Tumor Growth is Stimulated by Muscarinic Receptors Agonism: Role of Autoantibodies from Breast Cancer Patients

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Abstract

In developed countries, cancer has replaced infectious diseases as a major cause of death. Currently, efforts in the immunoprevention of cancer are beginning to resemble ~~that~~ those presented by the prevention of infectious diseases by immunization a century ago. Breast cancer is the most frequent type of tumor in women and is the second leading cause of death by this illness, among them. Moreover, cancer incidence will grow during next years. Some findings in autoimmunity related to breast cancer in animal models have been important to clarify mechanisms that potentiate tumor growth. Clinical and experimental data now clearly indicate that chronic inflammation significantly contributes to cancer development. Emerging out of these studies is an appreciation that persistent humoral immune responses exacerbate recruitment and activation of innate immune cells in the neoplastic microenvironment where they regulate tissue remodeling, pro-angiogenic and pro-survival pathways that together potentiate cancer development. Generally, antigens involved in autoimmune response in breast cancer are modified self-proteins or over-expressed normal proteins that induce autoantibodies (autoAbs) formation, which exhibit tumor promoting actions. Very frequently muscarinic acetylcholine receptors (mAChR) are up-regulated in different types of tumors appearing in different species **Does this mean species of animal, or isoforms of proteins?**. mAChR have the ability to act as autoantigens for tumor bearers. This article will review recent results concerning to the ability of mAChR expressed in transformed breast cells to trigger auto-Abs formation either in experimental models or in breast cancer patients. We will also discuss the action of these antibodies as agonists of mAChR.

Keywords: Autoantibodies, muscarinic receptors, breast cancer, tumor growth.

Running title: Autoantibodies against mAChR and tumor growth

AN OVERVIEW OF TUMOR IMMUNOLOGY

~~The effect of preventing tumorigenesis by the stimulation of immune system has been anxiously searched by scientists~~ Scientists have long sought to prevent tumorigenesis by stimulation of the immune system in the same manner that microbiologists expected to immunize people against infective diseases a century ago. In spite of these efforts, many aspects of tumor immunology must be clarified to develop the most appropriate immunotherapeutic tools for cancer patients. It must be recognized that the immune system may be ineffective in preventing tumor growth. The theory of "immunosurveillance" that was proposed by Burnet in 1970~~,~~ has a limited role since although immune cells are activated in tumor bearing individuals, they are not able to promote effective anti-tumor actions [1, 2]. The "cancer immunoediting" hypothesis has enriched classical immunosurveillance theory and comprises three phases. The first phase, referred to as "elimination", encompasses our traditional understanding of cancer immunosurveillance, in which cells of the innate and adaptive immune systems recognize and destroy developing tumors, protecting host against cancer. The second phase is "equilibrium" in which ongoing tumor growth and immunosurveillance enter into a dynamic balance with one another. The third phase is "escape", where tumor variants that can avoid immune mediated destruction emerge and develop into a clinically ~~iv~~ stage neoplasm. Thus, in the long term tumor infiltration by immune cells, rather than exerting functions to protect the tumor host, produces great amounts of growth factors, oxidative molecules and pro-angiogenic mediators that can actually favor tumor development [3]. Although most tumors are antigenic, only a small fraction is spontaneously immunogenic. This is due to several escape mechanisms developed by tumor cells as alterations in processing or presentation of antigens, loss or down-regulation of class I and II major histocompatibility molecule expression, absence of B7 co-stimulatory molecules with consequential induction of anergy and/or tolerance [4]. Another important aspect of tumor escape either in animal models or in humans is the liberation of immunosuppressive cytokines such as prostaglandin E_2 (PGE_2), transforming growth factor β, interleukin-10 and vascular endothelial growth factor (VEGF), among others, that~~,~~ prevent cytotoxic T cell specific activation against tumors [3]. In addition, VEGF inhibits antigen presentation by mature dendritic cells and stimulates angiogenesis and/or lymphangiogenesis triggering tumor growth [5].

Most of the antigens identified in human tumors are self proteins, without mutations but inappropriately expressed or over-expressed. As a consequence, instead of acting as tumor rejection~~ns~~ antigens, they

induce tolerance or autoimmune responses to normal tissue [6-8]. In relation to the latter, it has been reported that the presence of antibodies against autoantigens during tumor development by B cells could be triggered by apoptotic tumor cells in situ, but they also can induce peripheral mechanisms that lead to tolerance. **This sentence is not clear—does this refer to a B cell tumor, or to autoAbs produced by B cells? And how does the source of antigen relate to mechanisms of peripheral tolerance in the context of antibodies?** The collective studies of T and B cell immunity in cancer patients, clearly demonstrate incomplete immunological tolerance against normal proteins. If genes are activated in tumors or expressed at unusually high levels, the gene products appear to gain access to the antigen presentation machinery leading to the induction of T cell immunity and the production of antibodies [9]. It has been postulated that B cell tolerance differs from T cell tolerance since the selection of autoreactive B clones is highly specific and appears when repetitive sequences are expressed in antigens, as has been observed for nuclear or neuronal proteins [10-12].

Breast cancer is the most frequent class of tumor in women and is the second leading cause of death from cancer ~~by this illness~~. Diagnosis is based mainly on clinical examination, mammography, cytology, and a few specific molecular markers, and there are no other conclusive methods for establishing diagnosis and/or prognosis for this pathology [13].

Several lines of evidence indicate that stromal cell responses in pre-malignant mammary tissue may "promote" progression to a malignant tumor. Cellular components of tumor stroma include (myo) fibroblasts, vascular cells, infiltrating leukocytes and specialized mes~~s~~enchymal support cells unique to each tissue microenvironment. A growing body of evidence has recently implicated tumor-infiltrating lymphocytes in tumor progression [14]. Hansen *et al*. [15] reported that tumor infiltrating B cells in patients with breast medullary carcinoma locally produce antibodies against autoantigens exposed in membranes as a consequence of cellular apoptosis. Moreover, several studies reviewed by Fernández Madrid [16] indicate that a plethora of autoantibodies (autoAbs) with different specificities has been found in breast cancer patients. These autoAbs directed to tumor associated antigens could be detected in the sera of patients months or years before clinical diagnosis and are potentially useful biomarkers for early detection of breast malignancies.

This article will revise **should be review?** recent results concerning the ability of muscarinic acetylcholine receptors (mAChR) expressed in transformed cells, to trigger autoAb~~s~~ formation either in

experimental models or in breast cancer patients. We will also discuss the action of these antibodies as agonists for mAChR functions.

mAChR AND BREAST CANCER

The mAChRs mediate most of the actions of the neurotransmitter acetylcholine in the central nervous system (CNS) and peripheral nervous system, as well as in the end organs of parasympathetic nerves. In mammals, five distinct mAChR subtypes (M_1–M_5) have been identified, with each receptor subtype being the product of a different gene. The mAChRs belong to the superfamily of seven-transmembrane receptors, which activate signal transduction pathways through their interactions with heterotrimeric guanine nucleotide binding regulatory proteins (G proteins). The M_1, M_3, and M_5 receptor subtypes are efficiently coupled to the pertussis toxin-insensitive Gαq/11 and Gα13 subtype G proteins, leading to, for example, activation of phospholipase (PL) C, PLA_2 and PLD. while M_2 and M_4 receptors preferentially couple to pertussis toxin-sensitive Gi and Go proteins, leading to the inhibition of adenylyl cyclase and/or to calcium channels gating [17, 18]. mAChRs regulate a large number of central functions including cognitive, behavioral, sensory, motor and autonomic processes. Changes in mAChR levels and activity have been implicated in the pathophysiology of many major diseases of the CNS including Alzheimer's disease, Parkinson's disease, depression and schizophrenia. The actions of acetylcholine on mAChR expressed in effector tissues include, for example, decreases in heart rate (mediated by M_2 receptors) and increases in smooth-muscle contractility and glandular secretion, predominantly mediated by M_3 receptor subtype.

The components of cholinergic system (mAChR, nicotinic receptors, acetylcholine, and the enzymes involved in its **does "its" refer to Ach or other components of the system?** synthesis and degradation) were gradually eventually detected in non-neuromuscular cells and tissues. Today the entire set of cholinergic molecules and their functions in cell-to-cell communication, mediated by intracellular ion dynamics, is almost entirely established. It has been demonstrated that mAChR expression and/or function are significantly increased in different types of tumor cells, such as colon, lung and erythroleukemia, in comparison to cells derived from the same normal tissues [19]. On this point, we demonstrated that mAChRs are constitutively expressed in different murine breast cancer cell lines (LM2, LM3 and LMM3) derived from BALB/c mice mouse tumors, while they are absent in normal murine mammary cells [20]. We also showed that the expression of mAChRs increased with the metastatic ability of tumors, since LMM3 cells that were obtained from a lung metastasis of M3 tumor up-regulate

mAChR expression by 50 fold in comparison with LM3 cells [21]. Moreover, we evaluated the functionality of mAChR in these cell lines and observed that the synthetic cholinergic agonist carbachol increased proliferation in all tumor cell lines. In LM3 cells, this agonist promoted proliferation via M_3 receptor activation by inositol 1,4,5-triphosphate (IP3) and nitric oxide (NO) production. Carbachol-induced LM2 cells proliferation needed both M_2 and M_1 receptor activation increasing PGE_2 liberation and arginase catabolism respectively [22]. In addition, carbachol stimulated LM2 and LM3-induced angiogenesis and tumor growth. This activation followed different patterns. In LM2 tumor, M_1 and M_2 receptors activation stimulated neovascularization via arginase II and cyclooxygenase-2 (COX-2)-derived products, while M_1 and M_3 receptors mediated carbachol-induced tumor growth by the same effector enzymes. In LM3 tumor, we observe that M_1 and M_2 receptors are involved in agonist-stimulated angiogenesis by COX and nitric oxide synthase (NOS) 1-derived products while tumor growth is stimulated by M_3 and M_2 receptors activation and COX-2-derived prostanoids [22].

Afterwards, we analyzed mAChR expression in human breast cancer tissue. Samples were surgically obtained from six patients with tumors corresponding to different histological grades. By Western blot using specific commercial anti-mAChR antibodies, in homogenates of breast tumor tissue, major expression of M_2 and M_3 receptor subtypes were was observed, and growing amounts increased levels of both proteins correlated with the malignancy and invasive capacity of those breast carcinomas. Breast samples obtained from patients with breast benign fibroadenoma (BFA) or free of illness from healthy donors lack of mAChR protein expression at all [23]. In addition, we provided direct evidence on the constitutive expression of functional mAChR in MCF-7 cells, a hormone-sensitive cell line derived from an invasive ductal human breast carcinoma that represents an early stage human breast cancer. We demonstrated for the first time, that MCF-7 cells express M_3 and M_4 receptor subtypes [24]. The activation of M_3 receptor with the cholinergic agonist carbachol increased MCF-7 cells proliferation since 4-DAMP, a selective M_3 receptor antagonist, but not tropicamide (M_4 selective antagonist), reduced carbachol action (Fig. 1A). Jiménez and Montiel [25] previously reported that in MCF-7 cells, mAChR stimulation with carbachol induced an increased of protein synthesis and cell proliferation, and these effects were prevented by PD098059, a specific inhibitor of the mitogen activated kinase kinase (MAPKK). They also demonstrated that mAChR downstream effectors PKC-zeta, PI3K, and Src family of tyrosine kinases, but not intracellular-free Ca^{2+} mobilization, are key molecules in the signal cascade leading to MAPK/ERK activation.

Taken together, these previous results reveal that the presence of mAChR in breast tumor cells and tissues, and the absence of these receptors in normal cells, could be assigning a central role to mAChR as promoters of malignant progression. In addition, the appearance of these receptors in transformed cells could be challenging the immune system to synthesize autoAbs. It has been described that mAChR can dimerize with nicotinic receptors ~~con~~forming an heterodimeric receptor that should **I would say "appears to be…"** be the target of autoAbs in patients with pemphigus vulgaris [26]. Also, M_2 and M_3 receptors can oligomerize forming dim~~m~~ers or trimers of higher molecular weight than native monomeric receptors [27]. Likewise the presence of heterodimeric mAChR with c-Myc protein that could increase its oncogenic and immunogenic ability has been described [28].

ANTIBODIES AGAINST mAChR FROM BREAST CANCER PATIENTES ACT AS RECEPTOR~~S~~ AGONISTS AND PROMOTE TUMOR PROGRESSION

The collective studies of T and B cell immunity in cancer patients clearly demonstrate incomplete immunological tolerance against normal proteins. If genes are activated in tumors or expressed at unusually high levels, the gene products appear to gain access to the antigen presentation machinery leading to the induction of T cell immunity and the production of antibodies. The antibody responses detected in cancer patients appear to be T cell dependent, since they are of IgG isotype and not of the T cell independent IgM isotype. An important question that has remained unresolved is the kinetics of immune responses against aberrantly expressed tumor antigens. Do the~~y~~ responses occur late in disease at a stage when tumor burden and antigen load is high, or do they occur early when the tumor load is small~~?~~? A study by Zhang *et al*. [29] has monitored tumor immunity in patients before and after the diagnosis of hepatocellular carcinoma. A ~~survey~~ longitudinal study of 17 patients showed that the transition from chronic hepatitis/cirrhosis to malignancy was associated with the appearance of antibody responses against protein expressed in the G2 and M phases of the cell cycle (p62). Interestingly, in one case these antibodies were detected before the clinical diagnosis of hepatocellular carcinoma. These data suggest that immune responses against these proteins can occur at early stages of the malignant process prior to clinical disease manifestation.

In breast cancer, while it is clear that acute activation of B cells may play a role in eradicating early neoplastic cells, or may participate in spontaneous regression of tumors through classical and well-studied immunoglobulin-mediated mechanisms, it has been postulated that chronic activation of B cells may paradoxically play a role in potentiating carcinoma development [8, 12]. During breast carcinogenesis,

mature B cells (including naïve cells, and activated cells) can be found in secondary lymphoid tissues as well as in tumor-associated stroma. As compared with healthy patients without evidence of cancer the sentinel lymph nodes of breast cancer patients contain proliferating and mature IgG+ B lymphocytes [30]. The percentage of B cells present in sentinel and axillary lymph nodes in these patients, reveals that their numbers correlate with disease stage when comparing stage I *vs.* stage II, and against total tumor burden [31, 32]. Similarly, B cells present in breast tumor-associated stroma may also play a role in disease progression. Several studies have reported that infiltrating B lymphocytes represent the predominant lymphoid population (in excess of T lymphocytes) in pre-malignant breast tissue, including hyperplasia and early ductal carcinoma in situ. Although the presence of chronically activated B cells and autoAbs in breast cancer patients is clear, the role of the latter in this disease progression is not clear. We had previously documented the presence of autoAbs in LM3 murine tumor bearers. LM3 is a malignant cell line derived from a murine mammary adenocarcinoma that spontaneously over-expressed mAChR. We described that IgG purified from early and late tumor bearers displaced tritiated quinuclidinyl benzilate binding to LM3 tumor cells, confirming the antibody~~y~~ies' interactions with mAChR, while IgG from normal mice did not modify the antagonist binding to receptors [33]. In addition, Abs detected a protein of 70 kDa on immunoblots ~~on~~ of murine tumor cell~~s~~ lysates and on heart homogenates; ~~that~~ a similar band was also recognized by a specific anti-M_2 receptor monoclonal antibody. ~~and~~ The autoAbs also **IS THAT CORRECT?** gave a positive immunofluorescence staining on LM3 cells membrane [34]. We also observed that IgG purified from early tumor bearers stimulated LM3 cells proliferation in a more effective manner than the muscarinic agonist carbachol. This effect was also observed when the IgG molecule was cleaved from its Fc fragment by pepsin digestion (unpublished data). The specificity of IgG action on mAChR was confirmed to prevent proliferative effects with the muscarinic antagonist atropine [33]. **THIS NEEDS CLARIFICATION—DOES ATROPINE REDUCE PROLIFERATION OF LM3 CELLS, AND THE AutoAbs BLOCK THIS EFFECT?**

Recently, we demonstrated the presence of autoAbs in the IgG fraction obtained from blood samples of breast cancer patients in stage I (T1N0Mx: tumor size <2 cm, without axillary node metastases) that exhibit the ability to activate mAChR expressed in MCF-7 cells, promoting cells proliferation, while IgG from BFA bearers or from healthy donors ~~patients free of illness~~ were ~~enable~~ unable to produce this action (Fig. 1B and C) [35]. The ~~action~~ proliferative stimulus of IgG from patients with T1N0Mx stage

breast tumors (T1N0Mx-IgG)on MCF-7 cells was reverted by 4-DAMP and tropicamide, revealing the participation of M_3 and M_4 receptors in this effect (Fig. 1B).

The presence of circulating antibodies AGAINST TUMOR AGs? in breast cancer patient-s' sera has been generally linked to a negative prognosis in this illness. By immunocytochemistry and Western blot it was demonstrated that Fab fragments of IgG from breast cancer patients recognize different mammary proteins in MCF-7 and MDA-MB-231 cells that were not recognized by the same fragment derived from normal IgG. Fab fragments from serum IgG from patients with breast cancer also stimulate tumor cell proliferation *via* ERK2 [36].

It is well known that M_3 receptor activation leads to Gq/11 coupling and to IP3 formation and calcium mobilization via PLC. Regarding the latter, we observed that the proliferative action exerted by carbachol on MCF-7 cells was reverted when cells were treated previously with the PLC inhibitor 2-nitro-4-carboxyphenyl N,N-diphenylcarbamate (NCDC), N^Gmonomethyl-L-arginine (L-NMMA), a non selective inhibitor of NOS) or H-7 (inhibitor of PKC) (Fig. 2A). In addition we confirmed that MCF-7 cells express PLCβ2 enzyme (Fig 2B). PLC enzymatic activity was measured by determining IP1 levels, the final product derived from IP3, with a commercial ELISA kit in MCF-7 cells treated with T1N0Mx-IgG or carbachol, to verify this enzyme participation in their NOT CLEAR WHICH ARE "THEIR" EFFECTS AT THIS POINT effects. As it is shown in Figure 3A either T1N0Mx-IgG or carbachol increased IP1 formation by more than 100% in comparison to control (untreated tumor cells) (Fig. 3A). In addition, it has been demonstrated that PLC is involved in other steps of tumorigenesis. You et al. [37] reported that PLC is involved in tumor cells migration by analyzing different human breast cancer cell lines with distinct metastatic potential. Although we described the expression of PLCβ2 in MCF-7 cells, we used a nonselective PLC inhibitor to test IP1 formation induced by carbachol or IgG, so other isoforms of this enzyme may be involved. Precisely, Alvarez-Breckenridge et al. [38] reported the expression of PLCγ in MCF-7 cells and pointed to the involvement of its products in tumor progression.

The presence of calcium-dependent isoforms NOS1 and NOS3 was documented by us in MCF-7 cells [35]. NO is considered an important mediator in carcinogenesis and depending on its levels it can exert opposite actions. NO liberated by calcium-dependent isoforms NOS1 and NOS3 promotes tumorigenesis, while NOS2-derived NO generally induces antitumor actions, cell cycle arrest, and apoptosis [39]. When NOS activity was quantified by determining nitrite (NO_2^-) production by Griess reaction, we observed that both T1N0Mx-IgG and carbachol increased NO formation. This effect was not

modified by preincubating cells with aminoguanidine, a NOS2 selective inhibitor, confirming the absence of this isoform in MCF-7 cells. It is important to note that T1N0Mx-IgG was more potent to stimulate NOS activity (7 fold) than carbachol (2 fold ~~increment~~ increase in NO_2^- formation) (Fig. 3B). The latter could be due to different mechanisms of action; ~~that~~ these could include different coupling to effectors which trigger this signal transduction pathway, and/or the dimerization/oligomerization of receptors with stabilization and permanent activation, promoting tumor cell~~s~~ migration. Schulze W. et al. [40] have reviewed the binding of autoAbs to epitopes of β_1 or M_2 receptors that belong to the GPCR family in various cardiovascular diseases. ~~In spite of this~~ While interaction is not completely understood, it has been shown that agonistic stimulation by autoAbs shifts the receptor to a dimeric state, stabilizes this conformation, and activates the signal cascade.

CONCLUSIONS

In recent years many scientists have been attempting to explain the mechanisms underlying the interactive relation between immune cells and tumor progression. Abundant evidence indicates that the immune system plays a major role in this interaction and under chronic tumor stimulation, begins to facilitate tumor growth. AutoAbs against mAChR have been described in other pathologies such as HIV infection, Chagas disease, Sjögrem Syndrome, and pemphigus vulgaris, all of them with an important autoimmune component [41-43, 26]. Humoral responses against mAChR have not been described in other tumors, but in breast cancer anti-mAChR antibodies reveal that autoimmunity is present in this illness, promoting tumor progression by producing agonistic actions that favors different steps of tumorigenesis. The presence of these antibodies at early stages of disease could predict a worse prognosis in this illness. Whether these autoAbs "appear" prior to tissue malignancy requires further investigation in order to achieve a clear predictive value for them.

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Legends of Figures

Fig. (1): Action of the maximal effective concentration of carbachol or T1N0Mx-IgG on MCF-7 cells proliferation. MCF-7 cells were treated with (A) carbachol (10^{-9} M), (B) T1N0Mx-IgG (2 mg/ml), (C) normal or benign fibroadenoma (BFA) IgG (2 mg/ml) **ABs IN FIG ARE LISTED IN MICROGRAMS/ML** in the absence or presence of 10^{-6} M: atropine (AT; ~~not~~ non-selective mAChR antagonist), 4-DAMP (M_3 receptor selective antagonist), or tropicamide (TROP; M_4 selective antagonist). Values are mean ± SEM of three experiments performed in triplicate. ###p<0.001, ##p<0.01 vs. cut-off value (represented by a dotted line). **p<0.001, *p<0.01 vs. carbachol or T1N0Mx-IgG **HOW WAS PROLIFERATION MEASURED? WHAT IS THE CUT-OFF VALUE, AND HOW IS IT DETERMINED? ALSO, GIVEN THIS PRESENTATION, IT SEEMS LIKE CARBACHOL AND THE PATIENT ABs ACTUALLY INHIBIT CELL GROWTH COMPARED TO CONTROL?**

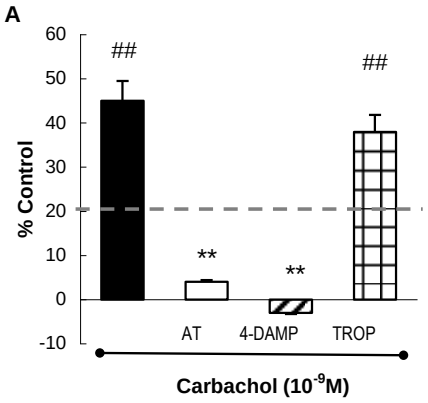
Fig. (2): Participation of phospholipase C (PLC)/ protein kinase C/ Nitric oxide synthase (NOS) in the proliferative action of carbachol on MCF-7 cells. A) Cells were treated during 1 h with carbachol (10^{-9} M) in the absence or presence of 2-nitro-4-carboxyphenyl N,N-diphenylcarbamate (NCDC) (10^{-6}M), N^Gmonomethyl-L-arginine (L-NMMA) (10^{-4}M) or H-7 (10^{-7}M). Values are mean ± SEM of 5 experiments performed in triplicate. * p<0,05; ** p<0.01 vs. control (cells without treatment) **SHOULDN'T THE CARBACHOL BAR GO ALL THE WAY ACROSS? AND AGAIN, THIS**

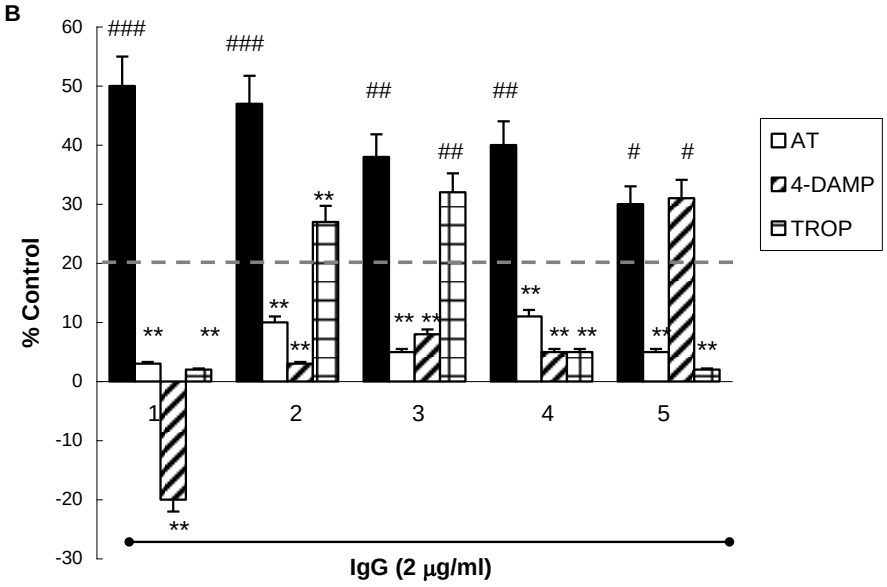
PRESENTATION STYLE MAKE IT LOOK LIKE CARBACHOL IS IHNIBITORY. B) Western blot assays to detect PLCβ2 and NOS isoforms. Molecular weights of proteins are indicated on the right. One representative experiment of three is shown. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that was used as loading control.

Fig. (3): A) Inositol monophosphate (IP1) production by MCF-7 cells. Cells were treated with carbachol (CARB) or T1N0Mx-IgG in the absence or presence of 10^{-5}M 2-nitro-4carboxyphenyl N,N-diphenylcarbamate (NCDC) or 10^{-9}M atropine (AT) or 4-DAMP. Values are mean I PRESUME THIS IS +/-? SEM of three experiments performed in duplicate. *p<0.001 vs. control (cells without treatment). B) Nitrite (NO_2^-) production by MCF-7 cells. Cells were treated with carbachol (CARB) or T1N0Mx-IgG in the absence or presence of 10^{-4}M N^Gmonomethyl-L-arginine (L-NMMA), 10^{-3}M aminoguanidine (AG) or 10^{-9}M atropine (AT) or 4-DAMP. Values are mean SEM of three experiments performed in duplicate. *p<0.001 vs. control (cells without treatment).

Fig. (4): Model of anti-muscarinic acetylcholine receptors (mAChR) antibodies action on tumor cells. CARB (carbachol) stimulates mAChR and via Gq/11 protein increases phospholipase C (PLC) activity. ~~These~~ This enzyme hydrolyzes phosphoinositides producing myoinositol 1,4,5-trisphosphate (IP3) and finally IP1. The latter increases intracellular calcium levels that in turn activate~~s~~ nitric oxide synthase (NOS) activity that produces nitric oxide (NO). The agonist and autoantibodies (auto-Abs) also increase the synthesis and liberation of vascular endothelial growth factor-A (VEGF-A). AutoAbs could resemble agonist action.

Fig.1


A
60
50
40
30
20
10
0
-10
% Control
##
##
**
**
AT
4-DAMP
TROP
Carbachol (10-9M)


B
60
50
40
30
20
10
0
-10
-20
-30
% Control
###
###
##
##
##
#
#
**
1
2
3
4
5
AT
4-DAMP
TROP
IgG (2 μg/ml)

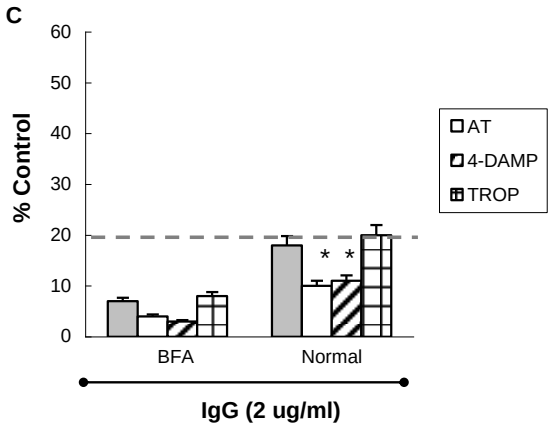

C
60
50
40
30
20
10
0
% Control
* *
BFA
Normal
AT
4-DAMP
TROP
IgG (2 ug/ml)

Fig. 2

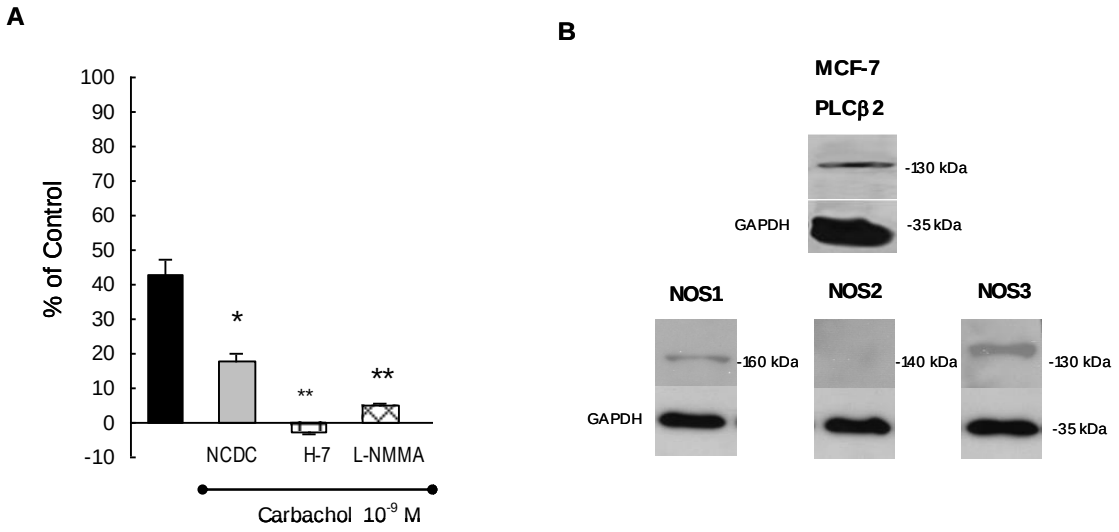

A
% of Control
100
90
80
70
60
50
40
30
20
10
0
-10
*
**
**
NCDC
H-7
L-NMMA
Carbachol 10-9 M
B
MCF-7
PLCβ2
-130 kDa
GAPDH
-35 kDa
NOS1
NOS2
NOS3
-160 kDa
-140 kDa
-130 kDa
GAPDH
-35 kDa

Fig. 3

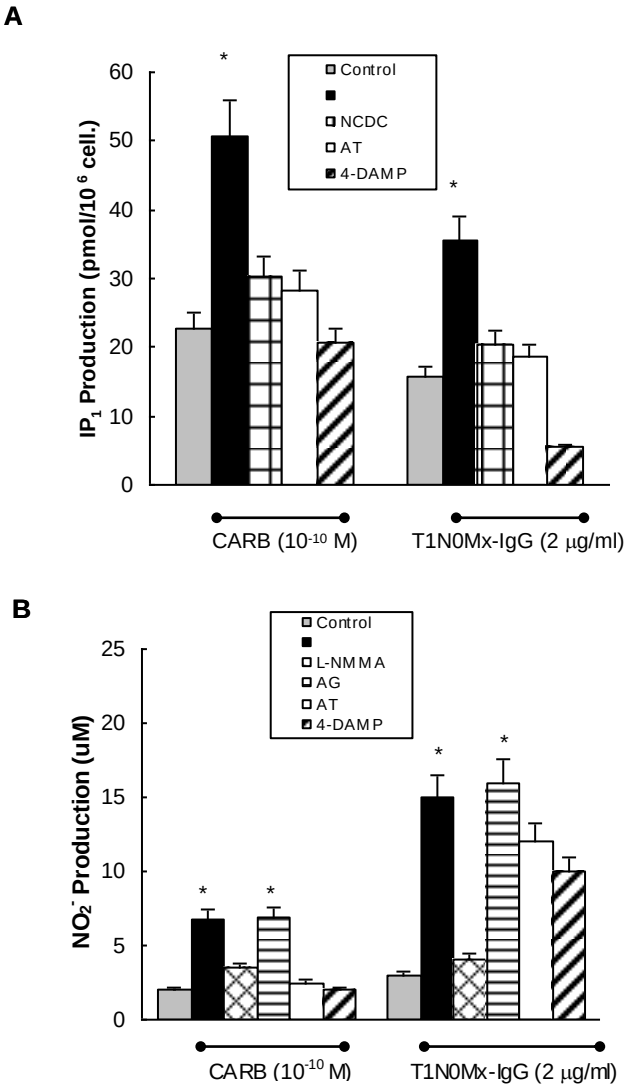
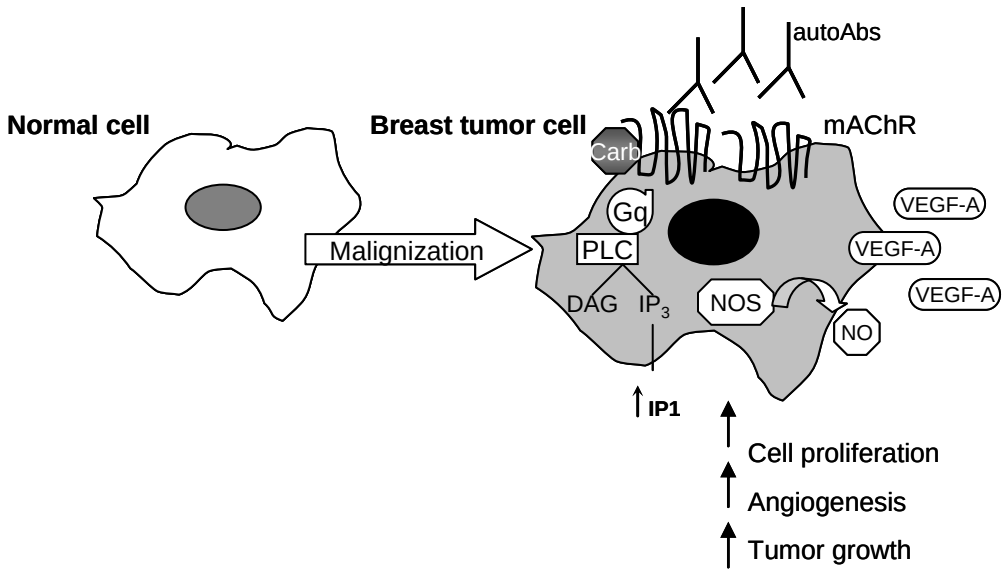

A
Control
NCDC
AT
4-DAMP
IP1 Production (pmol/10 6 cell.)
60
50
40
30
20
10
0
*
*
CARB (10-10 M)
T1N0Mx-IgG (2 μg/ml)
B
Control
L-NMMA
AG
AT
4-DAMP
NO2- Production (uM)
25
20
15
10
5
0
*
*
*
*
CARB (10-10 M)
T1N0Mx-IgG (2 μg/ml)

Fig. 4


autoAbs
Normal cell
Breast tumor cell
mAChR
Carb
Gq
PLC
Malignization
VEGF-A
VEGF-A
VEGF-A
DAG
IP3
NOS
NO
IP1
Cell proliferation
Angiogenesis
Tumor growth