless, both AG and L-NAME can decrease the multiplicity and the average dimension of squamous-cell carcinomas and this may partially contribute to the anti-angiogenic effect of iNOS inhibitors [10,22]. Also, the suppression of dysplasia and squamous-cell carcinoma is associated with an increase in the quantitative values for epithelial hyperplasias, suggesting that these inhibitors prevent not only the progression of dysplasia to squamous-cell carcinoma but also that of hyperplasia to dysplasia.

AG has been found to suppress the formation of intestinal polyposis in Apc (Min/+) mice [43] and L-NAME has been reported to reduce the azoxymethane-induced colonic aberrant crypt foci formation in rat [44]. The inhibitory effects of both AG and L-NAME on DMBA-induced hamster pouch model in the current study is compatible with these observations [44,45], as well as with other animal studies on cancers of different anatomic sites [8]. Various regimens to introduce iNOS

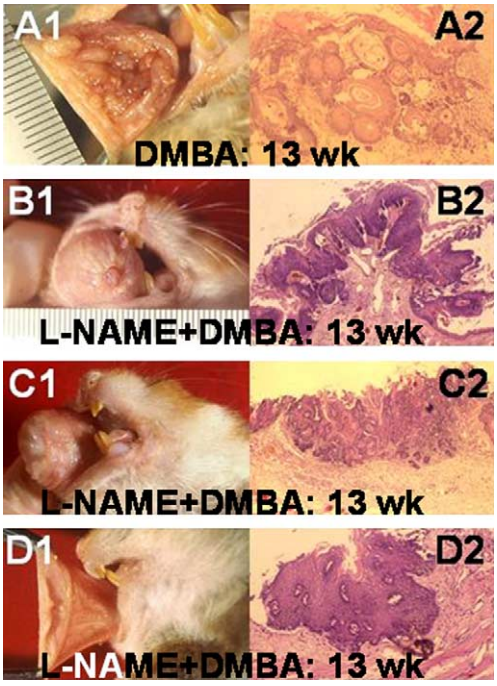

Fig. 3. Representative examples of effects of L-NAME in 13-week DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis. Gross (A1) and histological pictures of squamous-cell carcinoma (A2, hematoxylin eosin stain, original magnification 40×) of DMBA-treated pouch mucosa. Gross (B1) and histological pictures of squamous-cell carcinoma (B2, hematoxylin eosin stain, original magnification 40×) of pouch mucosa treated with L-NAME (30 μmol/ml) prior to DMBA. Gross (C1) and histological pictures of epithelial dysplasia (C2, hematoxylin eosin stain, original magnification 40×) of pouch mucosa treated with L-NAME (30 μmol/ml) prior to DMBA. Gross (D1) and histological pictures of epithelial hyperplasia (D2, hematoxylin eosin stain, original magnification 100×) of pouch mucosa treated with L-NAME (30 μmol/ml) prior to DMBA.

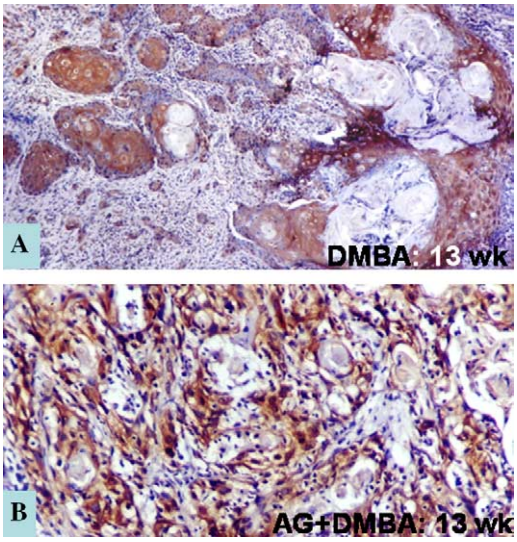

Fig. 4. Representative examples of iNOS immunoactivity in (A) squamous-cell carcinoma in hamster buccal–pouch treated with DMBA for 13-week (original magnification 40×) and in (B) squamous-cell carcinoma in hamster buccal–pouch mucosa treated with AG (30 μmol/ml) prior to DMBA for 13-week (original magnification 40×).

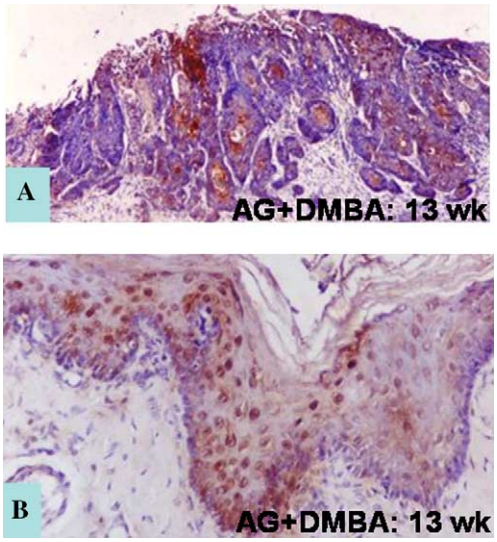

Fig. 5. Representative examples of iNOS staining in epithelial dysplasia (A, original magnification 40×) and epithelial hyperplasia (B, original magnification 100×) in hamster buccal–pouch mucosa treated with AG (30 μmol/ml) prior to DMBA for 13-week.

inhibitors, such as via drinking water and food, have been used in other animal models [8,43–45]. A protocol of topical application of the iNOS inhibitors to the hamster pouch has been employed in the present study to have a direct comparison with the positive control (DMBA-treated) group. A similar protocol has recently been used by Chun et al. [46]. However, this methodology could raise a possibility that iNOS inhibitors could inhibit the inflammatory and carcinogenic actions of DMBA through a physical barrier which was made by iNOS inhibitors themselves, but not through iNOS specific inhibition. To rule out this possibility, a negative control such as D-NAME which is virtually inactive for iNOS inhibition but possess a physicochemical property resemble to L-NAME should be employed.

The acetamidine 1400W is a very potent, irreversible iNOS inhibitor with greater than 5000- to 200-fold selectivity for iNOS compared with eNOS, respectively [47]. The use of 1400W resulted in a significant reduction of animal cancer metastasis (by up to 70%) [48], however, the toxicity of this compound may limit use in human [18,42]. Partial inhibition of NOS may be a better option than complete inhibition. Therefore, a less selective iNOS inhibitor (AG) [47] and a non-selective NOS inhibitor [24] have been used in the present study. Our results indicate that the inhibitory effect of AG is similar to that of L-NAME. Basically, these inhibitors suppress the iNOS catalytic activity but occasionally attenuate iNOS expression probably as NO has a property to induce iNOS expression via an autocrine loop.

The immunoreactivity of iNOS in the current study has been consistent with our previous study on hamster pouch model [30]. Furthermore, we found that the reduction of squamous-cell carcinoma/dysplasia with

increased concentrations of AG/L-NAME treatment was paralleled by a decrease in iNOS protein expression in DMBA-induced hamster pouch carcinogenesis, implying that iNOS protein expression was associated with the level of severity (progression from epithelial hyperplasia through dysplasia to squamous-cell carcinoma) of disease in this animal model. Consequently, the pharmacologic suppression of NO should contribute to the chemoprevention of chemically induced oral experimental carcinogenesis.

Leveling of iNOS protein expression was observed with concentrations of 20 and 30 μmol/ml of both AG and L-NAME for 9-week DMBA-induced hamster pouch carcinogenesis, whereas with the same concentrations over a 13-week period, leveling of iNOS protein expression was not found. This suggests that a higher concentration should have been employed to attain leveling of iNOS protein expression. It also indicates that a higher concentration of iNOS inhibitor is required to suppress the development of squamous-cell carcinoma than dysplasia. On the other hand, it has been reported that iNOS could enhance its ability to promote tumor growth in cooperation with cyclooxygenase-2 (COX-2) during DMBA-induced hamster buccal–pouch carcinogenesis [49]. Whether the inhibitory effect of both AG and L-NAME on iNOS is also associated with the suppressive effect of COX-2 inhibitor such as celecoxib [50] is warranted for future study. Furthermore, the induction of hyperorthokeratosis by the treatment with the NOS inhibitors, as demonstrated in the current study, may be due to the reactive response of the pouch mucosa to the irritation of the inhibitors and hence no iNOS immunoreactivity can be detected for these hyperorthokeratotic lesions.

In conclusion, the suppressive effects of AG and L-NAME have been established in DMBA-induced hamster buccal–pouch squamous-cell carcinogenesis. These results indicate that the DMBA-induced hamster buccal–pouch mucosa may serve as a suitable experimental model for investigation of the potential use of iNOS inhibitors as novel therapeutic tools for oral squamous-cell carcinogenesis.

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