Nitric oxide has diverse effects on head and neck cancer cell proliferation and glycolysis

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Abstract. Glycolysis is a key energy-providing process and one of the hallmarks of cancer. Nitric oxide (NO), a free radical molecule, regulates glycolysis in various cancers. NO can alter the cell cycle and apoptosis in head and neck squamous cell carcinoma (HNSCC) cells. However, the effect of NO on glycolysis in HNSCC cells remains unresolved. The present study investigated the effects of NO on cell proliferation, glucose transporter (GLUT) gene expression and glycolytic indicators in HNSCC cell lines. Two pairs of isogenic HNSCC cell lines, HN18/HN17 and HN30/HN31, were treated with a NO donor, diethylamine NONOate (DEA-NONOate), for 24, 48 and 72 h. Cell proliferation was assessed using MTT assay and NO concentration was measured using the Griess Reagent System. *GLUT1*, *GLUT2*, *GLUT3*, and *GLUT4* gene expression was analyzed using reverse transcription-quantitative PCR. Furthermore, hexokinase (HK) activity and lactate production were measured in NO-treated cells using colorimetric assay. NO exhibited concentration-dependent pro- and anti-proliferative effects on the HNSCC cell lines. Lower NO concentrations (5-200 μM) had pro-proliferative effects, whereas NO >200 μM had an anti-proliferative effect on HNSCC cells. NO (5 μM) promoted proliferation and glycolysis in HN18 cells by upregulating *GLUT1* and *GLUT2* gene expression and increasing HK activity and lactate levels. At 5-20 μM, NO-induced HN17 and HN30 cells demonstrated enhanced proliferation and *GLUT2*, *GLUT3* and *GLUT4* gene expression, whereas the glycolytic pathway was not affected. In conclusion, the present study demonstrated distinct proliferative effects of NO on HNSCC cells. NO may promote cell proliferation by stimulating glucose consumption and the glycolytic rate in HN18 cells. The effects of NO in other cell lines may be mediated by a non-glycolysis mechanism and require further investigation.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) has a high therapeutic failure rate, resulting in low 5-year survival rate (1). Alterations in metabolism, especially glycolysis, in cancer cells have been investigated to determine their potential as a cancer therapy target (2). Changes in glycolysis and associated signaling pathways has been associated with chemoresistance in various types of cancer (3).

Glycolysis is a key metabolic pathway in cancer cells that provides sufficient ATP, nucleotides, lipids, and amino acids for high tumor cell proliferation under aerobic conditions that contribute to tumor progression; this phenomenon is called the 'Warburg effect' (4). One of the hallmarks of cancer is a high glycolytic rate in cancer cells that is characterized by two key biochemical steps: Increased glucose uptake and conversion of glucose into lactate. The increased glucose uptake in cancer cells is facilitated by upregulating expression of glucose transporters (GLUTs) (5). GLUTs are a transmembrane protein family that is sub-divided into three phylogenetically distinct classes. Class 1 (GLUT1, 2, 3 and 4) has been extensively studied in mammalian cells (6,7). GLUT1 has a high affinity for glucose and is highly expressed in normal cells, including erythrocytes and endothelial cells, and overexpressed in breast (8), gastric (9), colorectal (10) and prostate (11) cancer. Moreover, GLUT1 upregulation is significantly associated with poorly differentiated cancer, positive lymph node metastasis, increased tumor size and worse overall and disease-free survival in patients with various types of cancers, such as gastric, colorectal, breast, pancreatic, liver, lung, ovarian and oral cancer (12). GLUT3 is primarily expressed in the nervous system and has a higher affinity for glucose than GLUT1 (13). GLUT3 is overexpressed in glioblastoma (14) and gastric (15) and non-small cell lung cancer (16). GLUT2 is constitutively expressed in the intestinal absorptive epithelial cells, hepatocytes and pancreatic β and kidney cells (17). GLUT2 is overexpressed in hepatocellular carcinoma cells (18) and colorectal cancer (19). GLUT4 is present in insulin-sensitive tissue, including adipose, heart and skeletal muscle. However, the expression of GLUT4 varies in ovarian and renal cancer (5).

Hexokinases (HKs), phosphofructokinase (PFK) and pyruvate kinase are rate-limiting enzymes in glycolysis and play important roles in catalyzing conversion of glucose to lactate (20). Increased activity of these glycolytic enzymes and upregulated lactate production are associated with tumor progression, such as tumor growth, chemoresistance and metastasis (21).

Nitric oxide (NO), a free radical that is highly reactive and diffusible, exhibits dual roles in the physiological maintenance and pathology of disease, including cancer (22). High NO levels cause nitrosative stress that affects homeostasis and alters protein function (23). Depending on the cancer type and NO concentration, NO modulates different aspects of cancer aggressiveness, including angiogenesis, apoptosis, cell cycle, invasion and metastasis (24). A high NO concentration induces HNSCC cell adaptability, such as survival, invasion and autophagy enhancement (25,26).

To the best of our knowledge, there is limited information on the effect of NO on glycolysis in cancer cells. In ovarian cancer, low NO concentration (≤100 nM) promote glycolysis, resulting in ATP production, oxidative defense and cell proliferation, whereas high NO concentration (≥500 nM) inhibits glycolysis and tumor progression (27). Based on the aforementioned roles of NO on tumor progression in HNSCC, it was hypothesized that NO might drive these tumorigenic behaviors via its effects on glycolysis. To the best of our knowledge, however, there is no evidence of the effect of NO on glycolysis in HNSCC. Therefore, the aim of the present study was to investigate the effects of NO on HNSCC cell proliferation and glycolytic intermediates including *GLUT1-4* gene expression, HK activity and lactate production.

Materials and methods

Cell culture. The isogenic primary and metastatic HNSCC cell lines were from the same patient and were initially established by Cardinali *et al* (28) and provided by Professor Silvio Gutkind (Moores Cancer Center, Department of Pharmacology, UC San Diego, USA). HN18 cells were obtained from primary tongue lesions and the HN17 cells were isolated from neck dissections (T2N2M0 stage). HN30 cells were obtained from primary pharynx lesions and HN31 cells were isolated from lymph node metastases (T3N1M0 stage). The cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a 5% CO_2 atmosphere.

MTT assay. HNSCC cell lines were cultured in 96-well plates (2,000 cells/well) at 37˚C for 24 h. Based on preliminary experiments (data not shown), diethylamine NONOate(DEA-NONOate) concentrations that affected the mean % cell proliferation within the 95% confidence interval were selected for investigation of the proliferative effect on each cell line in the present study. NO donor, DEA-NONOate (Sigma-Aldrich; Merck KGaA) concentrations 0.5, 5.0, 100.0 and 500.0 μM were selected to treat HN18 cells and 0.5, 10.0, 100.0 and 500.0 μM were selected to treat HN17, 30 and 31 cells at 37˚C for 72 h.

The minimum concentration that generated the highest cell proliferation was used as the effective dose for each cell line in subsequent experiments. The effective doses of 0.5, 10.0 and 100.0 μM DEA-NONOate were used to treat HN18 and HN17, HN30 and HN31 cells, respectively, at 37˚C for 24, 48 and 72 h. Cells in DMEM without DEA-NONOate served as control. The cell proliferation in each group was determined using MTT (Sigma-Aldrich; Merck KGaA) assay as previously described (29). Three independent experiments were performed.

NO determination. HNSCC cell lines were cultured and treated as aforementioned. The conditioned media from each condition was collected and stored at -80˚C until analysis. NO secretion was determined using the Griess Reagent System (Promega Corporation) per the manufacturer's instructions. Three separate experiments were conducted.

Reverse transcription-quantitative (RT-q)PCR. HNSCC cells were seeded in 6-well plates (200,000 cells/well) at 37˚C for 24 h. The HN18 and HN17, HN30 and HN31 cells were treated with 0.5, 10.0 and 100.0 μM DEA-NONOate, respectively, at 37˚C for 2 h. Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). The total RNA was converted into complementary DNA using a PrimeScript 1st strand cDNA Synthesis kit (Takara Bio, Inc.) per the manufacturer's instructions. The relative *GLUT1, 2, 3* and *4* mRNA expression levels were determined using the KAPA SYBR® FAST qPCR Kit Master Mix (Kapa Biosystems) in the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, Inc.). The primers were designed by Primer-BLAST online (National Center for Biotechnology Information; ncbi.nlm.nih.gov/tools/primer-blast/; Table I). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing at 60˚C for 60 sec. The constitutive expression was calculated using the $2^{-\Delta Cq}$ equation (30). β-*actin* was used as a housekeeping gene. ΔCq was calculated as *GLUT* Cq-β-*actin* Cq. The percentage of *GLUT* expression in each cell line was calculated as follows: *GLUT* expression (%)=[(mean $2^{-\Delta Cq}$ of *GLUT*)/(total $2^{-\Delta Cq}$ of *GLUTs*)] x100. To determine DEA-NONOate-induced *GLUT* expression in HNSCC cell lines, the relative expression of each *GLUT* was evaluated using the $2^{-\Delta\Delta Cq}$ method (30) as follows: ΔCq=Cq (treated cells)-Cq (untreated cells). Three independent experiments were conducted.

Lactate quantification. Cells were cultured in 25-cm^2 flasks at a density of 100,000 cells/ml for 24 h and treated as aforementioned. The cell suspension was centrifuged at 13,000 x g, 4˚C for 5 min to collect cell pellets. The cell pellets (1x10^6 cells) were collected and homogenized in 50 μl Lactate Assay buffer. Lactate Assay kit (cat. no. #MAK064; Sigma-Aldrich; Merck KGaA) was used for the lactate production test according to the manufacturer's instructions. Three independent experiments were performed.

HK activity evaluation. The cells were cultured in 25-cm^2 flasks at a density of 100,000 cells/ml at 37˚C for 24 h and treated as aforementioned. The cell pellets (1x10^6 cells) were

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Forward primer, 5'→3'	Reverse primer, 5'→3'	Product size, bp	Accession no.
GLUT1	TGGCATCAACGCTGTCTTCT	AACAGCGACACGACAGTGAA	123	NM_006516.4
GLUT2	GCCACACTCACACAAGACCT	AACTGGAAGGAACCCAGCAC	119	NM_000340.2
GLUT3	AGCTATCAAGTGTGCTTTAGCTTG	AAATGGGACCCTGCCTTACTG	100	NM_006931.3
GLUT4	TCTCCAACTGGACGAGCAAC	CAGCAGGAGGACCGCAAATA	101	NM_001042.3
β-*actin*	CTCACCATGGATGATGATATCGC	ATAGGAATCCTTCTGACCCATGC	165	NM_001101.5

GLUT, glucose transporter.

collected by centrifugation at 13,000 x g, 4˚C for 5 min and homogenized in 100 μl ice-cold HK Assay buffer (Hexokinase Colorimetric Assay kit; cat. no. #MAK091; Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. HK activity was determined by its oxidized product (NADH). The samples were measured at an absorbance of 450 nm at the initial time [$T(A_{450})_{initial}$] in a MULTISKAN Sky microplate reader (Thermo Fisher Scientific, Inc.). The plates were then incubated at room temperature, and measurements were taken every 5 min for 30 min. A_{450} at 30 min was defined as final time [$T(A_{450})_{final}$]. The change in the measurement from $T_{initial}$ to T_{final} for each sample (ΔA_{450}) was calculated as follows: $\Delta A_{450}=(A_{450})_{final}-(A_{450})_{initial}$.

The amount of NADH generated between $T_{initial}$ and T_{final} was determined by comparing the ΔA_{450} of each sample to a standard curve. HK activity of each sample was determined as follows: HK activity=[NADH x dilution factor/($T_{final}-T_{initial}$) x sample volume]. Three independent experiments were performed.

Statistical analysis. All data are presented as the mean ± SEM from three independent experiments. Multiple group comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Unpaired t test was applied to compare two independent groups. The statistical analyses were performed using Prism GraphPad 9.0 software (GraphPad Software, Inc.; Dotmatics). $P\leq0.05$ was considered to indicate a statistically significant difference.

Results

Effect of DEA-NONOate on HNSCC cell proliferation. HNSCC cell lines were treated with 0.5-500.0 μM DEA-NONOate for 72 h. DEA-NONOate (10-500 μM) decreased HN18 cell proliferation in a dose-dependent manner (Fig. 1A). DEA-NONOate at 0.5 and 5.0 μM demonstrated no significant difference in proliferation in HN18 cell when compared with control. By contrast, DEA-NONOate treatment did not significantly change proliferation in HN17 cells (Fig. 1B). DEA-NONOate significantly increased proliferation in HN30 (10 and 100 μM) and HN31 (100 μM) cells compared with control (Fig. 1C and D). Moreover, 500 μM DEA-NONOate had a cytotoxic effect on HN18, HN30 and HN31 cells (Fig. 1A, C and D).

DEA-NONOate at 0.5, 10.0 and 100.0 μM was used to test proliferation at 24-72 h in HN18 and HN17, HN30 and HN31 cells, respectively. The proliferation significantly increased in DEA-NONOate-treated HN18 and HN17 cells at 24 h compared with control (Fig. 2A and B), however, proliferation significantly declined at 48 to 72 h. DEA-NONOate significantly induced proliferation in HN30 and HN31 cells at 72 h compared with control (Fig. 2C and D). By contrast, at 24-48 h, DEA-NONOate did not affect proliferation of these cell lines.

Therefore, 0.5, 10.0 and 100.0 μM DEA-NONOate was selected as the effective concentration for HN18 and HN17, HN30 and HN31 cells, respectively, in the subsequent experiments.

Determination of NO in DEA-NONOate-treated HNSCC cells. The cells were treated with their effective DEA-NONOate concentration for 24-72 h. NO was secreted from the DEA-NONOate-induced cells and significantly increased in HN18, HN30 and HN31 cells from 24 to 72 h compared with control (Fig. 3A, C and D). The amount of NO released by DEA-NONOate-treated cells was 6.3-14.5 μM in HN18, 12.6-20.3 μM in HN30 and 169.4-201.0 μM in HN31 cells. Although DEA-NONOate significantly induced NO secretion in HN17 cells at 24 and 48 h compared with control (Fig. 3B), its levels remained constant until 72 h. NO secreted from DEA-NONOate-treated HN17 cells was 5.1-5.6 μM.

Gene expression profiling of GLUTs in HNSCC cells. The constitutive expression of *GLUT1*, *2*, *3* and *4* genes in HNSCC cell lines was evaluated before DEA-NONOate induction. The relative expression of *GLUT1* was 91.3, 95.1 and 97.3% in HN17, HN30 and HN31 cells, respectively (Fig. 4). The *GLUT2* expression (65.9%) was the highest among the *GLUTs* in HN18 cells.

DEA-NONOate induces GLUT gene expression in HNSCC cells. After DEA-NONOate induction, *GLUT1* and *2* expression was significantly increased in HN18 cells compared with control (Fig. 5A and B). *GLUT2* expression was significantly increased in DEA-NONOate-induced HN17 cells compared with control (Fig. 5B). Moreover, *GLUT3* and *4* expression was significantly increased in DEA-NONOate-induced HN30 cells compared with control (Fig. 5C and D). However, there was no alteration in *GLUT1*, *2*, *3* and *4* expression following DEA-NONOate induction in HN31 cells (Fig. 5).

Effect of DEA-NONOate on lactate amount and HK activity in HNSCC cells. The lactate amount in DEA-NONOate-treated

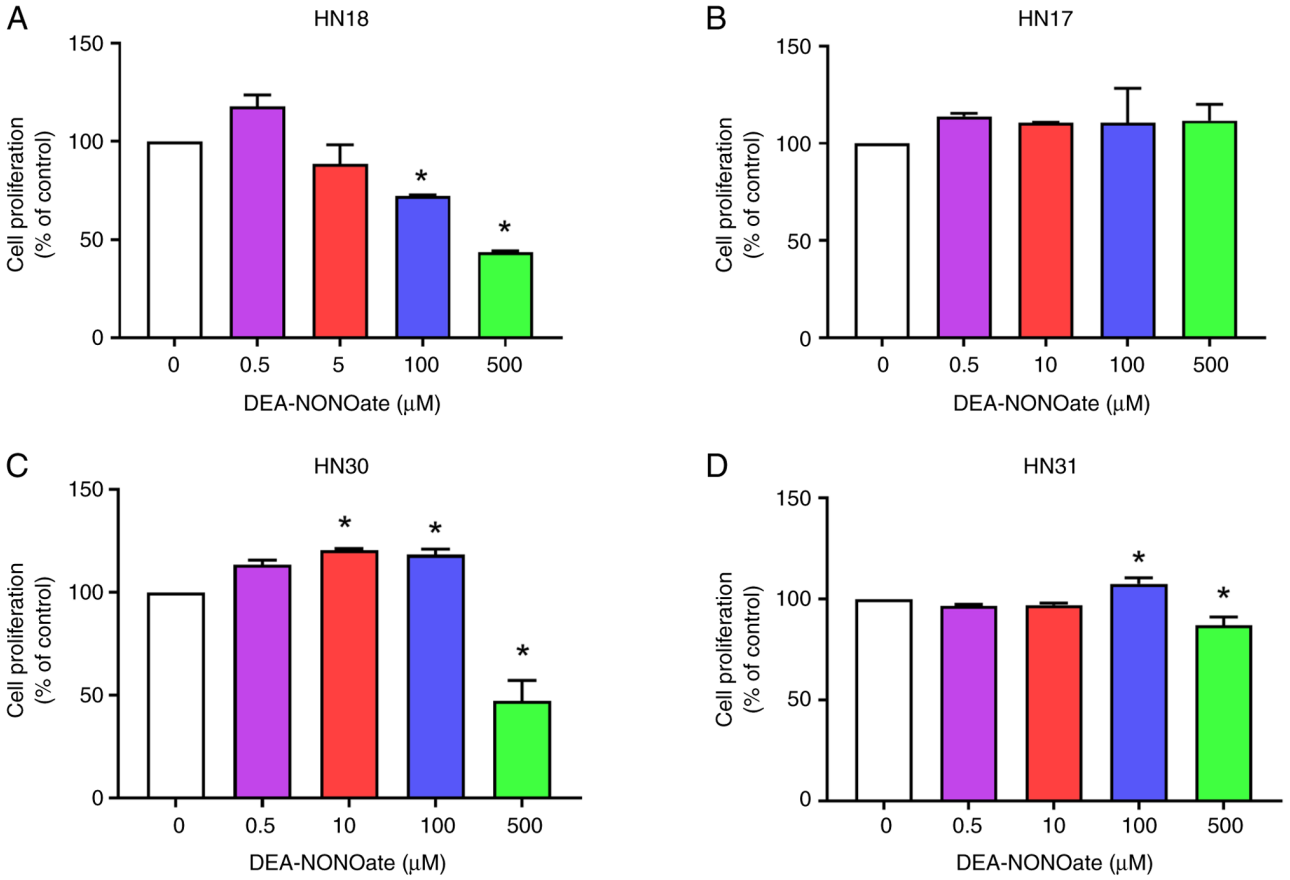

Figure 1. Effect of DEA-NONOate on HNSCC cell proliferation. HNSCC cell lines were treated with DEA-NONOate for 72 h. Cells cultured in growth media without DEA-NONOate served as control. MTT assay was used to evaluate proliferation in (A) HN18, (B) HN17, (C) HN30 and (D) HN31 cells. *P<0.05 vs. 0. DEA-NONOate, diethylamine NONOate; HNSCC, head and neck squamous cell carcinoma.

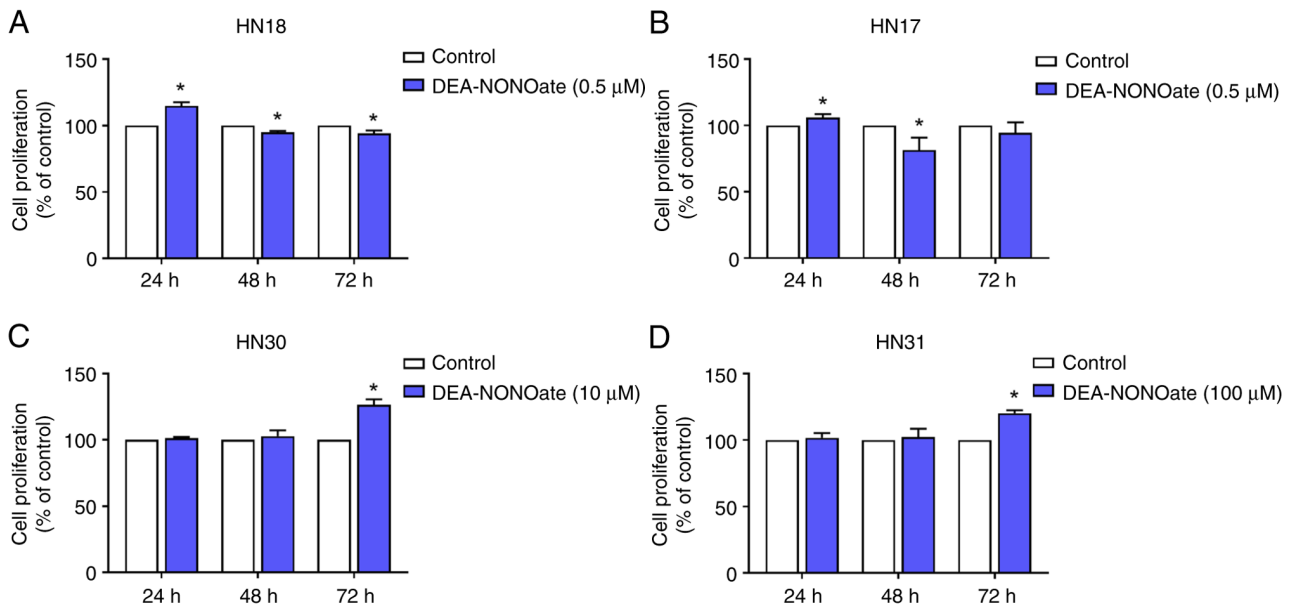

Figure 2. Effect of DEA-NONOate on HNSCC cell proliferation over time. Cells cultured in growth media without DEA-NONOate served as control. MTT assay was used to evaluate proliferation in (A) HN18, (B) HN17, (C) HN30 and (D) HN31 cells. *P<0.05 vs. control. DEA-NONOate, diethylamine NONOate; HNSCC, head and neck squamous cell carcinoma.

HN18 cells significantly increased to 5.15 pmol or 2.22-fold of control (Fig. 6A). However, the lactate levels in HN17, HN30 and HN31 cells were not affected by DEA-NONOate. Furthermore, DEA-NONOate significantly induced HK

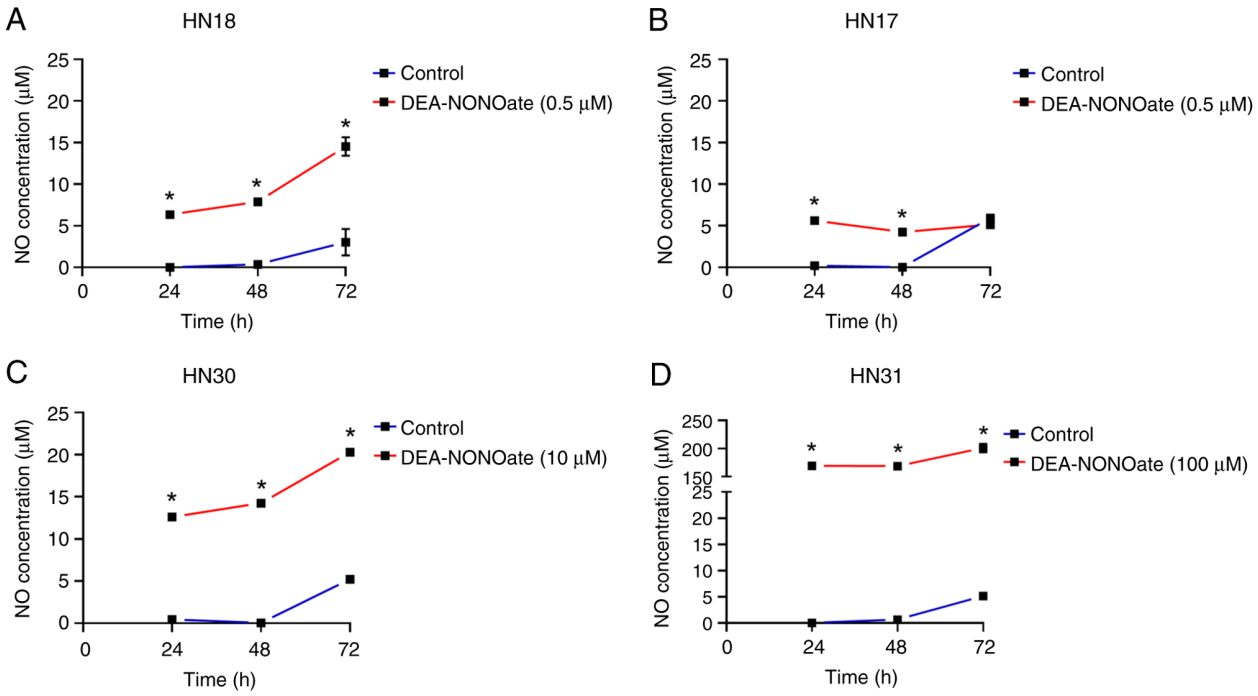

Figure 3. Effect of DEA-NONOate on NO secretion in HNSCC cells. Cells cultured in growth media without DEA-NONOate served as control. Griess reagent system was used to determine secreted NO in (A) HN18, (B) HN17, (C) HN30 and (D) HN31 cells. *P<0.05 vs. control. DEA-NONOate, diethylamine NONOate; HNSCC, head and neck squamous cell carcinoma.

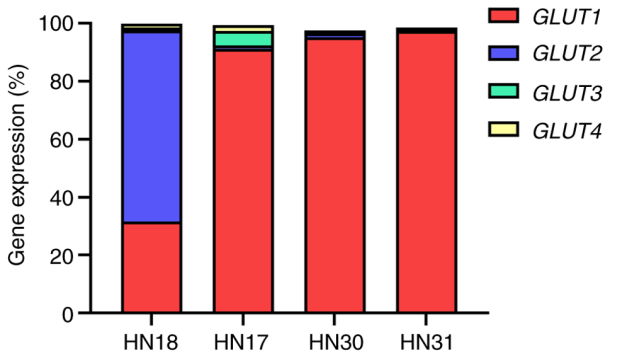

Figure 4. Baseline *GLUT* gene expression distribution in HNSCC cells. Reverse transcription-quantitative PCR was employed to evaluate *GLUT1*, *2*, *3* and *4* gene expression in HN18, HN17, HN30 and HN31 cells. HNSCC, head and neck squamous cell carcinoma; GLUT, glucose transporter.

activity in HN18 cells to 20.36 milliunits/ml or 1.20-fold of control (Fig. 6B). By contrast, the HK activity in HN17, HN30 and HN31 cells significantly decreased following DEA-NONOate treatment.

Discussion

The present study investigated the effects of DEA-NONOate on proliferation and glycolysis in HNSCC cell lines. Based on its concentration, DEA-NONOate exhibited disparate effects on proliferation, *GLUTs* expression, HK activity and lactate production between HNSCC cell lines. NO has a biphasic function in the tumor microenvironment (31). NO exhibits pro- and anti-tumor mechanisms depending on its concentration and cancer type (24). NO at 10-30 nM promotes pro-tumorigenic mechanisms, such as ERK pathway activation; NO >1 μM increases nitrative stress and apoptosis in a breast cancer cell line (32). In ovarian cancer, NO ≤100 nM promotes glycolysis and cell proliferation, whereas NO ≥500 nM exhibits antitumorigenic effects (27).

In the present study, DEA-NONOate was used as a NO donor to induce proliferation and glycolysis in HNSCC cells. DEA-NONOate significantly promoted HNSCC cell proliferation at different concentrations and induction periods; 0.5 μM DEA-NONOate increased proliferation in HN18 and HN17 cells at 24 h, whereas 10 and 100 μM DEA-NONOate induced proliferation in HN30 and HN31 cells at 72 h. Moreover, overall, the anti-proliferative concentration of DEA-NONOate for HNSCC cells was >200 μM. These effective concentrations of NO on HNSCC cells were relatively higher than those of other cancers, as aforementioned. Moreover, the present study demonstrated that the effect of NO on HNSCC cell proliferation might depend on TNM staging and NO concentration.

Although NO-associated oncogenic signaling has been described with respect to NO concentration in certain types of cancer cells (24), to the best of our knowledge, there is no information on the impact of NO on the metabolic pathways that modulate cancer cell activity. Glycolysis is the primary metabolic pathway that supports tumor progression, which is known as the 'Warberg effect' (4). GLUT1-4 are upstream proteins that facilitate glucose entry into the glycolysis pathway, providing high levels of ATP in cancer cells (17). GLUT1 upregulation is widely detected in cancer, including oral cancer tissue, and is significantly associated with poorly differentiated cancer, positive lymph node metastasis, increased tumor size and worse overall survival in patients (12). GLUT2 is overexpressed in hepatocellular

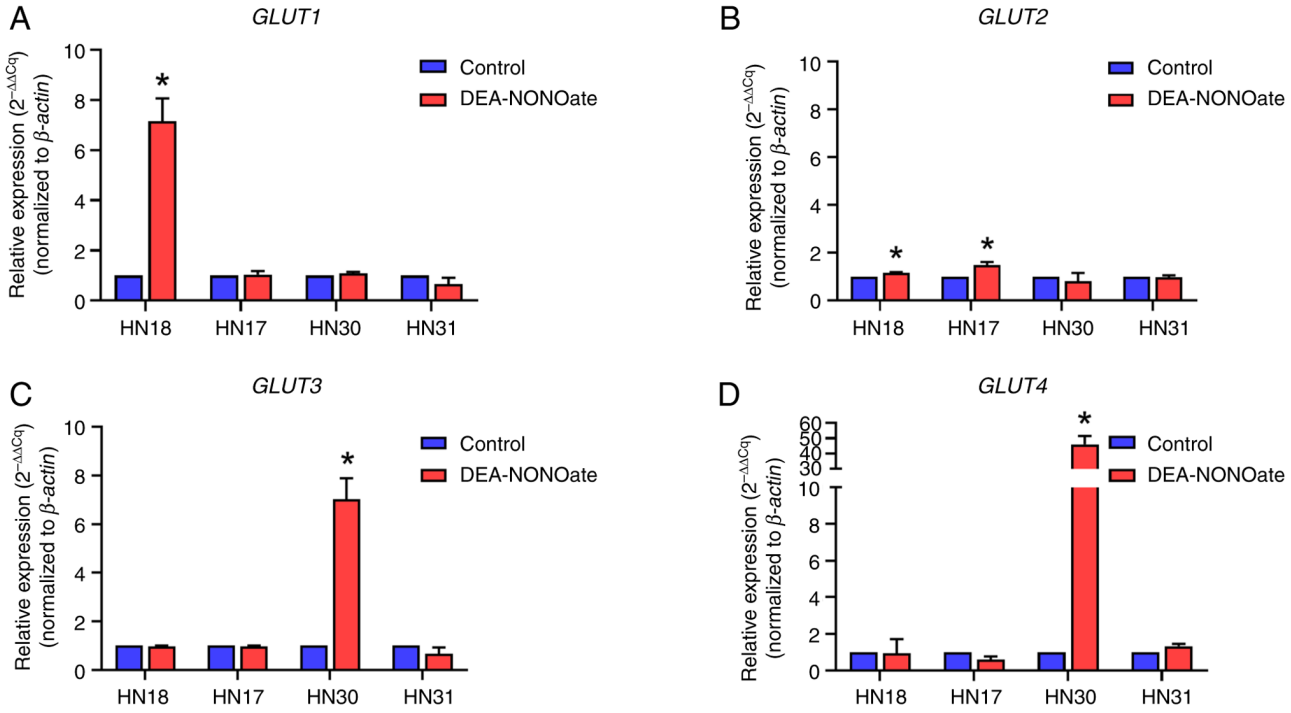

Figure 5. Effect of DEA-NONOate on *GLUTs* gene expression in HNSCC cells. Cells were treated with DEA-NONOate at their effective dose for 2 h. Reverse transcription-quantitative PCR was used to evaluate *GLUT* (A) *1*, (B) *2*, (C) *3* and (D) *4* gene expression in HN18, HN17, HN30 and HN31 cells. Cell cultured without DEA-NONOate served as control. *P<0.05 vs. control. DEA-NONOate, diethylamine NONOate; HNSCC, head and neck squamous cell carcinoma.

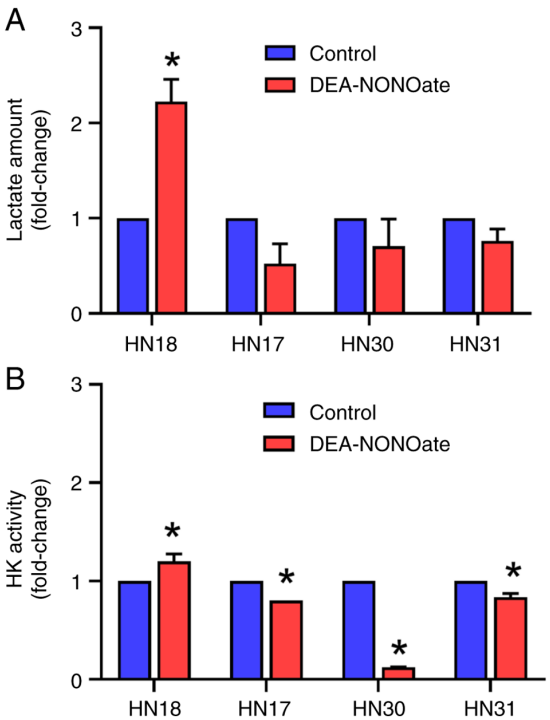

Figure 6. Effect of DEA-NONOate on lactate amount and HK activity in HNSCC cells. Cells were treated with DEA-NONOate at their effective dose for 24 h. (A) Lactate production and (B) HK activity were evaluated. Cell cultured without DEA-NONOate served as control. *P<0.05 vs. control. DEA-NONOate, diethylamine NONOate; HK, hexokinase; HNSCC, head and neck squamous cell carcinoma.

carcinoma cells (18) and colorectal cancer (19). In the present study, the *GLUT1* gene had relatively high expression levels in HN17, HN30 and HN31 cells compared with the other *GLUTs*. *GLUT2* gene showed the highest expression in HN18 cells. However, the present study had some limitations. *GLUTs* expression were verified at the mRNA level but not the protein level. In addition, only two pairs of cell lines were tested; the study of other HNSCC cell lines is necessary to validate the findings within the broader spectrum of HNSCC.

A previous study reported that NO promotes glycolysis in ovarian cancer by increasing gene expression of *GLUT1*, *HK*, *PFK* and lactate dehydrogenase (27). The present study partially confirmed that DEA-NONOate induced glycolysis in HN18 cells by increasing the gene expression of *GLUT1* and *GLUT2*, HK activity and lactate production. Although the *GLUT2*, *GLUT3* and *GLUT4* genes were upregulated, HK activity decreased and the lactate amount did not significantly change in DEA-NONOate-treated HN17 and HN30 cells. GLUT2 has a very low affinity for glucose and fructose (33). In the present study, the higher expression of *GLUT2* in the DEA-NONOate-induced HN18 cells might be sufficient for increased glucose influx and glycolysis compared with HN17 cells. Previous studies found that NO induces breast cancer cell proliferation via non-glycolysis pathways, such as EGF receptor (EGFR), PI3K/AKT and MAPK (34-36). Notably, EGFR is constitutively expressed in the HNSCC cell lines used in the present study (28). To confirm the effect of NO on HNSCC cell proliferation, the glucose uptake and non-glycolysis pathways should be assessed in additional studies. GLUT4 is the insulin-responsive glucose transporter, therefore, glucose uptake is dependent on insulin stimulation in cancer cell lines (5). GLUT4 expression levels are highly associated with insulin-like growth

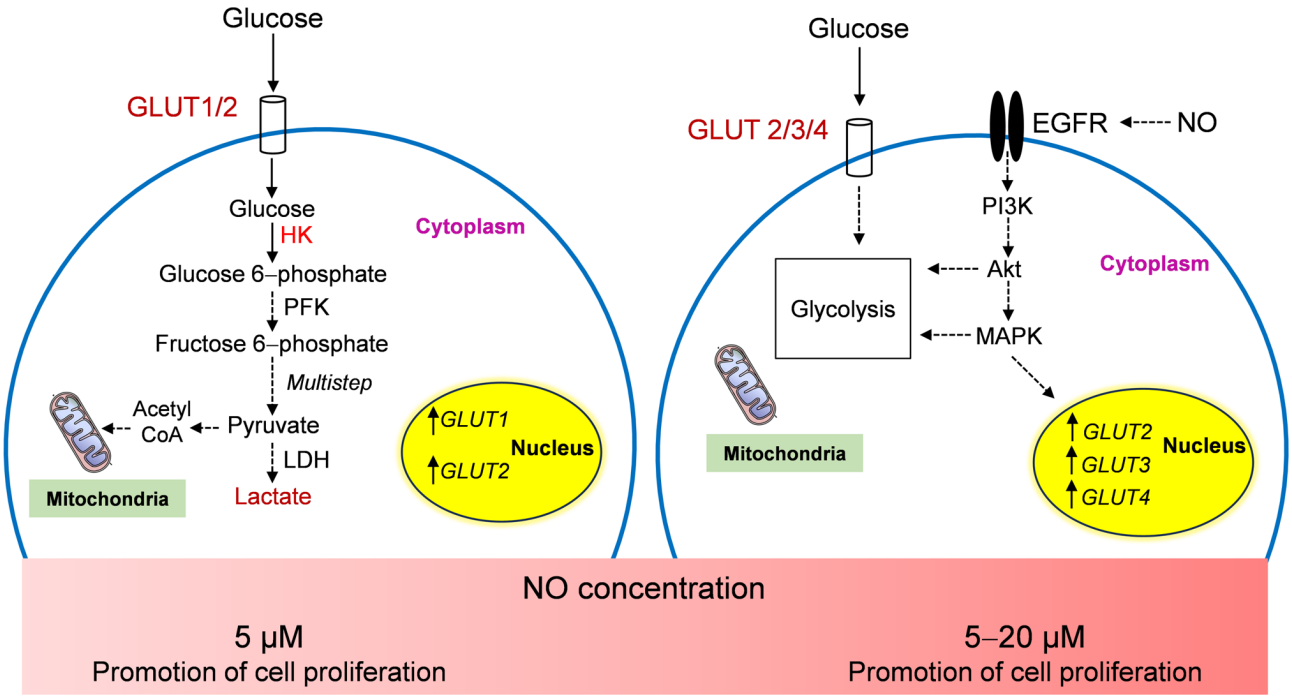

Figure 7. Proposed mechanism of NO-induced proliferation in HNSCC cells. NO promotes proliferation via glycolysis and non-glycolysis mechanisms in HNSCC cell lines. NO induces HN18 cell proliferation by upregulating *GLUT1* and *2* gene expression and increasing HK activity and lactate amount. NO increases *GLUT2*, *GLUT3* and *GLUT4* gene expression in HN17 and HN30 cells. The crosstalk between the EGFR signaling pathway and glycolysis may contribute to cell proliferation. Dashed lines indicate hypothesized mechanism. HK, hexokinase; PFK, phosphofructokinase; LDH, lactate dehydrogenase; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase; HNSCC, head and neck squamous cell carcinoma; GLUT, glucose transporter.

factor (IGF) and associated with the survival of patients with colorectal cancer (37). Based on the present data, the crosstalk between GLUT4 and the IGF signaling pathway should be evaluated prior to interpreting the effect of NO on HN30 cell proliferation.

In conclusion, the present study demonstrated the effects of DEA-NONOate on HNSCC cell proliferation and glycolysis. DEA-NONOate exhibited pro-and anti-proliferative effects in HNSCC cell lines depending on its concentration and TNM staging. NO at lower concentrations (10-200 μM) had pro-proliferative effects, whereas >200 μM had an anti-proliferative effect on the HNSCC cells. NO (5 μM) promoted proliferation and glycolysis in HN18 cell by upregulating *GLUT1* and *GLUT2* gene expression and increasing HK activity and lactate amount. At 5-20 μM, NO-induced HN17 and HN30 cells demonstrated increased proliferation and *GLUT2*, *GLUT3* and *GLUT4* gene expression, however, the glycolytic pathway was not affected. The proposed proliferative mechanism of NO in HNSCC cells is presented in Fig. 7. However, the pro-proliferative concentration of NO (200 μM) induced HN31 cell proliferation without glycolysis activation. Therefore, the pro-tumorigenic effects of NO on HNSCC cells on glycolysis and non-glycolysis mechanisms should be evaluated. Further investigation into crosstalk between the proliferation-related signaling pathways and glycolysis in HNSCC cells is needed.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KU and SK designed the experiments. KU and PK performed the experiments. KU analyzed the data and wrote the manuscript. KU and SK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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