REVIEW

GABA (γ-aminobutyric acid), a non-protein amino acid counters the β-adrenergic cascade-activated oncogenic signaling in pancreatic cancer: A review of experimental evidence

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GABA is a bioactive constituent of fruits, vegetables, cereals and is believed to play a role in defense against stress in plants. In animals, it acts as an inhibitory neurotransmitter in brain while also expressed in non-neuronal cells. Studies have implicated the regulator of fight or flight stress responses, β-AR signaling cascade, as mediators of cancer growth and progression in in vitro and in vivo models of pancreatic malignancies. Pancreatic cancer is the fourth leading cause of cancer mortality in western countries. This malignancy is generally unresponsive to conventional radio- and chemotherapy, resulting in mortality rate near 100% within 6 months of diagnosis. We review a series of experiments from our laboratory and those of others examining the contribution of this signaling network to pancreatic and other human malignancies. Stimulation of the β-adrenergic receptor by lifestyle and environmental factors, as well as a pre-existing risk of neoplasm, activates downstream effector molecules that lead to pro-oncogenic signaling and thereby aid cancer growth. GABAergic signaling mediated by the serpentine receptor $GABA_B$ acts as an antagonist to β-adrenergic cascade by intercepting adenylyl cyclase. These evidences enhance the pharmacological value of human diets rich in GABA for use as an adjuvant to standard therapies.

Keywords:
β_1AR / Cancer / Cell signaling / GABA / Prevention

1 Introduction

Cancer is responsible for approximately 13% of deaths worldwide [1] and remains the second leading cause of death in the United States, accounting for nearly one in every four deaths. The American Cancer Society estimated 569 490 cancer-related deaths in the United States in 2010 [2]. Cancer development is a dynamic, long-term process that involves many complex factors in its initiation, promotion, and progression. During this process, accumulation of genetic and epigenetic alterations leads to the progressive transformation of a normal cell into a malignant cell, which then grows uncontrollably throughout the body. Cancer cells acquire immunity against physiologically imposed restrictions to growth and division by their ability to posses: (i) self-sufficiency in growth signals, (ii) insensitivity to antigrowth signals, (iii) evasion of programmed cell death (apoptosis), (iv) limitless replicative

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Abbreviations: β-AR, β-adrenergic receptor; **cAMP,** cyclic adenosine 3′5′-monophosphate; **CREB,** cAMP responsive element binding protein; **GABA,** γ-aminobutyric acid; **GPCR,** G protein-coupled receptor; **nAChR,** nicotinic acetyl choline receptor; **PDAC,** pancreatic ductal adenocarcinoma; **PKA,** protein kinase A

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potential, (v) sustained angiogenesis, and (vi) tissue invasion and metastasis [3]. Cells and tissues are complex organizations with critical checkpoints to ensure normal growth. Normally, the division, differentiation, and death of cells are carefully regulated. All cancers start as a single cell that has lost control of its normal growth and replication processes.

About 5–10% of cancers result directly from inheriting the genes associated with cancer risk [4], but the majority involves genetic alterations accumulated over time. Cell growth involves regulation of both transcription and translational processes that are orchestrated by complex signaling pathways often interwoven. The spatial regulation of myriad cell signaling processes significant for normal (physiologically desired) cellular growth are subjected to environmental cues. However, in cancer cells, these cues may alter inherent constraints and lead to detrimental cross-talk, thereby allowing cells to acquire potential for indefinite proliferation. For example, smoking is not only a risk factor for lung cancer [5], but is also a risk factor for other malignancies such as pancreatic and gastric cancer [6, 7]. As such, smoking represents the most established correlation of environmental and lifestyle factors with cancer risk and incidence.

Smoking, diabetes mellitus, and pancreatitis from any etiology, including alcohol abuse, are risk factors for pancreatic ductal adenocarcinoma (PDAC) [6]. Pancreatic cancer is one of the most deadly neoplastic diseases, as it is typically asymptomatic until it has reached an advanced stage when effective treatments are unavailing. At the time of diagnosis, most pancreatic cancers are therefore inoperable and have metastasized to distant organs. In addition, this malignancy is generally unresponsive to conventional radio- and chemotherapy, resulting in a mortality rate near 100% within 6 months of diagnosis [8]. Pancreatic cancer arises from endocrine or exocrine pancreatic cells, with more than 95% of all pancreatic cancers demonstrating histological features of PDAC [9].

Although our current understanding of signaling cascades involved in the induction, promotion, and progression of cancer has advanced in the recent years, it is still a complex issue to trace the imbalance of physiologically essential and impaired cellular signaling that drive the neoplastic process. Studies pioneered by our laboratory suggest that neurotransmitter receptors of the nicotinic (nAChR), β-adrenergic (β-ARs), and γ-aminobutyric acid (GABA) families act as central regulators of cancer growth and progression. The present review seeks to summarize the research work conducted in our laboratory that implicates β-adrenergic signaling cascades in cancer cell proliferation; this information may provide vital leads for therapeutic interventions in neoplastic progression with reference to pancreatic cancer. We will also provide evidence for the role of this pathway in various other human malignancies and discuss a potential therapeutic alternative of intercepting the β-adrenergic cascade by GABA counter signaling.

2 Physiological action mechanism and constitutive elements of the β-AR pathway

β-Adrenergic receptors are constitutively expressed in most mammalian cells and are associated with regulatory pathways operating under conditions of stress, classically defined as ''fight or flight'' responses [10]. There are three subtypes of β-AR – namely, β1-AR, β2-AR, and β3-AR – and each of these, either alone or in a concerted manner, responds to stress stimuli, resulting in the pharmacological and physiological effects observed in an individual cell. However, the distribution and degree of expression of these subtypes may vary from tissue to tissue and in a given tissue from species to species [11]. The ubiquitous presence of β-AR in almost all mammalian cell types has attracted considerable research interest toward the complex array of mechanisms and functions distinct from their classically defined physiological actions. Evidence emerging from recent studies from our laboratory and those of others implicates β-ARs as important mediators of cancer growth and/or invasiveness in a number of cancers, including cancers of the lung, prostate, colon, stomach, breast, and ovary [12–17]. Their stimulation is thought to be related to the growth and differentiation of tumor cells [18], thus making β-ARs a promising target for the prevention and treatment of all of these cancer types.

β-Adrenergic receptors are expressed in almost all tissue types, and they modulate different physiological functions upon stimulation by a ligand. Furthermore, they are representative of the G protein-coupled receptor (GPCR) superfamily, a well-defined transduction machinery involved in signal trafficking from an external stimulus to the interior of the cell. β-Adrenergic receptors, like all typical GPCRs, have seven transmembrane segments (α helices) spanning across as three intracellular and three extracellular loops, which interact to form functional domains for ligand binding and interact with stimulatory $G\alpha_s$ protein [19]. The interaction of a β-adrenergic agonist with the receptor leads to a signaling cascade, which manifests into diverse physiological responses. The endogenous agonists of these receptors are norepinephrine and epinephrine. The stress neurotransmitters norepinephrine (product of tyrosine) and epinephrine (methylated norepinephrine) are catecholamines, which are synthesized and released into systemic circulation by neurons in response to nicotinic acetylcholine receptor (nAChR) stimulation in the central and peripheral nervous system and in the adrenal medulla [20, 21]. Epinephrine preferentially binds to β2-ARs, whereas norepinephrine binds with higher affinity to β1-ARs [22]. Binding of an agonist to these receptors activates the stimulatory G-protein $G\alpha_s$. In turn, $G\alpha_s$ activates adenylyl cyclase, the rate-limiting enzyme for the formation of intracellular cyclic adenosine 3′,5′-monophosphate (cAMP). Cyclic AMP then binds to the regulatory subunit of protein kinase A (PKA) to release the catalytic subunit that then phosphorylates a

number of intracellular proteins. Some of these proteins are enzymes that are activated when phosphorylated. A major component of the pathway is cAMP response element binding protein (CREB). When phosphorylated by PKA, CREB binds to the cAMP response element in the regulatory part of genes and stimulates the transcription of a number of genes. The phosphorylation of various other transcription factors by PKA may induce transactivation of distinct pathways, establishing a cross talk that potentially leads to synergistic responses.

3 GABA (γ-aminobutyric acid) signaling cascade

γ-Aminobutyric acid (GABA) is a non-protein amino acid synthesized by decarboxylation of glutamate by the enzyme glutamic acid decarboxylase [23]. The central nervous system (CNS) contains uniquely high concentrations of GABA, and GABA serves as a major inhibitory neurotransmitter along with another monocarboxylic amino acid, glycine [24, 25]. However, little attention has been devoted to the function of peripheral GABA, as the levels of GABA in most peripheral tissues are low compared with levels in the brain. γ-Aminobutyric acid has been long known to exist in plants and bacteria, where it serves a metabolic role in the Krebs cycle (the "GABA shunt"), suggesting that it is likely to be involved in metabolism rather than signaling. However, the high concentrations of GABA existing in the pancreatic islets, the oviduct, and the myenteric plexus of the gut are comparable to brain levels [26], and the GABA receptors found in various peripheral organs expanded the conventional wisdom from GABA as only a metabolic substrate and neurotransmitter to GABA as a signaling molecule with its own network of GABAergic receptors functioning in both neuronal and non-neuronal tissues [27, 28]. The biological effects of the amino acid neurotransmitter GABA are mediated by ionotropic GABA-A receptors, a family of ion channels, and by the metabotropic GABA-B receptor, a receptor coupled to the inhibitory G-protein [29]. The anti-oncogenic signaling of GABA seems to be mediated by GABA-B-R through reduction in β-adrenergic stimulated cAMP signaling via Gi-mediated inhibition of adenylyl cyclase [30].

4 Expression of β-adrenergic receptors in human exocrine ductal pancreatic adenocarcinoma cells

The most common type of pancreatic cancer is PDAC, which comprises 75% of all exocrine pancreatic cancers [9]. Smoking is an established risk factor for pancreatic cancer [6, 31], and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) causes exocrine ductal pancreatic cancer in laboratory rodents [32]. It is well established that NNK is metabolically converted into metabolites that react with DNA molecules to form DNA methyl and pyridyloxobutyl adducts [33]. One of these adducts, O^6-methylguanine, has been associated with the expression of an activating point mutation in codon 12 of the Ki-*ras* gene [34]. This activating point mutation (75%) and p53 mutations (50%) are common in exocrine ductal pancreatic carcinomas [35]. These mutations trigger carcinogenic events and then enter a progressive phase when they are stimulated by cell signaling pathways that, although performing their evolved, physiologically desired roles, switch to replenish the microenvironment required for the growth and proliferation of cancer cells [36]. Studies both in vitro and in vivo have documented the role of the β-adrenergic pathway in growth regulation of neoplastic disease [37, 38]. The correlation of smoking as a risk factor for pancreatic cancer [31] and NNK being classified as a high affinity agonist for β-AR [37] provides an interesting linkage between lifestyle factors, pancreatic cancer, and β-adrenergic cellular signaling. In order to obtain experimental evidence of this link, our laboratory investigated the ability of pancreatic cancer cells to express β-AR using two cell lines of human pancreatic adenocarcinoma, Panc-1 and BXPC-3 [39].

RT-PCR studies revealed the expression of mRNA for β1- and β2-AR in both cell lines (Fig. 1A and B). The β1 primers

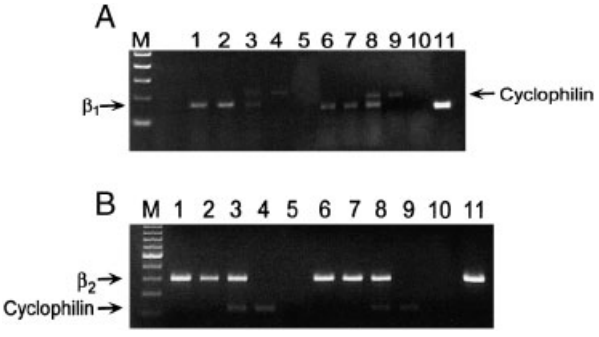

Figure 1. (A) Expression of mRNA for β1-adrenergic receptors in human pancreatic cancer cell lines BXPC-3 and Panc-1. The β1 primer amplified a 159 bp fragment. Lanes 1 and 2, BXPC-3 with β1 primers; lane 3, BXPC-3 with β1 and cyclophylin primers; lane 4, BXPC-3 with cyclophylin primer; lane 5, BXPC-3 negative control without M-MLV reverse transcriptase; lanes 6 and 7, Panc-1 with β1 primers; lane 8, Panc-1 with β1 and cyclophylin primers; lane 9, Panc-1 with cyclophylin primer; lane 10, Panc-1 negative control without M-MLV reverse transcriptase; lane 11, transfected CHO cell line Rex 50 with β1 primers (positive control). (B) Expression of mRNA for β2-AR in the human pancreatic cancer cell lines BXPC-3 and Panc-1. The β2 primer amplified a 401 bp fragment. Lanes 1 and 2, BXPC-3 with β2 primers; lane 3, BXPC-3 with β2 and cyclophylin primers; lane 4, BXPC-3 with cyclophylin primer; lane 5, BXPC-3 negative control without M-MLV reverse transcriptase; lanes 6 and 7, Panc-1 with β2 primers; lane 8, Panc-1 with β2 and cyclophylin primers; lane 9, Panc-1 with cyclophylin primer; lane 10, Panc-1 negative control without M-MLV reverse transcriptase, lane 11, transfected CHO cell line NBR29 with β2 primers (positive control). Reproduced from [39] by permission of Oxford University Press.

amplified a 159 bp fragment, whereas the β2 primers amplified a 401 bp fragment. Moreover, the PCR fragments amplified by the human β1 primers in the pancreatic cancer cells were 100% identical to the published sequence (GenBank accession no. J03019, bases 747–887). Similarly, the PCR fragments amplified by the human β2 primers in pancreatic cancer cells were also 100% identical to the published sequence (GenBank accession no. M15169, bases 1677-2060). Radio-receptor assays performed with crude membrane preparations from Panc-1 and BXPC-3 cells further supported the presence of these receptors in the tested cell lines. Increasing concentrations of β2-adrenergic antagonist ICI118.551 or β1-adrenergic antagonist atenolol competed with the broad-spectrum β-adrenergic ligand [^{125}I] CYP for the β-adrenergic binding sites under steady-state conditions. Non-linear regression analysis for two-site binding isotherms yielded biphasic curves, suggesting the presence of two populations of receptors (one with high and other with low affinity for the antagonist under steady state) in each cell line with β2 receptors predominating over β1 receptors (Panc-1: 70% β2, 30% β1; BXPC-3: 60% β2, 40% β1). These findings demonstrated for the first time that human exocrine PDAC cell lines express a β-AR-controlled signal transduction mechanism that might be contributing to pro-oncogenic signaling when stimulated by associated risk factors.

5 Certain dietary agents potentiate the proliferation of human pancreatic duct epithelial cells through PKA (a downstream effector molecule of the β-AR pathway)-dependent mitogenic signaling

Vitamin A (retinol) is formed in mammalian cells from β-carotene, contained in numerous vegetables. The classic concept of vitamin A as an essential protector of epithelial cell integrity [40] has prompted investigations into the potential preventive and therapeutic effects of retinoids on pancreatic cancer. The results of these studies, most of which have been conducted with dietary supplements of retinoids that yield high systemic concentrations, remain controversial. It has thus been shown that retinoids reduced the progression of premalignant lesions to overt pancreatic cancer in a rat model of azaserine-induced acinar cell carcinoma [41]. By contrast, four different retinoids tested as high- or low-dose dietary supplements showed tumor promoting effects on PDAC induced in hamsters by *N*-nitroso(2-oxopropyl)-amine [42]. Similarly, dietary dried cabbage, which yields low systemic concentrations of β-carotene, significantly promoted the development of PDAC induced in hamsters by this same nitrosamine [43]. All *trans*-retinoic acid (ATRA, 10 μM), a major metabolite of vitamin A, inhibited the proliferation of the human PDAC cell line Capan-1 but enhanced cell invasion [44]. The same vitamin A metabolite (0.10–10 μM) inhibited proliferation while inducing apoptosis in ten different human PDAC cell lines [45], whereas another laboratory reported cell cycle arrest in one of these cell lines, panc-1, only when ATRA concentrations of 1–50 μM were used [46]. High concentrations (10 μM–10 M) of the retinoid ATRA or 9-*cis*-retinoic acid (9-*cis*-RA) also significantly reduced cell numbers of human pancreatic cancer cell lines [47]. However, clinical trials in patients with advanced pancreatic cancer showed no response to 13-*cis*-retinoic acid (13-*cis*-RA) when administered at a dose of 1 mg/kg per day, which approximates a systemic concentration of 46.6 μM in a person of 70 kg (~154 lb) bodyweight and a mean total blood volume of 5 L [48]. On the other hand, another trial with a similar regimen reported prolonged stable disease [49], while the administration of 20 mg/day of β-carotene in the Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) trial in male smokers had no statistically significant effect on the incidence or mortality of pancreatic cancer [50]. Studies in cell lines derived from human PDAC and normal duct epithelia have shown that these cells are regulated by β-adrenoreceptors [39, 51]. The nitrosated carcinogenic nicotine-derivative NNK has been identified as a high affinity agonist for these receptors [37, 39], suggesting that this potent carcinogen may directly interfere with the β-adrenoreceptor regulation of PDAC.

Our laboratory thus proposed to explain the possible reason for the disappointment with the ATBC trial and the β-carotene and retinoid efficacy trial (CARET), which had to be aborted due to a significant increase in mortality [52, 53]. Treatment of human pancreatic duct epithelial cells with NNK or a classic β-adrenergic agonist stimulated cell proliferation via increase in intracellular cAMP, resulting in the activation of PKA and transactivation of the EGFR and its downstream effectors ERK1/2 [51, 54]. These findings suggest that agents that increase cAMP may stimulate the proliferation of pancreatic duct epithelial cells and the tumors arising from them, thus contributing to the development of pancreatic cancer. We investigated the effects of low concentrations of β-carotene on cell proliferation, intracellular cAMP concentration, activation of PKA, EGFR-tyrosine kinases, and ERK1/2 in an immortalized cell line derived from human pancreatic duct epithelial cells [55]. The study demonstrated that β-carotene led to an increase in cellular proliferation (Fig. 2), which was associated with an increase in cyclic-AMP levels (Fig. 3) and the activation of PKA (Fig. 4). The effect was attributed to the stimulation of the β-AR pathway by the dietary agent, resulting in elevated systemic levels of cAMP and the downstream activation of PKA. These findings are consistent with the ''classic'' concept of β-AR signaling via $G\alpha_s$ adenylyl cyclase, cAMP, PKA, CREB, and transactivation of EGFR. Our data are supportive of the idea that dietary agents that possess anticancer activity should be used as chemopreventive agents in a marker-guided approach. For example, systemic cAMP levels can be used as a marker for cancer types that are stimulated by cAMP signaling, and chemopreventive

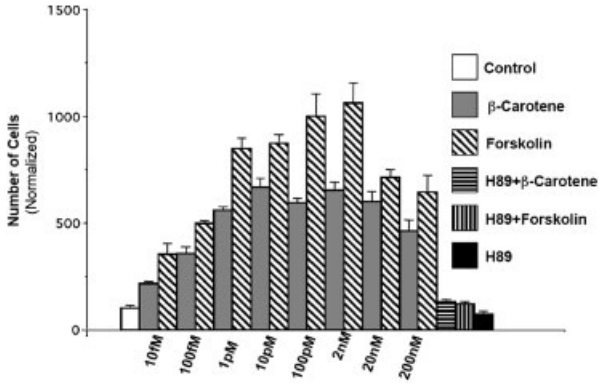

Figure 2. Results of MTT assays, illustrating the effects of β-carotene and forskolin on the proliferation potential of HPDE6-c7 cells. Both agents yielded significant ($p<0.001$) stimulation at all concentrations tested, an effect completely blocked by the PKA inhibitor H89 (1 μM). Data are mean values and standard errors of four samples per treatment group. Reproduced from [55] by permission of the International Institute of Anticancer Research.

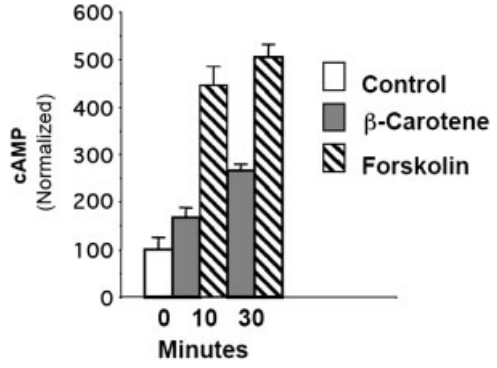

Figure 3. Data of cAMP immunoassays, showing a significant ($p<0.001$) increase in intracellular cAMP in cells exposed to β-carotene (20 nM) or forskolin (200 nM) for 10 or 30 min. Data are mean values and standard errors of triplicate samples per treatment group. Reproduced from [55] by permission of the International Institute of Anticancer Research.

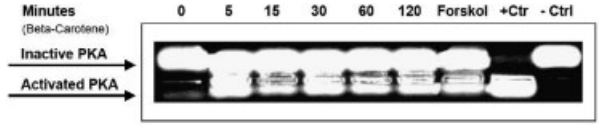

Figure 4. Levels of activated PKA in HPDE6-c7 cells in response to β-carotene (20 nM) or forskolin (200 nM) were assessed using a nonradioactive Pep Tag assay kit. β-Carotene significantly ($p<0.001$) induced PKA activation at all time intervals tested. The classic inducer of cAMP, forskolin, served as positive control. Additional positive and negative controls (+Ctrl, −Ctrl) were provided by the kit. Reproduced from [55] by permission of the International Institute of Anticancer Research.

dietary agents that inhibit the pathway are recommended as adjuvants to standard therapy, while those having a stimulatory effect should be used with caution.

6 β-AR antagonist propranolol prevents the NNK-induced development of PDAC in the experimental pancreatitis model of hamsters

β-Adrenergic activity of NNK resulting in mitogenic and/or anti-apoptotic signaling has been reported by us and others in several cell lines derived from human small airway-derived adenocarcinomas, immortalized human small airway epithelia [56, 57], and in colon cancer cells [58]. In addition, it has been shown that the migration and invasiveness of adenocarcinomas of the colon, prostate, breast, and ovaries are under β-adrenergic control [59–62]. Studies from this laboratory have earlier investigated the NNK-induced effects on the signal transduction pathways downstream of both β1- and β2-adrenergic receptors in immortalized human pancreatic HPDE6-c7 cells [51]. The HPDE6-c7 cells are developed from normal pancreatic duct epithelial cells, which are the putative cells of origin of PDAC. MTT cell proliferation assays demonstrated that NNK and the classic β-adrenergic agonist isoproterenol increased cell proliferation in HPDE6-c7 cells. Western blot and cyclic AMP assays demonstrated that NNK treatments also resulted in: (i) transactivation of the epidermal growth factor receptor EGFR, (ii) an increase in intracellular cyclic AMP accumulation, and (iii) phosphorylation of mitogen-activated protein kinase Erk1/2. The proliferative response to NNK and isoproterenol was inhibited by the use of β-blockers (propranolol), and inhibitors of adenylyl cyclase (SQ 22536), EGFR-specific tyrosine kinase (AG 1478), and Erk (PD 98059). In analogy to the increased risk of smokers and individuals with alcohol-induced pancreatitis, studies in our laboratory have further shown that pregnant hamsters given ethanol in the drinking water and treated with NNK give birth to offspring that developed a high incidence of pancreatitis-associated PDAC while the offspring of females given ethanol alone developed pancreatitis [63, 64]. Molecular analysis of the hamster PDACs showed upregulated expression levels of p-CREB and p-ERK1/2, suggestive of a hyperactive cAMP-dependent regulatory pathway [65]. This animal model was used in another study that tested the hypothesis that the general antagonist for β-ARs (synonym: β-blocker), propranolol, may prevent the development of PDAC by blocking this signaling pathway [66]. Indeed, cancer preventive treatment with propranolol significantly ($p<0.001$ by log-rank test) increased the survival time of hamsters receiving prenatal treatment with ethanol and NNK for the development of pancreatic cancer (Fig. 5). Our data show that the β-blocker propranolol has strong cancer preventive effects on PDAC induced by prenatal exposure to ethanol/NNK in hamsters, an effect that involved the reversal of increases in EGF/VEGF and of CREB/ERK phosphorylation (Fig. 6). These findings are in accordance with in vitro studies that have shown the activation of CREB and PKA-dependent transactivation of EGFR downstream from β-ARs following ligand-binding of NNK to β-ARs in

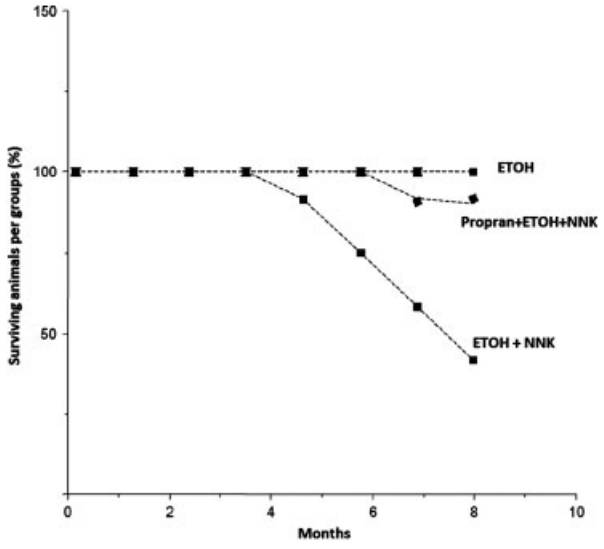

Figure 5. Survival curves of hamsters expressed as percent surviving animals per treatment group ($n = 12$ in each group) over time. Cancer preventive treatment with propranolol significantly ($p < 0.001$ by log-rank test) increased the survival time of hamsters treated prenatally with ethanol and NNK for the development of pancreatic cancer. Reproduced from [66] by permission of Elsevier.

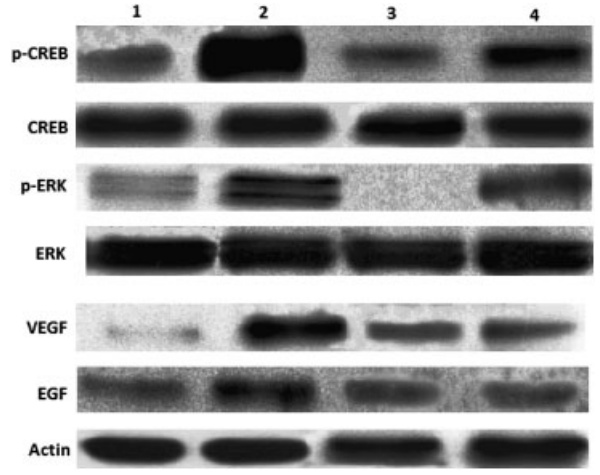

Figure 6. Western blots showing induction of p-CREB (2.9-fold, $p < 0.001$), p-ERK1/2 (2.2-fold, $p < 0.001$), VEGF (2.9-fold, $p < 0.001$), and EGF (1.8-fold, $p < 0.001$) in cells harvested from ETOH/NNK-induced PDACs and inhibition of these responses ($p < 0.001$) by treatment with propranolol. Each Western blot was conducted thrice with lysates from three separate samples per treatment group and yielded similar data. Reproduced from [66] by permission of Elsevier.

cell lines derived from human PDACs or lung adenocarcinomas and their respective cells of origin [37, 39, 51, 57]. More recently, it has been shown that NNK additionally increases the systemic levels of the stress neurotransmitters noradrenaline and adrenaline in hamsters [65, 67]. Since noradrenaline and adrenaline are physiological agonists for β-ARs, this effect of NNK further intensifies β-adrenergic signaling. The observed simultaneous increases in VEGF and EGF (Fig. 6) in PDAC cells and their reduction by propranolol are in accordance with the concept that both are stimulated by β-adrenergic signaling [68–71].

The observed upregulation of α7nAChR protein in PDAC cells of the group treated with ETOH/NNK alone is also concurrent with the documented function of NNK as an nAChR agonist [72–74] and mirrors the paradoxical upregulation of this receptor reported upon chronic exposure to nicotine in the nervous system [75]. However, in the current animal model, a single dose of NNK injected ito pregnant hamsters 1 day before the delivery of the pups upregulated this receptor in the offspring. This may be the reflection of the higher affinity of NNK to α7nAChR or a greater sensitivity of the receptor to agonist-induced upregulation in fetal tissues. It has also been reported that cAMP positively regulates the subunit assembly of nAChRs, thereby increasing their protein expression [76]. The NNK-induced direct and indirect stimulation of cAMP signaling downstream of β-ARs may thus have contributed to the observed upregulation of α7nAChR. Conversely, inhibition of cAMP formation by propranolol may have triggered the observed reduction in expression levels of this receptor below the levels in controls. In light of the fact that α7nAChR stimulates the synthesis and release of noradrenaline and adrenaline in the nervous system [77, 78] and colon cancer cells [68], the observed induction of p-CREB and p-ERK1/2 in PDAC cells were in part caused by stress neurotransmitter-induced stimulation of β-adrenergic signaling. This interpretation is supported by our earlier finding that NNK-treated hamsters have significantly increased systemic levels of noradrenaline and adrenaline [65, 67]. Thus, our studies demonstrated a cancer preventive effect of the β-blocker propranolol on PDAC via inhibition of several important targets of β-adrenergic signaling that drive the development and progression of this cancer. These findings further emphasize the importance of cAMP signaling downstream of β-ARs in the regulation of PDAC. They suggest that interference with this signaling cascade is a promising target for PDAC prevention, a strategy that may also be applicable to other cancers that are stimulated by stress neurotransmitters and β-AR signaling, including adenocarcinoma of the lung, prostate, colon, stomach, breast, and ovary [12–17].

7 Evidence for the activation of β-AR signaling as an important mechanism in stimulating the growth of cancer

In the preceding sections, we have described the involvement of the β-AR pathway in driving cancer cell growth in PDAC cancer models. However, we give below studies from other laboratories, which also give evidence for

a similar mechanism operating in cancers of various other organs:

(i) Breast cancer CG-5 cells show increased proliferation when exposed to different concentrations of clenbuterol, a β2-AR agonist, without involving steroid hormone receptors [79], suggesting that β-AR stimulation elicits CG-5 cell proliferation through a mechanism that probably differs from that of estradiol. Indeed, the second messenger cAMP was found to be a growth promoter for mouse, rat, and human mammary epithelioma [80], and its levels are elevated in several breast carcinomas [81]. Huang et al. reported a possible association between β3-AR polymorphism and susceptibility to breast cancer [82].

(ii) Nicotine and NNK significantly enhanced cell proliferation in human gastric cancer cells. Treatment of cells with propranolol (β-AR antagonist) blocked NNK-induced cell proliferation [83]. This may explain why smokers have twice the risk of getting gastric cancer than non-smokers [84]. Furthermore, atenolol and ICI 118 551, β1- and β2-adrenoceptor antagonists, respectively, reversed the stimulatory action of nicotine on the ERK1/2 phosphorylation together with cell proliferation [15]. Also, β-AR-induced activation of PKA may result in the transactivation of the EGFR pathway through downstream phosphorylation of ERK1/2.

(iii) In one study, colon cancer HT-29 cell growth was reported to be stimulated by the nonselective adrenergic agonist noradrenaline, and more effectively by the β-selective agonist isoproterenol [58]. The level of cyclic AMP, the second messenger for β-adrenoceptor activation, was elevated by isoproterenol treatment. β 2-Adrenoceptor blockade with ICI 118 551, in contrast, significantly decreased cell proliferation.

(iv) Studies have shown marked-up regulation (>4-fold) of the β2-adrenergic receptor mRNA in Kupffer cells being implicated for the metastatic potential of colorectal cancer to liver, suggesting the β-adrenergic receptor signaling pathway serves as a modulatory mechanism in cancer growth and metastasis [85].

(v) DNA microarray analyses of ten ovarian carcinomas identified 266 human transcripts that were differentially expressed in tumors from patients with elevated biobehavioral risk factors (high depressive symptoms and low social support) relative to grade- and stage-matched tumors from low-risk patients [86]. Promoter-based bioinformatic analyses indicated increased activity of several β-adrenergically linked transcription control pathways, including CREB/ATF, NF-kB, and STAT family transcription factors. Consistent with increased β-adrenergic signaling, high biobehavioral risk patients also showed increased intratumor concentrations of norepinephrine.

(vi) Another study showed that isoproterenol, a β-AR agonist, stimulated rat C6 glioma cell proliferation, while propranolol, a β-AR blocker, greatly reduced the proliferative effect of TNF-α on C6 cells. The gene and protein expressions of both β1- and β2-ARs were enhanced in C6 cells after TNF-α treatment [87].

(vii) Prostate tissue has been demonstrated to be a rich source of β-AR [88]. Biochemical studies have shown that β-AR agonists are able to stimulate adenylyl cyclase and raise cAMP levels in the rat prostate [89, 90]. Accumulation of cAMP and differentiation of neuroendocrine cells have been observed in human prostate cancer cell lines treated with forskolin, dibutyryl cAMP, and catecholamine-like agonists (isoproterenol and epinephrine) [91]. These effects were reversible upon withdrawal of these agents [92]. The pharmacological properties of β-AR agonists with respect to the receptor binding and cAMP generation were examined in PC-3 cells, a human prostate androgen-independent cell line [93]. The results of these studies indicated that PC-3 cells contained a large population of β2-ARs and that corresponding signaling plays a significant role in tumorigenesis. Further, β2-AR has also been identified as the major subtype-mediating catecholamine-induced cAMP changes in LNCaP prostate cancer cells [94].

8 Non-protein dietary amino acid GABA intercepts β-adrenergic oncogenic signaling

In light of the findings that correlate cancer growth and development with the hyperactivation of β-AR signaling, agents having the potential to counter such activation could act as lead molecules for developing anticancer drugs. The antagonists for β-ARs (β-blockers) have shown significant antitumorigenic effects in preclinical studies of adenocarcinoma of the lungs, prostate, colon, and breast [13, 14, 16, 38], but their clinical use for the prevention and treatment of human adenocarcinomas has been limited by the observation that these β-blockers have significant cardiovascular effects; their chronic use results in the sensitization of β-ARs, rendering these receptors more sensitive to agonists. Accordingly, long-term treatment with such agents may promote the development and progression of cancers under positive growth control by cAMP signaling. Therefore, an interesting concept is to neutralize the outcome of the stimulation rather than blocking the activation. GABA is the major inhibitory neurotransmitter in the CNS and controls the excitatory effects of cAMP signaling by inhibiting adenylyl cyclase via activation of the inhibitory G-protein ($G\alpha_i$)-coupled γ-aminobutyric acid receptor ($GABA_BR$) [95]. Studies from our laboratory have investigated the potential of GABA in cancer chemoprevention by counteracting hyperactive cAMP signaling [96]. Using four representative human PDAC cell lines and immortalized human pancreatic duct epithelial cells, we have shown that the proliferation, migration, and apoptosis of these cells are regulated by β-adrenoreceptors via cAMP-dependent signaling, resulting in the activation of PKA/CREB, transactivation of the epidermal growth factor receptor, and

activation of caspase-3 [39, 51, 97]. In addition, these studies showed that the powerful tobacco carcinogen NNK is a high-affinity agonist for β-adrenoreceptors that stimulates PDAC cells via activation of these signaling pathways. While these findings provided the first direct link between a tobacco-specific carcinogen and the regulation of PDAC, they also suggested that a systemic increase in catecholamines adrenaline/noradrenaline might contribute to the development and progression of PDAC. The amino acid neurotransmitter GABA is the major inhibitory neurotransmitter in the CNS that counteracts the stimulatory catecholamine neurotransmitters by binding to the GABAB receptor that inhibits adenylyl cyclase via Gai [98]. Recent studies have shown that GABA was suppressed in 29 of 30 investigated human PDACs, whereas noradrenaline, PKA, p-CREB, and p-ERK were overexpressed [97]. In addition, GABA significantly inhibited base-level and β-adrenoreceptor-stimulated PDAC cell growth and migration in vitro, responses blocked by genetic silencing of the GABAB receptor [97]. These findings strongly suggest that GABA may have tumor suppressor function for PDAC. β Cells of the pancreatic islets are an important source of GABA in the pancreas [98]. The number of these cells is greatly reduced in people with diabetes and pancreatitis. In addition, smoking decreases GABA levels by as yet unidentified mechanisms [99]. The resulting deficit in inhibitory GABA signaling may create an environment favorable for the development of PDAC in smokers with pancreatitis or diabetes. In one of our studies, the observed strong inhibition of basal and nicotine-induced PDAC xenograft growth (Fig. 7) and cAMP signaling (Figs. 8 and 9) by GABA represented the first in vivo evidence for the potential usefulness of this neurotransmitter in the prevention and treatment of PDAC [96]. In addition, we showed that GABA inhibited cAMP signaling, cAMP-dependent transactivation of the EGFR pathway, cell proliferation, and cell migration in immortalized human pancreatic duct epithelial cells and in human PDAC cell lines Panc-1 and BXPC-3 [97]. GABA has also been reported to inhibit the metastatic potential of colon cancer by a similar mechanism that involved GABA-B-receptors and downregulation of cAMP signaling [100]. These observations suggested that the development and progression of PDAC is subject to significant modulation by changes in the levels of stimulatory stress neurotransmitters and inhibitory GABA and that GABA may be suitable for the prevention of PDAC in individuals at risk (Fig. 10).

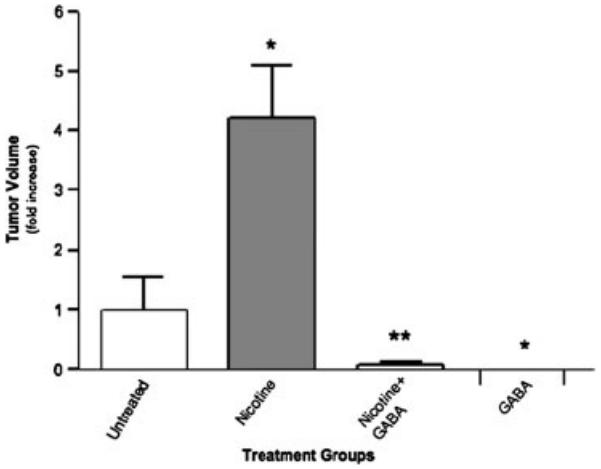

Figure 7. Effects of nicotine and GABA on Panc-1 xenografts. Athymic nude mice were subcutaneously injected with human PDAC cells Panc-1 and observed for 30 days. Controls received no pharmacological treatment, one group was given nicotine in the drinking water for 30 days, one group received nicotine in the drinking water and additional intraperitoneal injections with GABA (five times per week) and one group was treated by intraperitoneal injections with GABA alone. At the end of the observation period, nicotine had significantly ($p<0.001$) increased xenograft size, a response significantly ($p<0.001$) inhibited by GABA. Data were mean values and standard errors (expressed as fold increase over untreated animals) of the eight tumor-bearing mice in the untreated and nicotine treated groups and of the two mice that developed small xenografts in the group treated with nicotine plus GABA. None of the animals given GABA alone developed xenografts. *Significantly ($p<0.001$) different from untreated group; **Significantly ($p<0.001$) different from group treated with nicotine alone. Reproduced from [96] by permission of Oxford University Press.

9 GABA as a constituent of human food

Multiple epidemiological and animal studies have shown that consumption of foods rich in fruits and vegetables decreased the occurrence of cancers [101–105]. One of the earliest such studies conducted by Professors Doll and Peto, as an epidemiological survey for the World Health Organization, suggested that appropriate nutrition could prevent approximately 35% of cancer deaths and that up to 90% of certain cancers could be avoided by dietary enhancement [106, 107]. In the recent years there has been special interest in GABA as a health-related compound because it is a bioactive constituent of various fruits (such as strawberries and grapes), vegetables (such as tomato and soy), and cereals (such as rice and barley) [108–113]. Accumulation of GABA is a metabolic response of plant systems to stress such as salinity, anoxia, hypoxia, drought, heat, and chilling [114, 115]. Many researchers are following the strategy to use this inherent mechanism of plants to accumulate GABA under stressful conditions to develop GABA-rich foods for humans [116]. An interesting study was carried out by Kawabata et al. [117] in which the effect of dietary administration of GABA-enriched, defatted rice germ was investigated on azoxymethane-induced colon carcinogenesis in rats. The results from the study demonstrated that GABA-enriched rice germ significantly inhibited the growth of colonic (aberrant crypt foci) ACF and suppressed the progression of pre-neoplasia to a malignant neoplasm. In Japan, Tsushida et al. [118] accidentally found that a large amount of GABA accumulates in green tea under anaerobic conditions. They examined further

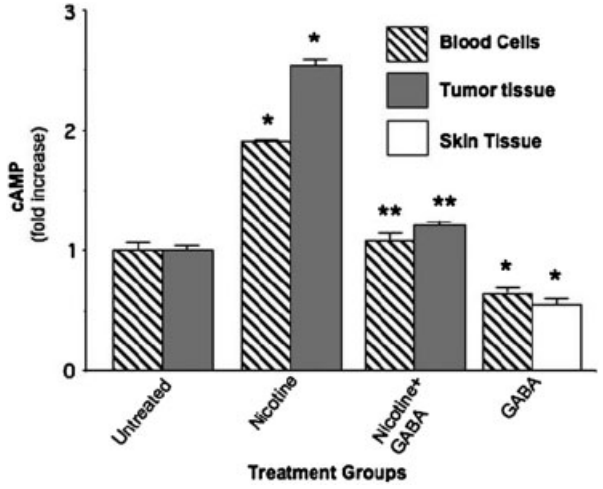

Figure 8. Results of immunoassay for the determination of intracellular cAMP in the cellular fraction of blood samples in tumor tissue and in the skin of mice without tumors after treatment with GABA alone. Treatment with nicotine increased intracellular cAMP 1.86-fold in blood cells and 2.6-fold in xenograft tissue, a response completely blocked by GABA. Treatment with GABA alone significantly ($p<0.001$) reduced cAMP levels below the base levels in untreated animals. Data were mean values and standard errors of triplicate samples per treatment group expressed as fold increase over the levels in untreated animals. *Significantly ($p<0.001$) different from untreated group; **Significantly ($p<0.001$) different from group treated with nicotine alone. Reproduced from [96] by permission of Oxford University Press.

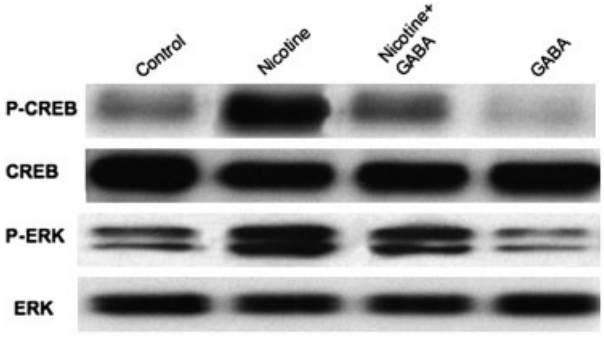

Figure 9. Western blots showing protein expression of phosphorylated and unphosphorylated CREB and ERK1/2 in xenografts of untreated mice (controls), animals treated with nicotine or with nicotine plus GABA, and in skin samples from the group treated with GABA alone. Nicotine significantly ($p<0.001$) induced the expression levels of p-CREB and p-ERK1/2. These effects were inhibited ($p<0.001$) by GABA. Reproduced from [96] by permission of Oxford University Press.

the GABA content of green, oolong, and black tea made under anaerobic conditions and found that GABA accumulated in all teas. Since then there have been a number of publications implicating both GABA and teas as preventive dietary regimens with pharmacological properties [119]. GABA tea has been produced on a commercial basis for

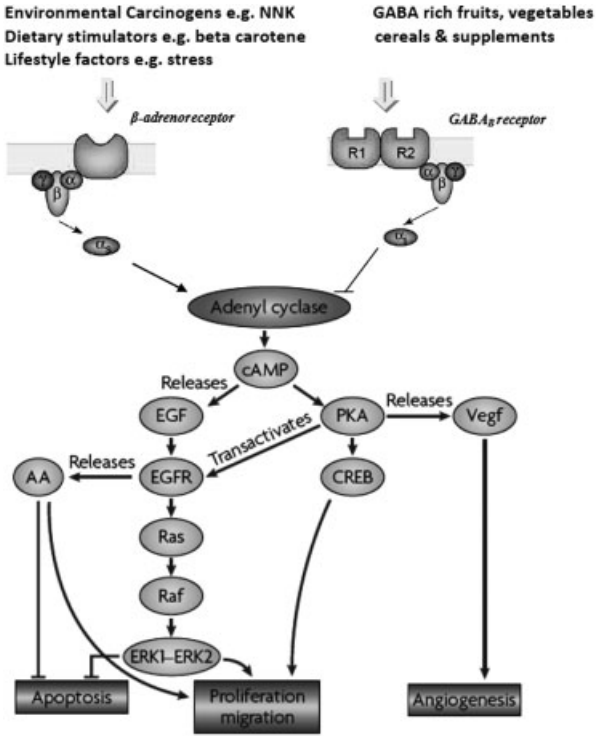

Figure 10. Schematic presentation of β-adrenergic pathway stimulation in cancer cells. Adenylyl cyclase activation downstream of β-AR induces the cyclic AMP–PKA–CREB pathway, transactivates EGFR, and induces the release of EGF, arachidonic acid (AA), and VEGF. The inhibitory neurotransmitter GABA inhibits this signaling cascade at the level of adenylyl cyclase.

people with hypertension. The only difference in processing steps between the two kinds of tea is that green tea is blanched and dried right after fresh-leaf picking, while the GABA tea undergoes anaerobic fermentation during tea-making. The analyses of their constituents show that GABA tea is almost the same as green tea in terms of the bioactive compounds while GABA tea also contains a high level of GABA [119]. Further, researchers in the area of food science and technology have been able to develop GABA-enriched foods by modifying the conditions of growth and ripening of fruits and vegetables as well as their fermentation process [108, 110, 120]. While commercially, accumulation of bioactive products may be undesirable, GABA-enriched foods possess pharmacological potential as adjuvants to standard chemotherapies against cancer, and their use as supplements by individuals at higher risk could be helpful in reducing the incidence of the disease.

10 Concluding remarks

Environmental and lifestyle factors as well as diet and preexisting risk of neoplasm may influence the physiologically essential signaling cascades to acquire a hyperactive state

and initiate a cross talk that leads to the breakdown of normal regulatory machinery, thereby allowing the cells to develop neoplastic potential. As reviewed above, the identification of therapeutically relevant hotspots of such signaling cascades in a complex disease like cancer is an essential step toward chemoprevention. Studies from this laboratory have strongly implicated β-adrenergic signaling in growth and progression of pancreatic cancer and further provide evidence for neutralizing the effect by inhibiting the effector mechanism of adenylyl cyclase using GABA. GABA is an FDA-approved dietary supplement. Moreover, several in vivo studies have indicated that pharmacological value of a GABA-enriched diet can be utilized for stimulation of immune system [121] and prevention of disorders such as hypertension [122] and diabetes [123]. Our data suggest that consuming a GABA-rich diet and other dietary factors that possess the ability to modulate β-adrenergic signaling, as adjuvants to standard cancer therapy, may have significant beneficial effects on the outcome of cancer by inhibiting the proliferation and migration of cancer cells. In addition, the regular intake of GABA as a dietary supplement may significantly reduce the incidence in individuals at a higher risk of neoplastic disease.

Over the last decade or more, based on the epidemiological studies implicating a strong correlation between dietary habits and the incidence of cancer worldwide, the pharmacological value of the constituents of human diets has been significantly enhanced as evidenced by the increase in research and development of so-called 'functional foods' or 'neutraceuticals' [124]. Many of the traditional spices, condiments, vegetables, and fruits used around the world are also important sources of anti-tumour agents [125]. It is thus believed that dietary constituents derived from plant sources have the ability to modify the process of carcinogenesis, thus relating the foodstuffs, beyond their basic nutritional benefits, to disease prevention [126].

The authors have declared no conflict of interest.

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