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Atorvastatin modulates anti-proliferative and pro-proliferative signals in Her2/neu-positive mammary cancer

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ABSTRACT

The widely used anticholesterolemic drugs statins decrease the synthesis of cholesterol and the isoprenylation and activity of small G-proteins such as Ras and Rho, the effectors of which are often critical in cell proliferation. Thanks to this property, it has been hypothesized that statins may have anti-tumor activities. We investigated this issue in BALB-neuT mice, which developed Her2/neu-positive mammary cancers with 100% penetrance, and in TUBO cells, a cell line established from these tumors.

Contrary to the mammary glands of BALB/c mice, the tumor tissue from BALB-neuT animals had constitutively activated Ras and ERK1/2. These were reduced by the oral administration of atorvastatin, but the statin did not prevent tumor growth in mice nor reduce the proliferation of TUBO cells, although it lowered the activity of mevalonate pathway and Ras/ERK1/2 signaling. By decreasing the mevalonate pathway-derived metabolite geranylgeranyl pyrophosphate and the RhoA/RhoA kinase signaling, atorvastatin activated NF-κB, that sustained cell proliferation. Unexpectedly Her2-positive cells were much more sensitive to the inhibition of RhoA-dependent pathways than to the suppression of Ras-dependent pathways elicited by atorvastatin. Only the simultaneous inhibition of RhoA/RhoA-kinase/NF-κB and Ras/ERK1/2 signaling allowed the statin to decrease tumor cell proliferation.

Our study demonstrates that Her2-positive mammary cancers have redundant signals to sustain their proliferation and shows that statins simultaneously reduce the pro-proliferative Ras/ERK1/2 axis and activate the pro-proliferative RhoA/RhoA-kinase/NF-κB axis. The latter event dissipates the antitumor efficacy that may arise from the former one. Only the association of statins and NF-κB-targeted therapies efficiently decreased proliferation of tumor cells.

1. Introduction

Statins, which inhibit the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR; EC 1.1.1.34) enzyme, are the most effective drugs in the reduction of intracellular synthesis of cholesterol [1]. They also decrease the synthesis of isoprenoids, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), impairing the isoprenylation and activity of small G-proteins, like Ras and Rho. When isoprenylated, monomeric G-proteins bind and hydrolyse GTP, activating downstream effectors which in turn may modulate cell proliferation, cytoskeleton remodeling, motility and angiogenesis [2]. High expression of the enzymes of the mevalonate pathway has been found in human breast cancer samples and has been correlated with the Ras-driven oncogenic transformation [3].

By preventing the activation of Ras/Rho proteins, it has been proposed that statins may exert pleiotropic benefits on the cardiovascular apparatus, as well as on diseases of the colon, lung, kidney, bone and central nervous system; for the same reasons they have also been proposed as potential adjuvant drugs in anticancer therapy [4]. In different experimental models statins have reduced tumor cell proliferation [5–7] and enhanced the cytotoxic efficacy of conventional chemotherapeutic drugs [8,9]. Few and conflicting data exist about the efficacy of statins in preventing tumor onset: atorvastatin has not decreased the incidence of methylnitrosourea-induced breast cancers in rats [10]; in humans some epidemiologic evidence has hypothesized a positive correlation between the long term use of statins and the

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onset of breast cancers [11], some others have shown little or no reduction of the incidence of breast malignancies in patients taking HMGCoAR inhibitors [12]. Her2-positive breast tumors represent 20–30% of total breast cancers and are often associated with poor prognosis and chemo-radio-resistance [8]. Following the constitutive activation of the Her2 downstream transducers, which include the Ras/Raf/mitogen-activated kinase (MAPKs) pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, these tumors are characterized by a high proliferation rate and resistance to apoptosis [8,13]. An active mevalonate pathway seems critical for the growth of Her2-positive tumors: indeed the amounts of plasma membrane cholesterol affect Her2 conformation and activity and the levels of isoprenoid dolichol regulate the Her2 glycosylation. Furthermore, an adequate supply of FPP is mandatory for the activity of Ras protein and for the subsequent activation of Ras-downstream effectors [8]. The association of statins and specific Her2-targeting therapies has achieved a good anti-proliferative effect [8,9]. However, it has also been reported that statins increase the activity of the MAPK members extracellular signal-regulated kinase 1/2 (ERK1/2) [14], which induces pre-invasive lesions in the mammary epithelium [15].

BALB/c mice transgenic for the transforming rat *Her2/neu* oncogene under the transcriptional control of the mouse mammary tumor virus promoter (BALB-neuT mice), develop breast cancer with 100% penetrance [16]. Starting from atypical hyperplasia (at week 3), mammary glands display at 8 weeks multiple in situ carcinomas, which become invasive tumors between weeks 17 and 22 [17].

In this study we investigated the effects of atorvastatin, at a dose equivalent roughly to 40 mg/day in human treatment, that falls in the range adopted for anticholesterolemic therapy (10–80 mg/day) [18], on the growth of breast tumors in BALB-neuT mice and on the pathways that are controlled by the drug in the transformed mammary cells.

2. Materials and methods

2.1. Chemicals

Fetal bovine serum (FBS), penicillin–streptomycin (PS) and RPMI 1640 were supplied by Sigma Chemical Co (St. Louis, MO), plastic ware for cell culture was from Falcon (BD Biosciences Discovery Labware, Bedford, MA). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA), the protein content of cell monolayers and cell lysates was assessed with the bicinchoninic acid kit from Sigma Chemical Co. Atorvastatin was purchased from Sequoia Research Products (Pangbourn, UK). FTI-I and GGTI-286 were acquired from Calbiochem (San Diego, CA). When not otherwise specified, all the other reagents were purchased from Sigma Chemical Co.

2.2. Cells

TUBO cells are a cloned line generated from a BALB-neuT mouse mammary gland carcinoma and express large amounts of Her2/neu protein [19]. Cells were cultured in DMEM with 10% v/v FBS, 1% v/v PS in a humidified atmosphere at 37 °C and 5% CO_2.

2.3. Mice and in vivo treatments

Six-week-old female BALB/c mice were supplied by Charles River Italia SpA (Calco, Italy). Virgin female BALB-neuT mice transgenic for the transforming activated rat *Her2/neu* oncogene under the transcriptional control of the mouse mammary tumor virus promoter were bred under specific pathogen-free conditions by Charles River. Mice were treated according to the guide lines established in Guide for the Care and Use of Laboratory Animals and in Principles of Laboratory Animal care (directive 86/609/EEC). All the experiments were approved by the institutional Ethical Committee.

Mice were randomly assigned to control and treatment groups and all groups were treated concurrently. Atorvastatin was brought into suspension in PBS at 12 μg/day (roughly equivalent to 40 mg/day in human treatment), and a 0.2 ml volume was administered via oral gavage. Atorvastatin administration occurred once daily, 5 days per week for 8 weeks, starting from week 10 until week 17. Control mice were treated with PBS only.

BALB/c mice were challenged s.c. in the right flank with 0.2 ml of a suspension containing the minimal lethal dose of TUBO cells (10^5) [19].

Mice were inspected weekly to monitor the appearance of autochthonous mammary tumors. Neoplastic masses were measured with calipers in two perpendicular diameters and the average value was recorded. Progressively growing masses (>1 mm mean diameter) were regarded as tumors. According to our ethical protocol mice were killed when a first tumor exceeded 10 mm mean diameter.

2.4. Mammary gland isolation

BALB/c and BALB-neuT mice were sacrificed at week 18, when the atorvastatin treated groups were at the end of the 8 week atorvastatin treatment period. The thoracic and abdominal mammary glands were isolated, incubated for 48 h at 4 °C in RNA Later (Quiagen, Hilden, Germany), drained and stored at −80 °C for 1 day and than homogenized in MLB buffer (125 mM Tris–HCl, pH 7.4, 750 mM NaCl, 1% v/v NP40, 10% v/v glycerol, 50 mM $MgCl_2$, 5 mM EDTA, 25 mM NaF, 1 mM $NaVO_4$, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin and 1 mM phenylmethanesulfonylfluoride, PMSF).

2.5. Mevalonate pathway activity

TUBO cells were incubated for 24 h with 1 μCi of [^{3}H]acetate (3600 mCi/mmol; Amersham GE Healthcare, Little Chalfront, UK), then washed with PBS and transferred to glass microcentrifuge tubes. The intracellular synthesis of cholesterol, FPP and GGPP was measured by the methanol/hexane extraction method, followed by thin layer chromatography [20]. Standard solutions of cholesterol, FPP and GGPP (1 mg/ml; Sigma Chemical Co.) were also loaded on the chromatography gel. After the separation, the gel was exposed to an iodine-saturated atmosphere. Each spot was cut and solubilised and the radioactivity incorporated measured by liquid scintillation counting (Ultima Gold, PerkinElmer, Waltham. MA). The results were expressed as fmol/10^6 cells, according to the titration curve previously obtained.

2.6. NF-κB activity

TUBO cells and mammary gland extracts were rinsed with 0.5 ml lysis buffer A (10 mM HEPES, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol DTT, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.1% v/v NP40, pH 7.6), incubated for 10 min on ice, vortexed and centrifuged for 30 s at 13,000 × *g* to pellet nuclei, which were re-suspended in 0.2 ml wash buffer B (25 mM HEPES, 2 mM KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 2 μg/ml leupeptin, pH 7.6) and incubated at 4 °C for 10 min. An equal volume of buffer C (25 mM HEPES, 0.1 mM EDTA, 20% v/v glycerol, pH 7.6) was added and after 10 min the mix was centrifuged at 13,000 × *g* for 15 min at 4 °C. The activity of NF-κB in the supernatants containing the nuclear

extracts was assessed by the TransAM™ Flexi NFκB Family kit (Active Motif, Rixensart, Belgium), adding 1 pmol of the biotinylated NF-κB consensus site 5′-GGGACTTTCC-3′ to 10 μg of nuclear extracts and following the manufacturer's instructions. The absorbance at 450 nm was measured with a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). For each set of experiments, a blank was prepared with distilled water, and its absorbance was subtracted from that obtained in the presence of nuclear extracts. To assess the procedure specificity, a competition assay was performed by adding 40 pmol of wild type non-biotinylated oligonucleotide to nuclear extracts derived from TUBO cells treated 24 h with 20 μg/ml lipopolysaccharide from *E. coli* (Sigma Chemical Co.). Data were expressed as mU absorbance/mg cell proteins.

2.7. Cell proliferation assays

TUBO cells were seeded at a density of 5×10^3/ml into flat bottom 96-well microplates in 100 μl complete DMEM medium. After 6 h the medium was replaced with serum-free medium and cells were incubated 24 h with the appropriate stimuli (2.5, 20, 50, 100 and 200 μM atorvastatin; 10 μM parthenolide; 50 μg/ml mytomicin-C). After 12 h, 10 μl Br-deoxyuridine (Br-dU; Cell Proliferation ELISA BrdU kit, Roche, Basel, Switzerland) was added to every well and after a further 12 h, cell proliferation was assessed, reading the sample absorbance at 370 nm, in accordance with the producer's instructions. Growth of TUBO cells was represented as optical density (O.D.). The proliferation index was calculated as the ratio between the O.D. of each other experimental point and the O.D. obtained in the presence of the higher concentration (200 μM) atorvastatin.

2.8. G-proteins activity

Ras-GTP binding, considered an index of monomeric G-protein activation [21], was measured in pull-down assays using the Ras Assay Reagent (Raf-1 RBD, agarose conjugate; Millipore, Billerica, MA), as reported in [22]. RhoA activation was detected with the G-LISA™ RhoA Activation Assay Biochem Kit (Cytoskeleton Inc., Denver, CO), according to the manufacturer's instructions. Data were expressed as mU absorbance/mg cell proteins.

2.9. RhoA kinase activity

RhoA kinase activity was measured using the CycLex Rho Kinase Assay Kit (CycLex MBL International, Woburn, MA), a single site binding immunoassay, as previously reported [22]. Data were expressed as mU absorbance/mg cell proteins.

2.10. Western blot analysis

TUBO cells and mammary gland homogenates were lysed in the MLB buffer, sonicated on crushed ice (with two 10 s-bursts, using a Hielscher Ultrasonics GmbH Instrument, Teltow, Germany) and centrifuged at 13,000 × *g* for 10 min at 4 °C. 20 μg cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene fluoride membrane sheets (Immobilon-P, Millipore) and probed with the following antibodies: anti-phospho-(Thr202/Tyr204, Thr185/Tyr187)-extracellular signal-regulated kinase 1/2 (ERK1/2; diluted 1:1000 in TBS-milk 5% w/v, Millipore); anti-ERK1/2 (diluted 1:500 in TBS-milk 5% w/v, Millipore); anti-phospho-Ser(473)-Akt (diluted 1:200 in TBS-milk 5% w/v, Millipore); anti-Akt (diluted 1:500 in PBS–BSA 1% w/v, Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-Ras (diluted 1:250 in TBS-milk 5% w/v, Millipore); anti-RhoA (diluted 1:250 in PBS–BSA 1% w/v, Santa Cruz Biotechnology Inc.); anti-phospho-Ser(176/180)-IKKα/β (diluted 1:500 in PBS–BSA 1% w/v, Cell Signaling Technology Inc., Danvers, MA); anti-IKKα/β (diluted 1:500 in PBS–BSA 1% w/v, Santa Cruz Biotechnology Inc.); anti-IκB-α (diluted 1:500 in PBS–BSA 1% w/v, Santa Cruz Biotechnology Inc.); anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; diluted 1:500 in PBS–BSA 1% w/v, Santa Cruz Biotechnology Inc.). Membrane was washed in TBS-Tween 0.1% v/v, subjected for 1 h to a peroxidase-conjugated anti-rabbit or anti-mouse IgG (diluted 1:3000 in TBS-milk 5% w/v, Bio-Rad), washed again with TBS-Tween 0.1% v/v. Proteins were detected by enhanced chemiluminescence (PerkinElmer).

In vitro IKK kinase assay was performed as reported previously [22], using an anti-IKKγ antibody (diluted 1:200 in PBS–BSA 1% w/v, Santa Cruz Biotechnology Inc.) to isolate the IKK complex. 100 μg of the immuno-precipitated proteins were incubated with 1 mM ATP and 30 μg of immunopurified IκB-α protein. Reaction was carried over at 30 °C for 30 min and stopped with 30 μl of Laemli buffer (60 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 5% w/v β-mercaptoethanol, 0.01% w/v bromophenol

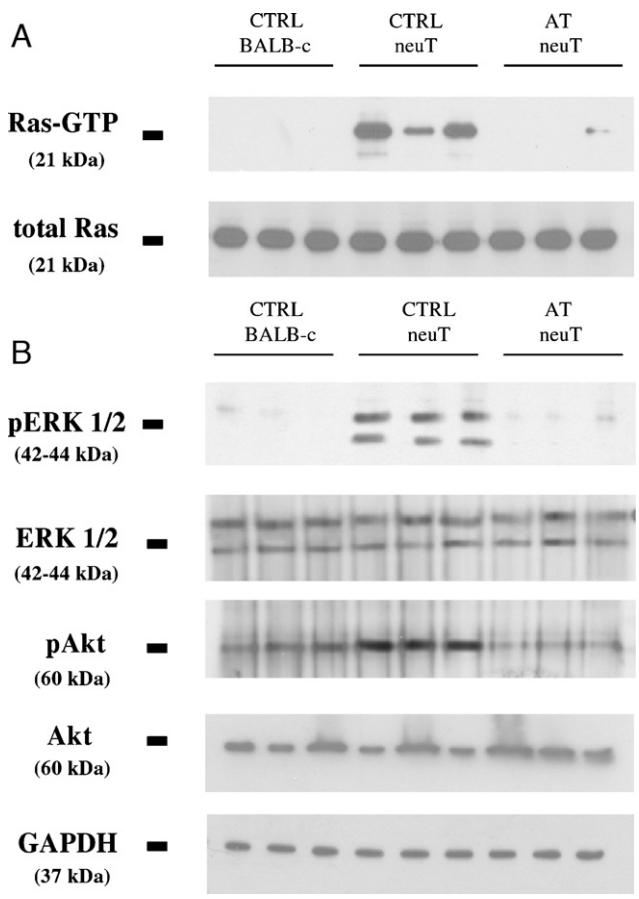

Fig. 1. Effect of atorvastatin on Ras, ERK1/2 and Akt activity in mammary glands of BALB/c and BALB-neuT mice. Transgenic BALB-neuT mice ($n = 5$) were treated with atorvastatin (*AT*) for 8 weeks as reported in Section 2; then mammary glands were isolated for protein extraction. An equal number of transgenic BALB-neuT mice and wild type BALB/c mice were used as control (*CTRL*). The experiment was replicated three times. A. Ras-GTP pull down. Mammary gland homogenates were lysed and the expression of Ras-GTP and total Ras was analyzed as described in Section 2. The figure is representative of 3 mice in each group of treatment; similar results were obtained in the other animals of each group; B. Western blot detection of phospho-ERK1/2, ERK1/2, phospho-Akt, Akt. Mammary gland homogenates were analyzed by Western blotting with an anti-phospho-(Thr202/Tyr204, Thr185/Tyr187)-ERK1/2, an anti-ERK1/2, an anti-phospho-Ser(473)-Akt or an anti-Akt antibody, as reported in Section 2. The expression of GAPDH was used to check the equal protein loading. The figure is representative of 3 mice in each group of treatment; similar results were obtained in the other animals of each group.

blue), then samples were subjected to SDS-PAGE and probed with an anti-IκB-α antibody, to check the total amount of IκB-α loaded in each assay, and with an anti-phospho-Ser(32)-IκB-α antibody (diluted 1:250 in PBS–BSA 1% w/v, Santa Cruz Biotechnology Inc.), to detect the amount of IκB-α phosphorylated by the active IKK complex.

The densitometric analysis of Western blots was performed using ImageJ software (http://rsb.info.nih.gov/ij/) and was expressed as arbitrary units, where "1" is the mean band density of untreated cells.

2.11. RhoA silencing

1×10^5 TUBO cells were treated for 6 h with 1 ml siRNA transfection medium (Santa Cruz Biotechnology Inc.), containing 5 μl siRNA transfection reagent (Santa Cruz Biotechnology Inc.) and 50 pmol RhoA siRNA (Santa Cruz Biotechnology Inc.). In each set of experiments, one dish was treated with 50 pmol of Control siRNA-A (Santa Cruz Biotechnology Inc.), a non-targeting 20- to 25-nucleotide siRNA designed as a negative control, instead of RhoA siRNA. After 72 h the silencing efficacy was verified by Western blotting for RhoA protein. Cell toxicity was assessed by monitoring the extracellular release of lactate dehydrogenase enzyme, as reported [23], and was super-imposable on that of untreated cells (not shown).

2.12. Statistical analysis

All data in text and figures are provided as means ± SE. The results were analyzed by a one-way Analysis of Variance (ANOVA). Survival curves were analyzed by a Chi square Test and the tumor growth was analyzed by Student's *t* Test. A $p < 0.05$ was considered significant. r^2 coefficient in linear regression analysis was calculated using Fig.P software (Fig.P Software Inc., Hamilton, Canada).

3. Results

3.1. Atorvastatin abolishes the activation of Ras and ERK1/2 in BALB-neuT mice mammary gland tumors

Female BALB/c mice and BALB-neuT mice were sacrificed at 18 weeks of age, a time at which in BALB-neuT mammary glands

A

Latency (mm)	Atorvastatin treated mice (days after tumor challenge)	Untreated mice (days after tumor challenge)	
2.5	19±4	18±4	p= 0.158 n.s.
5	31±8	25±6	p= 0.263 n.s.
7	43±19	28±5	p= 0.260 n.s.

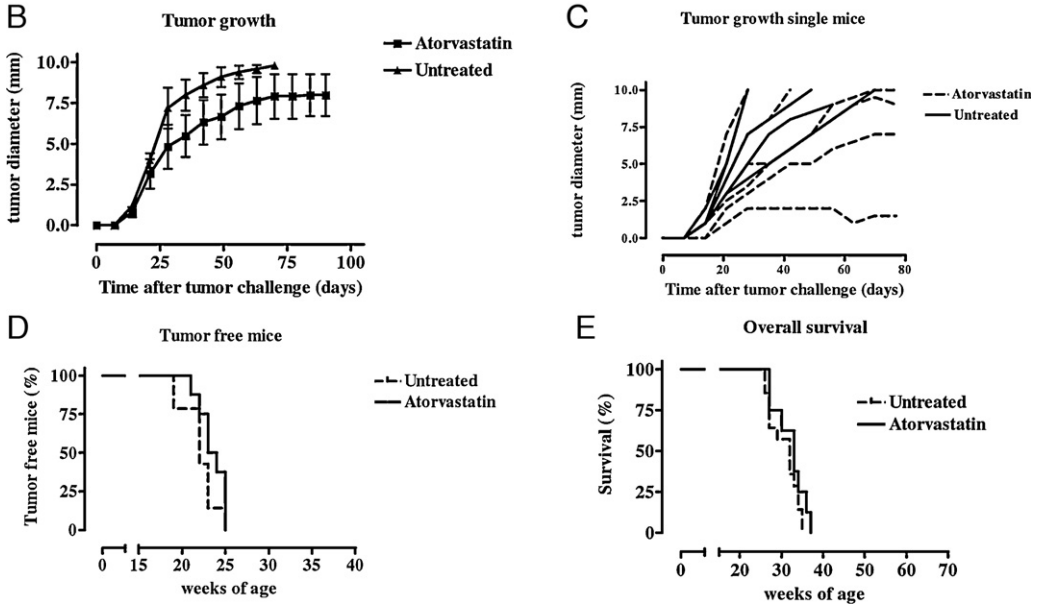

Fig. 2. Effect of atorvastatin on the growth of Her2/neu over-expressing tumors. A. Tumor latency (time required from the challenge to a tumor of the indicated mean diameter) in mice challenged with Her2/neu over-expressing TUBO cells and treated with atorvastatin. BALB/c mice were challenged with a lethal dose (10^5 cells) of syngenic TUBO mammary tumor cells and orally treated with atorvastatin administered for 8 weeks as described in Section 2 (atorvastatin group: $n = 6$). Tumor diameters were not significantly different in the atorvastatin treated mice compared with the control (PBS-treated) mice (control group: $n = 4$); B. Tumor growth (mean ± SE, mm) in function of time (days) in control and atorvastatin-treated BALB/c mice of point A. C. Graph representing tumor growth in single BALB/c mice challenged with TUBO cells. D. Percentage of tumor free animals, in control ($n = 14$) and atorvastatin-treated ($n = 8$) BALB-neuT mice. Chi square test: 2.160 (not significant). E. Overall survival of BALB-neuT mice of point D. Chi square test: 1.609 (not significant).

multiple tumors develop [24]. A sub-group of BALB-neuT mice was treated with atorvastatin as reported in Section 2. Mammary glands were collected and examined for the activity of the downstream effectors of Her2/neu. The expression of total Ras did not change between BALB/c and BALB-neuT mice (Fig. 1A), whereas significant differences were detected in Ras-GTP binding, an index of Ras isoprenylation and activity [21]: Ras-GTP was in fact undetectable in non transformed mammary glands of BALB/c mice, but present in tumoral glands of BALB-neuT mice. Interestingly, in BALB-neuT mice treated with atorvastatin, the activation of Ras was prevented (Fig. 1A).

We then analyzed the expression and activity of MAPKs ERK1 and ERK2, which are downstream effectors of Ras, and of Akt, which can also be activated independently from Ras [13]. In keeping with the Ras-GTP activity, phosphorylated active ERK1/2 were hardly detected in BALB/c mammary glands and constitutively present in tumor glands of BALB-neuT mice; again atorvastatin strongly reduced the phosphorylation of ERK1 and ERK2 in BALB-neuT tumor extracts (Fig. 1B). Akt was basally phosphorylated in BALB/c mice; the phosphorylation was higher in BALB-neuT mice and slightly reduced by the statin (Fig. 1B). The total amount of ERK1/2 and Akt did not change under any experimental conditions (Fig. 1B).

3.2. Atorvastatin administration does not inhibit the growth of implanted TUBO cells in BALB/c mice and the spontaneous tumor development in BALB-neuT mice

Implanted TUBO cells, a cell line established from BALB-neuT mice mammary tumors, grow fast in wild-type BALB/c mice and form a palpable tumor mass in about 2 weeks [25]. To evaluate the effects of atorvastatin on the growth of implanted tumors, female BALB/c mice were challenged s.c. with 10^5 TUBO cells. On the same day, oral treatment with atorvastatin was started. No significant differences were observed in the growth of TUBO tumors in treated vs. control mice (Fig. 2A and B). Only in 2/6 atorvastatin treated mice was a delay in tumor growth observed (Fig. 2C).

Female BALB-neuT mice develop mammary tumors in all their mammary glands [25]. In the BALB-neuT control group at least one palpable carcinoma (>1 mm mean diameter) was evident in each mouse by week 24 (Fig. 2D), while by week 35 a tumor mass was palpable in all 10 mammary glands or the tumor burden was such that the mice were sacrificed for ethical reasons (Fig. 2E). Oral administration of atorvastatin from week 10 to week 17 did not delay tumor progression in female BALB-neuT mice. All treated mice had at least one palpable tumor by week 25 (Fig. 2D) and at 37 weeks all animals were dead (Fig. 2E).

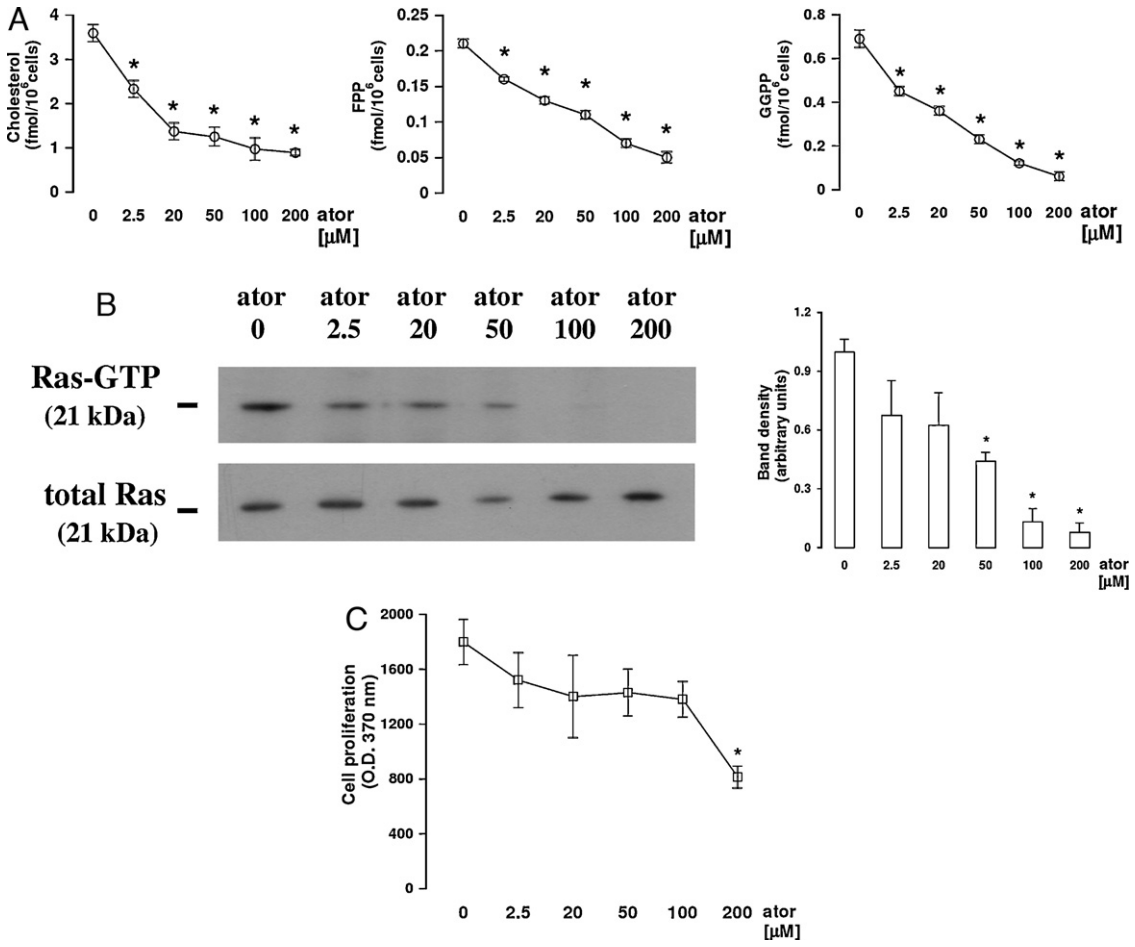

Fig. 3. Dose-dependent effects of atorvastatin on mevalonate pathway, Ras activity and proliferation of TUBO cells. Cells were incubated in the absence (0) or presence of different concentrations of atorvastatin (2.5, 20, 50, 100, 200 μM; *ator*) for 24 h, then subjected to the following investigations. A. Measurement of the mevalonate pathway activity. Cells were grown in a medium containing 1 μCi of [^{3}H]-acetate, then lysed and subjected to lipid extraction to measure the rate of synthesis of cholesterol, FPP and GGPP, as reported in Section 2. Data are presented as means ± SE (n = 3). vs. "0": $^*p < 0.02$. B. Ras-GTP pull down. The expression of Ras-GTP and total Ras was analyzed in pull-down assays (see Section 2). The figure is representative of 3 experiments with similar results. The band density ratio between Ras-GTP and total Ras was expressed as arbitrary units. vs. "0": $^*p < 0.005$. C. Evaluation of the effects exerted by atorvastatin at different concentrations on the proliferation of TUBO cells. As internal control, the cell proliferation blocker mytomicin C (50 μg/ml) was used and yielded 190.00 ± 10.28 O.D. Data are presented as means ± SE (n = 3). vs. "0": $^*p < 0.05$.

3.3. Atorvastatin decreases the FPP levels and the Ras activity but not the proliferation of mammary Her2-positive cancer cells

To investigate the molecular basis of atorvastatin low efficacy in preventing tumor growth, despite the reduction of the pro-proliferative signals acting downstream Her2 oncogene (like Ras and ERK1/2), we first verified whether atorvastatin was able to exert its "canonical" metabolic effects, that is to reduce the activity of the mevalonate pathway in TUBO cells. The drug regularly worked as an HMGCoAR inhibitor in our model, since it dose-dependently reduced the *de novo* synthesis of cholesterol and of the intermediate isoprenoid metabolites FPP and GGPP (Fig. 3A). In keeping with these results, the drug also decreased the amount of GTP-bound Ras (Fig. 3B). On the other hand, the proliferation of TUBO cells was not affected, except in the presence of high micromolar concentrations of atorvastatin (Fig. 3C), thus reproducing the refractoriness to the anti-proliferative effect previously observed *in vivo* (Fig. 2).

3.4. In Her2-positive cancer cells atorvastatin increases the activation of NF-κB, that is negatively correlated with the amount of isoprenoids

A correlation is known to exist between the mevalonate pathway and the activation of the transcription factor NF-κB [26,27]. In TUBO cells increasing concentrations of atorvastatin, which progressively reduced the activity of the mevalonate pathway (Fig. 3A), elicited an increasing activation of NF-κB (Fig. 4A). Such activation was inversely correlated with the amount of FPP (Fig. 4B) and GGPP (Fig. 4C), suggesting that isoprenoids may exert inhibitory effects on the activity of NF-κB in TUBO cells.

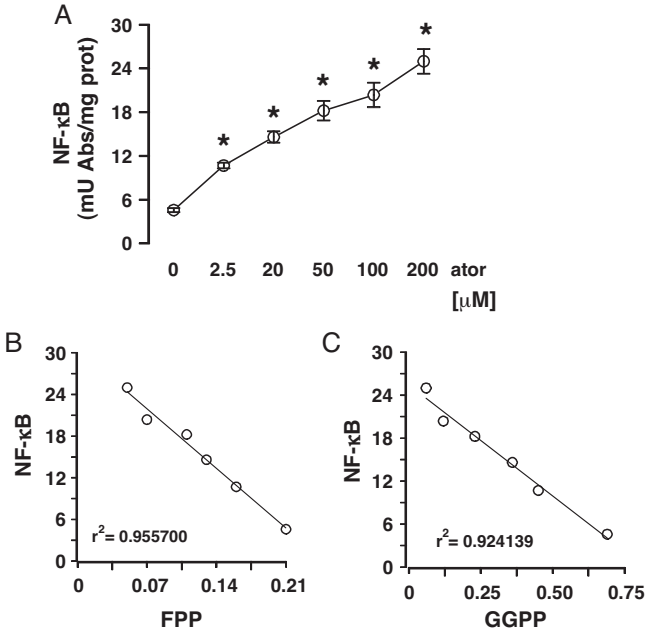

Fig. 4. Dose-dependent effects of atorvastatin on NF-κB activation in TUBO cells. Cells were incubated in the absence (*0*) or presence of different concentrations of atorvastatin (2.5, 20, 50, 100, 200 μM; *ator*) for 24 h. A. NF-κB activation by atorvastatin. The activity of NF-κB was detected in the nuclear extracts measuring the amount of NF-κB bound to its target sequences on DNA, with a DNA binding assay (see Section 2 for details). In the competitions assay, performed with an excess of non-biotinylated oligonucleotide and used as a control of the assay specificity, the activity of NF-κB in cells treated with *E. coli* lipopolysaccharide was 2.06 ± 0.45 mU/mg prot. Measurements were performed in duplicate and data are presented as means ± SE ($n = 3$). vs. "0": $^{*}p < 0.001$. B. Linear regression analysis between FPP synthesis and NF-κB activity. FPP levels (Fig. 3A) were plotted vs. NF-κB activation (expressed in A), to obtain r^2 coefficient. C. Linear regression analysis between GGPP synthesis and NF-κB activity. GGPP levels (Fig. 3A) were plotted vs. NF-κB activation (A), to obtain r^2 coefficient.

3.5. Her2-positive cancer cells have a different sensitivity toward the effects of atorvastatin on Ras-dependent and RhoA-dependent pathways

Although atorvastatin decreased the rate of synthesis of FPP at low micromolar concentrations (Fig. 3A), a clear reduction in Ras activity was obtained only at high micromolar concentrations in TUBO cells (Fig. 3B). According to this observation, the activity of the Ras-downstream effectors ERK1/2 and of Akt was not affected by low concentration (2.5 μM) atorvastatin (Fig. 5A and C). A 50 μM (Fig. 5B and D) or a higher concentration (data not shown) of the statin was necessary to decrease the activity of ERK1/2 and to reduce – although to a lesser extent – the activation of Akt. Such effects were likely to have been dependent on the diminished farnesylation of Ras, since they were abrogated by the cell-permeable FPP analogue farnesol and were mimicked – with a stronger potency – by the inhibitor of farnesyl transferase FTI-I (Fig. 5B and D).

Surprisingly, 2.5 μM atorvastatin was sufficient to elicit a remarkable decrease of RhoA and RhoA kinase activity, to an extent super-imposable on the geranylgeranyl transferase inhibitor GGTI-286 (Fig. 6A). In parallel atorvastatin induced the activation of NF-κB (Fig. 6B), by increasing the phosphorylation (Fig. 6C) and activity (Fig. 6D) of IκB kinase (IKK)α/β complex and by decreasing the amount of the inhibitor protein IκB-α (Fig. 6C). The effect of atorvastatin on NF-κB (Fig. 6B) and on IKK/IκB-α axis (Fig. 6C) was likely dependent on the reduced activity of RhoA, since it was lost in the presence of the cell-permeable GGPP analogue geranylgeraniol. On the contrary the activation of NF-κB was obtained if we inhibited the geranylgeranyl transferase enzyme with GGTI-286 (Fig. 6B). The activation of IKK/IκB-α axis was also achieved in cells knocked down for RhoA protein, a situation which made geranylgeraniol devoid of effects (Fig. 6C and D). Farnesol (10 μM for 24 h) on the contrary did not prevent the effects of atorvastatin on NF-κB in TUBO cells (data not shown).

3.6. Atorvastatin decreases the activity of RhoA/RhoA kinase and activates NF-κB in BALB-neuT mice mammary gland tumors

A similar pattern of RhoA-dependent activation of NF-κB was observed in the tumor mammary glands of mice. We did not detect any difference in the basal activity of RhoA, RhoA kinase (Fig. 7A), IKK/IκBα proteins (Fig. 7B) and NF-κB activity (Fig. 7C) in BALB/c and BALB-neuT animals, whereas atorvastatin significantly reduced the activation of RhoA and of its downstream effector in treated BALB-neuT mice (Fig. 7A). In parallel the drug increased the phosphorylation of IKK, decreased the amount of the constitutively present IκB-α (Fig. 7B) and elicited the binding of NF-κB on its target sequences on DNA (Fig. 7C).

3.7. The simultaneous co-administration of a NF-κB inhibitor allows atorvastatin to reduce Her2-positive cancer cell proliferation

Since NF-κB is a crucial factor for cell proliferation and survival [28], we hypothesize that the lack of anti-proliferative effects of atorvastatin, despite the down-regulation of Ras/MAPKs signaling, could depend on the simultaneous activation of NF-κB with a RhoA-dependent mechanism. We thus examined the effects of the statin in the presence of a NF-κB inhibitor such as parthenolide, which reduced per se the basal activity of NF-κB (Fig. 8A) and the proliferation of TUBO cells (Fig. 8B).

If associated with parthenolide, atorvastatin – at a concentration that reduced the Ras signaling (Fig. 3B), but did not decrease cell proliferation (Fig. 3C) – was prevented to activate the transcription factor (Fig. 8A). Only in this condition did the drug effectively reduce the proliferation of TUBO cells; interestingly it had additive effects with parthenolide (Fig. 8B).

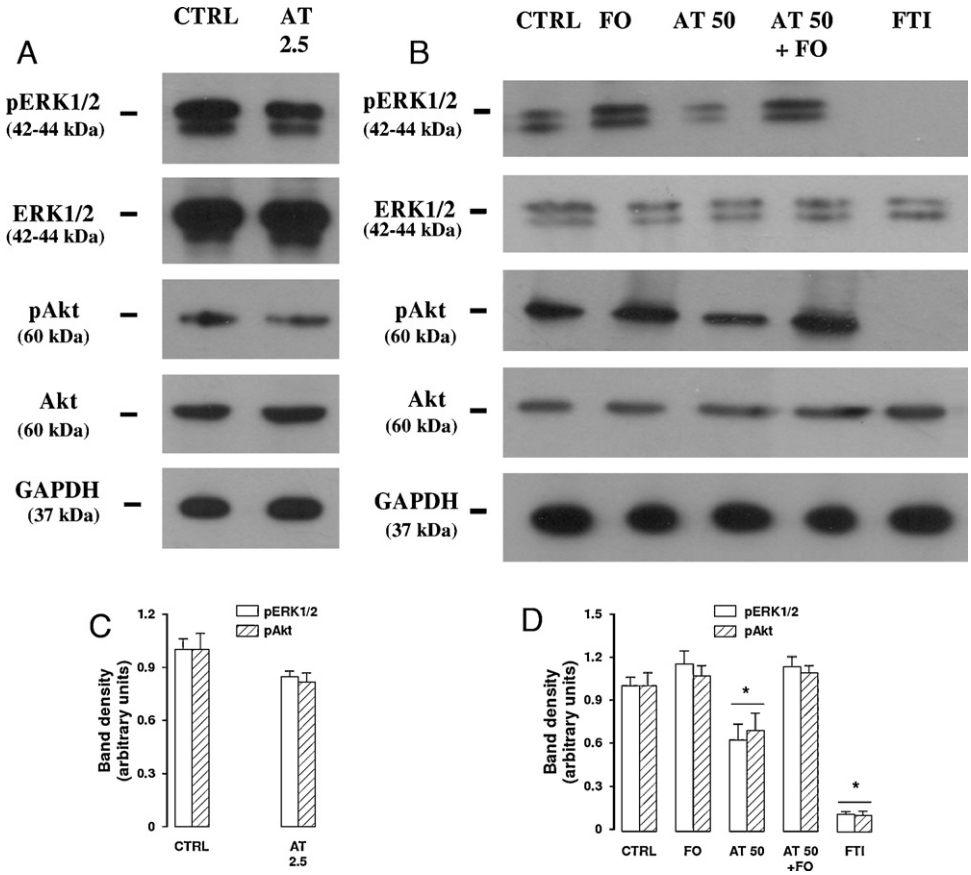

Fig. 5. Effects of low vs. high concentrations of atorvastatin on ERK1/2 and Akt activity in TUBO cells. Cells were incubated for 24 h in fresh medium (*CTRL*) and in the presence of a low atorvastatin concentration (2.5 μM, *AT*; panel A), or of a high atorvastatin concentration (50 μM, *AT*), with or without farnesol (10 μM, *FO*), a cell-permeable analogue of FPP (panel B). The farnesyl transferase inhibitor FTI-I (10 μM for 24 h, *FTI*) was added as a positive control of Ras inhibition. Cells were lysed and analyzed by Western blotting with an anti-phospho-(Thr202/Tyr204, Thr185/Tyr187)-ERK1/2, an anti-ERK1/2, an anti-phospho-Ser(473)-Akt or an anti-Akt antibody, as reported in Section 2. The expression of GAPDH was used to check the equal protein loading. The figure is representative of 3 experiments with similar results. C and D. The band density ratio between pERK1/2 and total ERK1/2 and between pAkt and Akt for each experimental point of panel A and B was expressed as arbitrary units. vs. "*CTRL*": $^{*}p < 0.02$.

4. Discussion

Cancer represents a putative field of application for statins, since most activities of transformed cells, such as proliferation, matrix invasion and angiogenesis, are controlled by the isoprenylated small G-proteins of Ras and Rho family, that are inhibited by statins [2].

The subgroup of Her2 overexpressing breast cancers is particularly interesting, because one of the most active downstream transducers of the Her2 oncogene is Ras [8].

In our work we found that untransformed mammary glands of BALB/c mice were devoid of active GTP-bound Ras, which was instead detected in tumor glands of Her2-transgenic BALB-neuT and suppressed by treatment with atorvastatin. The striking difference in Ras activity between wild-type and transgenic mice led us to the hypothesis that this G-protein has a prominent role in the tumorigenesis and/or in the tumor growth in BALB-neuT mice.

In cells with constitutively activated EGF receptors, the MAPKs signaling enhances proliferation [5]. Indeed the activation of ERK1 and ERK2 kinases in our experimental model strictly followed the activity of Ras. The activity of the Akt signaling displayed minor changes: phospho-Akt was detectable also in non transformed mammary glands, was slightly increased in BALB-neuT animals and slightly reduced by atorvastatin. These results however are not surprising: also in Her2-negative MCF-7 cells, Akt is constitutively phosphorylated [29], probably in consequence of the multiple activating signals converging on kinase B, besides those dependent on Her2 and EGF receptors. By reducing Ras/ERK1/2 and Akt activation in the mammary glands of BALB-neuT mice, atorvastatin should have at least two requisites to reduce cells proliferation [5].

Unexpectedly, we did not observe any growth reduction of tumors implanted in statin-treated BALB/c mice nor a delay in the growth of the autochthonous mammary tumors in BALB-neuT animals, a result that is in contrast with other experimental works [5,30]. To clarify whether the lack of efficacy of atorvastatin, despite its ability to reduce the Her2-dependent signal transduction, was due to reasons residing within the tumor cell, we investigated if the drug exerted the "classical" metabolic effects of statins in the epithelial TUBO cell line, derived from the BALB-neuT mice mammary tumors. Here atorvastatin decreased the rate of synthesis of cholesterol, FPP and GGPP already at low micromolar concentrations, in keeping with the statin's K_i for HMGCoAR, that is in nanomolar range [31]. At these concentrations atorvastatin did not reduce the proliferation rate, but was able to increase the activity of NF-κB, a transcription factor critical for proliferation and inhibition of apoptosis [28]. A strong negative correlation between isoprenoids levels and NF-κB activation was found in TUBO cells, suggesting that statins may up-regulate NF-κB activity through the decreases of some isoprenylated products.

The effects of statins on the NF-κB pathway are highly variable depending on cell type [32–34]. In breast cancer cells, both inhibitory [6] and stimulating [35] effects of statins on NF-κB target genes have been reported. The activity of NF-κB can be reduced by FPP [27] and GGPP [26], and in some experimental

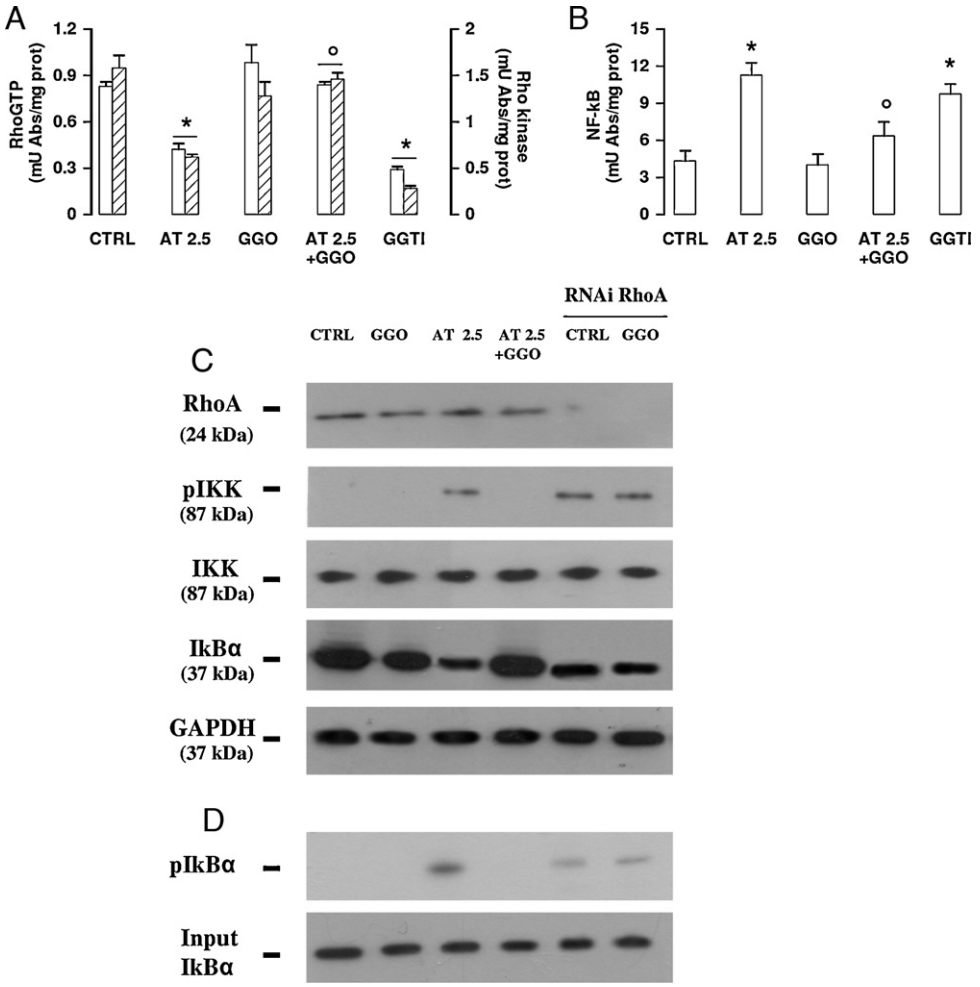

Fig. 6. Effects of low concentration of atorvastatin on RhoA/RhoA kinase/NF-κB pathway in TUBO cells. Cells were grown 24 h in the absence (*CTRL*) or presence of atorvastatin (2.5 μM, *AT*), geranylgeraniol (10 μM, *GGO*), a cell-permeable analogue of GGPP, or both. GGTI-286 (10 μM, *GGTI*), a specific inhibitor of geranylgeranyl transferase, was added as a positive control of RhoA inhibition. A. Samples were subjected to ELISA assays to measure the amount of RhoA-GTP (*open bars*) and the activity of RhoA kinase (*hatched bars*). The experiments were performed in duplicate, as described in Section 2. Data are presented as means ± SE ($n = 3$). vs. CTRL: $^{*}p < 0.005$; vs. AT: $^{\circ}p < 0.01$. B. NF-κB activation. The activity of NF-κB was detected in the nuclear extracts measuring the DNA-binding capacity of NF-κB with a DNA-binding assay (see Section 2). Measurements were performed in duplicate and data are presented as means ± SE ($n = 3$). vs. CTRL: $^{*}p < 0.005$; vs. AT: $^{\circ}p < 0.05$. C. TUBO cells were treated as reported above; an aliquot of cells was subjected to RhoA silencing (*RNAi RhoA*), as reported in Section 2, and grown 24 h after the end of transfection in the absence (*CTRL*) or presence of geranylgeraniol (10 μM, *GGO*). Samples were analyzed by Western blotting with an anti-RhoA, an anti-phospho-Ser(176/180)-IKKα/β, an anti-IKKα/β and an anti-IκB-α antibody, as reported in Section 2. The expression of GAPDH was used to check the equal protein loading. The figure is representative of 3 experiments with similar results. D. IKK activity. From cell extracts incubated as reported at point C, IKK complex was isolated by immunoprecipitation using an anti-IKKγ antibody and incubated with purified IκB-α protein. The amount of IκB-α phosphorylated by the active IKK complex was detected with an anti-phospho-Ser(32)-IκB-α antibody (*pIκBα*). The extracts were also probed with an antibody for total IκB-α, to check that equal amounts of the protein were used in each kinase assay (*input IκBα*). The figure is representative of 2 experiments with similar results.

models NF-κB is inhibited by RhoA activation [36,37]. In malignant mesothelioma and colon cancer RhoA and RhoA kinase negatively regulate the activity of the IKK complex, and prevent the phosphorylation of the inhibitory protein IκB-α, which in this way maintains NF-κB inactive in cytosol. On the contrary, statins or RhoA silencing activate the IKK kinase and allow NF-κB to translocate into the nucleus [22,23]. Such a RhoA-operated inhibition of NF-κB occurred also in TUBO cells treated with atorvastatin. Since geranylgeraniol prevented the effects of the statin on IKK/IκB-α axis in wild-type but not in RhoA-silenced cells, we can conclude that no geranylgeranylated proteins other than RhoA are involved in the control of NF-κB activation in TUBO cells.

What is most surprising is the observation that the sensitivity to statin of Ras-dependent pathways was lower than the sensitivity of RhoA-dependent pathways. The presence of the constitutively active Her2 oncogene which maintains Ras in a permanent activated status could make difficult for atorvastatin to suppress such activation, except at high concentrations. The low sensitivity of TUBO cells to drugs affecting the Her2/Ras signaling was confirmed by the observation that low micromolar concentrations of farnesol did not reduce the activation of NF-κB elicited by atorvastatin. On the other hand the strong negative correlation between FPP level and NF-κB activation suggests that also FPP, as well as GGPP, exerted a suppressive role on NF-κB. Considering the low sensitivity of Ras-dependent pathways and the constitutively high activation of Ras in TUBO cells, it seems unlikely that FPP prevents the activation of NF-κB through a further increment of Ras activity. FPP could act instead with an indirect mechanism, e.g. being the precursor of GGPP, that suppresses the activation of NF-κB in a RhoA/RhoA kinase-dependent way.

If compared with other tumor histotypes [22,38], RhoA and RhoA kinase have a very low basal activity in our Her2-positive mammary cancer cells. Therefore, also a small reduction of RhoA activity, such as that elicited by low micromolar concentrations of

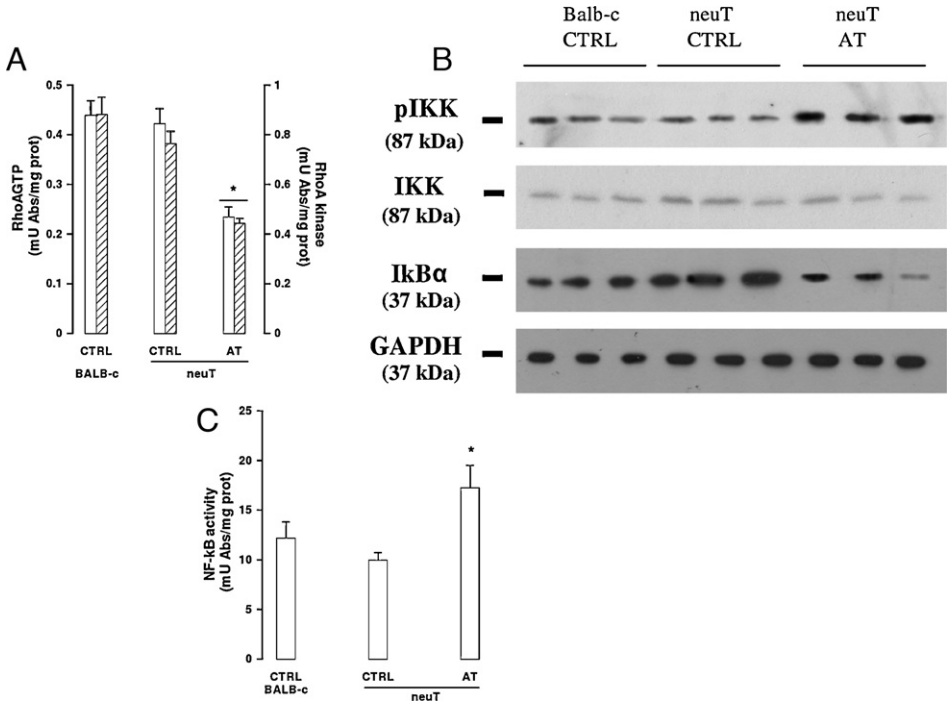

Fig. 7. Effects of atorvastatin on RhoA, RhoA kinase and NF-κB activation in mammary glands of BALB/c and BALB-neuT mice. Transgenic BALB-neuT mice (n = 5) were treated with atorvastatin (*AT*) for 8 weeks as reported in Section 2; then mammary glands were isolated for protein extraction. An equal number of transgenic BALB-neuT mice and wild type BALB/c mice were used as control (*CTRL*). A. RhoA-GTP and RhoA kinase activity. Samples were subjected to ELISA assay to measure the amount of RhoA-GTP (*open bars*) and the activity of RhoA kinase (*hatched bars*). The experiments were performed in duplicate, as described in Section 2. Data are presented as means ± SE. vs. CTRL BALB-neuT: $^{*}p < 0.005$. B. Western blot detection of phospho-Ser(176/180)-IKKα/β, IKKα/β and IκB-α protein in cytosolic extracts from mammary glands. The expression of GAPDH was used to check the equal protein loading. The figure is representative of 3 mice in each group of treatment; similar results were obtained in the other animals of each group; C. The activity of NF-κB was detected in nuclear extracts as the capacity of the translocated NF-κB to bind its target sequences on DNA (as reported in Section 2). Measurements were performed in duplicate and data are presented as means ± SE. vs. CTRL BALB-neuT: $^{*}p < 0.05$.

atorvastatin, was likely sufficient to significantly affect the molecular events downstream RhoA. Second, the V_{max} of FPP synthase (1.08 μmol/min/mg prot) [39] is higher than the V_{max} of GGPP synthase (0.24 μmol/min/mg prot) [40], that can represent a limitating factor for RhoA geranylgeranylation. When atorvastatin further lowered the amount of GGPP, the activation of RhoA may be hampered much more than the activation of Ras. Similarly K_{cat} of farnesyl transferase (0.09 s^{-1}) [41] is higher than K_{cat} of geranylgeranyl transferase (0.02 s^{-1}) [42]. In the presence of a decreased amount of substrates, as occurred with atorvastatin, it is expected that the less efficient enzyme, i.e. geranylgeranyl transferase, would be more affected.

Taken together, our data depict two scenarios in Her2-positive cells exposed to statin: at low micromolar concentrations, atorvastatin did not reduce the activity of Ras/ERK1/2 pathway and up-regulated the IKK/NF-κB pathway; at high micromolar concentrations, atorvastatin was able to decrease the activation of Ras-dependent pathways, but simultaneously maintained NF-κB active. The latter scenario occurred in animals treated with atorvastatin, since in their mammary glands Ras/ERK1/2 pathway resulted as suppressed, while NF-κB signaling resulted as activated.

Besides preserving mammary cells trophism, NF-κB up-regulates several genes involved in the inhibition of apoptosis and/or in cell proliferation (such as Bcl2, Bcl-xl, cyclin D1, cyclin D2, c-Myc, c-Myb) and increases the synthesis of cytokines and growth factors, which sustain tumor proliferation and progression with autocrine mechanisms [28]. These mechanisms may explain why atorvastatin was not able to elicit any antiproliferative effect in mammary tumors of BALB-neuT mice, although it turned down Her2-dependent proliferation signals. Only if we prevented the activation of NF-κB with parthenolide, did the statin become able to reduce cell proliferation at lower concentrations, sufficient to decrease Ras-dependent signaling. Interestingly also parthenolide alone reduced TUBO cell proliferation, suggesting that NF-κB has a strong and autonomous role in sustaining the growth of Her2-positive cells. Such hypothesis is in accordance with previous observations reporting that IKKα regulates the proliferation of mammary cells and plays a pivotal role in the carcinogenesis of Her2/neu-positive mammary tumors [43].

In summary our work underlines that in Her2-positive cancers the Her2-dependent pathways could be not the only signals important for cell proliferation, but other Her2-independent mechanisms can critically sustain tumor growth in cooperation with Her2 oncogene. Such a redundancy of pro-proliferative signals may explain the aggressive phenotype of Her2-over-expressing tumors, a feature frequently observed in clinical practice.

Furthermore we have shown that statins can contemporaneously modulate multiple signaling pathways in Her2-positive cancer cells: such pleiotropism, which in other contexts has made statin drugs potentially useful in several diseases, may paradoxically dissipate the expected beneficial effects of HMGCoAR inhibitors in terms of antiproliferative activity. The identification of the pathways controlled by statins in each tumor is mandatory, in order to take advantage of the use of these drugs in association with other tumor-specific and selective targeted therapies. In this perspective the association of atorvastatin and NF-κB inhibitors may be regarded as a potential new therapeutic approach in Her2-positive tumors, with results often refractory to conventional anticancer therapies.

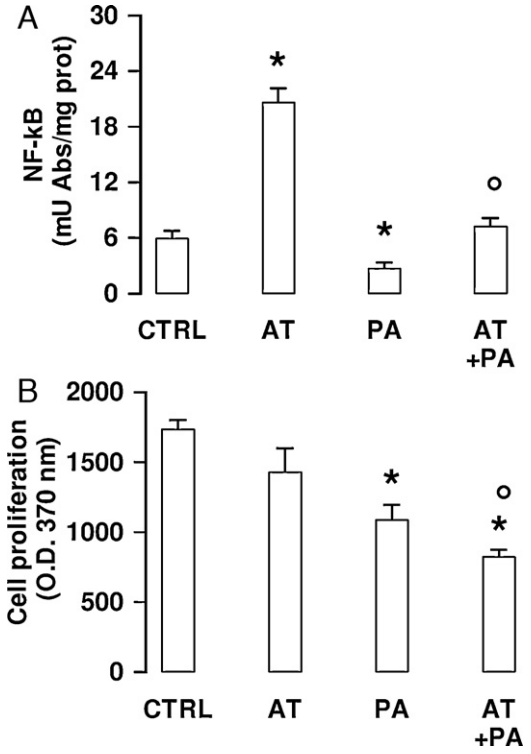

Fig. 8. Effective inhibition of TUBO cells proliferation by associating atorvastatin and parthenolide. Cells were cultured 24 h in fresh medium (*CTRL*) or in medium containing atorvastatin (50 μM, *AT*), in the absence or presence of the inhibitor of NF-κB parthenolide (10 μM, *PART*). A. NF-κB activation. The activity of NF-κB was detected in the nuclear extracts using a DNA binding assay as reported previously. Measurements were performed in duplicate and data are presented as means ± SE ($n = 3$). vs. CTRL: $^{*}p < 0.005$; vs. AT: $^{\circ}p < 0.005$. B. Proliferation of TUBO cells was measured by the BrdU assay, as described in Section 2. Measurements were performed in quadruplicate and data are presented as mean ± SE ($n = 3$). vs. CTRL: $^{*}p < 0.02$; vs. AT: $^{\circ}p < 0.05$.

Conflict of interest

None.

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