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Regulation of non small-cell lung cancer stem cell like cells by neurotransmitters and opioid peptides

Jheelam Banerjee, Arokya M. S. Papu John, and Hildegard M. Schuller

Experimental Oncology Laboratory, Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA

Abstract

Non small-cell lung cancer (NSCLC) is the leading type of lung cancer and has a poor prognosis. We have shown that chronic stress promoted NSCLC xenografts in mice via stress neurotransmitter-activated cAMP signaling downstream of beta-adrenergic receptors and incidental beta-blocker therapy was reported to improve clinical outcomes in NSCLC patients. These findings suggest that psychological stress promotes NSCLC whereas pharmacologically or psychologically induced decreases in cAMP may inhibit NSCLC. Cancer stem cells are thought to drive the development, progression and resistance to therapy of NSCLC. However, their potential regulation by stress neurotransmitters has not been investigated. In the current study, epinephrine increased the number of cancer stem cell like cells (CSCs) from three NSCLC cell lines in spheroid formation assays while enhancing intracellular cAMP and the stem cell markers sonic hedgehog (SHH), aldehyde dehydrogenase-1 (ALDH-1) and Gli1, effects reversed by GABA or dynorphin B via $G\alpha_i$ -mediated inhibition of cAMP formation. The growth of NSCLC xenografts in a mouse model of stress reduction was significantly reduced compared with mice maintained under standard conditions. Stress reduction reduced serum levels of corticosterone, norepinephrine and epinephrine while the inhibitory neurotransmitter γ -aminobutyric acid (GABA) and opioid peptides increased. Stress reduction significantly reduced cAMP, VEGF, p-ERK, p-AKT, p-CREB, p-SRc, SHH, ALDH-1 and Gli1 in xenograft tissues whereas cleaved caspase-3 and p53 were induced. We conclude that stress neurotransmitters activate CSCs in NSCLC via multiple cAMP-mediated pathways and that pharmacologically or psychologically induced decreases in cAMP signaling may improve clinical outcomes in NSCLC patients.

Keywords

non small-cell lung cancer; cancer stem cell like cells; cancer stem cell markers; cAMP signaling; neurotransmitters; opioid peptides

Introduction

Non small-cell lung cancer (NSCLC) is the leading type of lung cancer and has a poor prognosis¹. Although smoking is a risk factor for all lung cancers, NSCLC develops in a

significant number of nonsmokers² and the significant decrease in smokers during the past five decades has not yielded a decrease in incidence or mortality of NSCLC². In fact, one histological subtype of NSCLC, adenocarcinoma, continues to rise in incidence, accounting for 80% of NSCLC cases in never smokers today²⁻⁴. Together, these observations suggest that factors unrelated to smoking significantly impact the development and progression of NSCLC, particularly adenocarcinoma.

Cancer stem cells are thought to drive the development, progression and resistance to therapy of cancer, including NSCLC^{5, 67-9}. Cancer stem cells only constitute a small population of cells within NSCLC tissues and cell lines and have the ability for self-renewal and differentiation. The maintenance of cancer cell lines in serum free stem cell-selective medium selects for the renewal of cancer stem cells as three dimensional floating aggregates (spheroids) and repeated subculture of spheroids in this environment is widely used to generate cell populations enriched in cancer stem cells from cancer cell lines^{5, 10}. It has been shown that the renewal of cancer stem cells from NSCLC cell lines is activated by the sonic hedgehog (SHH)/Gli pathway⁸. In addition, aldehyde dehydrogenase 1 (ALDH-1)-dependent signaling has been associated with the clonogenicity and tumorigenicity of cancer stem cell like cells from NSCLC⁷.

In vitro studies with NSCLC cell lines, which are comprised of mixed populations of differentiated and stem cells, have shown that beta-adrenergic receptor agonists increased cell proliferation and migration via cAMP-dependent signaling that included transactivation of the epidermal growth factor receptor (EGFR) pathway, resulting in the activation of ERK, Src and AKT signaling proteins¹¹⁻¹³. These responses were inhibited by gene knockdown of the β 1-adrenergic receptor¹⁴ or by treatment with the inhibitory neurotransmitter γ -aminobutyric acid (GABA), which activated the inhibitory G-protein G_{α_i} coupled to GABA-B receptors, thus inhibiting the formation of cAMP¹⁵. Moreover, it has been shown that low concentrations of agonists for delta and kappa-opioid receptors (DORs, KORs) that are also coupled to G_{α_i} ^{16, 17} inhibited the growth of NSCLC cell lines^{18, 19}.

The physiological agonists for beta-adrenergic receptors, norepinephrine and epinephrine, are released from sympathetic nerves of the autonomic nervous system and from the adrenal medulla in response to psychological stress and are generally referred to as stress neurotransmitters²⁰. In addition to such classic stress responses, chronic psychological stress suppresses GABA^{21, 22}. The G_{α_i} -coupled GABA-B receptors (GABA-B-R1, GABA-B-R2) maintain cAMP homeostasis by inhibiting the activation of adenylyl cyclase, the single rate-limiting step required for the formation of intracellular cAMP²³. GABA-B-Rs have tumor suppressor function in NSCLC cell lines and in normal airway epithelial cells by inhibiting the formation of cAMP¹⁵ and NSCLC patients with high levels of GABA-B-R expression had improved clinical outcomes²⁴. We have reported that chronic social stress in mice significantly promoted the growth of NSCLC xenografts, a response mediated by multiple cAMP-driven pro-proliferation and anti-apoptotic signaling cascades downstream of G_{α_s} -coupled beta-adrenergic receptors activated by norepinephrine and epinephrine and reversed by treatment of the animals with GABA²⁵. Another laboratory showed that chronic treatment of mice with epinephrine or chronic exposure of the animals to behavioral stress degraded the tumor suppressor gene p53 via beta-2 adrenergic receptor signaling^{26, 27}. The

translational value of these preclinical data has been confirmed by clinical studies that revealed improved clinical outcomes in NSCLC patients with incidental beta-blocker therapy²⁸ and in individuals whose NSCLCs overexpressed GABA-B receptors²⁴. Collectively, these findings suggest that chronic psychological stress may increase the risk and mortality of NSCLC via beta-adrenergic cAMP-dependent signaling and that pharmacological as well as psychological strategies that decrease the activity of this signaling cascade may diminish NSCLC growth and improve clinical outcomes in NSCLC patients. This hypothesis would only prevail in the clinical setup if cAMP were not only the driving force of differentiated NSCLC cells but would additionally regulate the cancer stem cell population in these cancers. However, neither the effects of stress neurotransmitters and GABA nor opioid peptides on lung cancer stem cells or cancer stem cell markers has been studied to date.

Using in vitro studies with cancer stem cell like cells (CSCs) enriched by spheroid formation assays from three NSCLC cell lines in conjunction with environmental and enrichment strategies reported to generate the “calm mouse”²⁹, an animal model for stress reduction and well-being, in athymic mice carrying NSCLC xenografts, our current data provide evidence, for the first time, that stress neurotransmitters significantly increase the number of CSCs via cAMP-driven stimulation of the SHH pathway and ALDH-1 and that these responses are inhibited by pharmacologically or psychologically-induced increases in GABA or opioid peptides.

Materials and Methods

A. In vitro studies with NSCLC cancer stem cell like cells

NSCLC cell lines (histological category: adenocarcinoma) NCI-H322 (European Collection of Cell Cultures, Health Protection Agency Porton Down, Salisbury, Wiltshire, UK, expresses activating point mutations in *K-ras*), NCI-H1299 and NCI-H441 (American Type Culture Collection ATCC, Manassas, VA, USA, do not express *ras* mutations) were authenticated at an independent commercial laboratory (IDEXX BioResearch, Columbia, MD, USA) by 9-marker short tandem repeat genotyping and species-specific PCR evaluation immediately at the end of all experiments.

Spheroid formation assay—Cells were seeded in 6-well plates at a low density of 2,000 cells per well and grown in serum free N2B27 medium mix, consisting of 200ml DMEM/F-12, 200ml of RPMI 1640 basal media, 1ml N2-Medium supplement, 2ml Glutamax, 4ml B27 supplement and 728µl of beta-mercaptoethanol. The cells were maintained in these conditions for 21 days with subculture of 2000 cells per well from manually dissociated spheroids every 7 days. The effects of epinephrine (10nM) on spheroid numbers was tested in the presence and absence of the Gα_i-coupled GABA-B-R and KOR agonists GABA (1µM) and dynorphin B (1µM). Treatment started 24 hours after seeding of the cells and was continued for 20 days with replenishing of drugs daily and media every 3 days (100µl). Each treatment group consisted of five replicates. Spheroids were counted with a hemocytometer and expressed as mean values and standard deviations of spheroids per 1000 seeded cells. Statistical analysis of data was by Kruskal-Wallis ANOVA followed by Mann-Whitney

tests. Spheroids were then harvested and protein was isolated for further analyses as described below.

Determination of the cancer stem cell markers SHH, ALDH-1 and Notch-1 in NSCLC spheroids—SHH and ALDH-1 were measured in spheroids from all three NSCLC cell lines. SHH was measured using a Sonic Hedgehog Human ELISA kit (Abcam, Cambridge, MA) in accordance with the vendors' recommendations. ALDH1A was measured with an ELISA kit (Antibodies-Online Inc., Atlanta, GA) using the vendors' recommendations. NCI-H322 spheroids were additionally tested for the stem cell marker Notch-1 following the vendor's instructions (Notch-1 human ELISA kit, BioVision, Inc., Milpitas, CA). Absorbances were read with an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT). In all ELISA assays five samples per treatment group were measured and data were subjected to statistical analysis by Kruskal-Wallis ANOVA and Mann-Whitney tests.

Assessment of the SHH effector Gli1 in NSCLC spheroids by Western blotting—Cells were lysed in lysis buffer (50mmol/L Tris-HCl, 1% NP-40, 150mmol/L NaCl, 1mmol/L phenylmethylsulfonylfluoride, 1 mmol/L Na₃VO₄, 1mmol/L NaF and 1µg/ml of aprotinin, leupeptin and pepstatin) and the supernatants were obtained. Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL). After heat denaturation, protein samples (20µg) were electrophoresed using 10% SDS gels (Invitrogen, Carlsbad, CA) and blotted onto membranes. The membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature. Blots were probed with Gli 1 (Santa Cruz Biotechnology, Dallas, TX) and Beta-Actin (Abcam, Cambridge, MA). The membranes were then washed (0.5% Tween 20/TBS) and incubated with their respective fluorescent secondary antibodies for 1 hour. Protein bands were then visualized with enhanced chemiluminescence reagent (Pierce ECL plus Western Blotting Detection).

Assessment of intracellular cAMP in NSCLC spheroids—At the end of the 21-day spheroid formation assay, spheroids were treated with IBMX (1mM) for 30 min. Proteins were isolated using the manufacturer's recommendation for the cAMP assay kit. Five samples per treatment group were analyzed with a cAMP ELISA kit (Enzo Life Sciences, Farmingdale, NY) following the vendors' recommendations. Absorbance of samples was measured using an Epoch Microplate spectrophotometer (BioTek, Winooski, VT). Five samples per treatment group were analyzed. Statistical evaluation of data was by Kruskal-Wallis ANOVA and Mann-Whitney tests.

B. In vivo studies

The animal experiment was approved by the University of Tennessee Knoxville Institutional Animal Care and Use Committee (IACUC). Male, 6-week-old athymic nude mice (Harlan Sprague Dawley Inc.) were randomly assigned to two groups (n=20). Control mice were housed under standard conditions recommended by IACUC for the maintenance of mice: 4 animals per cage in polycarbonate cages (189 × 297 × 128mm) provided with standard corn cob bedding, autoclaved tap water and irradiated food ad libitum. In accord with published procedures²⁹, stress reduction was achieved in the treatment group by housing the same

number of mice in larger polycarbonate cages (257mm × 483mm × 152mm) provided with the following enrichment items: two polycarbonate tunnels (Bio-Serv) to allow tunneling behavior, two paper nest boxes (Bio-Serv) and nesting material (compressed Enviro-dry Eco-bedding paper strips, Fibercore) for shelter and enhancement of nesting behavior. All enrichment items were autoclaved prior to use. When the mice had been maintained under these conditions for seven days, they were subcutaneously inoculated in the flank region with cells (3×10^6 in 0.2 ml of PBS, viability >95%) from the human NSCLC cell line NCI-H322. Control mice maintained under standard conditions were injected with identical numbers of cancer cells on the same day. All animals were observed for 30 days after inoculation with cancer cells, while being monitored daily. Body weights were recorded weekly. Two perpendicular diameters (length and width) of each xenograft were measured with a caliper weekly, and tumor volumes were calculated $(\text{length}/2) \times (\text{width}^2)$. At the end of the 30-day observation period, the animals were euthanized by CO₂ inhalation. Mean values and standard deviations of xenograft volumes over time as well as tumor incidence (percentage of animals per group with detectable xenografts) were calculated and graphed using Prism 6 Graphpad software (Graphpad Software Inc., San Diego, CA, USA). Statistical significance of xenograft volumes for each week was determined by Mann-Whitney tests. Significant differences of tumor incidences among controls and animals with stress reduction were assessed by Chi-square test from contingency tables. Blood samples and xenografts were harvested for additional analyses.

Assessment of serum levels of stress mediators, GABA and opioid peptides—

The serum levels of norepinephrine, epinephrine (2-CAT ELISA assay kit, Rocky Mountain Diagnostics, Colorado Springs, CO), corticosterone (Corticosterone ELISA assay kit, B-Bridge International, Cupertino, CA) and GABA (GABA-Research ELISA kit, Rocky Mountain Diagnostics) were determined by ELISA assays following the instructions of the vendors. The serum levels of the opioid peptides met-enkephalin, dynorphin A and dynorphin B were also measured by ELISA assays (Peninsula Laboratories LLC, San Carlos, CA). Five random serum samples per group were used for each ELISA assay. Statistical analysis of data was conducted by Mann-Whitney tests.

Determination of cAMP, vascular endothelial growth factor (VEGF), SHH and ALDH-1 in xenograft tissues—Gα_i-coupled GABA-B receptors²³ and opioid peptide receptors³⁰ reduce cAMP levels by inhibiting the activation of adenylyl cyclase whereas stress neurotransmitters increase cAMP levels by activating adenylyl cyclase downstream of Gα_s-coupled beta-adrenergic receptors³¹. We therefore measured the levels of cAMP in xenograft tissues from control mice and mice with stress reduction using an ELISA assay kit (Enzo Life Sciences, Farmingdale, NY) in accordance with the vendor's instructions. VEGF (VEGF Elisa assay kit, Enzo Life Sciences International), SHH (SHH Elisa assay kit, Abcam, Cambridge, MA) and ALDH-1 (ALDH1A Human ELISA kit, Antibodies-online Inc, Atlanta, GA) were also measured. All Elisa assays of xenograft tissues were conducted on five randomly chosen xenografts per treatment group. Statistical significances between the two animal groups were determined by Mann-Whitney tests.

Western blotting of signaling proteins associated with cell proliferation, apoptosis and cancer stem cell self renewal in xenograft tissues—Protein samples (20µg) were electrophoresed using 10%, 12% and 14% SDS gels (Invitrogen, Carlsbad, CA, USA) and blotted onto membranes. The membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature. The blots were probed with phosphorylated and unphosphorylated signaling proteins associated with cell proliferation AKT, ERK 1/2, CREB or SRC, the pro-apoptotic proteins for the tumor suppressor gene p53 and cleaved and uncleaved Caspase-3 (Cell Signaling Technology, Danvers, MA, USA) and the SHH effector Gli1 (Santa Cruz Biotechnology, Dallas, TX) at 4°C overnight. Beta-Actin (Abcam, Cambridge, MA, USA) at 4°C served as loading control. The membranes were then washed (0.5% Tween 20/TBS) and incubated with their respective fluorescent secondary antibodies for 2 hours. Protein bands were visualized with enhanced chemiluminescence reagent (Pierce ECL plus Western Blotting Detection Substrate). Each protein was assayed from three randomly chosen xenografts per treatment group and two densitometric readings per band were taken using NIH ImageJ. Statistical analysis of data was by unpaired two-tailed t tests.

Results

A. In Vitro studies

Assessment of CSC numbers by spheroid formation assays identified the stress neurotransmitter epinephrine as a strong stimulator of spheroid formation ($p < 0.0001$) in all three investigated NSCLC cell lines (Figures 1A–1D). This effect was significantly inhibited in all three cell lines by dynorphin B (NCI-H322: $p < 0.03$; NCI-H441: $p < 0.0001$; NCI-H1299: $p < 0.0001$) or GABA (NCI-H322: $p < 0.0001$; NCI-H441: $p < 0.0001$; NCI-H1299: $p < 0.0001$). Dynorphin B and GABA also significantly ($p < 0.05$) inhibited spheroid formation in each cell line in the absence of epinephrine stimulation (Figures 1A–1D). However, the inhibitory effects of dynorphin B on spheroid formation in the presence and absence of epinephrine was less pronounced in the cell line with activating point mutations in *K-ras* mutations (NCI-H322) than in the two cell lines without *ras* mutations (NCI-H441, NCI-H1299). Elisa assays for the assessment of SHH which regulates the self renewal of NSCLC stem cells⁸ and ALDH-1 which regulates the clonogenicity and tumorigenicity of NSCLC stem cells⁷ at the end of the spheroid formation assays showed significant (SHH: $p < 0.001$; ALDH-1: $p < 0.03$) increases of both stem cell markers in response to epinephrine in spheroids from all three NSCLC cell lines (Figures 2A–2C). These effects of epinephrine were completely blocked by dynorphin B or GABA. Moreover, treatment of the spheroids with dynorphin B or GABA alone significantly reduced the levels of both measured stem cell markers below control levels (SHH: $p < 0.02$; ALDH-1: $p < 0.03$) in spheroids from each of the investigated cell lines (Figures 1A–1C). In accord with a report that tumor cell survival of *K-ras*-induced lung adenocarcinomas depends strongly on Notch-1³², dynorphin B was less effective in reducing Notch-1 levels in NCI-H322 spheroids (Figure 2B), thus correlating with the responses of these treatment groups observed in the spheroid formation assays (Figure 1B).

The protein expression of the zinc finger transcription factor Gli1 that regulates the renewal of NSCLC stem cells in response to SHH⁸ was increased in Western blots of spheroids exposed to epinephrine from all three NSCLC cell lines (Figure 3A). By contrast, GABA treatment alone or in combination with epinephrine reduced Gli1 expression (Figure 3A).

Exposure of spheroids from all three NSCLC cell lines to epinephrine increased the levels of intracellular cAMP up to 3.7-fold ($p < 0.0001$) and this effect was completely blocked by simultaneous treatment of the spheroids with dynorphin B or GABA (Figure 3B).

B. In Vivo studies

Analysis of serum samples revealed that mice maintained with enrichment items in larger cages had significantly ($p < 0.03$) decreased serum levels of norepinephrine, epinephrine and corticosterone whereas GABA levels almost doubled ($p < 0.03$; Figure 4A). In addition, these animals showed significantly ($p < 0.01$) increased serum levels of the three measured opioid peptides (dynorphin A and B, met-enkephalin; Figure 4B). Bodyweights were not significantly different between the two treatment groups (data not shown).

Mice maintained in larger cages with enrichment items showed significantly ($p < 0.03$ weeks 2 and 3; $p < 0.01$ week 4) decreased xenograft volumes in weeks 2 through 4 (Figure 4C and inset Figure 4D) than the control animals. In addition, the number of detectable xenografts per group (tumor incidence) was only 60% ($p < 0.03$) in these animals as opposed to 100% in the controls (Figure 4C inset). In accord with the inhibitory effect observed on tumor growth, the levels of cAMP ($p < 0.03$), VEGF ($p < 0.03$) and the stem cell markers SHH ($p < 0.03$) and ALDH-1 ($p < 0.01$) were significantly reduced in xenograft tissues of animals in mice maintained in larger cages with enrichments than in the control group (Figure 4D), suggesting that in addition to cAMP-dependent pro-proliferative signaling pathways, angiogenesis and cancer stem cell pathways involved in cancer stem cell renewal, tumorigenicity and clonogenicity were inhibited.

Western analysis of proteins associated with NSCLC cell proliferation, migration, apoptosis and NSCLC cancer stem cell self renewal (Figures 5A–5D) from xenograft tissues revealed that maintenance of the mice in larger cages with enrichments inhibited the expression of all investigated phosphorylated proteins associated with the regulation of cell proliferation and migration (p -ERK: $p < 0.0001$; p -AKT: $p > 0.01$; p -CREB: $p < 0.0001$; p -Src: $p < 0.0001$) whereas the protein expression of pro-apoptotic cleaved caspase-3 and p53 as well as the SHH effector Gli1 were significantly reduced in xenografts of these animals (cleaved caspase-3: $p < 0.001$; p53: $p < 0.0001$; Gli1: $p < 0.001$).

Discussion

Our data provide evidence, for the first time, that CSCs from NSCLC cell lines are regulated by the opposing effects of stress neurotransmitters, GABA and opioid peptides, with stress neurotransmitters acting as “accelerators” and GABA and opioid peptides functioning as “brakes”. The stress neurotransmitter epinephrine significantly increased the number of spheroids while simultaneously enhancing the levels of the stem cell markers SHH, Gli1 and ALDH-1 and the downstream effector of beta-adrenergic receptors, cAMP, in spheroids

from all three investigated NSCLC cell lines. GABA and the opioid peptide dynorphin B each significantly inhibited all of these responses. Inhibition of spheroid formation from NCI-H322 cells appeared to depend primarily on Notch-1 as evidenced by the observed discrepancy of spheroid numbers with SHH and ALDH-1 levels. This interpretation is in accord with a report that Notch-1 is required for the survival of K-ras-driven lung adenocarcinoma ³².

Our findings are in accord with reports that the G α_i -coupled GABA-B receptors have tumor suppressor function in NSCLC ^{15, 25, 33} and that G α_i -coupled KORs and DORs inhibit the proliferation of NSCLC cells in vitro ^{18, 34}. The cited findings were generated with NSCLC cell lines that contained only small populations of cancer stem cells and therefore largely reflected the responses of differentiated cancer cells. Our current findings in CSC spheroids indicate that G α_i -signaling in response to GABA and opioid peptides also exerts significant inhibitory actions on CSCs. In accord with these in vitro findings, environmental manipulations which decreased serum levels of stress neurotransmitters and increased levels of GABA and opioid peptides in mice caused significant reductions in the growth of NSCLC xenografts (monitored by measurements of xenograft volumes) and the ability of injected NSCLC cells to establish tumors in vivo (measured as tumor incidence). In analogy to the in vitro experiments with CSC spheroids, xenograft tissues in this treatment group showed significant decreases in the cancer stem cell markers SHH and ALDH-1 along with reduced protein expression of the SHH effector Gli1. The additionally observed reductions in phosphorylated signaling proteins associated with cell proliferation and increases in pro-apoptotic proteins further indicate strong inhibiting effects of the environmental manipulations on differentiated NSCLC cells.

Current therapy of NSCLC includes traditional chemotherapy alone or in combination with agents that block individual signaling molecules that are often overexpressed in these cancers such as EGFR tyrosine kinases, p-Src, p-ERK and p-AKT either as single agent treatments or in combination, but with limited success ¹. More recent approaches additionally explore agents that inhibit stem cell specific pathways such as the SHH pathway ^{9, 35}. However, there is concern that due to the very small population of cancer stem cells present in any cancer success of this strategy may be limited and clinical trials targeting the SHH or Notch pathways have so far been disappointing ³⁶. It is noteworthy that in the current experiment stress reduction decreased NSCLC xenograft growth by the simultaneous inhibition of cancer stem cell markers as well as phosphorylated signaling proteins involved in the regulation of cell proliferation while additionally inducing pro-apoptotic proteins.

The adverse effects of chronic psychological stress or experimental treatment with stress neurotransmitters on cancer development and progression has gained considerable interest in recent years. Tumor promoting effects of experimentally induced psychological stress as well as in vitro treatments with norepinephrine or epinephrine have thus been reported for some of the most common human cancers, including cancer of the ovary ^{37, 38}, breast ^{39, 40}, pancreas ^{41, 42}, colon ⁴³ prostate ⁴⁴ and NSCLC ²⁵. In accord with these preclinical findings, some investigations have described improved clinical outcomes in such cancer patients who received incidental beta-blocker therapy ^{28, 45–47}. However, pending on the type, dose and

duration of beta-blocker treatment used, this class of agents may have tumor promoting effects due to reactive desensitization of beta-adrenergic receptors in response to general beta-blockers or upregulation of beta-2 adrenergic receptors in response to beta-1 blockade ⁴⁸. In addition, cAMP-driven pathways can be activated by a host of environmental and lifestyle factors independent of beta-adrenergic receptors, including exposure to estrogen ¹⁴, corticosteroids ⁴⁹, beta-carotene ^{50, 51} as well as phosphodiesterase inhibitors, including caffeine ⁵² and theophylline ⁵³. In addition, numerous over-the counter decongestants as well as prescription broncho-dilators increase cAMP levels via epinephrine-like effects on adrenergic receptors. The controlled experimental conditions of our current in vitro and in vivo experiments precluded the interference of such cAMP-enhancing factors with cancer inhibiting effects of GABA, Dynorphin B and stress reduction. Investigations aimed at improving clinical outcomes in NSCLC patients by psychological and pharmacological reduction in cAMP signaling therefore need to monitor systemic cAMP levels (e.g. in blood lymphocytes) and minimize incidental exposure of the patients to uncontrolled cAMP-enhancing factors.

Our current data identify pharmacological or psychological strategies that raise the levels of Gα_i-activating physiological inhibitors of cAMP signaling instead of blocking beta-adrenergic receptors as promising tools for the prevention and adjuvant therapy of NSCLC. The inhibition of cAMP formation by both, GABA and endogenous opioid peptides counteracts stress-induced hyperactivity of the sympathetic branch of the autonomic nervous system and hypothalamic pituitary adrenal gland activation ^{54–56} and also plays an important role in down-toning cardiac activity ⁵⁷. The endogenous opioid system participates in the mediation of pleasure ⁵⁸ a fact that lead to the abuse of opiates as recreational drugs. Similarly, GABA contributes to the neurobiology of positive emotions ⁵⁴. More recent studies have additionally shown that brain GABA levels increase in response to stress-reducing activities such as yoga ⁵⁹. It hence appears that individualized programs for the achievement of stress reduction and pleasurable emotions accompanied by regular monitoring of systemic cAMP levels (e.g. in blood lymphocytes) could significantly improve clinical outcomes in NSCLC patients. In addition, GABA has been widely used as a nutritional supplement without adverse side effects ⁶⁰ and could hence be used to reduce cAMP levels by pharmacological means when cAMP monitoring indicates that the psychological approach alone is not effective.

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Abbreviations used

NSCLC	non-small cell lung cancer
GABA	γ-aminobutyric acid
cAMP	cyclic adenosine monophosphate
CSCs	cancer stem cell like cells

DOR	delta opioid receptor
KOR	kappa opioid receptor
Epi	epinephrine
Nor	norepinephrine

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Novelty and Impact

Our data show, for the first time, that the stress neurotransmitter epinephrine increases the levels of cancer stem cell markers and cancer stem cell like cells (CSCs) in NSCLC cell lines via cAMP-driven activation of sonic hedgehog and aldehyde dehydrogenase pathways in vitro, that the inhibitory neurotransmitter γ -aminobutyric acid and endogenous opioid peptides counteract these effects and that stress reduction inhibits NSCLC growth in vivo by increasing systemic levels of GABA and opioid peptides that inhibited the cAMP-driven activation of CSCs.

Figure 1.

Epinephrine significantly increased the number of cancer stem cell like cells from three NSCLC cell lines in spheroid formation assays, an effect blocked by GABA or dynorphin B (Figures 1A–D). Significantly ($p < 0.05$) different from controls (asterix); significantly ($p < 0.03$) different from epinephrine treatment (double asterix).

Figure 2.

The levels of the stem cell markers SHH ($p < 0.001$) and ALDH-1 ($p < 0.03$) were significantly (asterix) increased by treatment of spheroids from all three NSCLC cell lines with epinephrine (Figures 2A, C, D). These responses were significantly (double asterix) decreased by simultaneous treatment of the spheroids with GABA ($p < 0.001$) or dynorphin B ($p < 0.001$). The cancer stem cell marker Notch-1 in spheroids of NCI-H322 was less responsive to the inhibitory effects of dynorphin B (Figure 2B). Significantly ($p < 0.05$) different from controls (asterix); significantly ($p < 0.03$) different from epinephrine treatment (double asterix).

Figure 3.

Western blots (Figure 3A) showing that epinephrine increased while GABA decreased the protein expression of the transcription factor Gli1 in spheroids from all three NSCLC cell lines. Figure 3B illustrates the modulation of intracellular cAMP levels in spheroids exposed to epinephrine in the presence and absence of GABA or dynorphin B. Significantly ($p < 0.0001$) different from control (asterix); significantly ($p < 0.001$) different from epinephrine treatment (double asterix).

Figure 4.

Serum levels of norepinephrine (Nor), epinephrine (Epi) and corticosterone (Cort) were significantly ($p < 0.03$) reduced while GABA ($p < 0.03$) and opioid peptides ($p < 0.01$) were increased by stress reduction (Figures 4A, 4B). Xenograft volumes ($p < 0.03$ - $p < 0.01$; Figure 4C, Inset Figure 4D) and xenograft incidence ($p < 0.03$; Figure 4C Inset) were significantly decreased by stress reduction. Stress reduction also significantly decreased the levels of cAMP ($p < 0.03$), VEGF ($p < 0.03$), SHH ($p < 0.03$) and ALDH-1 ($p < 0.01$) in xenograft tissues (Figure 4D). Significantly different from controls (asterix).

Figure 5.

Western blots showing reduced expression of phosphorylated signaling proteins p-ERK, p-AKT, p-CREB, p-Src and Gli1 by stress reduction whereas cleaved caspase-3 and p53 increased (Figure 5A, B). Quantitative densitometry (Figures 5C, D) indicate that the observed changes in protein expression were significant ($p < 0.1$ - $p < 0.0001$; asterix).

Figure 6.

Cartoon illustrating the proposed mechanisms of pharmacologically or psychologically induced inhibition of cAMP signaling and the resulting tumor suppressor effects on NSCLC.