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Prolactin Induces Chitotriosidase Expression in Human Macrophages Through PTK, PI3-K, and MAPK Pathways

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ABSTRACT

We previously reported that prolactin (PRL) induces chitotriosidase (CHIT-1) mRNA expression in human macrophages. In this investigation we determined the signaling pathways involved in CHIT-1 induction in response to PRL. The CHIT-1 induction PRL-mediated was reduced by wortmannin and LY-294002, inhibitors of phosphatidylinositol 3-kinase (PI3-K) and by genistein an inhibitor of protein tyrosine kinase (PTK). Pre-treatment of macrophages with SB203580, a specific inhibitor of the mitogen-activated kinases (MAPK) p38, or with U0126, an inhibitor of MAPK p44/42, prevented both basal and exogenous PRL-mediated CHIT-1 expression. No significant effects on CHIT-1 induction PRL-mediated were observed with a protein kinase C inhibitor (PKC), rottlerin, or with an Src inhibitor, PP2, or with JAK2 inhibitor, AG490. In addition, PRL induced a phosphorylation of AKT that was prevented both by the two MAPK inhibitors SB203580 and U0126 and by the PI3-K inhibitors wortmannin and LY-294002. In conclusion, our results indicate that PRL up-regulated CHIT-1 expression via PTK, PI3-K, MAPK, and signaling transduction components. J. Cell. Biochem. 107: 881–889, 2009.

KEY WORDS: CHITOTRIOSIDASE; PROLACTIN; SIGNALING PATHWAYS; HUMAN MACROPHAGES

Prolactin (PRL) is widely recognized as an important physiological modulator of the immune response. Accumulating evidence suggest that PRL regulates proliferation and survival of immune system cells. PRL serves as a cofactor for interleukin-2 (IL-2) stimulated T-cell proliferation [Clevenger et al., 1990]. Moreover, its immuno-neutralization blocked mitogenesis stimulated by IL-2 and interleukin-4 (IL-4) in cytokine responsive cell lines [Hartmann et al., 1989]. Human lymphocytes synthesize and secrete PRL [Pellegrini et al., 1992; Sabharwal et al., 1992] and express cell surface PRL receptors (PRLR) [Gagnerault et al., 1993]. The PRLR, expressed in the lympho-hemopoietic system, is a member of the hematopoietin/cytokine receptor superfamily, which also includes receptors for IL-2 and growth hormone [Cosman, 1993]. PRL in combination with IL-2, phytohemagglutinin, or *Staphylococcus aurieus cowan*, it enhances mitogenesis in natural killer and T and B lymphocytes [Matera et al., 1992]. PRL stimulates cytokines and antibody production [Hartmann et al., 1989] and supports interferon-gamma (IFN-γ) production through interferon regulatory factors [Schwarz et al., 1992]. In addition, the presence of PRL-R on macrophages [Gala and Shevach, 1993] indicates that PRL is involved in regulating monocyte/macrophage functions. Previous studies from our laboratory revealed that the addition of physiological concentrations of PRL to human macrophages cultures up-regulated chitotriosidase-1 (*CHIT-1*) gene expression and enhances Chit activity [Malaguarnera et al., 2004].

CHIT-1 is a member of chitinase protein family that cleaves and shows transglycosylation activity towards chitin, the linear polymer of *N*-acetyl-D-glucosamine present in coatings of many pathogens such as protozoan parasites, fungi, and nematodes [Aguilera et al., 2003]. In man, CHIT-1 is a chitinase expressed mainly in neutrophils and macrophages [Renkema et al., 1997]. Chit is present in normal plasma [Den Tandt et al., 1988] its activity increased more than 1,000-fold in some patients with Gaucher Disease [Hollak et al., 1994]. Plasma Chit activity is also elevated in other lysosomal storage diseases [Guo et al., 1995], thalassemia [Barone et al., 1999], and in macrophages of atherosclerotic plaques [Boot et al., 1999].

Chitinases are expressed in several organisms, including plants, in which they are thought to play a role in innate immunity and in particular in defense against fungal pathogens [Aguilera et al., 2003]. Accumulating evidence suggest that CHIT-1 may play a

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comparable role in man. CHIT-1 mRNA expression is up-regulated following stimulation by tumor necrosis factor-alpha (TNF-α), IFN-γ, and lipopolysaccharide (LPS) [Di Rosa et al., 2005; Malaguarnera et al., 2005]. In vitro studies with recombinant human CHIT-1 have demonstrated that CHIT-1 lyses the hyphael tip of *Mucor rouxii* and inhibits the growth of hyphae of *Candida albicans* [van Eijk et al., 2005]. In vivo, neutropenic mice challenged with lethal doses of *C. albicans* or *Aspergillosis* exhibit increased survival when treated with human recombinant chitotriosidase [van Eijk et al., 2005]. Moreover, CHIT-1 is capable of cooperating with the *Plasmodium falciparum* (malarial) chitinase to break down the chitin containing peritrophic membrane in the Anopheles mosquito midgut [Di Luca et al., 2007], further suggesting that human CHIT-1 has chitinase activity against natural substrates. Moreover, CHIT-1 has been implicated in various pathologies, including neurodegenerative diseases [Di Rosa et al., 2006], cardiovascular diseases [Karadag et al., 2008], and liver diseases [Malaguarnera et al., 2006a,b].

Recent finding showed an important constitutive *CHIT-1* gene regulatory function for C/EBPβ in differentiated macrophages [Pham et al., 2007]. Nevertheless the signaling pathways implicated in CHIT-1 induction have not extensively been studied.

For these reasons, a series of kinase inhibitors were utilized to elucidate possible contributions of signaling pathways involved in the up-regulation of *CHIT-1* gene expression PRL-mediated in human macrophages. Our results indicate that PRL up-regulated CHIT-1 expression via a protein tyrosin kinase (PTK), phosphatidylinositol 3-kinase (PI3-K), and mitogen-activated kinases (MAPK) signaling transduction components, providing evidence that there are different signal pathways activated which could have a cumulative effect.

MATERIALS AND METHODS

CELL CULTURE

Human monocyte-macrophages (HMMs) were isolated from fresh buffy coat of healthy volunteers (with the exclusion of women and atherosclerotic subjects). DNA analysis to search the subjects homozygous for the wild CHIT allele was previously performed in all donors. The buffy coat was diluted with phosphate-buffered saline (PBS) supplemented with 2.5 mM EDTA and layered onto Ficoll-Hypaque gradients (Invitrogen, Milan, Italy). After 30 min of centrifugation at 400*g* at room temperature, the mononuclear cells were collected, washed twice with PBS, and placed in plastic Petri dishes at a concentration of 1×10^6–2×10^6 cells/cm surface areas in Iscove's medium supplemented with 2 mM glutamine, and 50 mg/ml of penicillin/streptomycin. After 2 h incubations, the non-adherent cells were washed out using PBS. The adherent cells (monocytes) were cultured in Iscove's medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 1% of penicillin/streptomycin (Invitrogen).

CELL TREATMENTS

Cells were seeded in 100 mm Petri dishes and cultured for 4 days. The cells were divided into different groups. The different sets of macrophage cultures were pretreated with PI3-K inhibitor, wortmannin (200 nM) or LY-294002 (25 μM), or with PTK inhibitor genistein (10 μM), or with MAPK p-38 inhibitor, SB202190 (20 μM) or with MAPK p44/42 inhibitor, U0126 (10 μM), or with JAK2 inhibitor, AG490 (10 μM), or with protein kinase C (PKC) inhibitor, rottlerin (5 μM), or with c-Src tyrosine kinase inhibitor, PP2 (100 nM) alone or in combination with PRL (25 ng/ml).

Signal transduction inhibitors were purchased from Calbiochem (Milan, Italy), each dissolved in DMSO, were added to the culture media at 1 h, respectively, before the treatment of PRL for 4 h for gene expression studies and for 6 h for protein expression studies. In the experiment using signal transduction inhibitors, control cultures were treated with the solvent DMSO. Dose-defining experiments to optimize the dose of inhibitors used were performed (data not shown). PRL used in these studies was endotoxin free.

RT–PCR

Reverse transcription (RT) was carried out using the SuperscriptTM One-Step RT-PCR System (Invitrogen). Specific primers for the human CHIT-1 cDNA fragment were as follows: Forward 5′-ACCCTGTTAGCCATCGGAGGCTGG-3′; Reverse 5′-TGCACAGCAGCATCCACGTTGAGG-3′. PCR was performed using the AmpliTaq PCR (Perkin-Elmer Cetus Instruments). For each RT-PCR, a sample without reverse transcriptase was processed in parallel and served as a negative control. Cycling parameters for amplifying RT products were as follows: 95°C, 1 min; 60°C, 1 min; 72°C, 1–3 min, for 30 cycles, and then extended at 72°C for another 5 min. Specific primers for GAPDH cDNA fragment were as follows: Forward 5′-ACTCCCATTCCTCCACCTTT-3′; Reverse 5′-TTACTCCTTGGAGGCCATGT-3′ was used for normalization of data. After amplification, PCR products were electrophoresed on 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light.

WESTERN BLOT

The cells were harvested using a cell lysis buffer as described previously [Malaguarnera et al., 2002]. The lysates were collected for Western blot analysis. Equal amounts of protein (25 μg/ml) were separated by SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, Milan, Italy) using a semidry transfer apparatus (Bio-Rad). The non-specific binding sites of nitrocellulose membrane were blocked with a 5% non-fat dried milk solution in TBS 0.1% Tween-20 for 2 h at room temperature (22 ± 2°C). The blot was then probed with anti-polyclonal anti-Chit, or anti-GAPDH antibodies (Santa Cruz Biotechnology) for 1 h at room temperature with constant shaking. The phosphorylation of PKB or p38 MAPK and p44/42 MAPK was detected using polyclonal PKB and phospho-PKB antibody or polyclonal MAPK and phospho-MAPK antibody (BD, BD Biosciences). The filters were then washed and probed with horseradish peroxidase labeled rabbit or mouse IgG as secondary antibodies. The antibody-reactive bands were revealed by chemiluminescent detection (ECL Western detection kit, Amersham Pharmacia Biotech) according to the manufacturer's instructions. The bands of CHIT-1 were scanned and densitometrically analyzed with an imaging densitometer. Normalization of results was assured by running parallel Western blots with phosphorylation-independent antibodies or GAPDH antibody.

Chit ACTIVITY DETERMINATION

For Chit activity assay, cells were harvested using cell lysis buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, pH 8, 60 mM KCl, 1 mM PMSF, 0.5% NP40). The lysates were used for determination by fluorimetric method [Di Rosa et al., 2005]. Chit activity was measured by incubating 5 μl of undiluted lysates with 100 μl of a solution containing 22 μmol/L of the artificial substrate 4-methylumbelliferyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose (Sigma Chemical Co., Milan, Italy) in 0.5 M citrate-phosphate buffer, pH 5.2, for 15 min at 37°C. The reaction was stopped by using 2 ml of 0.5 mol/L Na_2CO_3-$NaHCO_3$ buffer, pH 10.7. The fluorescence was read by a Perkin-Elmer fluorimeter, on 365 nm excitation and 450 nm emissions. Chit activity was measured as nanomoles of substrate hydrolyzed per ml per hour (nmol/ml/h). Samples with a Chit activity >110 nmol/ml/h were measured again after a dilution of 10- or 50-fold with distilled water.

STATISTICAL ANALYSIS OF THE DATA

The densitometric data of RT-PCR and Western blot bands corresponding to the treated groups were expressed as a percentage of the means of the control values considered as 100%. The statistical analysis was performed using ANOVA. The threshold for statistically significant differences was set at $P < 0.05$.

RESULTS

EFFECT OF WORTMANNIN AND LY-294002 ON PRL-INDUCED EXPRESSION OF CHIT-1 mRNA AND OF Chit PROTEIN IN HMMs

As PRL has been shown to activate the signaling transduction element PI3-K [Berlanga et al., 1997; Bole-Feysot et al., 1998] was investigated the effect of the two PI3-K inhibitors, wortmannin and LY-294002 on the PRL induced CHIT-1 expression in HMMS. Since in our previous studies we observed that the induction of CHIT-1 was more significant with 25 ng/ml of PRL within 4 h of treatment, these experimental conditions were used in the following researches. Wortmannin and LY-294002 in a concentration of 200 nM and 25 μM, respectively, were able to prevent PRL-induced CHIT-1 expression (Fig. 1A, lanes 4 and 6). The densitometric analysis of PRL, wortmannin and LY-294002 effects on CHIT-1 RNA expression is shown in Figure 1B. To assess whether the inhibition of CHIT-1 mRNA was associated with the decrement in Chit protein, the effect of the two PI3-K inhibitors, wortmannin and LY-294002, was investigated also by Western blot analysis on cell lysates obtained from HMMs. Wortmannin and LY-294002 in a concentration of 200 nM and 25 μM, respectively, were able to prevent PRL-induced Chit protein (Fig. 1C, lanes 4 and 6). The densitometric analysis of PRL, wortmannin and LY-294002 effects on protein expression is shown in Figure 1D. Thus, PI3-K transduction element appears critical for PRL-mediated effect on CHIT-1 expression in HMMs.

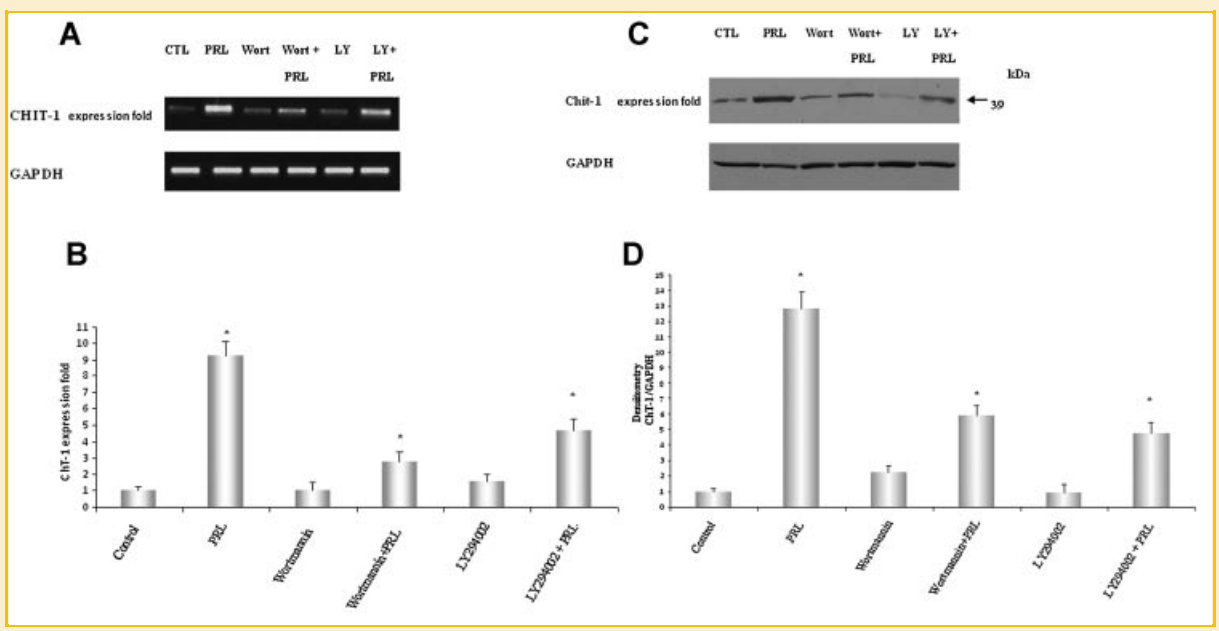

Fig. 1. A: Detection of CHIT-1 expression by RT-PCR of RNA obtained from HMMS pretreated with PI3-K inhibitors as indicated in Materials and Methods Section. Lanes: (1) Negative control; (2) PRL (25 ng/ml); (3) Wortmannin (200 nM); (4) Wortmannin (200 nM) + PRL (25 ng/ml); (5) LY-294002 (25 μM); (6) LY-294002 (25 μM) + PRL (25 ng/ml). B: The level of Chit mRNA normalized to GAPDH. C: Detection of Chit protein by Western blot from cell lysates of HMMS pretreated with PI3-K inhibitors as indicated in Materials and Methods Section. Lanes: (1) Negative control; (2) PRL (25 ng/ml); (3) Wortmannin (200 nM); (4) Wortmannin (200 nM) + PRL (25 ng/ml); (5) LY-294002 (25 μM); (6) LY-294002 (25 μM) + PRL (25 ng/ml). D: The level of Chit proteins normalized to GAPDH. Data are representative of three independent experiments. Statistical analysis was performed by Student's *t*-test; $^{*}P < 0.05$, of pretreated cells versus PRL group values.

EFFECT OF SB203580 AND U0126 ON PRL-INDUCED EXPRESSION OF CHIT-1 mRNA AND OF Chit PROTEIN IN HMMs

To determine whether MAPK activation is required for PRL-induced modulation of CHIT-1 expression, HMMs were treated with SB203580, a specific inhibitor of the MAPK p38, or with U0126, an inhibitor of MAPK p44/42. SB203580 and U0126 in a concentration of 20 and 10 μM, respectively, were able to prevent PRL-induced CHIT-1 expression (Fig. 2A, lanes 4 and 6). The densitometric analysis of PRL, SB203580, and U0126 effects on CHIT-1 RNA expression is shown in Figure 2B. The effect of SB203580 and U0126 was investigated also on PRL-induced Chit protein expression in HMMs. Pre-treatment with SB203580 (20 μM) and U0126 (10 μM), before the addition of PRL (25 ng/ml), reduced PRL-induced *CHIT-1* gene transcription (Fig. 2C, lanes 4 and 6). The densitometric analysis of PRL, wortmannin, and LY-294002 effects on Chit protein expression is shown in Figure 2D. This result confirmed that the MAPK pathway is also involved in PRL-induced CHIT-1 expression in HMMs.

EFFECT OF PRL ON MAPK PHOSPHORYLATION ON PRL-INDUCED EXPRESSION OF Chit PROTEIN IN HMMs

To confirm whether MAPK activation is required for the PRL-induced modulation of Chit expression, HMMs were treated with PRL (25 ng/ml) at different time intervals after 12 h of serum starvation. Western analysis using phospho- and non-phospho-MAPK antibody showed that phosphorylation of MAPK was induced at 15′ and 30′ PRL, which disappeared at 120′ (Fig. 3, lanes 3 and 4). This result suggested that this hormone activated MAPK in HMMs.

EFFECT OF ROTTLERIN AND OF GENISTEIN ON PRL-INDUCED EXPRESSION OF CHIT-1 mRNA AND OF Chit PROTEIN IN HMMs

To investigate if PKC is involved in PRL-induced modulation of CHIT-1 expression, the effect of rottlerin, an inhibitor of PKC, was investigated on PRL-induced CHIT-1 expression in HMMs. Rottlerin in a concentration of 5 μM was not able to prevent PRL-induced CHIT-1 expression (Fig. 4A). This result showed that the PKC pathway is not involved in PRL induced CHIT-1 expression in HMMs. The densitometric analysis of PRL, rottlerin effects on CHIT-1 expression is shown in Figure 4B. We evaluated also the involvement of PTK in PRL-induced modulation of Chit protein expression in HMMs. The PTK inhibitor, genistein (10 μM/ml) prevented PRL induced up-regulation of the enzyme (Fig. 4A), thus demonstrating that PRL induces CHIT-1 expression via PTK activation. The densitometric analysis of PRL and genistein effects on CHIT-1 expression is shown in Figure 4B. We next investigated the effect of rottlerin on PRL-induced Chit protein expression. Rottlerin in a concentration of 5 μM was not able to prevent PRL-induced Chit protein expression (Fig. 4C), confirming that the PKC pathway is not involved in PRL-induced Chit expression in HMMs. In contrast, the PTK inhibitor genistein (10 μM/ml) prevented PRL induced up-regulation of the enzyme (Fig. 4C). The densitometric analysis of PRL, rottlerin, and genistein effects on Chit protein expression is shown in Figure 4D.

EFFECT OF PP2 AND AG490 ON PRL-INDUCED EXPRESSION OF CHIT-1 mRNA AND OF Chit PROTEIN IN HMMs

To establish if Src family kinases are involved in the PRL-induced CHIT-1 expression, the selective Src inhibitor PP2 (100 nM) was

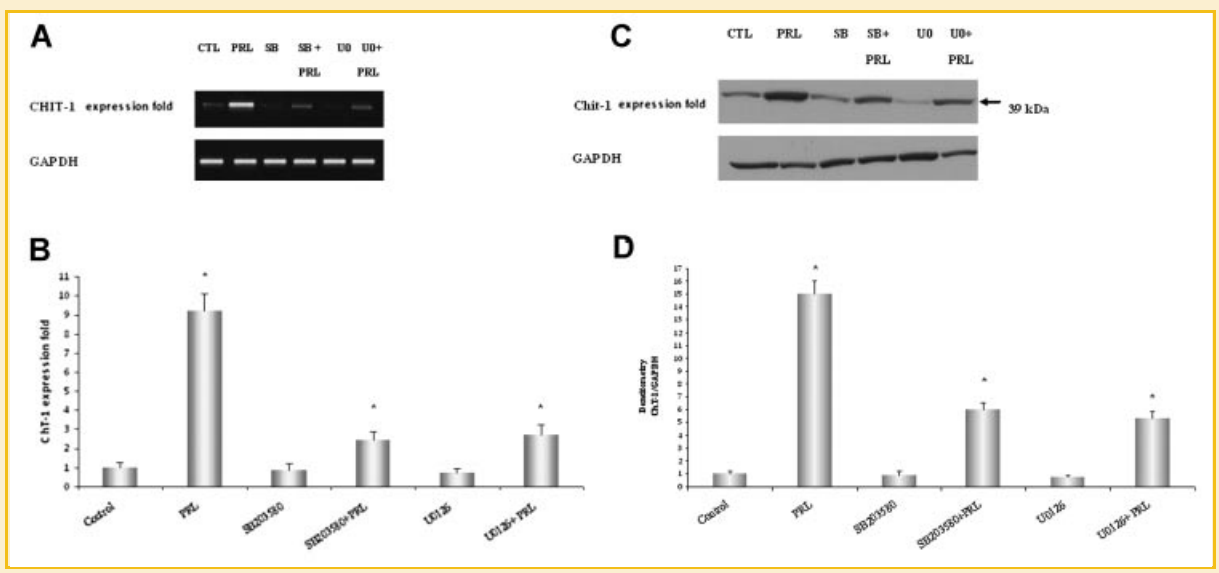

Fig. 2. A: Detection of CHIT-1 expression by RT-PCR of RNA obtained from HMMs pretreated with MAPK inhibitors as indicated in Materials and Methods Section. Lanes: (1) Negative control; (2) PRL (25 ng/ml); (3) SB202190 (20 μM); (4) SB202190 (20 μM) + PRL (25 ng/ml); (5) U0126 (10 μM); (6) U0126 (10 μM) + PRL (25 ng/ml). B: The level of Chit mRNA normalized to GAPDH. C: Detection of Chit protein by Western blot from cell lysates of HMMS pretreated with MAPK inhibitors as indicated in Materials and Methods Section. Lanes: (1) Negative control; (2) PRL (25 ng/ml); (3) SB202190 (20 μM); (4) SB202190 (20 μM) + PRL (25 ng/ml); (5) U0126 (10 μM); (6) U0126 (10 μM) + PRL (25 ng/ml). D: The level of Chit proteins normalized to GAPDH. Data are representative of three independent experiments. Statistical analysis was performed by Student's *t*-test; $^{*}P < 0.05$, of pretreated cells versus PRL group values.

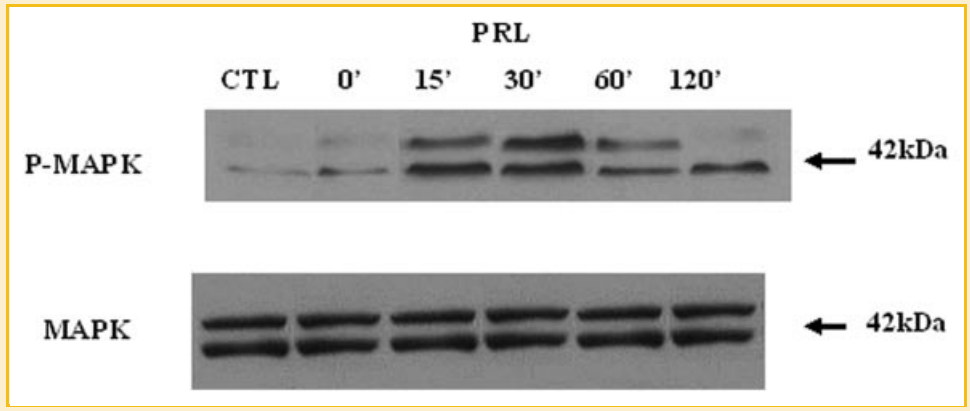

Fig. 3. Effect exerted by PRL (25 ng/ml) at 0, 5, 15, 30, 60, and 120 min on MAPK phosphorylation (upper blot) and on total MAPK (lower blot) in HMMs.

investigated in HMMs. PP2 was not able to reduce PRL CHIT-1 induced up-regulation (Fig. 5A). Given that PRL has been shown to activate the signaling pathway of Janus kinase/signal transducer and activator of transcription (Jak/Stat) the effect of a specific inhibitor of JAK/STAT pathway, AG490 was investigated PRL-induced CHIT-1 expression in HMMs. The pre-treatment of AG490 in a concentration of 10 μM was not able to prevent PRL-induced CHIT-1 expression (Fig. 4A). The densitometric analysis of PRL, PP2, and AG490 effects on CHIT-1 expression is shown in Figure 5B. The selective Src inhibitor PP2 (100 nM) was not able to reduce PRL Chit protein induced up-regulation (Fig. 5C). The pre-treatment of the specific inhibitor of JAK/STAT pathway, AG490 in a concentration of 10 μM was not able to prevent PRL-induced Chit protein level (Fig. 5C). The densitometric analysis of PRL, PP2, and AG490 effects on Chit expression is shown in Figure 5D.

EFFECT OF PRL, OF THE TWO MAPK INHIBITORS SB203580 AND U0126 AND OF THE TWO PI3-K INHIBITORS WORTMANNIN AND LY-294002, ON PRL-INDUCED PKB (AKT) PHOSPHORYLATION

As it has been demonstrated that PKB (Akt) can be the downstream target of both PI3-K and MAPK pathways [Baudhuin et al., 2002], the effect of PRL, of the MAPK inhibitors SB203580 and U0126 and of the two PI3-K inhibitors, wortmannin and LY-294002, on PKB phosphorylation was investigated. Phosphorylation of PKB was induced by PRL, this phosphorylation was prevented by MAPK inhibitors SB203580 and U0126 and by the two PI3-K inhibitors

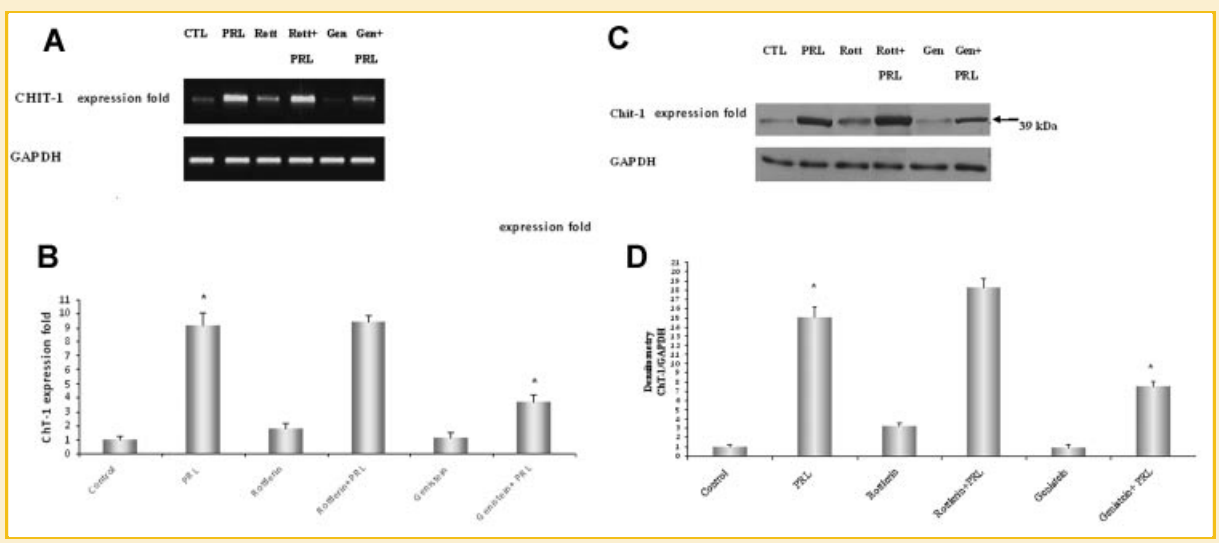

Fig. 4. A: Detection of CHIT-1 expression by RT-PCR of RNA obtained from HMMs pretreated with PKC and PTK inhibitors as indicated in Materials and Methods Section. Lanes: (1) Negative control; (2) PRL (25 ng/ml); (3) Rottlerin (5 μM); (4) Rottlerin (5 μM) + PRL (25 ng/ml); (5) genistein (10 μM); (6) genistein (10 μM) + PRL (25 ng/ml). B: The level of CHIT-1 mRNA normalized to GAPDH. Data are representative of three independent experiments. Statistical analysis was performed by Student's *t*-test; $^{*}P < 0.05$, of pretreated cells versus PRL group values. C: Detection of Chit protein by Western blot from cell lysates of HMMS pretreated with PKC and PTK inhibitors as indicated in Materials and Methods Section. Lanes: (1) Negative control; (2) PRL (25 ng/ml); (3) Rottlerin (5 μM); (4) Rottlerin (5 μM) + PRL (25 ng/ml); (5) genistein (10 μM); (6) genistein (10 μM) + PRL (25 ng/ml). D: The level of Chit proteins normalized to GAPDH. Data are representative of three independent experiments. Statistical analysis was performed by Student's *t*-test; $^{*}P < 0.05$, of pretreated cells versus PRL group values.

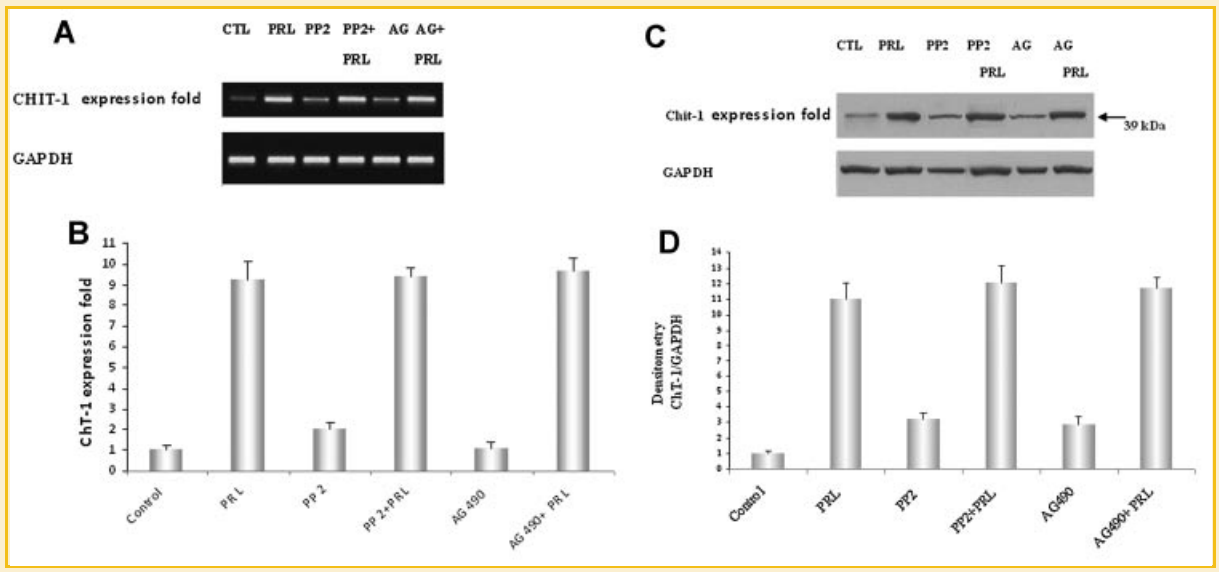

Fig. 5. A: Detection of CHIT-1 expression by RT-PCR of RNA obtained from HMMs pretreated with Src and JAK/STAT inhibitors as indicated in Materials and Methods Section. Lanes: (1) Negative control; (2) PRL (25 ng/ml); (3) PP2 (100 nM); (4) PP2 (100 nM); + PRL (25 ng/ml); (5) AG490 (10 μM); (6) AG490 (10 μM) + PRL (25 ng/ml). B: The level of CHIT-1 mRNA normalized to GAPDH. C: Detection of Chit protein by Western blot from cell lysates of HMMS pretreated with Src and JAK/STAT inhibitors as indicated in Materials and Methods Section. Lanes: (1) Negative control; (2) PRL (25 ng/ml); (3) PP2 (100 nM); (4) PP2 (100 nM); + PRL (25 ng/ml); (5) AG490 (10 μM); (6) AG490 (10 μM) + PRL (25 ng/ml). D: The level of Chit proteins normalized to GAPDH. Data are representative of three independent experiments. Statistical analysis was performed by Student's *t*-test.

wortmannin and LY-294002 at the concentrations of 20 μM, 10 μM, 200 nM, and 25 μM, respectively (Fig. 6A,B). These results showed that the PI3-K and MAPK pathways are involved in PRL-induced PKB activation in HMMs.

EFFECT OF WORTMANNIN, LY-294002, SB203580, U0126 GENISTEIN ON BASAL AND PRL-INDUCED Chit ACTIVITY IN HMMs

Experiments were carried out to verify whether also the pharmacological inhibitors of PI3-K, MAPK, and PTK inhibited the enzymatic Chit activity. In our previous studies we observed that Chit activity reached the maximum increase within 8 h of PRL stimulation [Malaguarnera et al., 2004], therefore we performed the assay using this point. As shown in Figure 7 the two selective inhibitors of PI3-K, wortmannin (200 nM) and LY-294002 (25 μM), the MAPK inhibitor SB203580 (20 μM) and U0126 (10 μM), and the PTK inhibitor genistein (10 μM) prevented PRL-induced Chit activity in lysates of HMMs. Taken together, these data confirm that PI3-K, MAPK, and PTK signaling pathways are involved in the PRL-mediated CHIT-1 induction. In contrast, rottlerin, AG490, and PP2, did not affect Chit activity.

DISCUSSION

Our previous studies have shown that in vitro treatment of HMMs with 25 ng PRL up-regulate *CHIT-1* gene expression reaching the maximum within 4 h of PRL treatment [Malaguarnera et al., 2004]. In this manuscript we investigated the signaling transduction pathway involved in PRL-mediated CHIT-1 induction in HMMs. Macrophages respond to infection or injury from a resting cellular

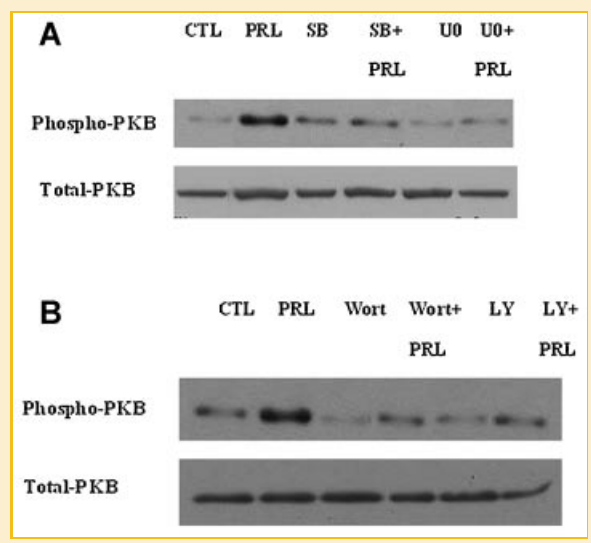

Fig. 6. Effects of the PRL and of PI3-K inhibitors, wortmannin, and LY-294002, and of the MAPK inhibitors, SB202190 and U 0126, on the PKB phosphorylation. A: Lanes: (1) Negative control; (2) PRL (25 ng/ml); (3) SB202190 (20 μM); (4) SB202190 (20 μM) + PRL(25 ng/ml); (5) U0126 (10 μM); (6) U0126 (10 μM) + PRL(25 ng/ml). B: Lanes: 1 Negative control; (2) PRL(25 ng/ml); (3) Wortmannin (200 nM); (4) Wortmannin (200 nM) + PRL(25 ng/ml); (5) LY-294002 (25 μM); (6) LY-294002 (25 μM) + PRL(25 ng/ml). Phosphorylation of PKB (upper blot) and on total PKB (lower blot) in HMMs. Each western blot is representative of three independent experiments.

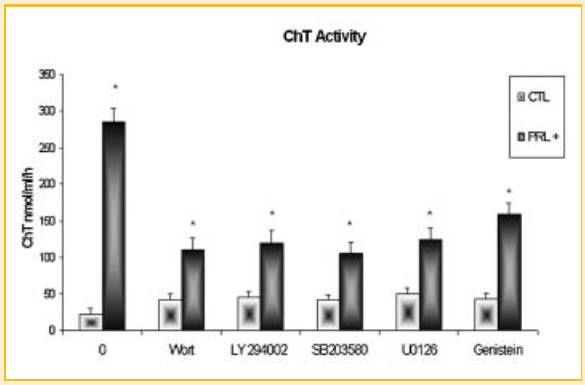

Fig. 7. Effect of wortmannin, LY-294002, SB203580, U0126 genistein on both basal and PRL-induced Chit activity in lysates of HMMs. Each bar is the mean ± SE of six values obtained in three different experimental sections: percentage values which are significantly different ($P < 0.05$) as compared with the control value; $^{*}P < 0.05$ versus PRL group values.

phenotype to an activated state that is characterized by cytotoxic effectors functions. The transition from resting to an activated state is regulated by cytokines and or pathogenic signals. Once activated, macrophages other than large amount of reactive nitrogen and reactive oxygen species produce CHIT-1; which is currently regarded as marker of macrophages activation. Activation of phagocitosis and subsequent superoxide anion (O_2^-) production as a killing mechanism of pathogens has been observed after PRL administration [Malaguarnera et al., 2004]. Additionally macrophages show an enhanced activity of CHIT-1 following PRL treatment [Malaguarnera et al., 2004]. The enhanced productions of pro-inflammatory cytokines are central in the regulatory role of CHIT-1 and induction of immune response [Malaguarnera et al., 2003, 2005; van Eijk et al., 2005]. The inducible nature of CHIT-1 suggests it can add to inflammatory responses, such as oxidative stress and other inflammatory mediators [Malaguarnera et al., 2005; Malaguarnera, 2006]. A lot of evidence points towards a role of CHIT-1 as a significant element of cellular resistance mechanism against external pathogens [van Eijk et al., 2005; Malaguarnera, 2006]. Other reports show that CHIT-1 can serve as a biomarker PKC [Hollak et al., 1994], or focused on the regulation of this intriguing enzyme [Boot et al., 1999; Pham et al., 2007; van Eijk et al., 2007]. Real functional data are lacking, which is in part caused by the factor that expression in men and mice differs. It has been shown that activation of several serine-threonine kinases including, MAPK [Tripathi and Sodhi, 2008], PKC [Gerlo et al., 2006], and S6 kinase [Bishop et al., 2006], PI3-K [Seriwatanachai et al., 2008] are implicated in PRL action. Therefore we explored the effect of PI3-K of MAPK and of PKC inhibitors. Our findings show that the PRL induced expression of *CHIT-1* gene was strongly reduced in HMMs pretreated with PI3-K inhibitor wortmannin and LY-294002. Therefore, the induction of CHIT-1 by PRL treated HMMs entailed the involvement of PI3-K signaling pathway. MAP kinases constitute an important parameter in the signal transduction pathway leading macrophages activation. The MAPKs are a group of protein kinases that mediate the nuclear response of cells to a wide variety of extracellular stresses such as inflammatory cytokines, growth factors, pathogens, and oxidative stress [Cowan and Storey, 2003; Kaminska, 2005; McCubrey et al., 2006; Monge et al., 2006; Kendrick and Bogoyevitch, 2007]. Although three distinct subfamilies have been described, extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38, there is significant cross-talk between the pathways as well as common downstream targets [Whitmarsh et al., 1995]. The inhibitor of MAPK p38, SB203580, and the inhibitor of MEK/ERK, U0126, interfered with the effect of PRL. This finding suggested that candidate upstream signaling pathways for CHIT-1 regulation are MAPKs. Meanwhile, it was seen that genistein a PTK inhibitor decreased the production of Chit induced by PRL, suggesting a possible involvement of PTK activity in the PRL-dependent increased expression of CHIT-1 enzyme in HMMs. Similar inhibitory effect of these pharmacological inhibitors was also observed at the transcriptional and translational level of *CHIT-1* gene, thus supporting the hypothesis that these transduction components of signal pathways play a crucial role in the regulation of CHIT-1 expression. In contrast, pre-treatment with rottlerin an inhibitor of PKC was unable to prevent the PRL induced expression of *CHIT-1* gene. Regarding the molecular mechanism triggered by the binding of the PRL to its own receptors, it has been shown that the activation of the extracellular domain of PRL receptors involves ligand-induced sequential dimerization of the extracellular transmembrane and intracellular domains [Constantinescu et al., 2001]. This ligand-mediated activation of PRL receptor results in the phosphorylation of tyrosine residues of the receptor itself and of numerous soluble and membrane associated cellular proteins like Src family kinases [Fresno Vara et al., 2001]. Nevertheless, in the present study, a specific Src tyrosine kinase-blocking agent PP2 [Salazar and Rozengurt, 2001] was ineffective to prevent the up-regulation of PRL-induced expression of CHIT-1. In addition to the PI3-K and MAPK pathway, the most extensively studied signal transduction mechanism associated with activation of the PRLR is the Jak family of tyrosyl kinases and their coupling to downstream Stat transcription factors. The Jak/Stat signaling pathway has been implicated in the regulation of several PRL-inducible genes [Tripathi and Sodhi, 2008]. Once activated, STAT transcription factors, as homo- or heterodimers, bind to γ-interferon-activation sequences (GAS) in PRL-responsive genes [Brockman et al., 2002]. In our finding, blocking Jak/Stat kinases with AG490, there was no significant reduction in PRL induced *CHIT-1* gene. This observation suggested that either PKC or Jak pathways are not involved in CHIT-1 expression induced by PRL. PKB (Akt) is a protein kinase that in PRL responsive cells has been identified as a pro-survival and -proliferative signal pathway [Alessi and Cohen, 1998; Coffer et al., 1998] and might be the downstream target of both PI3-K and MAPK. In the present report, both the inhibition of PI3-K with wortmannin and LY-294002, and of MAPK with SB203580 and U0126 affected the prevention of PRL-induced PKB phosphorylation, suggesting that PKB may be involved in the PRL-induced CHIT-1 up-regulation. However, our results did not allow us to clarify whether, in the chain of the transduction events triggered by PRL activation, MAPK action on PKB follows PI3-K activation, or vice versa. Nevertheless, it is possible that the signal pathways activated by PRL can activate PKB phosphorylation in an independent fashion. The modulatory effect

exerted in HMMs by PRL on the expression of CHIT-1 is completely mirrored by the increased or reduced Chit activity when the activation of PRL and its transduction mechanisms were stimulated or inhibited, respectively. Additionally, the result showing that PI3-K and MAPK transduction elements appear critical for PRL-mediated effect on CHIT-1 expression in HMMs suggests that the constitutive *CHIT-1* gene regulatory function for C/EBPβ in differentiated macrophages could be a potential target of the PI3-K pathway. In conclusion, in the current study, we confirm that PRL is a potent inducer of CHIT-1 and provide, for the first time, the evidence that multiple signaling pathways are involved. Given that several evidence point towards a role of CHIT-1 induction in host immunosurveillance upon exposure to diverse pathogens, understanding the signaling pathways involved in CHIT-1 induction could lead to the development of more selective molecules, with immunomodulatory capacity favoring control over infections and other types of diseases.

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