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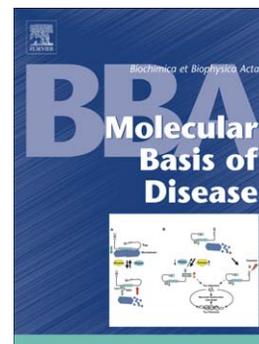
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Proliferative actions of muscarinic receptors expressed in macrophages derived from normal and tumor bearing mice.

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cyclooxygenase; protein kinase C

Abstract

Macrophages (Mps) are essential cellular components of the innate immune system. They are released from the bone marrow as immature monocytes and after circulating in the blood stream, migrate into tissues to undergo final differentiation into resident Mps. In general terms Mps behavior in breast tumors, was described as being either for or against tumor growth. Under certain well defined circumstances Mps are able to kill cells in two ways: direct tumor cytotoxicity or antibody dependent cytotoxicity. We had previously demonstrated that peritoneal Mps from LMM3 mammary tumor bearing mice (TMps) enhanced in vivo LMM3 induced angiogenesis, promoting tumor growth while Mps from normal BALB/c mice (NMps) did not. In this work, we demonstrate that Mps, expressing functional muscarinic acetylcholine receptors, are able to proliferate in vitro in response to the muscarinic agonist carbachol. These peritoneal cells use two distinct metabolic pathways: TMps are primed by tumor presence and they proliferate mainly by activating arginase pathway and by producing high levels of prostaglandin E₂ via M₁-M₃ receptors activation. In NMps, carbachol stimulates M₂ receptors function, triggering protein kinase C activity and induces moderate prostaglandin E₂ liberation via M₁ receptor.

1. Introduction

Macrophages (Mps) are essential cellular components of the innate immune system. They are released from the bone marrow as immature monocytes and after circulating in the blood stream, migrate into tissues to undergo final differentiation into resident Mps. These cells can be found in almost all tissues of the body and depending on the local microenvironment, acquire specialized phenotypic characteristics. Mps perform a multitude of functions essential for tissue remodelling, inflammation and immunity, including phagocytosis, cytotoxicity and secretion of a wide array of cytokines, growth factors, lysozymes, proteases, complement components, coagulation factors and prostaglandins [1]. The first description of the presence of leukocytes in human tumors was done by Virchow in 1864 and it was thought to reflect the onset of cancer at sites of previous chronic inflammation. It has now been established that the majority of malignant tumors contain numerous Mps as a major component of the host leukocytic infiltrate [2]. Although most of these Mps are derived from peripheral blood monocytes into the tumor mass from the circulation, there are evidences of local proliferation of Mps [3].

In general terms Mps behaviour in breast tumors was described as being either for or against tumor growth. Because multiple pathways are activated, it is likely that a complex mix of effects would be occurring. Under certain well defined circumstances, Mps are capable of killing cells in two ways: direct tumor cytotoxicity or antibody dependent cytotoxicity. We had previously demonstrated that peritoneal Mps from LMM3 mammary tumor bearing mice (TMps) enhance in

vivo LMM3 induced angiogenesis promoting tumor growth while Mps from normal BALB/c mice (NMps) did not [4]. In addition, we demonstrated that TMps from LMM3 tumor bearers themselves induce a potent angiogenic response that is augmented by carbachol. Muscarinic acetylcholine receptors (mAChR) activation triggers arginine metabolism, prostaglandin E₂ (PGE₂) synthesis and vascular endothelial growth factor production, promoting neovascularization [5]. Here we evaluate the participation of different mAChR subtypes in NMps and TMps proliferation, as well as the activation of muscarinic effector enzymes: protein kinase C (PKC), arginase and cyclooxygenase (COX), involved in carbachol proliferative action.

2. Materials and Methods

2.1. Animals and tumor cell line

Female BALB/c mice, 12 weeks old, from our Animal Division were used. Animal care was provided in accordance with the Guide for Care and Use of Laboratory Animals (NIH Publication, 1986). The tumor cell line LMM3 was previously selected from a spontaneous syngeneic mammary adenocarcinoma MM3 [6]. Cell suspensions with more than 90% viability (assessed by Trypan blue) were used. Tumor-bearing mice were obtained by subcutaneous inoculation of 4×10^5 LMM3 cells into the flank of BALB/c female mice.

2.2. Purification of peritoneal macrophages

Peritoneal cells from normal and tumor bearing mice (7 days post inoculation, with similar palpable tumors) were obtained by washing the peritoneal cavity with 5 ml D-MEM supplemented with 10% fetal calf serum (FCS). The adherent cells from normal and tumor bearers were purified by adhesion to plastic for 2 hours. After

being washed twice with PBS, adherent cells were scrapped and resuspended in culture medium. Only cell suspensions with more than 95% viability were used.

2.3. Proliferation assay

NMps and TMps (10^4 cell/well) were seeded by triplicate in 96 well plates in 200 μ l D-MEM without FCS. Cells were stimulated during 1 hour with carbachol in the absence or presence of 1 μ M atropine, 1 μ M pirenzepine, 1 μ M methoctramine or 1 μ M 4-diphenylacetoxy-N-methylpiperidine (4-DAMP), 1 μ M 2-nitro-4-carboxyphenyl-N,N-diphenyl carbamate (NCDC), 100 μ M N^G-monomethyl-L-arginine (L-NMMA), 100 nM H-7, 100 μ M N^ω-hydroxy-L-arginine (NOHA), 50 mM valine, 1 μ M indomethacin or 100 μ M NS-398. As a positive control we included the action of lipopolysaccharide (LPS) (0,5 mg/ml) from E. Coli plus interferon gamma (IFN γ) (100 U/ml) added during 18 hours. Then, medium was replaced by fresh D-MEM medium containing 10 μ l of [³H]dT tritiated thymidine diluted 1:25 (sp.Act. 5.0 Ci/mmol, NEN, Life Science Products). Cells were cultured for 48 hours at 37°C in a humidified 5% CO₂ air atmosphere. Then supernatants were discarded and cells were washed twice with PBS. Cells were lysed in 200 μ l NaOH 0.2 M and the content of each well was added to 1 ml of biodegradable scintillation cocktail (Hisafe, Wallac) and counted with about 50% efficiency. Results were expressed as [³H]-Thymidine uptake in counts per minute.

2.4. Detection of muscarinic acetylcholine receptor subtypes by Western blot

Purified Mps (10^6 cells) were lysed with 0.5 ml 0.5% NP-40, 10 mM Tris-HCl, pH 7.4 and 5 mM MgCl₂, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 0.1 mM orthovanadate and the following protease inhibitors: 10 μ g/ml aprotinin, 10

$\mu\text{g/ml}$ leupeptin, 5 mM PMSF, 50 $\mu\text{g/ml}$ soybean trypsin inhibitor. Lysates were sonicated during 30 sec and centrifuged at 3,000 rpm for 10 min at 4°C. Supernatants were centrifuged at 10,000 rpm for 20 min at 4°C. Resulting supernatants were stored at -80°C. Protein concentration was determined by the method of Lowry [7].

Samples were subjected to 7.5 % SDS-PAGE minigel electrophoresis seeding 30 μg protein in each lane. Standards of known molecular weight were also seeded. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad) and washed with distilled water. The nitrocellulose strips were blocked in buffer 20 mM Tris-HCl, 500 mM NaCl, 0,05% Tween 20 (TBST) with 5% skimmed milk, for 1 hour at 20°C to 25°C and subsequently incubated overnight with goat anti-M₁, anti-M₂ or anti-M₃ polyclonal antibodies or rabbit anti-M₄ or anti-M₅ polyclonal antibodies (Santa Cruz Biotechnology) diluted 1:100 in TBST. After several rinses with TBST, strips were incubated with the second antibody (goat anti mouse or rabbit anti mouse IgG conjugated with alkaline phosphatase and diluted 1:4000 in TBST) at 37°C during 1 hour. Bands were revealed with a mixture of nitroblue tetrazolium chloride and 5-bromo-4chloro-3-indolylphosphate-p-toluidine salt (NBT/BCIP). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as loading control. Quantification of the bands was performed with a computerized densitometer connected to an image analyzer (Bio-Rad GS700) and is expressed in optical density units per mm^2 [8].

2.5. Protein kinase C assay

Mps were incubated alone or in the presence of carbachol (100 nM) for 10 min in the absence or presence 100 nM H-7 to inhibit PKC activity or muscarinic antagonists: 1 μ M atropine, 1 μ M pirenzepine, 1 μ M methoctramine or 1 μ M 4-DAMP and were immediately frozen at a concentration of 10⁷ cells/sample in liquid N₂. PKC was purified from sub-cellular fractions as previously described [9]. Enzyme activity was assayed by measuring the incorporation of ³²P from [11-³²P]-ATP into histone H₁. Incubations were conducted in a final volume of 85 μ l at 30°C for 30 min. In the final concentrations, the assay mixture contained 25 mM ATP (0.4 mCi), 10 mM magnesium acetate, 5 mM β -mercaptoethanol, 50 mg histone H₁, 20 mM HEPES, pH 7.5, and, unless otherwise indicated, 0.2 mM CaCl₂ and 10 mg/ml of phosphatidylserine vesicles. The incorporation of [³²P] phosphate into histone was linear for at least 30 min. The reaction was stopped by the addition of 2 ml ice-cold 5% trichloroacetic acid with 10 mM H₃PO₄. The radioactivity retained on GF/C glass fiber filters and after filtration was determined by counting the filters in 2 ml scintillation fluid. PKC activity was determined after subtracting the ³²P incorporation in the absence of Ca²⁺ and phospholipids. Data were calculated as picomoles of phosphate incorporated into the substrate per minute and per 10⁷ cells. Enzyme activity was expressed as % of activity in membrane or cytosol fractions.

2.6. Prostaglandin E₂ assay

PGE₂ production by Mps was determined by radioimmunoassay (RIA) according to a previously described method [10]. Purified Mps (2x10⁶ cells/sample) were

incubated during 1 hour at 37°C in a Dubnoff bath with carbogen in 1 ml MEM without or with 100 nM carbachol in the absence or presence of 1 µM atropine, 1 µM methoctramine, 1 µM 4-DAMP, 1 µM indomethacin or 10 µM NS-398. After incubation cells were centrifuged for 10 min at 200 g and supernatants were frozen at -80° C until the assay was carried out. For PGE₂ RIA, 100 µl samples or standards were incubated for 30 min with 500 µl rabbit anti-PGE₂ antiserum (Sigma) at 4°C. Then 5 pg [³H]-PGE₂ (specific activity: 154 Ci/mmol; New England Nuclear) were added to each tube. All dilutions were done in 0.01 M PBS, pH 7.4, containing 0.1% bovine serum albumin and 0.1% sodium azide. After incubation, a dextran-coated charcoal suspension was added to separate the bound from the free fraction. The supernatants were removed from each tube and added to 1 ml of scintillation solution (Optiphase Hisafe 3 Wallac) to determine the amount of radioactivity present. Results are expressed in picograms per 10⁶ cells.

2.7. Detection of cyclooxygenase isoforms by Western blot

Purified Mps were washed twice in cold PBS and then resuspended in 300 µl of lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 10 µg/ml dithiothreitol, 100 µg/ml Soybean trypsin inhibitor, 1 mg/ml benzamidine). After 1 hour, lysates were centrifuged at 5,000 rpm during 10 min. Resulting supernatants were stored at -80°C. Protein concentration was determined by the method of Lowry [7].

Samples were subjected to 7.5 % SDS-PAGE minigel electrophoresis seeding 30 µg protein in each lane. Standards of known molecular weight were also seeded. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-

Rad) at 4°C during 18 hours. Membranes were then washed with distilled water and incubated with blocking solution (5 % skimmed milk in TBST) for 1 hour at 20°C to 25°C. Membranes were incubated with rabbit polyclonal anti COX-1 or COX-2 antibodies (Cayman Chemical) in TBS for 90 min at room temperature. Then secondary anti-rabbit IgG antibody conjugated with alkaline phosphatase was added for 1 hour at 37°C. Proteins were visualized with NBT/BCIP and quantified by a densitometric analysis [11]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as loading control.

2.8. Arginase activity assay

Arginase activity was determined in cell lysates according to previously described methods [12]. Briefly, 10^5 cells treated or not with 100 nM carbachol in the absence or presence of 100 μ M NOHA, 1 μ M atropine, 1 μ M pirenzepine, 1 μ M methoctramine or 1 μ M 4-DAMP. After being washed, cells were lysed with 0.5 ml 0.1% Triton X-100, 25 mM Tris-HCl pH 7.4, containing 5 mM $MnCl_2$. The enzyme was then activated by heating at 56°C for 10 min. Arginine hydrolysis was performed by incubating 25 μ l of the activated lysate with 25 μ l of 0.5 M arginine, pH 9.7, at 37°C for 60 min. The reaction was stopped in acid medium. Urea concentration was measured at 540 nm with a microplate reader. Results are expressed as micromoles of urea per hour and per 10^6 cells.

2.9. Detection of arginase isoforms by Western blot

Mps were rinsed twice with ice-cold PBS and then scrapped into 300 μ l lysis buffer: 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 0.1 mM PMSF. Lysis was completed by sonication. Samples (25 μ g)

were subjected to 10 % SDS-PAGE as it was stated in previous methods [13]. Nitrocellulose membranes were incubated overnight with a monoclonal anti-mouse arginase I antibody (BD Transduction Laboratories) or with a rabbit anti-arginase II antibody (gently gifted by Dr. Masataka Mori). The secondary antibody anti-mouse or anti-rabbit IgG conjugated with alkaline phosphatase was added for 1 hour at 37°C. Proteins were visualized using a mixture of NBT/BCIP and quantified by densitometric analysis of the bands. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as loading control.

2.10. Determination of cAMP Levels

Mps (10^6 cells) were incubated in 1 ml of D-MEM in the presence of 1 mM isobutyl methyl xantine during 30 min with or without 1 μ M isoproterenol to induce cAMP formation. Isoproterenol-stimulated Mps were treated with 100 nM carbachol in the absence or presence of 1 μ M methoctramine or 1 μ M tropicamide. Then samples were centrifuged at 1,000 r.p.m and liberation of cAMP was assayed in supernatants. Samples (100 μ l) were treated with triethylamine:acetic anhydride (2:1) and after 10 min, 100 μ l of [125 I]-cAMP (Specific Act: 600 Ci/mmol) and 100 μ l of a rabbit polyclonal anti cAMP antibody (gently gifted by Dr. Parlow NIDDK's National Hormone and Pituitary Program, USA) were added and mixed with shaking at 4°C overnight. Then 50 μ l of 2% bovine seroalbumine in distilled H₂O and 2 ml of ethanol (96%) were added to each tube and mixed. After 10 min samples were centrifuged at 3,000 r.p.m and supernatants were discarded. Radioactivity in pellets was detected in a gamma counter. cAMP concentration in samples is expressed in femtomoles per 10^6 cells per ml [14].

2.11. Drugs

All drugs were purchased from Sigma-Aldrich unless otherwise stated. Solutions were prepared fresh daily.

2.12. Statistics

Results are given as means \pm SEM for at least three independent experiments. The statistical significance of differences between groups was analyzed by ANOVA or Kruskal-Wallis, using GraphPad Prism 4; $p < 0.05$ was considered statistically significant.

3. Results

3.1. Proliferative actions of carbachol on macrophages

We had previously documented the presence of non neuronal cholinergic receptors in Mps with the ability to control angiogenesis. As shown in Figure 1 A, treatment with carbachol stimulates NMps as well as TMps proliferation only at 100 nM concentration. In NMps, this concentration of carbachol increased [3 H]-Thymidine incorporation by 90% in relation to basal (cells without treatment). These results were confirmed by counting cell number in a Trypan blue exclusion test (basal: $(1.10 \pm 0.01)10^4$ cells/well; 100 nM carbachol: $(1.98 \pm 0.02)10^4$ cells/well; $n=4$). TMps were less sensitive to the agonist, since it stimulated by 62% [3 H]-Thymidine uptake respect to untreated cells. These results were coincident with cell counting (basal: $(1.30 \pm 0.01)10^4$ cells/well; 100 nM carbachol $(2.10 \pm 0.02)10^4$ cells/well; $n=5$). In addition we tested the action of carbachol in the presence of different muscarinic antagonists and we observed that NMps proliferation was totally reverted by 1 μ M methoctramine, a M_2 receptor blocker and partially reduced by the same concentration of the M_1 selective antagonist pirenzepine. In TMps not only

pirenzepine and methoctramine diminished carbachol stimulatory action but also the M₃ antagonist 4-DAMP (1 μM) was able to reduce muscarinic agonist stimulation. The non-selective muscarinic antagonist, atropine (1 μM) totally blunted carbachol proliferative actions on both populations of Mps confirming muscarinic receptors participation. The addition of LPS plus IFN γ as a positive control, increased [³H]-Thymidine uptake in NMps by 65% (basal: 1004,5±98,5 cpm; LPS plus IFN γ :1655,5± 154,5 cpm; p<0,05).

By Western blot and densitometric analysis of the bands, we confirmed a major expression of M₁ receptor in both populations of Mps (Fig. 1 C). As it is also shown in Figure 1 C that TMps express lower amounts of M₁ and M₃ receptor proteins than NMps. On the contrary, TMps express higher amounts of M₂ receptor subtype in comparison with NMps. In NMps as well as in TMps M₄ receptors are almost undetectable while M₅ receptor protein was absent.

3.2. Protein kinase C and cyclooxygenase are involved in the proliferative action of carbachol on normal macrophages

Since carbachol-stimulated NMps proliferation was mediated by M₁ receptors activation, we investigated whether phospholipase C-PKC-nitric oxide synthase signaling pathway, classically coupled to those receptors, was involved in that effect. It can be seen in Figure 2 A that the stimulatory action of carbachol on NMps was only reverted by preincubating cells with 100 nM H-7. The phospholipase C inhibitor, NCDC or L-NMMA a non-selective nitric oxide synthase inhibitor, did not reduce carbachol action on NMps. We evidenced that PKC activation was induced by carbachol because 100% of the active enzyme was

totally located to the cell membrane after treating NMps during 10 min with 100 nM carbachol (Fig.2B). This effect was completely reverted by preincubating cells with 100 nM H-7. We also observed that the addition of 1 μ M methoctramine partially reduced carbachol action on PKC translocation in NMps (cytosol: $52\pm 4\%$, membrane: $48\pm 5\%$). The addition of 1 μ M pirenzepine or 4-DAMP did not modify carbachol action on PKC translocation.

In general, M_2 receptors are linked via G_i protein to adenyl cyclase, reducing cAMP accumulation. We measured this nucleotide levels in NMps stimulated by 1 μ M isoproterenol. As shown in Table 1 the addition of 100 nM carbachol didn't modify the increment in cAMP liberation induced by isoproterenol (carbachol+isoproterenol: 825.4 ± 35.1 , n=3; isoproterenol: 966.6 ± 28.1 , n=3).

COX-derived prostaglandins are important mediators in different biological cell activities such as: immune response, inhibition of cell apoptosis and proliferation. We tested the participation of COX-derived PGE_2 in carbachol-induced proliferation on NMps. In Figure 2C, is shown that in spite COX-1 expression is higher than COX-2 in NMps, the preincubation of cells with 1 μ M indomethacin or 10 μ M NS-398 significantly reduced carbachol action on NMps proliferation. The addition of carbachol significantly increased PGE_2 liberation by these cells in comparison with basal (untreated cells) (Basal: 424.5 ± 48.3 (n=4); carbachol: 746.4 ± 65.8 ; n=4) (Fig.2 D). When NMps were previously treated with 1 μ M atropine, 1 μ M pirenzepine or 1 μ M 4-DAMP, PGE_2 liberation was reverted to basal values. On the contrary, 1 μ M methoctramine was unable to modify carbachol-stimulated PGE_2 synthesis (768.3 ± 65.4 ; n=3). We also observed that preincubation of cells with 100

nM H-7 potentiates carbachol stimulatory action on PGE₂ liberation by TMps (920.4±89.0) (n=3).

3.3. Arginase and cyclooxygenase are involved in the proliferative action of carbachol on macrophages derived from mammary tumor bearing mice

Arginase is the enzyme that converts L-arginine to urea and ornithine; the latter is needed for polyamine synthesis and cell proliferation. As shown in Figure 3 A, both isoforms of the enzyme are expressed in TMps and the proliferative action of 100 nM carbachol on TMps is significantly reduced by the non selective arginase inhibitor NOHA as well as by valine, an amino acid that interferes arginase pathway, while this effect was not observed in NMps. We confirmed that arginase is linked to carbachol-stimulated TMps proliferation since a 3.5 fold increase in urea formation is observed in comparison with untreated TMps (Fig. 3B). Muscarinic agonist stimulation on urea formation was totally reduced not only by 1 μM atropine but also by 1 μM pirenzepine. Methoctramine partially reduced carbachol-stimulated urea synthesis while 4-DAMP was ineffective.

In addition we observed that COX isoforms are up-regulated in TMps in comparison with NMps (Fig 3C) and treatment of TMps with 1 μM indomethacin or 10 μM NS-398 significantly reduced carbachol-stimulated [³H]-thymidine incorporation by TMps. When we measured PGE₂ formation in TMps we observed that 100 nM carbachol dramatically increased its liberation by 100 fold in comparison with basal (Fig. 3D). Only the preincubation of cells with 1 μM atropine or 1 μM 4-DAMP was able to reduce carbachol action on PGE₂ liberation, indicating that M₃ receptor subtype is mediating this effect.

Since we observed that M₂ receptor protein was up-regulated in TMps, we evaluated the function of this receptor subtype by measuring carbachol action on isoproterenol-stimulated cAMP levels. It is shown in Table 1 that isoproterenol-stimulated cAMP synthesis was reduced to basal values by the addition of 100 nM carbachol (Basal: 885.3±34.6; isoproterenol (10⁻⁶M): 2060.3±14.4; CARB+isoproterenol: 921.7±13.6; n=3). This action was reverted by adding 1 μM atropine or 1 μM methoctramine previously to carbachol but not by the same dose of the M₄ receptor antagonist tropicamide.

Preincubation of TMps with NCDC, L-NMMA or H-7 did not modify carbachol action on cell proliferation (data not shown).

4. Discussion

Mps perform multiple functions that are essential in tissue remodelling, wound healing, inflammation and immunity. Recently, it has been reported the expression of mRNAs encoding the five mAChR subtypes M₁-M₅ in immune cells, including Mps, leading to modulation of their function [15]. We observe that M₁, M₂ and M₃ receptor proteins are mainly expressed in Mps derived from normal and LMM3 tumor bearing mice; but while M₁ and M₃ receptors are down regulated in TMps, M₂ subtype is up-regulated in this population of Mps. Stimulation of mAChR with 100 nM carbachol triggers thymidine uptake and Mps proliferation. This effect was also observed in different normal cell types as rat thyroid epithelial cells or scleral chondrocytes generally by activating diverse metabolic pathways [16, 17]. We had previously reported the presence of mAChR in mouse and human mammary tumor cells that also stimulate cell proliferation [18, 19]. Our results indicate that while

both populations of Mps equally incorporate [³H]-Thymidine in basal conditions, NMps are more sensitive to the agonist action at 100 nM than TMps. The latter could be due to a major expression of M₁ and M₃ proteins in NMps than in TMps. The lack of response to 1 μM carbachol exhibited by Mps was also observed by us and others in different cell types under similar experimental conditions [20, 21]. Desensitization of mAChR and lack of biological responses, produced by high doses of agonist (≥1 μM) and/or long incubation periods (≥ 1 hour) has been largely documented [21, 22].

Our results indicate that Mps purified from normal or tumor bearing mice, majorly express M₁ receptors. It has been extensively documented that these receptors are classically coupled via Gq/11 protein to phospholipase C-inositol polyphosphates hydrolysis that could in turn activate nitric oxide production by nitric oxide synthase and on the other hand, diacyl-glycerol-PKC pathway [23]. Surprisingly we observed that neither phospholipase C inhibitors nor nitric oxide synthase inhibitors were able to reduce carbachol-stimulated proliferation in NMps, indicating that PKC could be linked to another metabolic pathway and/or that could be activated itself by another mAChR subtype. The latter was confirmed by the reduction of PKC translocation from cytosol to membrane in NMps, only by methoctramine, a M₂ receptor antagonist. PKC_ε, an atypical PKC isoform, could be activated by βγ subunit from Gi protein linked to M₂ receptors but not to other receptors (M₄ or α_{2C}) [24]. This PKC isoform is fully activated by short time stimulation and lacks of diacyl-glycerol binding site. Other authors have postulated that in murine Mps, the inhibition of PKC activity increased PGE₂ synthesis by two different mechanisms:

regulation of phospholipase A₂ activity and induction of COX-2 protein [25]. Concordantly we observed that PGE₂ liberation induced by carbachol in NMps via M₁ and M₃ receptors activation is enhanced by preincubating cells with H-7 revealing a negative regulatory action of PKC on phospholipase A₂ or COX activity. The fact that H-7 blocked carbachol-induced proliferation but enhanced PGE₂ liberation by 30% could be indicating two different time-depending mechanisms, because carbachol stimulation of PGE₂ synthesis is a short term effect, probably due to an increase in PLA₂ activity; while H-7 inhibition of proliferation was detected after NMps were cultured by 48 h, thus it could be acting by inhibiting other cell signalling steps that must be studied further.

Synthesis of PGE₂ via COX was related to mAChR-G protein stimulation of phospholipase A₂ [26]. Murine peritoneal Mps expressed both COX proteins as it was stated recently by Luo C et al. [27]. COX-1 and COX-2 protein levels are higher in TMps than in NMps. Particularly the inhibition of [³H]-Thymidine uptake due to NS-398 is major than that induced by indomethacin only in TMps and the action of carbachol on PGE₂ synthesis is dramatically higher than that induced on NMps, pointing to a major role of COX-2 derived PGE₂ on TMps proliferation.

In addition, Mps are part of the innate immune system and Mps-derived PGE₂ is considered a very important soluble mediator that acts as a modulator of immune response generally as an immunosuppressant. PGE₂ production in these cells is due to M₃ receptors activation. It has been documented that these receptors mediate proliferative actions in normal and transformed cells. This effect is also due to an up-regulation of COX-2 derived PGE₂ production [28]. It must be considered that in spite M₃ receptor is expressed at lower level than M₁ and M₂

subtypes in TMps, it exerts an important role in cell PGE₂ production. Similar results were obtained in our laboratory when we characterized mAChR expression and function in rat ileal tissue. M₃ receptor is the minor fraction of mAChR expressed in the ileum, but controls tissue motility via IP₃ formation and calcium mobilization; while M₂ subtype, which is mainly expressed in rat ileum, inhibits cAMP synthesis facilitating ileal motility. [23].

On the other hand, it has been extensively studied that the activation of M₂ receptors, reduced cAMP accumulation by the coupling of α 1 subunit to adenylyl cyclase [22]. The addition of carbachol to NMps, was not able to reduce isoproterenol-stimulated cAMP levels confirming uncoupling of this signalling pathway in NMps. By the contrary M₂ receptors that are up-regulated in TMps, are linked to the inhibition in cAMP concentration produced by carbachol treatment, since it was reverted by methoctramine. The lack of effect observed with tropicamide, indicate that although M₄ receptors could be expressed at very low levels in TMps, they are not coupled to the inhibition of adenylyl cyclase.

Mps are the major component of the mononuclear leukocyte population of some solid tumors [2, 29]. Although the origin of tumor-infiltrating Mps has been discussed extensively, evidences support both recruitment from the circulating pool of monocytes and proliferation of the local Mps population induced by stimuli such as growth factors or low oxygen tension as well as soluble tumor antigens [30]. We had demonstrated that arginase I and II expression and function are up-regulated in TMps in comparison with NMps [4]. Here we confirmed that under muscarinic stimulation arginase metabolites are needed for TMps proliferation. We had

previously reported that TMps co-inoculated with LMM3 tumor cells promote tumor growth, in part by stimulating angiogenesis. This effect was also enhanced by carbachol [4, 5]. We cannot discard that urea and ornithine produced by Mps could be used by tumor cells proliferation and/or endothelial cells sprouting. We also observed that carbachol dramatically increased PGE₂ liberation in TMps via M₃ receptor activation. This effect could be due to a different coupling of the latter receptor to the effector enzyme. It must be taken into account that H-7 did not modify carbachol-TMps induced proliferative action or PGE₂ production by these cells.

Our results demonstrate that stimulation of mAChR expressed in Mps triggers proliferation by using distinct metabolic pathways depending on their previous activation. On the one hand, NMps proliferation depends on M₂-PKC phosphorylation signals that turn down the synthesis of PGE₂. On the other hand TMps that are primed by tumor presence, proliferate mainly by activating arginase pathway via M₁ and M₂ receptors and by up-regulating muscarinic-PGE₂ synthesis linked to M₃ receptor subtype. These signals could be favouring tumor growth and host immunosuppression during tumor progression.

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ACCEPTED MANUSCRIPT

Legend of Figures

Figure 1. Muscarinic receptors participation in the proliferation of macrophages (Mps). A) Normal or LMM3 mammary tumor bearer macrophages (NMps or TMps) were treated with different concentrations of carbachol (* $p < 0.01$ vs. Basal) in the absence or presence B) of muscarinic antagonists: 1 μ M atropine (AT), 1 μ M pirenzepine (PIR) or 1 μ M 4-DAMP. Then cells were pulsed with [6-³H]dT-tritiated thymidine. Results are expressed as incorporated cpm. Values are mean \pm SEM of four experiments performed in triplicate. * $p < 0.01$ # $p < 0.05$ vs. carbachol C) Western blot of muscarinic receptors expressed in NMps or in TMps and densitometric analysis of the bands expressed in optical density units per mm^2 (O.D./ mm^2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as loading control. One representative experiment of three is shown. N.D. (not detected)

Figure 2. A) Participation of protein kinase C and cyclooxygenase in the proliferation of normal macrophages (NMps). A) Cells were treated with 100 nM carbachol in the absence or presence of 1 μ M 2-nitro-4-carboxyphenyl-N,N-diphenyl carbamate (NCDC); 100 μ M M N^G-monomethyl-L-arginine (L-NMMA), 100 nM H-7 or C) cyclooxygenase (COX) inhibitors 1 μ M indomethacin (INDO) or 10 μ M NS-398. Western blot of COX-1 and COX-2 in NMps. Densitometric analysis of the bands was expressed in optical density units per mm^2 (O.D./ mm^2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein was used as loading control. One representative experiment of three is shown B) Protein kinase C enzymatic assay in membrane or cytosol fractions from NMps stimulated with

carbachol (100 nM) in the absence or presence of: 1 μ M atropine (AT), 1 μ M pirenzepine (PIR) or 1 μ M 4-DAMP; one representative experiment of three is shown D) Prostaglandin E₂ (PGE₂) determination in the culture supernatants of NMps treated with carbachol in the absence or presence of cyclooxygenase inhibitors. Values are mean \pm SEM of 4 experiments performed in duplicate. *p<0.05 vs. basal.

Figure 3. Arginase and cyclooxygenase participation in tumor bearer macrophages (TMps) proliferation. A) Cells were treated with carbachol (100 nM) in the absence or presence of A) 100 μ M N^ω-hydroxy-L-arginine (NOHA) or 50 mM valine. Western blot of arginase (A) I and II in TMps. One representative experiment of three is shown C) TMps were treated with cyclooxygenase (COX) inhibitors 1 μ M indomethacin (INDO) or 10 μ M NS-398 previous to carbachol. Western blot of COX-1 and COX-2 in TMps. Densitometric analysis of the bands was expressed in optical density units per mm² (O.D./mm²). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein was used as loading control. One representative experiment of three is shown B) Arginase activity in TMps stimulated with carbachol (100 nM) in the absence or presence of: 1 μ M atropine (AT), 1 μ M pirenzepine (PIR) or 1 μ M 4-DAMP. D) Prostaglandin E₂ (PGE₂) determination in culture supernatants of TMps treated with carbachol in the absence or presence of COX inhibitors and muscarinic antagonists. Values are mean \pm SEM of 4 experiments performed in duplicate. *p<0.05 vs. basal.

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Table 1

cAMP production by macrophages

cAMP (fmol/10⁶ cells.ml)	NMps	TMps
Basal	172.2 ± 3.4	885.3±34.6
Isoproterenol	966.6± 28.1*	2060.3 ±14.4#
CARB + Isoproterenol	825.4±35.1*	921.7±13.6
AT + CARB + Isoproterenol	1083.9±14.5*	2174.3±19.5#
MET+ CARB + Isoproterenol	1143.5±2.9*	1800.4±12.8#
TROP + CARB +Isoproterenol	998.8±7.8*	978.7±11.7

cAMP was determined in macrophages (10⁶ cells/ml) from normal (NMps) or tumor bearing mice (TMps) incubated during 30 min in the absence or presence of 1 μM atropine (AT), 1 μM methoctramine (MET) or 1 μM tropicamide (TROP). Isoproterenol (1 μM) and 100 nM carbachol (CARB) were added for 15 and 25 min respectively. cAMP concentration in samples was expressed in femtomoles per 10⁶ cells and per ml. Values are mean ± SEM of 3 experiments performed in duplicate.*p<0.05 vs. NMps without treatment; #p<0.05 vs. TMps without treatment.

Figure 1

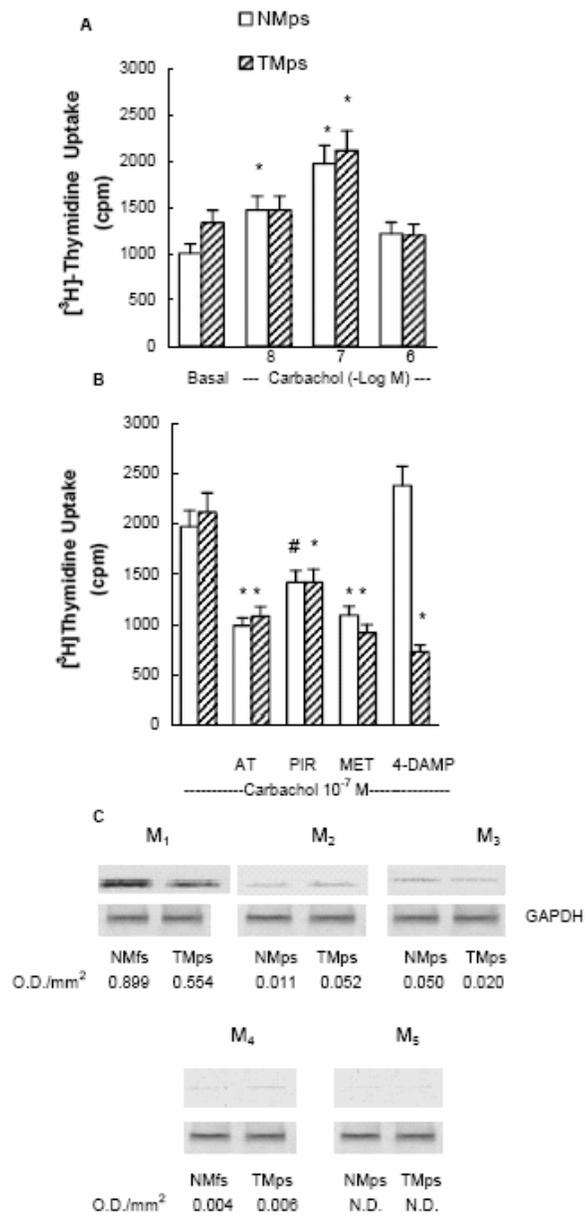


Figure 1

Figure 2

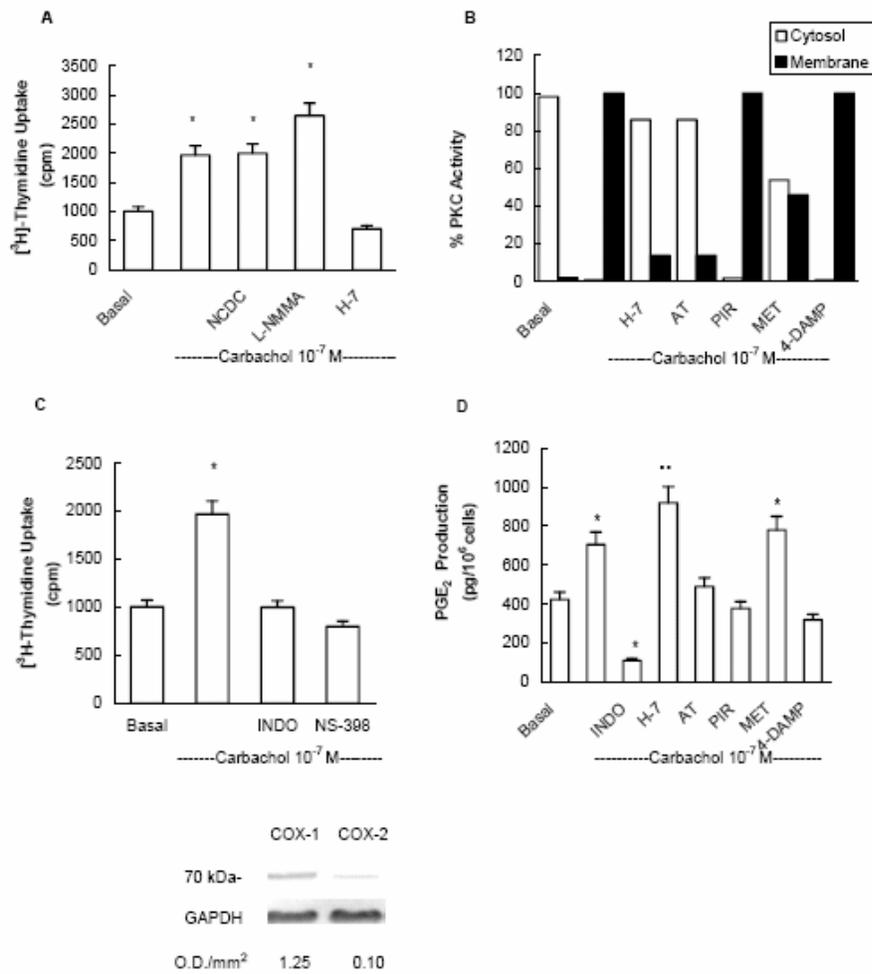


Figure 3

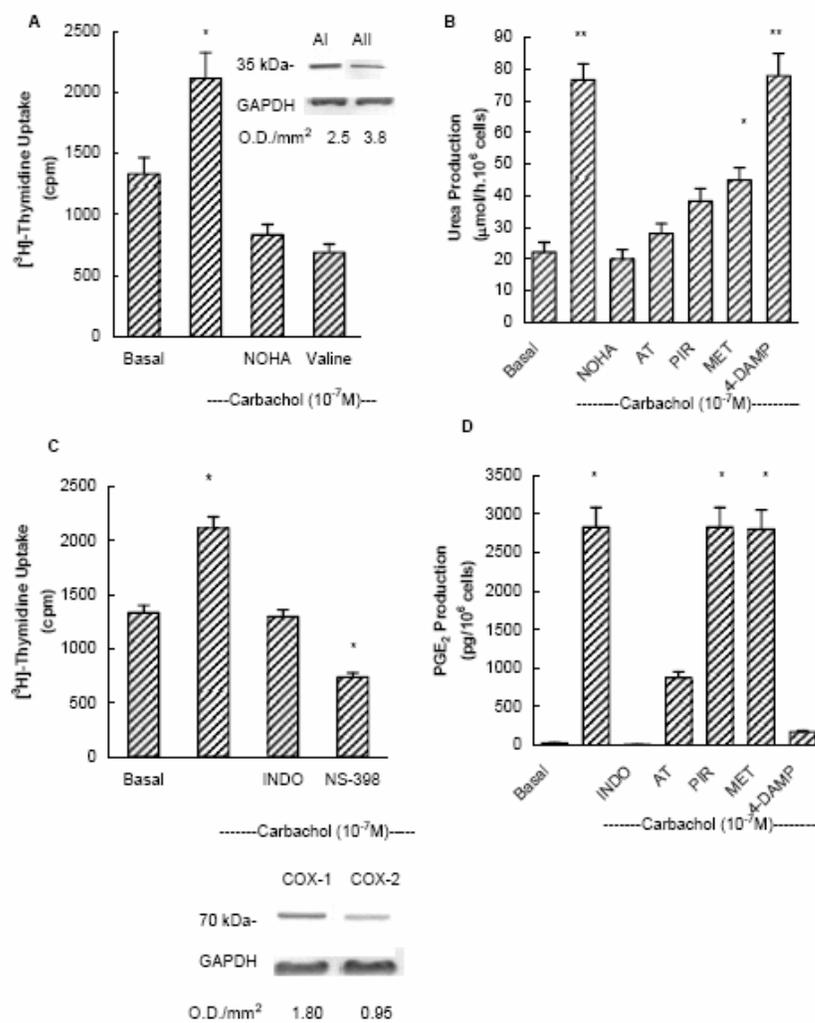


Figure 3