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Proton-sensing G protein-coupled receptors: detectors of tumor acidosis and candidate drug targets

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Cells in tumor microenvironments (TMEs) use several mechanisms to sense their low pH (<7.0), including via proton-sensing G protein-coupled receptors (psGPCRs): GPR4, GPR65/TDAG8, GPR68/OGR1 and GPR132/G2A. Numerous cancers have increased expression of psGPCRs. The psGPCRs may contribute to features of the malignant phenotype via actions on specific cell-types in the TME and thereby promote tumor survival and growth. Here, we review data regarding psGPCR expression in tumors and cancer cells, impact of psGPCRs on survival in solid tumors and a bioinformatics approach to infer psGPCR expression in cell types in the TME. New tools are needed to help define contributions of psGPCRs in tumor biology and to identify potentially novel therapeutic agents for a variety of cancers.

First draft submitted: 5 November 2019; Accepted for publication: 18 December 2019; Published online: 2 March 2020

Keywords: acidosis • bioinformatics • cancer-associated fibroblasts • G protein-coupled receptors (GPCRs) • ion channels • pH • solid tumors • tumor microenvironment

The regulation of cellular acidity (pH) is a critical feature of cell and tissue physiology. Numerous physiological and biochemical processes interact to maintain pH homeostasis and its impact on enzymes, ion channels, transporters and other cellular proteins. An increase in acidity, in other words, lowering of pH, occurs in the extracellular milieu in a number of pathophysiological settings.

Malignant tumors are a key example of a setting with low pH. Multiple mechanisms have been identified that generate protons (H^+), transport them out of cells and mediate their effects on the tumor microenvironment (TME). The TME includes cancer cells and other cell types that contribute to the altered function in tumors and include: cancer-associated fibroblasts (CAFs; which generate extracellular matrix [ECM], cytokines and other entities), immune cells and vascular-associated cells (e.g., endothelial cells and pericytes). A key feature of the TME is its low pH (<7.0 but sometimes <6.5) with heterogeneous spatial distribution of pH levels within tumors [1–3].

Numerous factors contribute to the low extracellular pH (acidosis) in tumors, including increased glycolysis and mitochondrial respiration. One contributor is the Warburg effect [4]: rapidly growing cells in tumors have a prominent increase in glucose uptake and production of ATP and lactic acid (derived from glycolysis); the increase in lactic acid occurs even in the presence of oxygen and fully functional mitochondria [5,6]. This metabolic activity leads to formation and release of lactic acid from tumor cells and acidification of the extracellular environment. Protons in tumors are also released by CO_2 produced by mitochondrial respiration, such that the complete conversion of one molecule of glucose yields six HCO_3^- and six H^+ , potentially severalfold more than derives from 'anaerobic' glycolysis [7,8]. The release of lactate and H^+ by cancer cells into the extracellular space is aided by their upregulation of a Na^+/H^+ -antiporter but also occurs via other proton transporters (e.g., V-ATPase, monocarboxylate transporters and carbonic anhydrase) [6,9,10].

Tumors have other properties that contribute to a decrease in extracellular pH, including poor vascular perfusion and limited supply of oxygen and nutrients. Vascular perfusion can be limited by the growth of blood vessels in tumors and pressure within the tumors, factors that contribute to hypoxia in the TME, anaerobic metabolism and lactic acid production. The increase in pressure can result from tumor growth and accompanying ECM

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accumulation (which has been termed 'solid stress') that can compress vessels within tumors and by the leakiness of vessels (termed 'intratumor fluid pressure') [11].

Acidosis can exert a variety of actions on tumors. Genetic changes that occur with the development of malignant tumors are associated with phenotypic changes (the 'hallmarks of cancer' [12]). Extracellular acidosis can contribute to phenotypic changes, for example, cancer cell somatic evolution, progression to malignancy, tumor growth, metastasis, metabolic rewiring and decreased immune surveillance [13–17]. Accordingly, proton pump inhibitors and buffers such as TRIS, citrate and NaHCO_3 , in addition to acid-activated drugs/nanotherapeutics, have been proposed as potential therapeutic approaches to help restore normal pH and blunt the consequences of persistent acidosis [14,15,18].

What are the mechanisms that mediate effects of acidosis/low pH in tumors and that lead to the features of the malignant phenotype? Stated differently, how does acidosis/low pH alter cells and other tumor constituents to produce such effects? Protonation of residues on proteins (e.g., ECM and plasma membrane proteins) and of substrates/ligands in the extracellular space, can alter functional activities; multiple examples with relevance for cancer biology have been identified (e.g., [18–22]).

Numerous types of ion channels may mediate effects of extracellular low pH/acidosis. These include: TWIK-related acid-sensitive potassium channels (TASK1-3), which belong to the family of two-pore domain (K[2P]) potassium channels [23]; other pH-sensing ion channels, such as transient receptor potential vanilloid-1 (TRPV1, which preferentially responds to $\text{pH} < 5.0$); acid-sensing ion channels; and voltage-independent channels (which mainly conduct Na^+ but are also activated by pH's between 4.0 and 7.0 and may be mechanosensors) [24–26]. Other acid-sensitive ion channels have been suggested as detectors of acidosis, including other TRP family members, P2X receptors, inward-rectifier K^+ channels, L-type Ca^{2+} channels, hyperpolarization-activated cyclic nucleotide-gated channels, gap junction channels and chloride channels [24,27,28].

The focus of this review is on another class of signaling entities that respond to acidosis/low pH: a family of proton-sensing G protein-coupled receptors (psGPCRs). This family of class A/rhodopsin-like G protein-coupled receptors (GPCRs) is comprised of four members: GPR4, GPR65 (also known as T-cell death-associated gene [TDAG8]), GPR68 (also known as ovarian cancer G protein-coupled receptor [OGR1]) and GPR132 (also known as G2A).

General features of GPCRs & psGPCRs

GPCRs, the largest membrane protein receptor family in the human genome (>800 GPCRs) and many other genomes, are proteins with seven membrane-spanning domains. GPCRs detect a wide variety of extracellular signals and chemical entities. GPCRs are also the largest family of targets for currently approved drugs [29,30]. GPCRs couple to one or more heterotrimeric ($\alpha\beta\gamma$) GTP binding (G) proteins of four major classes: G_s , $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$, each of which preferentially regulates certain enzymes, ion channels or other proteins involved in signaling cascades. G_s and G_i were originally named for their ability to respectively stimulate or inhibit adenylyl cyclase activity and as a result, to alter intracellular levels of cyclic AMP (cAMP). $G_{i/o}$ also regulates a variety of ion channels. $G_{q/11}$ activates phospholipase C_β to generate inositol trisphosphate and diacylglycerol; $G_{12/13}$ promotes exchange of GTP for bound GDP on RhoA, a low molecular weight G protein. Agonist-activated GPCRs can be phosphorylated by GPCR kinases and bind β -arrestins, proteins that can initiate receptor internalization and serve as scaffolds for signaling entities. Use of GPCRomic methods (which identify and quantify GPCRs) have revealed that various cell types, including cancer cells, express a large number (generally >100) of different GPCRs [31–33], which via 'downstream' signaling pathways and networks, regulate many cellular responses, including general functions (e.g., metabolism, migration, growth and death) as well as cell-specific activities (e.g., secretion, enzyme induction/de-induction, contraction/relaxation, etc.).

Each of the four psGPCRs couples to one or more G proteins: GPR4 (G_s , $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$), GPR65 (G_s), GPR68 (G_s and $G_{q/11}$) and GPR132 (G_s and $G_{12/13}$) [34]. Studies involving the use of transient transfection have suggested that GPR68 and GPR132 can have constitutive inverse agonist activity via G_i [35] and for GPR132, also via $G_{q/11}$ activation. GPR132 has two functionally active splice variants that differ in this activity [36]. It has also been suggested that dimerization of GPR68 and GPR132 can enhance proton-induced calcium signaling [37].

psGPCRs in cancer

The psGPCRs have been implicated in a wide variety of functional responses and cancers [38–50]. We recently reviewed such findings for GPR68 [51].

Figure 1. Proton-sensing G protein-coupled receptor expression in solid tumors. (A) Median mRNA expression (Log2 TPM) of psGPCRs in 45 types of solid tumors. **(B)** Data for select tumors with high expression for one or more psGPCRs, shown as median with upper and lower quartiles. These data are adapted from analysis [54] of TCGA data [55].

ACC: Adrenocortical carcinoma; BCNM: Bronchoalveolar carcinoma non-mucinous; BLCA_np: Non-papillary bladder urothelial carcinoma; BLCA_p: Papillary bladder urothelial carcinoma; BRCA_IDC: Breast invasive carcinoma invasive ductal carcinoma; BRCA_ILC: Breast invasive carcinoma invasive lobular carcinoma; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; COAD: Colon adenocarcinoma; EndoAd: Endocervical adenocarcinoma; ESCA_AD: Esophageal cancer adenocarcinoma; ESCA_SQC: Esophageal cancer squamous cell carcinoma; HR+: Hormone receptor positive; INT: Intestinal; KICH: Kidney chromophobe; KIRC: Kidney clear cell carcinoma; KIRP: Kidney papillary cell carcinoma; LIHC: Liver hepatocellular carcinoma; LSQC: Lung squamous cell carcinoma; LUAD: Lung adenocarcinoma; MucAd: Mucinous adenocarcinoma; NOS: Not otherwise specified; OV: Ovarian serous cystadenocarcinoma; PDAC: Pancreatic ductal adenocarcinoma; psGPCR: Proton-sensing G protein-coupled receptor; PRAD: Prostate adenocarcinoma; SKCM: Skin cutaneous melanoma; SQC: Squamous cell carcinoma; STAD: Stomach adenocarcinoma; STAD_Signet: Signet ring STAD; TCGA: The Cancer Genome Atlas; TGCT: Testicular germ cell tumors; THCA: Thyroid carcinoma; TPM: Transcripts per million; UCS: Uterine corpus endometrial carcinoma.

Figure 1A shows data for psGPCR expression (from RNA-sequencing data in The Cancer Genome Atlas [TCGA]) for 45 solid tumors and Figure 1B shows data for selected tumor types, in which psGPCRs are relatively highly expressed. Expression values are normalized in transcripts per million (TPM); in general, GPCRs expressed >1 TPM and especially those >10 TPM are expected to be functionally relevant [32]. Overall, *GPR68* is the most widely expressed GPCR in TCGA tumors but *GPR4* and *GPR132* are also widely expressed among the tumor types. *GPR65* is typically lower expressed in most solid tumors. *GPR68*, which has recently been identified in pancreatic and colon cancer CAFs [52,53], is also higher expressed in breast, lung and gastric adenocarcinomas, tumors that are associated with substantial fibrosis.

Figure 2 compares the expression of psGPCRs for the tumors in TCGA with that in normal tissues (from The

Figure 2. mRNA expression of proton-sensing G protein-coupled receptors (log2 fold-change) in solid tumor types compared with corresponding normal tissue. All nonzero fold changes are statistically significant (false discovery rate <0.05). Positive values indicate increased expression in tumors. These data are adapted from analysis presented in [54] of TCGA data [55].

ACC: Adrenocortical carcinoma; BCNM: Bronchoalveolar carcinoma non-mucinous; BLCA_np: Non-papillary bladder urothelial carcinoma; BLCA_p: Papillary bladder urothelial carcinoma; BRCA_IDC: Breast invasive carcinoma invasive ductal carcinoma; BRCA_ILC: Breast invasive carcinoma invasive lobular carcinoma; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; COAD: Colon adenocarcinoma; EndoAd: Endocervical adenocarcinoma; ESCA_AD: Esophageal cancer adenocarcinoma; ESCA_SQC: Esophageal cancer squamous cell carcinoma; HR+: Hormone receptor positive; INT: Intestinal; KICH: Kidney chromophobe; KIRC: Kidney clear cell carcinoma; KIRP: Kidney papillary cell carcinoma; LIHC: Liver hepatocellular carcinoma; LSQC: Lung squamous cell carcinoma; LUAD: Lung adenocarcinoma; MucAd: Mucinous adenocarcinoma; NOS: Not otherwise specified; OV: Ovarian serous cystadenocarcinoma; PDAC: Pancreatic ductal adenocarcinoma; PRAD: Prostate adenocarcinoma; SKCM: Skin cutaneous melanoma; SQC: Squamous cell carcinoma; STAD: Stomach adenocarcinoma; STAD_Signet: Signet ring STAD; TCGA: The Cancer Genome Atlas; TGCT: Testicular germ cell tumor; THCA: Thyroid carcinoma; UCS: Uterine corpus endometrial carcinoma.

Figure 3. mRNA expression (log2 transcripts per million) of proton-sensing G protein-coupled receptors in the Cancer Cell Line Encyclopedia cell lines of tumor types from different tissues. The data are adapted from a prior analysis [63].

CLL: Chronic lymphocytic leukemia and other lymphocytic and T-cell leukemias; DLBC: Diffuse large B-cell lymphoma and other B-cell lymphomas.

Genotype-Tissue Expression database [56]). These comparisons reveal differential expression (DE) of psGPCRs in multiple tumor types. *GPR132*, *GPR68* and *GPR65* show frequent DE whereas *GPR4* rarely shows such DE. Breast, ovarian and pancreatic tumors appear to have the most pronounced DE of these three psGPCRs, as compared with corresponding normal tissue. Further analysis reveals that psGPCRs do not appear to be mutated or have altered copy number in solid tumors [54].

Expression data for tumors encompasses findings for multiple cell types. Cancer cells themselves are likely a major contributor to such results. Figure 3 shows data for the expression of psGPCRs in cancer cell lines (in the Cancer Cell Line Encyclopedia [57]). *GPR68* and *GPR132* are the most widely expressed psGPCRs in tumor cell lines; *GPR4* and *GPR65* are lower expressed. This result implies that if expressed in tumors, *GPR4* and *GPR65* are likely not localized in cancer cells but instead in cells in the TME. *GPR68* and *GPR132* are potentially expressed in multiple cell types. *GPR65* is primarily expressed in certain hematopoietic cancers, consistent with data indicating expression and actions of *GPR65* in lymphoid cells [58–62]. Expression of *GPR65* in chronic lymphocytic leukemia patients correlates with that of several anti-apoptotic (but not pro-apoptotic) proteins and has been implicated in adaptation of chronic lymphocytic leukemia cells to acidic microenvironments [47]. Other data have suggested that *GPR65* is a contextual tumor suppressor that may be downregulated in hematopoietic malignancies [48]. Studies with primary B-cell lymphoblastic leukemia samples identified an increase in *GPR132* expression in BCR-ABL

Figure 4. Association of proton-sensing G protein-coupled receptor mRNA expression with markers for cell types in the tumor microenvironment. Gene symbols for markers used for the different cell types are as follows: cancer cells (*E-Cadherin*); CAFs (*COL1A1*); general immune cells (*CD45*); T cells (*CD3G*); monocytes (*CD14*); myeloid cells (*CD33*); endothelial Cells (*VWF*). The dashed line indicates the threshold for the association to be statistically significant. CAF: Cancer-associated fibroblast; VWF: Von Willebrand factor.

negative patients (i.e., patients without the fusion of the *BCR* and *ABL* genes) and have suggested that the transcription factor Ikaros may regulate GPR132 in such patients [50]. Recent data suggest that GPR132 may be a target in at least one other type of leukemia, acute myelocytic leukemia, based on studies with a novel therapeutic agent ONC212, an imipridone [42].

Limited experimental data are available for other cell types in the TME. CAFs isolated from several types of tumors (e.g., pancreatic cancer, colon cancer, appendiceal cancer and gastrointestinal stromal tumors) are enriched in the expression of *GPR68* but not the other psGPCRs [51–53].

Bioinformatic analysis provides a means to determine the cell types within tumors that express specific GPCRs (including psGPCRs): one identifies the association between mRNA expression of a gene of interest with that of known markers for such cells [54]. Figure 4 shows such associations for psGPCRs in pancreatic ductal adenocarcinoma (PDAC). The strength of association was calculated from the log₁₀ of the inverse of the false discovery rate, as a measure of the statistical significance of the association for each GPCR and corresponding cellular markers. Associations <0.01 false discovery rate are considered significant. None of the psGPCRs are strongly associated with tumor epithelial cells in PDAC but *GPR68* is strongly associated with CAFs, a result consistent with experimental data [52]. *GPR65* is strongly associated with expression of several immune cell (including T cell) markers, consistent with previous data indicating high expression of *GPR65* in immune cells [58–62]. This type of analysis can enhance understanding of the localization and implicate roles for psGPCRs within the TME.

Does the expression of psGPCRs influence prognosis and survival? Figure 5 shows examples of tumor types from TCGA, in which psGPCR expression has a statistically significant ($p < 0.05$) impact on survival. These data are based on analysis of an effect on survival of expression of all GPCRs [54]. High expression of *GPR68* reduces survival in kidney renal clear cell carcinoma. *GPR4* has a similar effect in nonpapillary Bladder Cancer while *GPR132* has this effect in classical thyroid cancer and in kidney renal clear cell carcinoma. By contrast, high expression of *GPR132* is a positive prognostic marker in hormone receptor-positive breast cancer and cervical squamous cell carcinoma; *GPR65* expression has a similar effect in hepatocellular carcinoma (Figure 5).

Discussion

This review highlights information regarding the low pH in TME's and data for the expression of the four psGPCRs (GPR4, GPR65, GPR68 and GPR132) in solid tumors and cancer cell lines. We also provide findings for cellular

Figure 5. Impact of proton-sensing G protein-coupled receptors on survival of patients with certain cancers. A Kaplan–Meier survival curve for GPR68 in KIRC is shown as an example; instances of tumor types in which psGPCRs have a significant effect on survival is also shown. Statistics were calculated based on the Peto–Peto method for analyzing Kaplan–Meier curves. The data shown are adapted from [54].

BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive adenocarcinoma; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; GPCR: G protein-coupled receptor; KIRC: Kidney renal clear cell carcinoma; LIHC: Liver hepatocellular carcinoma; psGPCR: Proton-sensing G protein-coupled receptor; THCA: Thyroid carcinoma.

localization (in particular, for PDAC) of the psGPCRs and the potential impact of altered expression of psGPCRs on patient survival in various cancers. Our goal has been to help guide future studies regarding functional roles for psGPCRs in tumors and efforts directed at psGPCRs as therapeutic targets. A limited number of compounds are currently available to aid in such studies (e.g., [59,64,65]). We envision that small molecules and perhaps antibodies may be useful to define functional activities and therapeutic potential of the psGPCRs. Evidence that GPR68 is enriched in CAFs from several tumor types and that GPR65 is enriched in immune (e.g., T cells and myeloid cells) implies that it may be possible to selectively target particular psGPCRs and certain cell types in the TME (Figure 4).

The mechanisms that alter the expression of psGPCRs in cancers are not well-defined and may involve transcriptional and/or post-transcriptional events. Co-culture studies, perhaps using cancer cell organoids [66,67] grown in 3D cultures, plus together with other cell types from the TME, may prove helpful in defining the key cells and mechanisms involved in regulating expression and activity of psGPCRs. Tumor necrosis factor- α induces *GPR68* expression in pancreatic CAFs but has not been tested in other cancers [52]. Recent data suggest that endogenous peptides may modulate activity of GPR68 [68] but studies are needed to confirm these results and determine if peptides interact with other psGPCRs. Certain metabolites (e.g., lactate) and lipids (e.g., oxylipids, lysophosphatidylcholine and commendamide) have been implicated in the regulation of signaling and functional activity of psGPCRs and may influence those receptors in tumors (e.g., [41,69–71]).

As noted in the introduction, numerous ion channels can respond to low pH. In addition, function of ion channels and their potential as therapeutics has been implicated in numerous cancers (e.g., [72–77]). Such channels may act in concert with psGPCRs to modulate functional activities in the TME. Moreover, interactions may involve macromolecular complexes between psGPCR (e.g., GPR68 and GPR132 [37]) or with other proteins (e.g., troponin [78]). Further efforts, such as co-immunoprecipitation and microscopic approaches, including cryo-electron microscopy [79], are needed to confirm and extend such findings.

Based on the increase in expression of certain psGPCRs in tumors, agents that inhibit expression or activity of those psGPCRs may be useful in blunting low pH-mediated effects on tumor biology. Well-validated such agents are currently unavailable but might be receptor antagonists, inverse agonists or negative allosteric modulators. Negative allosteric modulators would increase the concentration of H^+ required to activate psGPCRs. Certain psGPCRs

might also be targeted with inhibitors that show ‘biased signaling’ since GPR68, for example, can link to both G_s and $G_{q/11}$ but functional activities related to cytokine production appear to be mediated by G_s /cAMP/protein kinase A, and cAMP response element-binding protein [51,52]. $G_{q/11}$ and Ca^{2+} signaling have been implicated in mechanosensing by GPR68 [80–82]. *In vivo*, such actions possibly may contribute to tumor biology via sensing stiffness in the TME associated with ‘solid stress’ or ‘intratumor fluid pressure’, as noted in the introduction. Therapeutic approaches directed at psGPCRs will likely be one component of drug combinations, for example, together with chemotherapeutics that target cancer cells and inhibitors of immunosuppression. One might also consider the use of agents to neutralize the low pH of the TME as a means to remove H^+ as an agonist for the psGPCRs [14].

Future perspective

In conclusion, a growing body of data has revealed that psGPCRs have increased expression in a wide variety of cancers and has implicated psGPCRs as mediators of effects induced by low pH in different cell types in the TME. Much remains to be learned about these receptors. New tools (e.g., tool compounds, validated antibodies and experimental models) are needed to aid in acquiring that knowledge and to help reveal the previously unrecognized potential of psGPCRs as therapeutic targets for numerous cancers.

Executive summary

Acidosis in the tumor microenvironment

- Multiple properties of tumors, in particular the Warburg effect and hypoxia from decreased perfusion, create an acidotic (low pH) tumor microenvironment (TME).
- Acidosis impacts on properties of tumors via effects on multiple cell types in the TME.
- A variety of mechanisms have been implicated as mediating effects of acidosis in the TME.
- Proton-sensing G protein-coupled receptors (psGPCRs) – GPR4, GPR65, GPR68 and GPR132 – are a GPCR family that contributes to such effects.

Properties of psGPCRs

- psGPCRs are activated by protons/ H^+ and couple to one or more heterotrimeric GTP binding (G) proteins, which in turn regulate effectors and cellular functions.
- The selective expression of psGPCRs on particular cell types induces cell-specific functions that impact on physiology and pathophysiology.

psGPCRs in cancers

- Each psGPCR has different patterns of expression in solid tumors and cancer cell lines.
- psGPCRs show increased expression in numerous tumors compared with that in the normal tissues from which the tumors are derived.
- Using markers for particular cell-types in the TME, bioinformatic approaches can identify the association/localization of psGPCRs (and other GPCRs) with those cell-types.
- High expression of particular psGPCRs are associated with altered survival of patients with certain tumors.

Financial & competing interests disclosure

The authors would like to acknowledge that the work in their laboratory on this topic has previously been supported by the research and training grants from the National Institutes of Health, a Padres Pedal the Cause Award and a David Lehr Research Award from the American Society for Pharmacology and Experimental Therapeutics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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