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Activated α_2 Macroglobulin Induces Matrix Metalloproteinase 9 Expression by Low-Density Lipoprotein Receptor-Related Protein 1 Through MAPK-ERK1/2 and NF-κB Activation in Macrophage-Derived Cell Lines

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ABSTRACT

Macrophages under certain stimuli induce matrix metalloproteinase 9 (MMP-9) expression and protein secretion through the activation of MAPK-ERK and NF-κB signaling pathways. Previously, we demonstrated that activated α_2-macroglulin (α_2M^*) through the interaction with its receptor low-density lipoprotein receptor-related protein 1 (LRP1) induces macrophage proliferation mediated by the activation of MAPK-ERK1/2. In the present work, we examined whether α_2M^*/LRP1interaction could induce the MMP-9 production in J774 and Raw264.7 macrophage-derived cell lines. It was shown that α_2M^* promoted MMP-9 expression and protein secretion by LRP1 in both macrophage-derived cell lines, which was mediated by the activation of MAPK-ERK1/2 and NF-κB. Both intracellular signaling pathways activated by α_2M^* were effectively blocked by calphostin-C, suggesting involvement of PKC. In addition, we demonstrate that α_2M^* produced extracellular calcium influx via LRP1. However, when the intracellular calcium mobilization was inhibited by BAPTA-AM, the α_2M^*-induced MAPK-ER1/2 activation was fully blocked in both macrophage cell lines. Finally, using specific pharmacological inhibitors for PKC, Mek1, and NF-κB, it was shown that the α_2M^*-induced MMP-9 protein secretion was inhibited, indicating that the MMP production promoted by the α_2M^*/LRP1 interaction required the activation of both signaling pathways. These findings may prove useful in the understanding of the macrophage LRP1 role in the vascular wall during atherogenic plaque progression. J. Cell. Biochem. 111: 607–617, 2010.

KEY WORDS: α-MACROGLOBULINS; LOW-DENSITY LIPOPROTEIN RECEPTOR; PROTEINASE; CELL SIGNALING

Alpha-2-macroglobulin (α_2M) is a plasma proteinase inhibitor with broad specificity that is structurally formed by a tetramer composed of two non-covalently associated dimmers of disulfide-linked identical subunits (~180 kDa). α_2M is characterized by a proteolysis-sensitive bait region sequence and an internal β-cysteinyl-γ-glutamyl thiol ester bond per subunit, which are susceptible to cleavage at the bait region by endopeptidases and to nucleophilic attack by monoamines at the thiol ester bonds [Chu and Pizzo, 1994]. Consequently, α_2M undergoes an extensive conformation, alteration, and compacting to become an activated form, designated α_2M^*, which recognizes the specific cell surface receptor, low-density lipoprotein receptor-related protein 1 (LRP1), a member of the LDL receptor gene family. However, α_2M^* only recognizes LRP1 and not any other LDL receptor members [Hussain et al., 1999]. In addition, α_2M^* is internalized by LRP1, implying that both molecules are involved in the modulation of the extracellular activity of several proteases [Sanchez et al., 1998; Herz and Strickland, 2001; Ramos et al., 2002].

LRP1 is a cell surface glycoprotein synthesized as a 600-kDa proreceptor and post-translationally processed into 515-kDa α-subunit and 85-kDa β-subunit that remain associated through non-covalent interactions [Herz and Strickland, 2001]. It is a typical scavenger receptor, which in addition to binding α_2M^*, also binds and internalizes multiple structurally and functionally diverse ligands

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including *Pseudomonas* exotoxin, lipoprotein lipase, apolipoprotein E-enriched lipoproteins, urokinase- and tissue-type plasminogen activator (either alone or in complexes with plasminogen inhibitors), tissue factor pathway inhibitor, lactoferrin, thrombospondin, and lipoprotein(a). These ligands do not compete with each other for binding, except for the receptor-associated protein (RAP), which blocks the binding of all known ligands to the receptor [Herz and Strickland, 2001]. Furthermore, it has been reported that LRP1 promotes intracellular signaling, which downstream mediates proliferation, migration, and differentiation of different types of cells, including macrophages, vascular smooth muscle cells, and neurons [Chiabrando et al., 2002; Barnes et al., 2003; Strickland and Ranganathan, 2003; Hu et al., 2006; Bonacci et al., 2007]. Related to this, we have previously demonstrated that α_2M^*/LRP1 interaction induces cellular proliferation of macrophages, which is mediated by MAPK-ERK1/2 activation [Bonacci et al., 2007]. Several other studies have shown that LRP1 is involved in the formation and development of atherosclerotic lesions, although it is still disputed whether LRP1 expressed in macrophages has detrimental or protective atherogenic properties [Boucher et al., 2003; Zhu et al., 2003; Spijkers et al., 2005; Hu et al., 2006].

An important amount of evidence has indicated that macrophages under certain stimuli induce matrix metalloproteinase 9 (MMP-9) expression and protein secretion through the activation of MAPK-ERK and NF-κB signaling pathways [Benbow and Brinckerhoff, 1997; Moon et al., 2004; Han et al., 2006]. MMP-9 expression in inflammatory cells, particularly in macrophages, mediates cell migration and proliferation by promoting extracellular matrix remodeling [Brown et al., 1995; Blankenberg et al., 2003]. Considering that α_2M^*/LRP1 interaction induces macrophage proliferation mediated by the activation of intracellular signaling pathways, we hypothesize that α_2M^* may also regulate MMP-9 expression in macrophages. Thus, in the present work, we examined whether α_2M^*/LRP1 interaction could induce MMP-9 production in J774 and Raw264.7 macrophage-derived cell lines. Our results demonstrated that α_2M^* promoted MMP-9 protein secretion in both macrophage cells mediated by LRP1. In addition, we also showed that α_2M^*/LRP1 interaction implied PKC activation and intracellular calcium mobilization, which downstream mediated MAPK-ERK1/2 and NF-κB activation as well as MMP-9 production.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Mouse J774 and Raw264.7 macrophage-derived cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 mg/ml) at 5% CO_2, 95% humidity, and 37°C. α_2M was purified from human plasma following a procedure previously described [Chiabrando et al., 1997]. The activated form of α_2M (α_2M^*) was generated by incubating α_2M with 200 mM methylamine–HCl for 6 h at pH 8.2, as previously reported [Chiabrando et al., 2002]. An expression construct, encoding RAP as a glutathione *S*-transferase (GST) fusion protein (GST-RAP), was kindly provided by Dr. Guojum Bu (Washington University, St Louis, MO). GST-RAP was expressed and purified as described elsewhere [Bu et al., 1995] and used without further modification. Immunoblots were performed with the following primary antibodies: mouse monoclonal anti-phosphorylated ERK1/2 (anti-p-ERK1/2), anti-NF-κB/p65, anti-IκBα and anti-Grp78, as well as rabbit polyclonal anti-total ERK1/2, anti-HDAC1, anti-α-tubulin, and anti-β-actin, which were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-β-subunit LRP1 (clon 5A6) antibody was kindly donated by Dr. Dudley Strickland (University of Maryland, Scholl of Medicine, MD). Rabbit polyclonal antibody against the latent (pro-MMP-9) and active form of MMP-9 was purchased from Calbiochem Merck4 Biosciences (Darmstadt, Germany). The following inhibitors, calphostin-C (PKC inhibitor), PD98059 (Mek1-ERk1/2 inhibitor), and Bay 11-7082 were obtained from Sigma–Aldrich (St. Louis, MO). The calcium chelator BAPTA-AM and the fluorescent calcium indicator Fura 2-AM were also from Sigma–Aldrich.

GELATIN ZYMOGRAPHY

J774 and Raw264.7 cells (1×10^6 cells) were cultured in RPMI 1640 medium containing glutamine, penicillin, streptomycin, and 10% FBS, for 24 h at 37°C in a humidified CO_2 (5%) incubator as described above. After aspirating the medium, fresh serum-free medium was added and cultured for 2 h. Then, cells were cultured with various concentrations of α_2M^* for 24 h and the conditioned cell media (CCM) were collected. The cellular debris was eliminated by centrifugation and the MMP-9 activity was measured by gelatin zymography. The following inhibitors were evaluated: GST-RAP (50–400 nM), calphostin-C (100 μM), PD98059 (10 μM), and Bay 11-7082 (1 μM), which were added to the cell culture 30 min previous to α_2M^* stimulus. Cell extracts corresponding to the same CCM were prepared, and Western blot for total ERK1/2 was performed as control of the cellular amount. Aliquots of CCM containing MMP-9 were assayed by gelatin zymography in 7.5% SDS–PAGE gel containing 0.15% (w/v) gelatin [Kleiner and Stetler-Stevenson, 1994]. After electrophoresis, the gel was washed with 2.5% of Triton X100 and incubated with the enzyme buffer (50 mM Tris, 0.2 M sodium chloride, and 5 mM calcium chloride, pH 7.6) for 24 h to develop the zymography. Gelatinolytic activity of MMP-9 was visualized as clear bands against a blue-stained background, which were quantified by densitometric analysis using image software (UVP Vision Works ® LS Image Acquisition and Analysis Software, Upland, CA). The data were expressed as the densitometric intensity of MMP-9 activity relative to total ERK1/2, and compared to a control assay performed without α_2M^* stimulation.

WESTERN BLOT ASSAYS

J774 and Raw264.7 cells (1×10^6 cells) were cultured in RPMI 1640 medium containing glutamine, penicillin, streptomycin, and 10% FBS, for 24 h at 37°C in a humidifed CO2 (5%) incubator as described above. After aspirating the medium, fresh serum-free medium was added and cultured for 2 h. Then, cells were cultured with 20 nM α_2M^* for 15 min. Cell extracts were prepared using RIPA buffer [10 mM phosphate buffer saline (PBS), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycolate, 0.1% sodium dodecyl sulfate (SDS), 0.2% sodium azide (NaN_3), and 0.1% Nonidet NP-40 (octyl phenoxylpolyethoxylethanol)], and analyzed by SDS–PAGE. The membranes were probed, following the manufacturer's blotting instructions with primary specific antibodies for p-ERK1/2, NF-κB/p65 protein, and

IκBα. Treatments were normalized in parallel by assessment of the protein loading, using total ERK1/2, α-tubulin, HDAC1, or β-actin. The membranes were then incubated with secondary horseradish peroxidase-conjugated antibodies, before the specific bands were revealed by chemiluminescence reaction and quantified by densitometric analysis as mentioned above. To study the inhibitory effect on intracellular signaling of GST-RAP (200 nM), PD98059 (10 μM), calphostin-C (100 μM), Bay 11-7082 (1 μM), and BAPTA-AM (30 μM), these compounds were added for 30 min in serum-free medium prior to the addition of α_2M^*.

REVERSE TRANSCRIPTASE-PCR SPECIFIC FOR MMP-9 mRNA

To evaluate the effect of α_2M^* on the specific mRNA for MMP-9, J774, and Raw264.7 cells were plated into 24 wells and grown to 70–80% confluence. After aspirating the medium, a fresh serum-free one was added and cultured for 2 h. Then, cells were cultured with 60 nM α_2M^* for different periods of times (up to 24 h) at 37°C, before being lysated with a solution containing 38% (v/v) saturated phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium isothiocyanate, 0.1 M sodium acetate, and 0.5% (v/v) glycine. Total RNA was extracted by a single-step method using an RneasyR Mini kit (Qiagen, Chatsworth, CA), following the manufacturer's instructions. A reverse transcription polymerase chain reaction (RT-PCR) was used to measure the changes in the transcript level of MMP-9. Templates of total RNA were obtained using random hexaprimers. Specific primers for MMP-9 and GADPH were: MMP-9 sense primer 50-CTGCCCCAGTGAGAGACTCTACAC-30 and anti-sense primer 50-GAATGATCTAAGCCCAGCGCCAT-30; GADPH sense primer 50-TGAAGGTCGGTGTGAACGGA-30 and anti-sense primer 50-ATGC-CAAAGTTGTCATGGATGAC-30. The conditions for analyzing MMP-9 and GADPH mRNA were as previously described [Ceschin et al., 2009]. Briefly, the PCR conditions used for the mRNA MMP-9 analysis consisted of 27 cycles of amplification (annealing at 58°C for 60 s, extension at 72°C for 45 s, and denaturation at 94°C for 60 s). The PCR products were evaluated in a 2% agarose gel–ethidium bromide, and the intensity of the bands was analyzed using the UVP image analyzer as above.

MEASUREMENT OF INTRACELLULAR CALCIUM CONCENTRATION

$[Ca^{2+}]_i$ in J774 and Raw264.7 cells was measured using the fluorescent indicator Fura-2/AM [Grynkiewicz et al., 1985]. Cells (1×10^6) incubated overnight in RPMI 1640 medium were harvested and the cell suspension was cooled to room temperature. Then, Fura-2/AM (1–1.5 μM) was added and incubated at room temperature for 30 min in the dark. The cell suspension was washed twice with HHBSS (without calcium ions) before being placed in a quartz-glass cuvette (0.1 cm × 1.0 cm) with weak stirring and measured in a standard fluorometer with dual excitation (340 and 380 nm) and emission (450 nm). After collecting the baseline data, α_2M^* (20–60 nM final concentration) was added to the cuvette. The temperature was maintained at 37°C using an air curtain incubator. To obtain $[Ca^{2+}]_i$, the following equation was applied [Grynkiewicz et al., 1985]:

$$[(Ca^2+)_i] = kd \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{(F_{min})}{(F_{max})}$$

where $[(Ca^{2+})_i]$ is the intracellular calcium concentration; Kd is the dissociation constant of Fura 2-calcium ion complex; R represents the fluorescent intensity ratio (to 450 nm) obtained from dual 340 nm/380 nm excitation; R_{min} is the minimum ratio obtained when the extracellular calcium was totally complexed with 1 mM EGTA; R_{max} is the maximum ratio obtained when the extracellular calcium concentration was 1.0 mM and the cells were stimulated with 5 μM ionomycin; F_{min} represents the fluorescent intensity (to 450 nm) from excitation (to 380 nm) when the extracellular calcium was totally complexed with 1 mM EGTA; and F_{max} is the fluorescent intensity (to 450 nm) from excitation (to 380 nm) when the extracellular calcium concentration was 1.0 mM and the cells were stimulated with 5 μM ionomycin. To reach an extracellular calcium ion concentration of 1.0 mM, an adequate volume of calcium chloride stock solution (10.0 mM) was added to the cuvette. To examine whether the α_2M^* effect was mediated by LRP1, GST-RAP (final concentration of 200 nM) was added to the cuvette 1 min previous to the α_2M^* stimulus.

ANALYSIS OF NUCLEAR AND CYTOPLASMIC NF-κB AND IκBα DEGRADATION

J774 and Raw264.7 cells (1×10^6 cells) were cultured in RPMI 1640 medium containing glutamine, penicillin, streptomycin, and 10% FBS, for 24 h at 37°C in a humidifed CO_2 (5%) incubator as described above. After aspirating the medium, fresh serum-free medium was added and cultured for 2 h. Then, cells were cultured with 20 or 60 nM α_2M^* for different times, and NF-κB/p65 detection was examined in cytoplasmic and nuclear extracts. To obtain total cell lysates, J774 or Raw264.7 cells were resuspended in 50–200 μl of RIPA buffer, disrupted by passages through a 25-G needle, and incubated on ice for 30 min, followed by removal of DNA and cell debris by centrifugation at 10,000*g* for 20 min at 4°C. Nuclear and cytoplasmic extracts were obtained by subcellular fractionation, essentially as described elsewhere [Schreiber et al., 1989]. The supernatant containing cytoplasm was collected and frozen at 70°C or used immediately. The nuclear pellet was resuspended in 50 μl of ice-cold buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF), and the tube was vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a Microfuge at 4°C, and the supernatant was frozen in aliquots at 70°C or used immediately. Western blot for NF-κB/p65 in cytoplasmic and nuclear extracts, as well as for IκBα degradation in total cell lysates, was carried out as indicated above. To evaluate whether calphostin-C and GST-RAP abrogated the IκBα induced by α_2M^*, these compounds were added to the cell culture 30 min previous to α_2M^* stimulus.

STATISTICAL TREATMENT OF DATA

Comparison of quantitative data was performed using the nonparametric Mann–Whitney *U*-test.

RESULTS

α_2M^* INDUCES MMP-9 EXPRESSION THROUGH LRP1 INTERACTION IN MACROPHAGE-DERIVED CELL LINES

In order to test the possibility that α_2M^* is involved in MMP-9 expression in macrophages, we evaluated the MMP-9 protein

secretion in J774 and Raw264.7 macrophage-derived cell lines cultured in the presence of different concentrations of α_2M^* for 24 h. By using zymographic assays, it was demonstrated that α_2M^* induced a significant increase in MMP-9 activity in a dose-dependent manner, with more pro-MMP-9 being secreted than active MMP-9 (Fig. 1A). This increased activity of MMP-9 was confirmed by Western blot using a specific antibody against pro-MMP-9 and active MMP-9, which showed that α_2M^* induced MMP-9 protein secretion in a dose-dependent manner in J774 cells (Fig. 1B). In contrast, both zymography and Western blot showed that MMP-2 protein secretion was not modified when J774 or Raw264.7 cells were cultured in the presence of α_2M^* (data not shown). In addition, by RT-PCR, cells cultured in the presence of α_2M^* (60 nM) demonstrated a significant MMP-9 mRNA expression after 2 h of stimulus (Fig. 1C). We next evaluated whether α_2M^*-induced MMP-9 protein secretion was mediated by its interaction with LRP1, by pretreating macrophage cells with different concentrations of RAP. Figure 2 shows that RAP, from 200 nM, significantly blocked the MMP-9 protein secretion induced by α_2M^* in J774 cells. Similar results were also observed for Raw264.7 cell cultures (data not shown). Hence, taken together, from these results we can conclude that the α_2M^*/LRP1 interaction induced MMP-9 expression and protein secretion in J774 and Raw264.7 macrophage-derived cell lines.

MAPK-ERK1/2 ACTIVATION INDUCED BY α_2M^*/LRP1 INTERACTION IS MEDIATED BY PKC AND INTRACELLULAR CALCIUM MOBILIZATION

Previously, we showed that α_2M^* induced MAPK-ERK1/2 activation by LRP1 in J774 cells [Bonacci et al., 2007]. This intracellular signaling pathway activation was fully blocked by the presence of RAP and PD980059 (a Mek1 inhibitor). Moreover, an important amount of evidence suggests that the intracellular signaling activity of LRP1 is regulated by the association of the intracytoplasmic domain (ICD) of LRP1-β-subunit with distinct intracellular adaptor proteins [Gotthardt et al., 2000; Stolt and Bock, 2006]. Related to this, it has been previously demonstrated that the calcium-dependent PKCα isoform modulates the endocytic and signaling function of LRP1 by phosphorylation of its ICD [Ranganathan et al., 2004]. Hence, in the present work, we decided to evaluate whether PKC are involved in the α_2M^*-induced MAPK-ERK1/2 activation, by pretreating both J774 and Raw264.7 cells for 30 min with calphostin-C (100 μM), a broad inhibitor of all PKC isoforms, previous to the addition of α_2M^* (60 nM). Figure 3 shows that calphostin-C fully blocked the MAPK-ERK1/2 phosphorylation induced by α_2M^* in J774 and Raw264.7 cells. Thus, these results indicate that the MAPK-ERK1/2 phosphorylation induced by α_2M^*/LRP1 interaction was mediated by PKC activation.

Several reports have shown that α_2M^* can increase intracellular calcium in macrophages [Misra et al., 1994, 1996], although this

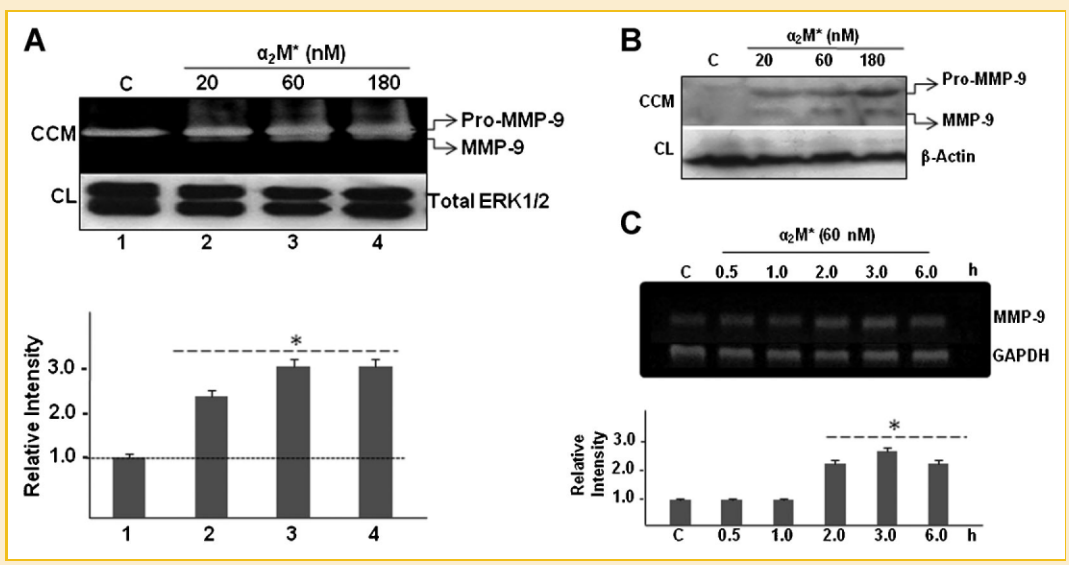

Fig. 1. A: Zymographic analysis of MMP-9 in the conditioned cell medium (CCM), obtained from J774 cells cultured in the presence of different α_2M^* concentrations for 24 h. Thirty microliters of CCM were loaded in SDS–PAGE 10% and zymography was performed as described in the Materials and Methods Section. The Pro-MMP-9 and active MMP-9 forms are indicated. Total ERK1/2 was used as the loading control of cell lysates (CL) for each plate well of the J774 cell culture, which was detected by Western blot using rabbit polyclonal anti-ERK1/2 and revealed by peroxidase-conjugated antibodies and enhanced chemiluminescence. The quantitative analysis by densitometry is shown below, where relative intensity represents the densitometric unit of the zymography band respect to the ERK1/2 band and normalized respect to control. Bars represent the mean ± SEM from triplicate experiments. The asterisk denotes statistical differences respect to control with a *P*-value <0.05. B: Western blot for MMP-9 of CCM from J774 cells cultured in the presence of different concentrations of α_2M^* for 24 h. Two milliliters of CCM were lyophilized and then reconstituted to 30 μl with sample buffer and loaded in SDS–PAGE 10%, and Western blot was performed as described in the Materials and Methods Section. The protein loading control of β-actin is also shown, which was analyzed from CL obtained from each plate well of J774 cells. C: Analysis of RT-PCR for MMP-9 in α_2M^*-treated J774 cells. Total RNA was extracted from J774 cells treated for 0.5, 1.0, 2.0, 3.0, and 6.0 h with serum-free medium or 20 nM α_2M^*, as described in the Materials and Methods Section. The PCR products were evaluated in a 2% agarose gel–ethidium bromide and the bars show the relative intensity of MMP-9/GAPDH with respect to controls, representing the mean ± SEM from triplicate experiments. The asterisk denotes statistical differences respect to control for a *P*-value <0.05.

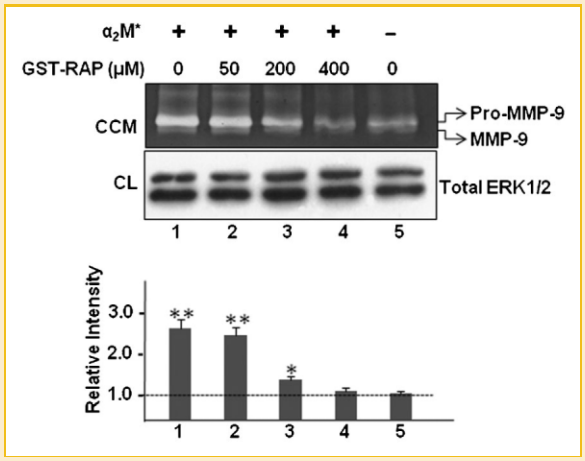

Fig. 2. Zymographic analysis of MMP-9 in the conditioned cell medium (CCM), obtained from J774 cells cultured in the presence of 20 nM α_2M^* for 24 h. Different concentrations of GST-RAP were added to the J774 cell culture 30 min previous to α_2M^* stimulation. Thirty microliters of CCM were loaded in SDS–PAGE 10%, and zymography was performed as described in the Materials and Methods Section. The Pro-MMP-9 and active MMP-9 forms are indicated. Total ERK1/2 was used as loading control of cell lysates (CL) of each plate well of the J774 cell culture, which was detected by Western blot using rabbit polyclonal and ERK1/2 and revealed by peroxidase-conjugated antibodies and enhanced chemiluminescence. The quantitative analysis by densitometry is shown below, where the relative intensity represents the densitometric unit of the zymography band respect to the ERK1/2 band and normalized respect to control. Bars denote the mean ± SEM from triplicate experiments. The asterisks show statistical differences respect to control for a *P*-value <0.05 (*) and <0.001 (**), respectively.

mechanism apparently involves a second α_2M^* receptor not inhibited by RAP, termed α_2M^* signaling receptor/Grp78 [Misra et al., 2005]. In the present work, we investigated whether α_2M^* increased the intracellular calcium concentration ($[Ca^{2+}]_i$) in macrophage-derived cell lines using a quantitative spectrofluorometric assay with fluorescent indicator Fura 2-AM. Different experiments were carried out in the absence or presence of extracellular calcium (1 mM $CaCl_2$) in the cellular medium. Figure 4A shows that α_2M^* added to cell cultures containing extracellular calcium produced a rapid rise in $[Ca^{2+}]_i$, reaching levels ~2-fold higher than that of cell controls. On the other hand, the absence of extracellular calcium α_2M^* did not produce a significant rise of $[Ca^{2+}]_i$ respect to control. In addition, when J774 cells were pretreated with RAP previous to α_2M^* stimulus in the presence of extracellular calcium, $[Ca^{2+}]_i$ was not significantly modified respect to control. Similar results were also obtained in Raw264.7 cells (data not shown). Thus, our data indicate that α_2M^* induces extracellular calcium influx in macrophage-derived cells, which was mediated by LRP1. In order to evaluate whether this calcium influx induced by α_2M^* was involved in the MAPK-ERK1/2 activation, we pretreated J774 and Raw264.7 cells with a cell-permeable intracellular calcium chelator, BAPTA-AM (30 μM), for 30 min, and then analyzed the ERK1/2 phosphorylation induced by α_2M^*. Figure 4B shows that BAPTA completely abrogated the α_2M^*-induced MAPK-ERK1/2 phosphorylation. Thus, we conclude that α_2M^*/LRP1 interaction induced the MAPK-ERK1/2 phosphorylation by calcium influx and PKC activation.

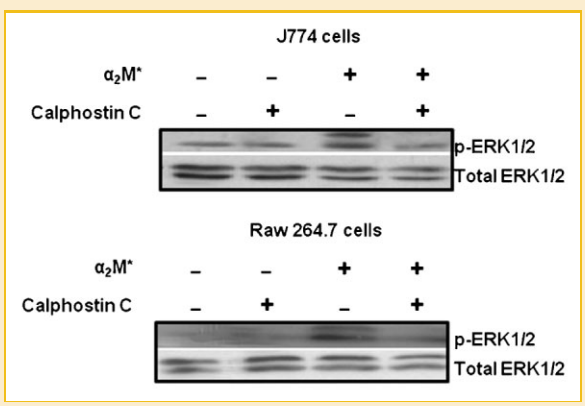

Fig. 3. Western blot for ERK1/2 of J774 and Raw 264.7 cells cultured in the presence of α_2M^* and calphostin C. Previous to α_2M^* stimulus (20 nM for 15 min) J774 cells were pretreated with 100 μM calphostin C. Cell lysates were loaded to SDS–PAGE 10% and electrotransferred to nitrocellulose membrane. Primary antibodies anti p-ERK1/2 and total ERK1/2 (protein loading control) were used, and Western blot was applied as described in the Materials and Methods Section.

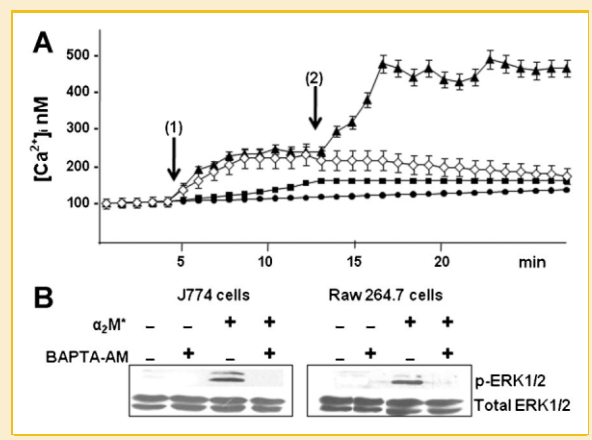

Fig. 4. A: Analysis of intracellular calcium concentration $[Ca^{2+}]_i$ in J774 cells stimulated with 20 nM α_2M^*. The $[Ca^{2+}]_i$ was monitored using the fluorescent calcium ion indicator Fura-2 as described in the Materials and Methods Section. The arrows indicate the time (1 and 2). The symbols represent: (●) baseline obtained in J774 cells in the absence of α_2M^* stimulus; (■) J774 cells incubated in the absence of extracellular calcium with α_2M^* stimulus at the times indicated in (1) and (2); (▲) J774 cells incubated in the presence of 1 mM Ca^{2+} and α_2M^*, stimulated at the times indicated in (1) and (2); (◇) J774 cells incubated in the presence of 1 mM Ca^{2+} with α_2M^* stimulus at the time indicated in (1). In (2), 400 nM GST-RAP was added 1 min previous to α_2M^* stimulus. The error bars are indicated and represent the mean ± SEM of triplicate experiments. B: Western blot for ERK1/2 of J774 and Raw 264.7 cells cultured in the presence of 20 nM α_2M^* for 15 min and BAPTA-AM. Previous to α_2M^* stimulus J774 cells were pretreated with 30 μM BAPTA-AM. Cell lysates were loaded in SDS–PAGE 10% and electrotransferred to the nitrocellulose membrane. Primary antibodies anti p-ERK1/2 and total ERK1/2 (protein loading control) were used, and Western blot was applied as described in the Materials and Methods Section.

α_2M*/LRP1 INTERACTION INDUCES THE NUCLEAR TRANSLOCATION OF NF-κB

MMP-9 expression and secretion is known to be enhanced by multiple mediators, although the upstream regulatory pathways are less well understood. Related to this, it has been previously demonstrated that MMP-9 expression and secretion can be regulated by the intracellular activation of MAPK-ERK1/2 and NFκB intracellular pathways [Van den Steen et al., 2002; Holvoet et al., 2003; Rhee et al., 2007a,b]. Although we have shown that α_2M*/LRP1 interaction can induce MAPK-ERK1/2 activation, the effect of α_2M* on NF-κB activation is still unknown. It is well established that the activity of NF-κB is tightly regulated by its inhibitors, the IκB family of proteins [Karin, 1999]. These inhibitory proteins bind to NF-κB dimers, thus masking their nuclear localization and resulting in the cytoplasmic retention of NF-κB [Baldwin, 1996]. Upon stimulation, IκB is phosphorylated and degraded via ubiquitination and the proteasome-mediated pathway, leading to the nuclear import of NF-κB, where it then binds to the cognate sequence in promoter regions of multiple genes. Thus, in the present work, we investigated whether the α_2M*/LRP1 interaction induced the nuclear translocation of NF-κB and IκB degradation in macrophage cells. Analysis of the cytoplasmic-nuclear shuttling of this transcription factor revealed a substantial decrease in NF-κB/p65 in the cytoplasmic fractions of α_2M*-treated cells compared with control (Fig. 5A). In contrast, there was an increase in NF-κB/p65 in the nuclear fractions of α_2M*-treated cells. Similar results were also observed in Raw264.7 cells (data not shown). As this nuclear translocation of NF-κB/p65 was associated with a decreased level of IκBα protein in α_2M*-treated cells (Fig. 5B), to evaluate whether LRP1 was mediating the α_2M*-induced IκBα degradation, J774 cells were pretreated with RAP. Figure 6 shows that RAP significantly blocked IκBα degradation in α_2M*-treated cells. Then, considering that PKC activation was mediating the α_2M*-induced ERK1/2 phosphorylation, we examined whether PKC was also involved in IκBα degradation in α_2M*-treated cells. Figure 6 shows that claphostin-C abrogated the IκBα degradation in J774 cells cultured in the presence of α_2M*. However, PD98059 did not interfere with the α_2M*-induced IκBα degradation, indicating that Mek1-ERK1/2 was not involved in NF-κB activation (Fig. 6). Similar results were also observed in Raw264.7 cells (data not shown). Thus, we conclude that α_2M*/LRP1 interaction activated the NF-κB signaling pathway in macrophage-derived cell lines, which may have involved the putative participation of PKC activation.

α_2M*-INDUCED MAPK-ERK1/2 AND NF-κB ACTIVATION DOWNSTREAM MEDIATES MMP-9 PRODUCTION IN MACROPHAGE CELLS

To gain insights into the mechanisms involved in α_2M*-induced MMP-9 protein secretion, we investigated the potential role of PKC, MAPK-ERK1/2, and NF-κB in this process activated by α_2M*. Both J774 and Raw264.7 cells were treated with the following pharmacological inhibitors: calphostin-C, PD98059, and Bay 11-

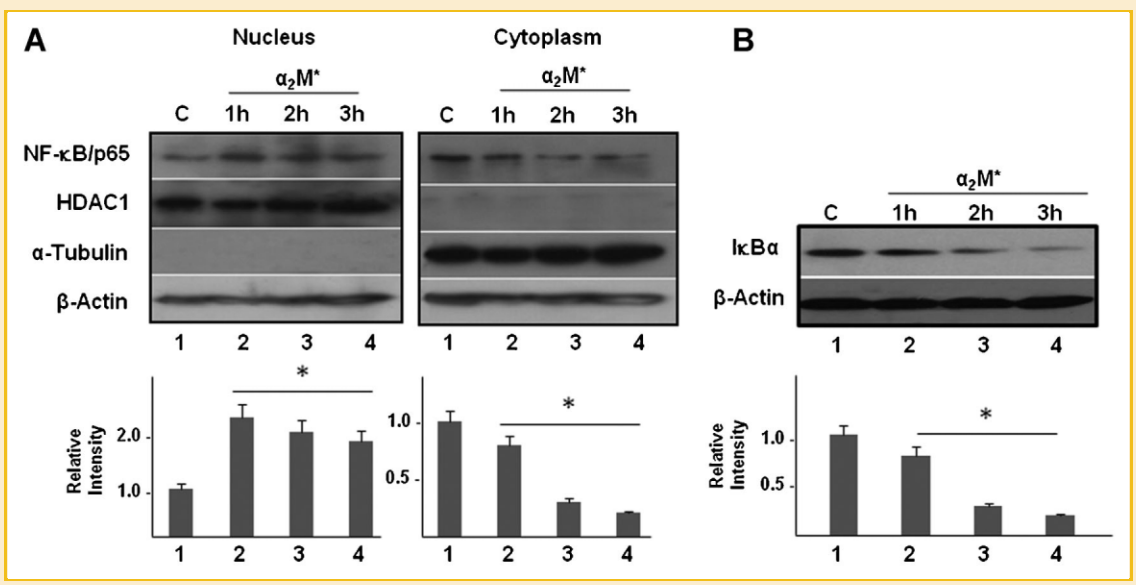

Fig. 5. A: Control of the cytoplasmic-nuclear shuttling of NF-κB by α_2M* in J774 cells. Cells were stimulated with 20 nM α_2M* for the indicated times. Nuclear and cytoplasmic cell extracts (40 μg) were used for immunodetection of NF-κB/p65. Anti-α-tubulin and HDAC1 antibodies were used to control the purity of subcellular fractions. Western blot was carried out as described in the Materials and Methods Section. Densitometric analysis of immunoreactive protein bands are expressed as relative intensity calculated from the densitometric profile of the NF-κB/p65 signal normalized to β-actin. Bars represent the mean ± SEM of triplicate experiments. The asterisk shows statistical differences respect to control with a *P*-value <0.05. B: Western blot for IκBα in J774 cells cultured in the presence of 20 nM α_2M* for the indicated times. Cell lysates were loaded to SDS–PAGE 10% and electrotransferred to the nitrocellulose membrane. The primary anti-IκBα antibody was used, and Western blot was applied as described in the Materials and Methods Section. β-actin is shown as the protein loading control. Densitometric analysis of the immunoreactive protein bands is expressed as relative intensity calculated from the densitometric profile of the IκBα signal normalized to β-actin. Bars represent the mean ± SEM of triplicate experiments. The asterisk shows statistical differences respect to control with a *P*-value <0.05 (*).

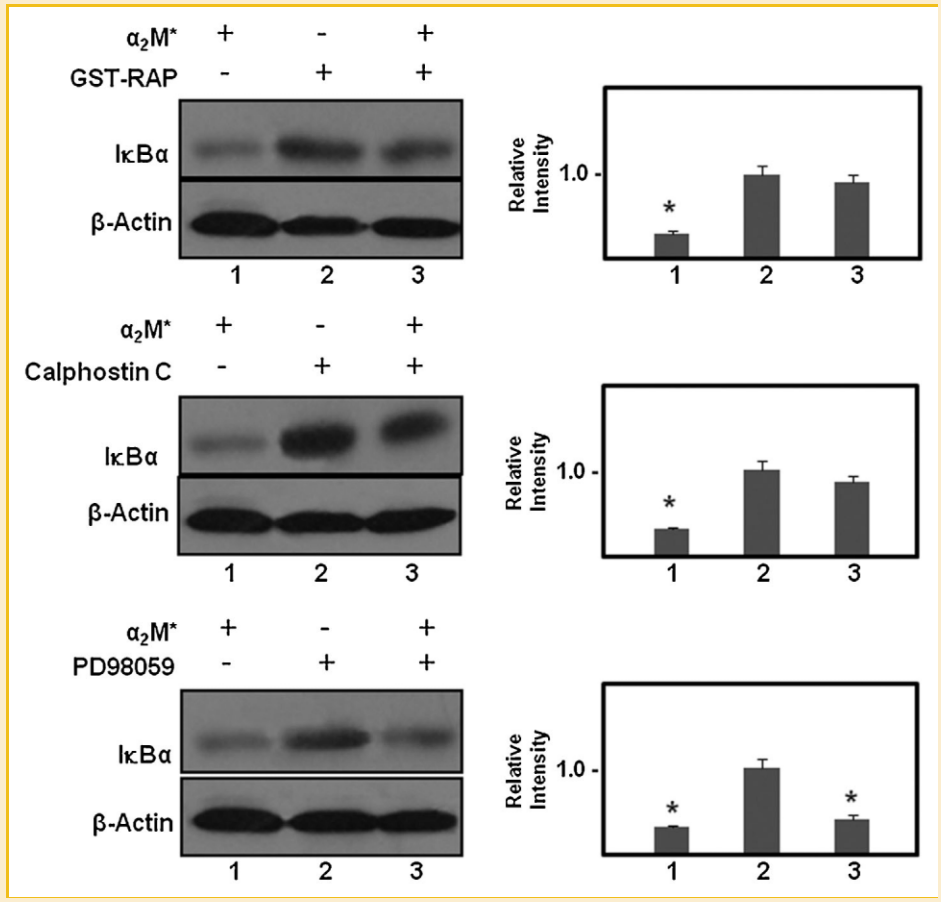

Fig. 6. Western blot for IκBα in J774 cells cultured in the presence of α_2M^* and GST-RAP, calphostin C, and PD98059. Previous to α_2M^* stimulus (20 nM for 1 h), J774 cells were cultured in the presence of 400 nM GST-RAP, 100 μM calphostin C, or 20 μM PD98059 for 30 min. Cell lysates were loaded in SDS–PAGE 10% and electrotransferred to the nitrocellulose membrane. The primary anti-IκBα antibody was used, and Western blot was applied as described in the Materials and Methods Section. β-actin is shown as the protein loading control. Densitometric analysis of the immunoreactive protein bands is expressed as relative intensity calculated from the densitometric profile of the IκBα signal normalized to β-actin and related to control without α_2M^* stimulus as 1.0. Bars represent the mean ± SEM of triplicate experiments. The asterisk shows statistical differences respect to control with a *P*-value <0.05.

7082 (a specific NF-κB inhibitor), previous to α_2M^* stimulus. Figure 7 shows a representative zymographic assay, where it can be observed that the PKC inhibitor significantly blocked the α_2M^*-induced MMP-9 protein secretion. A similar inhibitory effect on the α_2M^*-induced MMP-9 protein secretion was observed with PD98059. These data indicate that PKC and MAPK-ERK1/2 pathway activation are mediating the MMP-9 production induced by the α_2M^*/LRP1 interaction. In addition, when we evaluated the functional role of NF-κB in the α_2M^*-induced MMP-9 production, zymography revealed that Bay 11-7082 effectively blocked the MMP-9 protein secretion (Fig. 8). Moreover, the three inhibitors also abrogated the MMP-9 mRNA expression induced by α_2M^* (Fig. 8). Thus, taken together, these results show that the intracellular signaling pathway, activated by the α_2M^*/LRP1 interaction and characterized by a PKC-dependent activation of MAPK-ERK1/2 and NF-κB pathways, promoted an increased production of MMP-9 in macrophage-derived cell lines. Furthermore, both intracellular signaling pathways activated by the α_2M^*/LRP1 interaction were necessary for MMP-9 production in J774 and Raw264.7 macrophage-derived cell lines.

DISCUSSION

In a previous report, we demonstrated that the α_2M^*/LRP1 interaction induced MAPK-ERK1/2 activation, which downstream promoted an enhanced H^3-thymidine incorporation in the J774 macrophage-derived cell line [Bonacci et al., 2007]. In the present work, we showed that the α_2M^* promoted MMP-9 expression and protein secretion by LRP1 in the J774 and Raw264.7 macrophage-derived cell lines. In addition, this α_2M^*-induced MMP-9 production was mediated by multiple activation of intracellular signaling pathways, characterized by MAPK-ERK1/2 and NF-κB activation. Interestingly, both the intracellular signaling pathways activated by α_2M^* were effectively blocked by calphostin-C, which strongly suggests that the activation of PKC was involved in this signaling event and in MMP production. Furthermore, we demonstrated that α_2M^* produced extracellular calcium influx, thereby promoting a rapid rise of $[Ca^{2+}]_i$, via LRP1. However, when the intracellular calcium mobilization was inhibited by BAPTA-AM, the α_2M^*-induced MAPK-ER1/2 activation was fully blocked in both macrophage cells. Finally, using specific pharmacological inhibitors

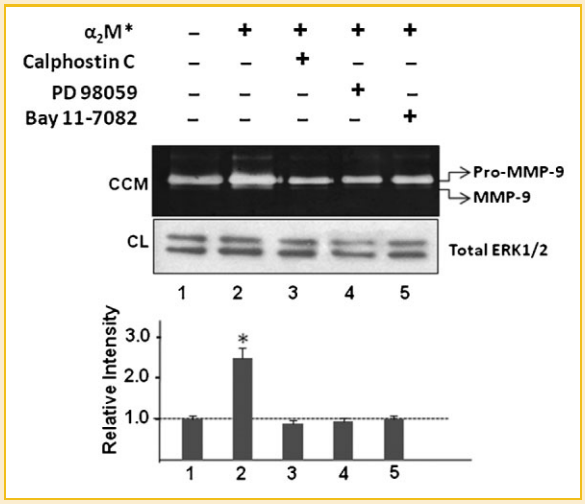

Fig. 7. Zymographic analysis of MMP-9 in the conditioned cell medium (CCM), obtained from J774 cells cultured in the presence of α_2M^* and calphostin C, PD98059, and Bay 11-7082. Previous to α_2M^* stimulus (20 nM for 24 h), 100 μM calphostin C, 20 μM PD98059, and 1 μM Bay 11-7082 were added for 30 min. Thirty microliters of CCM were loaded in SDS–PAGE 10% and zymography was performed as described in the Materials and Methods Section. The Pro-MMP-9 and active MMP-9 forms are indicated. Total ERK1/2 was used as the loading control of cell lysates (CL) of each plate well of the J774 cell culture, which was detected by Western blot using rabbit polyclonal and ERK1/2 and revealed by peroxidase-conjugated antibodies and enhanced chemiluminescence. The quantitative analysis by densitometry is shown below, where the relative intensity represents the densitometric unit of the zymography band respect to the ERK1/2 band and normalized respect to control. Bars denote the mean ± SEM of triplicate experiments. The asterisk shows statistical differences respect to control with a *P*-value <0.05.

for PKC, Mek1, and NF-κB resulted in the α_2M^*-induced MMP-9 protein secretion being inhibited, indicating that the MMP production promoted by the α_2M^*/LRP1 interaction required the activation of these signaling pathways.

MMP-9 expression is enhanced by multiple mediators which stimulate MMP-9 gene transcription, including PMA, TNF-α, and bacterial products such as LPS and CpG-ODN [Van den Steen et al., 2002; Holvoet et al., 2003; Rhee et al., 2007b]. Binding sites for the transcription factors AP-1, NF-κB, Ets, and Sp1 have been identified in the proximal promoter of the MMP-9 gene. Of these, the proximal AP-1 site is usually required for MMP-9 expression [Sato and Seiki, 1993], with the NF-κB and Sp1 sites also being necessary for the induction of the MMP-9 gene in response to PMA and TNF-α [Van den Steen et al., 2002]. However, the upstream regulatory pathways that control the expression and secretion of MMP-9 are very complex and not well understood. It has been established that the AP-1 transcriptional activity is specifically regulated by MAPK, which involves ERKs, JNKs, and p38 MAPK. In particular, MAPK-ERK1/2 plays a key role in the MMP-9 expression and protein secretion induced by homocysteine [Moshal et al., 2006]. Recently, it has also been reported that the glucocorticoid-induced TNF receptor family related protein ligand (GITRL), which is known to interact with its cognate receptor GITR, induced MMP-9 expression and protein secretion through the activation of MAPK-ERK1/2 and NF-κB [Bae et al., 2008]. This GITRL-induced MMP-9 production involved cross-talk activation between both signaling pathways, since NF-κB was dependent on previous MAPK-ERK1/2 activation. In the present work, we demonstrated that α_2M^* induced MAPK-ERK1/2 and NF-κB activation by LRP1 in J774 and Raw264.7 macrophage-derived cell lines. However, both signaling pathways were activated upstream by PKC, and the inhibition of the MAPK-ERK1/2 or NF-κB pathway effectively blocked the MMP-9 production induced by α_2M^*. Thus, our results suggest that α_2M^*/LRP1 interaction activates different transcriptions factors to induce MMP-9 expression in macrophage-derived cells, including NF-κB. However, further studies need to be carried out in order to investigate which MAPK-activated transcription factors are involved in this MMP gene expression induced by α_2M^*. In a previous report, we showed that the α_2M^*/LRP1 interaction also activated JNK [Bonacci et al., 2007]. Therefore, we suggest that the AP-1 transcription factor is involved, in addition to MAPK-ERK1/2 activation, in the MMP-9 production in macrophages.

An important amount of evidence has shown that several adaptor proteins, which harbor phosphotyrosine-binding (PTB) domains, bind the second or distal NPxY motif within the ICD of the LRP1 β-subunit [Boucher et al., 2002; Ranganathan et al., 2004]. Also, it has been reported that the conventional PKC isoform, PKCα, induces serine and threonine phosphorylations of the ICD of the LRP1 β-subunit, which are needed for tyrosine phosphorylation and interaction with the adaptor protein Shc [Lillis et al., 2005]. This allows the recruitment of the Ras small GTPase protein, with subsequent activation of MAP kinases. Interestingly, here we demonstrated that the signaling pathway activation induced by the α_2M^*/LRP1 interaction required a previous activation of PKC. This, together with the ability of α_2M^* to induce intracellular calcium mobilization, suggests that a calcium-dependent PKC isoform may be activated in this event. In this way, it has been reported that α_2M^* was able to increase the intracellular calcium in macrophages [Misra et al., 2002], although this mechanism involved a second α_2M^* receptor (not inhibited by RAP) termed α_2M^* signaling receptor/Grp78 [Misra et al., 2005]. However, in the present work, we demonstrated that α_2M^* increased $[Ca^{2+}]_i$, which was fully blocked by RAP. In addition, by Western blot, we were unable to detect Grp78 in the protein extracts of J774 and Raw264.7 cells, whereas LRP1 was detected in both types of cells (data not shown and Supplementary Fig. 1). Thus, our data indicate that LRP1 was mediating the calcium influx induced by α_2M^* in macrophage-derived cell lines. From these results, we propose that the PKC activation induced by the α_2M^*/LRP1 interaction in macrophages involves calcium influx. However, the mechanisms by which α_2M^*/LRP1 interaction can induce this PKC activation are not understood. Therefore, we are currently performing new studies to investigate these putative mechanisms.

It has been widely reported that different PKC isoforms are key mediators of MAPK-ERK1/2 and NF-κB activation [Das and White, 1997; Lallena et al., 1999; Catley et al., 2004; Holden et al., 2008]. In particular, conventional PKC isoforms can mediate the activation of the MAPK-ERK1/2 pathway. In the present work, we showed that MAPK-ERK1/2 phosphorylation as well as the IκB degradation induced by the α_2M^*/LRP1 interaction are inhibited by calphostin-C.

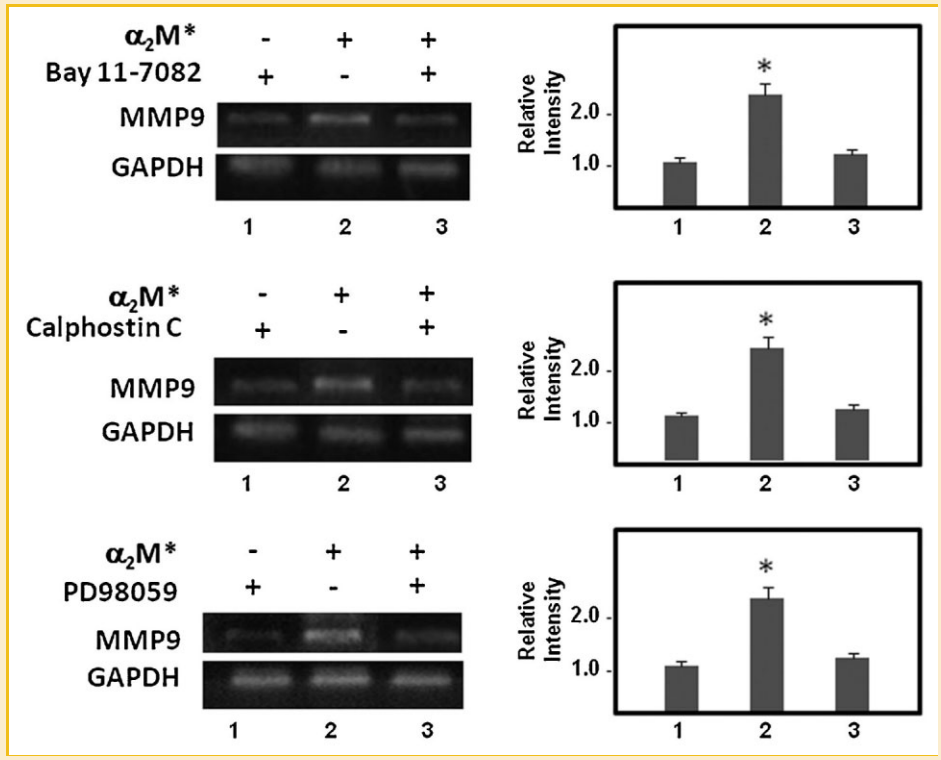

Fig. 8. Analysis of RT-PCR for MMP-9 in α_2M^*-treated J774 cells. Total RNA was extracted from J774 cells treated for 1 h with 20 nM α_2M^*. Previous to α_2M^* stimulus, 1 μM Bay 11-7082, 20 μM PD98059, and 100 μM calphostin C were added for 30 min. The PCR products were evaluated in a 2% agarose gel–ethidium bromide and the bars show the relative intensity of MMP-9/GAPDH with respect to controls, representing the mean ± SEM of triplicate experiments. The asterisk shows statistical differences respect to control with a *P*-value <0.05.

However, whereas Bay 11-11078 effectively blocked the IκB degradation induced by α_2M^*, the PD98059 Mek1 inhibitor was unable to affect this process, suggesting that: (i) the PKC activation induced by α_2M^* activated MAPK and NF-κB signaling pathways in an independent manner, and (ii) the Mek1-ERK1/2 activation did not modulate the NF-κB activation induced by α_2M^* in macrophages.

It is known that macrophages play a key role in atherogenesis due to the fact that these cells are tissue transformed to foam cells by the action of an increased internalization of oxidized lipoproteins [Linton and Fazio, 2003]. Furthermore, it is well established that macrophages are responsible for producing MMP-9 by the action of inflammatory mediators, which promotes extracellular matrix remodeling and atherogenic plaque progression [Gough et al., 2006]. However, it is still disputed whether LRP1 in macrophages has detrimental [Zhu et al., 2003; Spijkers et al., 2005] or protective [Boucher et al., 2003; Hu et al., 2006] atherogenic properties. Although α_2M and LRP1 can regulate the proteolytic activity of different proteinases, including MMPs [Arbelaez et al., 1997], it has been proposed that α_2M^* can act as a growth factor, having properties which activate macrophages [Misra et al., 1993]. From our results, we propose that α_2M^*/LRP1 induced MMP-9 production in macrophages, which could be crucial in the progression of atherosclerotic lesions.

In summary, we have demonstrated for the first time that α_2M^* induced MMP-9 production through interaction with LRP1 in macrophages, which was mediated by calcium influx and PKC followed by MAPK-ERK1/2 and NF-κB activation. These findings may prove useful in the understanding of the macrophage LRP1 role in the vascular wall during atherogenic plaque progression.

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