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Journal of Cellular Biochemistry 106:372–380 (2009)

Journal of Cellular Biochemistry

Insulin Induces the Low Density Lipoprotein Receptor-Related Protein 1 (LRP1) Degradation by the Proteasomal System in J774 Macrophage-Derived Cells

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ABSTRACT

Low-density lipoprotein receptor-related protein 1 (LRP1) is an endocytic receptor, which binds and internalizes diverse ligands such as activated α_2-macroglobulin (α_2M^*). LRP1 promotes intracellular signaling, which downstream mediates cellular proliferation and migration of different types of cells, including macrophages. Unlike the LDL receptor, LRP1 expression is not sensitive to cellular cholesterol levels but appears to be responsive to insulin. It has been previously demonstrated that insulin increases the cell surface presentation of LRP1 in adipocytes and hepatocytes, which is mediated by the intracellular PI_3K/Akt signaling activation. The LRP1 protein distribution is similar to other insulin-regulated cell surface proteins, including transferring receptor (Tfr). However, in macrophages, the insulin effect on the LRP1 distribution and expression is not well characterized. Considering that macrophages play a central role in the pathogenesis of atherosclerosis, herein we evaluate the effect of insulin on the cellular expression of LRP1 in J774 macrophages-derived cells using Western blot and immunofluorescence microscopy. Our data demonstrate that insulin induces a significant decrease in the LRP1 protein content, without changing the specific mRNA level of this receptor. Moreover, insulin specifically affected the protein expression of LRP1 but not Tfr. The insulin-induced protein degradation of LRP1 in J774 cells was mediated by the activation of the PI_3K/Akt pathway and proteasomal system by an enhanced ubiquitin–receptor conjugation. The decreased content of LRP1 induced by insulin affected the cellular internalization of α_2M^*. Thus, we propose that the protein degradation of LRP-1 induced by insulin in macrophages could have important effects on the pathogenesis of atherosclerosis. J. Cell. Biochem. 106: 372–380, 2009.

KEY WORDS: ATHEROSCLEROSIS; α-MACROGLOBULIN; ENDOCYTIC RECEPTORS; LDL RECEPTORS; UBIQUITIN

Low-density lipoprotein receptor-related protein 1 (LRP1) is a cell surface glycoprotein synthesized as a 600-kDa pro-receptor and post-translationally processed into 515- and 85-kDa subunits that remain associated through non-covalent interactions [Herz and Strickland, 2001]. LRP1 is an endocytic receptor which binds and internalizes multiple structurally and functionally diverse ligands including activated α_2-macroglobulin (α_2M^*), *Pseudomonas* exotoxin A, lipoprotein lipase, apolipoprotein E-enriched lipoproteins, urokinase- and tissue-type plasminogen activators alone or in complexes with plasminogen inhibitors, tissue factor pathway inhibitor, lactoferrin, thrombospondin, and lipoprotein(a) [Gotthardt et al., 2000]. However, 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]. Although generally considered to be an endocytic receptor, 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 (SMCs), and neurons [Barnes et al., 2003; Strickland and Ranganathan, 2003; Hu et al., 2006a; Bonacci et al., 2007]. These cellular events have been associated with different pathological processes such as atherosclerosis and Alzheimer's disease [Llorente-Cortes and Badimon, 2005; Boucher et al., 2007; Carter, 2007; Liu et al., 2007].

Grant sponsor: Secretaría de Ciencia y Tecnología, Universidad Nacional de Córdoba (SECyT); Grant numbers: 162/06, 69/08; Grant sponsor: Agencia Nacional de Ciencia de la República Argentina (FONCyT: BID 1728/OC-AR PICT); Grant numbers: 05-13945, 01207; Grant sponsor: Consejo de Investigaciones Científicas y Tecnológicas de la República Argentina (CONICET) PIP 05-06; Grant number: 5421.

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Received 11 September 2008; Accepted 12 November 2008 • DOI 10.1002/jcb.22014 • 2008 Wiley-Liss, Inc.
Published online 29 December 2008 in Wiley InterScience (www.interscience.wiley.com).

The regulation of LRP1 activity is complex. Unlike the LDL receptor, the LRP1 expression is not sensitive to cellular cholesterol levels, but appears to be responsive to hormones and growth factors [Kutt et al., 1989]. In this way, insulin stimulates recycling of LRP1 in adipocytes from an endosomal pool to the plasma membrane, which is regulated in a PI_3K-dependent manner without affecting the specific mRNA level for LRP1 [Ko et al., 2001; Zhang et al., 2004]. This receptor recycling is associated with an increased cell surface presentation, accompanied by a decrease in the LRP1 level in the low-density microsomes. The magnitude of the insulin-stimulated cell surface presentation of LRP1 is similar to that of transferring receptor (Tfr), but is much less than that of the glucose transporter protein, GLUT4 [Ko et al., 2001]. Recently, it was proposed that LRP1 plays a critical role in adipocytes as a regulator of energy homeostasis, since functional disruption leads to reduced lipid transport, increased insulin sensitivity, and muscular energy expenditure [Hofmann et al., 2007]. On the other hand, a rapid increase in the LRP1 mRNA level has been shown to occur when peritoneal macrophages were cultured in the presence of insulin for ~1 h of incubation [Misra et al., 1999]. This elevated expression was accompanied by an enhanced ability to bind α_2M^*, which was associated with an increased cell surface presentation of LRP1. However, in macrophages, in contrast to adipocytes, the insulin effect on the LRP1 distribution and expression is not well characterized.

An important number of studies have demonstrated that ubiquitination and subsequent protein degradation by the proteasomal system play a key role in the control and activity of several receptor-mediated endocytosis [reviewed in Dupre et al., 2004]. Related to this, it has been proposed that the cellular turnover of LRP1 is regulated by the proteasomal system [Melman et al., 2002]. In addition, it has been reported that insulin stimulates the ubiquitination and degradation of IRS-2 in multiple cell types via a PI_3K/Akt/mTOR-dependent pathway, which correlated closely with the inhibition of insulin signaling. This negative feedback mechanism might limit the magnitude and duration of IRS-2-mediated signals, and contribute to insulin resistance associated with hyperglycemia and hyperinsulinemia [Rui et al., 2001, 2002].

Cardiovascular diseases are the leading cause of morbidity and mortality in the Western world. The primary cause of cardiovascular diseases is atherosclerosis, which is characterized by lipid accumulation and inflammation [Hansson, 2005]. Macrophages play a central role in the pathogenesis of atherosclerosis through internalizing modified low-density lipoprotein (LDL) and producing cytokines and growth factors, thus stimulating the migration and proliferation of SMCs as well as plaque development and progression [Hansson, 2005]. It has been demonstrated that macrophage LRP1 deficiency in mice increased atherosclerosis, independent of plasma lipoproteins, suggesting that LRP1 protects against the development of atherosclerotic lesions [Hu et al., 2006b]. In addition, cardiovascular diseases have a high clinical correlation with metabolic syndrome and type II diabetes mellitus. These conditions are associated with insulin resistance in fat tissues, hyperglycemia, and hyperinsulinemia [Linton and Fazio, 2003; Amar et al., 2006; Schmitz and Grandl, 2008].

In the present work, we evaluate the effect of insulin on the cellular expression of LRP1 in macrophage. Herein, we demonstrated that insulin induces a significant decrease in the LRP1 protein content in J774 macrophage-derived cells, which was mediated by the activation of the proteasomal system. Our data have important connotations associated with the pathogenesis of atherosclerosis.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Mouse J774 macrophage-derived cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 μg/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 described [Chiabrando et al., 2002]. α_2M^* was conjugated with fluorescein isothiocyanate (FITC) following the manufacturer's procedure (Pierce Biotechnology, Inc., IL).

WESTERN BLOT ANALYSIS

To evaluate the effect of insulin on the LRP1 protein expression, J774 cells were plated into 24 wells and grown to 70–80% confluence. After aspirating the medium, fresh serum-free medium was added and cultured for 2 h. Then, J774 cells were cultured with 10 nM insulin (Sigma, St. Louis, MO) for different periods of times (up to 24 h) at 37°C. J774 cells were lysated using 10 mM PBS, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycolate, 0.1% SDS, 0.2% NaN3, and 0.1% NP40. Fifty micrograms of lysate was separated on 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane (Amersham Bioscience, Denmark). Non-specific binding was blocked with 5% non-fat dry milk in Tris–HCl saline buffer containing 0.01% Tween 20 (TBS-T) for 60 min at room temperature. The membranes were incubated overnight at 4°C with a mouse anti-β LRP1 monoclonal antibody (clone 5A6), which was generously provided by Dr. Dudley Strickland (University of Maryland School of Medicine, Rockville, MD). After washing three times with TBS-T, the membranes were incubated with a secondary horseradish peroxidase-conjugated antibody (Amersham Bioscience) for 1 h at room temperature. The specific bands were revealed by chemiluminescence reaction (Pierce, UK) and quantified by densitometric analysis using an image analyzer (UVP Life Science, CA).

To evaluate the participation of the PI_3K/Akt signaling pathway activated by insulin on the LRP1 protein expression, J774 cells were plated as above. Previous to the insulin treatment, the cells were incubated for 30 min with 10 μM LY294002 (Sigma). After the insulin treatment, the cells were lysated as above. For Western blot analysis of phosphospecific Akt (p-Akt) and α-actin, we used specific rabbit polyclonal antibodies purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibody and densitometry analyses were carried out following the conditions described above. To determine the participation of the proteasomal system on the insulin-induced LRP1 degradation, J774 cells were

incubated for 30 min with 20 μM MG132 (Sigma) previous to the addition of insulin. Then, the LRP1 protein expression was analyzed by Western blot as above.

RNA ANALYSIS

To evaluate the effect of insulin on the specific mRNA for LRP1, J774 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, the J774 cells were cultured with 10 nM insulin for different periods of times (up to 24 h) at 37°C. J774 cells were 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) according to the manufacturer's instructions. A reverse transcription polymerase chain reaction (RT-PCR) was used to measure the changes in the transcript level of LRP1. Templates of total RNA were obtained using random hexaprimers. Specific primers for LRP1 and GADPH were: LRP1 sense primer 5′-TGGAGCAGATGGCAATCGACT-3′ and anti-sense primer 5′-CGAGTTGGTGGCGTAGAGATAGTT-3′; GADPH sense primer 5′-TGAAGGTCGGTGTGAACGGA-3′ and anti-sense primer 5′-ATGC-CAAAGTTGTCATGGATGAC-3′. PCR conditions were optimized to evaluate both LRP1 and GADPH transcripts together. Twenty-seven cycles were used for the 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. The intensity of the bands was analyzed using the UVP image analyzer as above.

IMMUNOFLUORESCENCE MICROSCOPY

To evaluate the effect of insulin on the protein expression of Tfr and LRP1, J774 cells were grown in coverslips in 24-well plates and grown to 30–70% confluence. After stimulus with insulin for different times, the coverslips containing the cells were washed twice with PBS, fixed in 3% paraformaldehyde at 4°C for 30 min, washed with PBS, permeabilized with 0.1% Triton X-100, 200 mM glycine in PBS (10 min at 4°C), and incubated in PBS plus 2% BSA for 1 h at 37°C to block non-specific binding sites. The coverslips were then incubated overnight at 4°C with primary antibodies, washed five times with PBS plus 1% BSA, and exposed to secondary antibodies for 90 min at 37°C. The primary antibodies were mouse anti-β LRP1 monoclonal antibody (clone 5A6) diluted 1:50, and rabbit anti-Tfr polyclonal antibody (Santa Cruz Biotechnology, Inc.) diluted 1:20. Secondary antibodies were rhodamine-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Inc.) diluted 1:1,000 or Alexa^{546}-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) diluted 1:700. After final washes with PBS plus 1% BSA, coverslips were mounted in FluorSave (Calbiochem) and visualized in a Nikon TE2000-U epifluorescence microscope.

CONFOCAL MICROSCOPY

To evaluate the participation of the proteasomal system induced by insulin, J774 cells were grown in coverslips as above and incubated with insulin for 24 h at 37°C in the presence or absence of 20 μM MG132. After these incubations, the coverslips containing the cells were washed twice with PBS, fixed in 3% paraformaldehyde for 30 min at 4°C, washed with PBS, permeabilized with 0.1% Triton X-100, 200 mM glycine in PBS (10 min at 4°C), and incubated in PBS plus 2% BSA for 1 h at 37°C to block non-specific binding sites. These coverslips were then incubated overnight at 4°C with primary antibodies, washed five times with PBS plus 1% BSA, and exposed to secondary antibodies for 90 min at 37°C. The primary antibodies were mouse monoclonal anti-β subunit of LRP1 (clone 5A6) diluted 1:50, and rabbit polyclonal anti-ubiquitin (Santa Cruz Biotechnology, Inc.) diluted 1:20. Secondary antibodies were Alexa^{546}-conjugated goat anti-mouse antibody diluted 1:1,000 or rhodamine-conjugated donkey anti-rabbit antibody diluted 1:1,000 (Santa Cruz Biotechnology, Inc.). Confocal images were collected using a Carl Zeiss LSM5 Pascal laser-scanning confocal microscope. Excitation wavelengths and filter set for Alexa^{546} (green) and rhodamine (red) were used. Images on fixed cells were taken using 63 × 1.4 numerical aperture (Plan-Apochromat, Zeiss) objectives and the confocal pinhole of the microscope set to obtain an optical slice of 1.1 μm.

INTERNALIZATION ASSAY

J774 cells were grown in coverslips as above and incubated with insulin for 24 h at 37°C. After these incubations, the coverslips containing the cells were washed twice with PBS, immediately incubated with 5 nM α_2M^*-FITC for 30 min at 4°C (binding step) and then incubated for 10 min at 37°C (internalization step). After this time, the covers were kept at 4°C and washed with cold acid solution (50 mM glycine, 150 mM NaCl, pH 2.5 containing 0.1% FBS) in order to detach the remnant α_2M^*-FITC bond to cell membrane and not internalized. Then, the coverslips were washed twice with PBS, fixed in 3% paraformaldehyde at 4°C for 30 min, mounted in FluorSave and visualized with a Nikon TE2000-U epifluorescence microscope. The fluorescence intensity of internalized α_2M^*-FITC was measured using Image J software (on-line free version), and the number of endocytic vesicles internalized with α_2M^*(FITC)-LRP1 complex is represented as the average number of granules per cell.

RESULTS

INSULIN DECREASES THE PROTEIN LEVEL OF LRP1 IN J774 CELLS

Although it has been demonstrated that insulin can affect the protein expression of LRP1 in adipocytes, its effect on macrophages is not well characterized. Hence, to evaluate this effect on LRP1 expression, J774 macrophage-derived cells were cultured in the presence of insulin for different times and the protein content of LRP1 was determined by Western blot in cell lysates. Figure 1a shows that insulin decreased the protein level of LRP1 from 12 h of incubation with respect to control. The quantitative analysis demonstrated that the highest insulin-induced reduction of LRP1 was observed for 24 h of incubation. In contrast, using semiquantitative RT-PCR, we observed that insulin did not produce a substantial modification in the transcript level of LRP1 for these times of incubation (Fig. 1b), suggesting that insulin principally affected the protein stability of LRP1. To demonstrate whether LRP1 protein degradation can be reverted after withdrawing the insulin

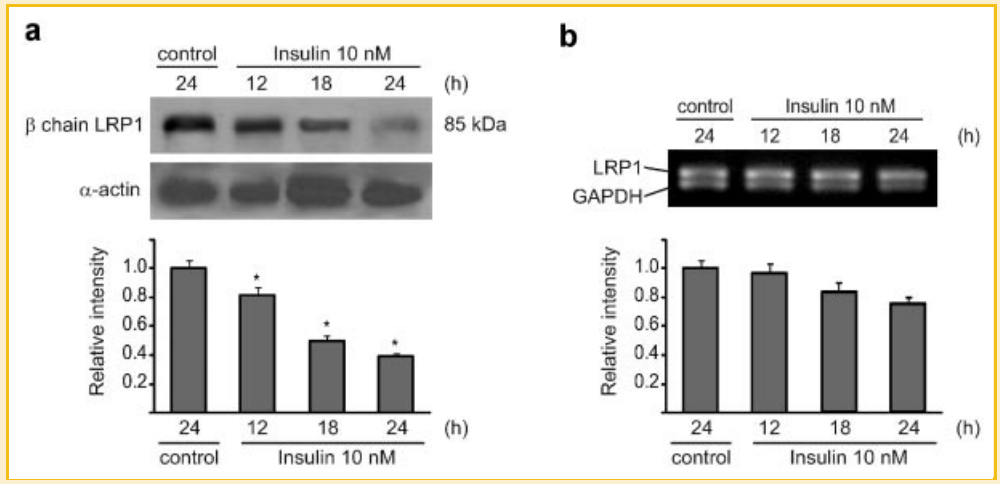

Fig. 1. a: Analysis of Western blot for LRP1 expression in insulin-treated J774 cells. Total proteins were extracted from J774 cells treated for 12, 18, and 24 h with serum-free medium or 10 nM insulin. After electrophoresis and electrotransfer to the nitrocellulose membrane, LRP1 was detected with mouse anti-β (85 kDa) subunit of LRP1 monoclonal antibody (clone 5A6) and revealed with a secondary horseradish peroxidase-conjugated antibody. The protein loading control of α-actin is also shown. The peroxidase-conjugated antibodies were detected by enhanced chemiluminescence. The bars show the relative intensity of LRP1 with respect to controls, representing the mean ± SE from triplicate experiments. The symbol (*) denotes *P* values (<0.01) significantly different to controls. b: Analysis of RT-PCR for LRP1 in insulin-treated J774 cells. Total RNA was extracted from J774 cells treated for 12, 18, and 24 h with serum-free medium or 10 nM insulin as described in Materials and Methods Section. The PCR products were evaluated in a 2% agarose gel-ethidium bromide and the bars show the relative intensity of LRP1/GAPDH with respect to controls, representing the mean ± SE from triplicate experiments.

from the cell culture, J774 cells previously treated with insulin were washed and cultured again in the absence of this hormone. By Western blot, we observed that under this culture condition the protein expression of LRP1 was fully restored after 24 h of incubation (data not shown). Thus, we conclude that the protein degradation of LRP1 induced by insulin in J774 macrophage-derived cells involves cellular mechanisms that affect the protein stability rather than the gene expression of this receptor.

It is known that both LRP1 and Tfr mediate endocytosis through clathrin-coated pits [reviewed in Stolt and Bock, 2006]. Moreover, in adipocytes, it has been previously demonstrated that insulin induces the protein translocation of LRP1 and Tfr from low-density microsomes to the cell membrane surface, without modifying the cellular content of both proteins [Ko et al., 2001]. Hence, in J774 cells, we evaluate the effect of insulin on LRP1 in comparison with Tfr expression by epifluorescence microscopy. Figure 2 shows a representative co-immunodetection of LRP1 and Tfr in J774 cells cultured in the presence of 10 nM insulin for 24 h. These data demonstrate that insulin decreased the protein level of LRP1 but not Tfr, which indicates that this hormone induces a differential protein regulation between both receptors in J744 macrophage-derived cells.

THE PROTEIN DEGRADATION OF LRP1 INDUCED BY INSULIN IS MEDIATED BY THE INTRACELLULAR PI_3K SIGNALING PATHWAY

It has been demonstrated that insulin mediates traffic and expression as well as proteasomal degradation of intracellular proteins by the activation of the intracellular PI_3K pathway [Rui et al., 2001, 2002]. Herein, we evaluate in J774 macrophage-derived cells whether the insulin effect on LRP1 degradation is mediated by PI_3K/Akt activation. For this purpose, we used LY294002 as a specific inhibitor of this intracellular signaling pathway. First, J774 cells were cultured for 24 h in the presence of 10 nM insulin previously incubated with 10 μM LY294002. After this time of incubation, the state of Akt phosphorylation (p-Akt) and the LRP1 level were analyzed by Western blot. Figure 3 shows that a sustained phosphorylation of Akt was observed in J774 cells treated for 24 h

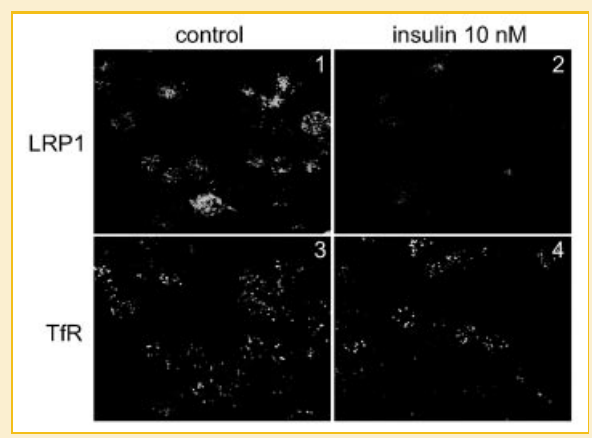

Fig. 2. Analysis of immunofluorescence microscopy for LRP1 and transferring receptor (Tfr) in insulin-treated J774 cells. The cells were incubated in the absence or presence of 10 nM insulin for 24 h. After the stimulus, coverslips were incubated together with mouse anti-β subunit of LRP1 monoclonal antibody (clone 5A6) and rabbit anti-Tfr polyclonal antibody, and were revealed with fluorescent secondary antibodies as described in Materials and Methods Section. Upper and lower panels represent the simultaneous immunodetection for LRP1 and Tfr in J774 cells treated (panels 2 and 4) and untreated (panels 1 and 3) with insulin, respectively.

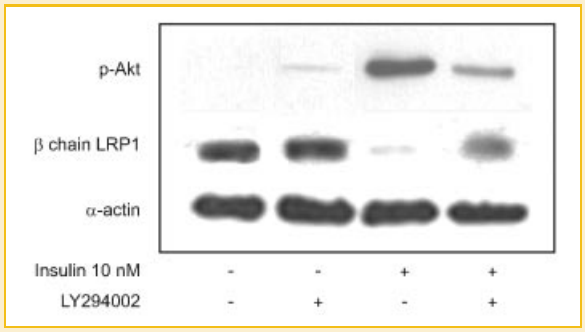

Fig. 3. Analysis of Western blot for Akt phosphorylation (p-Akt) and LRP1 in insulin-treated J774 cells. Total proteins were extracted from J774 cells treated with 10 nM insulin for 24 h. Previous to insulin stimulation, the cell cultures were incubated with 10 μM LY294002 for 30 min in serum-free medium. After electrophoresis and electrotransfer to the nitrocellulose membrane, p-Akt was detected with primary antibody and revealed with secondary horseradish peroxidase-conjugated antibody. Then, the same membrane was stripped and LRP1 was detected as described in Materials and Methods Section. The protein loading control of α-actin is also shown. The peroxidase-conjugated antibodies were detected by enhanced chemiluminescence.

with insulin. At the same time, an evident decrease in the LRP1 protein expression was also observed. In contrast, this insulin-induced Akt phosphorylation was attenuated in J774 cells previously incubated with LY294002, which correlated with a detectable level of LRP1 protein expression. On the other hand, the PI_3K inhibitor added alone without insulin did not affect the LRP1 expression compared to the control. Thus, these results demonstrate that insulin induces protein degradation of LRP1 in J774 macrophage-derived cells through the intracellular PI_3K pathway activation.

INSULIN INDUCES LRP1 PROTEIN DEGRADATION THROUGH THE PROTEASOMAL SYSTEM IN J774 CELLS

It has been demonstrated that the proteasomal system regulates the protein degradation and cellular turnover of LRP1. In this way, it has been reported that the proteasomal inhibitor, MG132, may prolong the half-life of LRP1 [Melman et al., 2002]. Herein, in an effort to determine whether insulin induces LRP1 protein degradation by activation of the proteasomal system, we treated J774 macrophage-derived cells with 20 μM MG132 for 24 h in the presence or absence of 10 nM insulin, and then evaluated LRP1 by Western blot. Figure 4 shows that J774 cells cultured in the presence of MG132 fully blocked the LRP1 protein degradation induced by insulin. The quantitative analysis demonstrated that MG132 incubated in the presence or absence of insulin produced a significant increase in the LRP1 level with respect to the amount of protein receptor contained in the basal cultures of J774 cells. Thus, our data indicate that insulin induces LRP1 protein degradation through the proteasomal system in J774 macrophage-derived cells.

It has been previously reported that the extracellular α-subunit of LRP1 can be ubiquitinated in macrophages, the ubiquitin-LRP1 conjugation being increased by the presence of MG132 [Misra and Pizzo, 2001]. To corroborate whether LRP1 is ubiquinated by the action of insulin, we cultured J774 cells in the presence of MG132 and then performed co-localization experiments by confocal microscopy using anti-β subunit LRP1 and anti-ubiquitin antibodies. The merged images indicate an absence of overlapping of

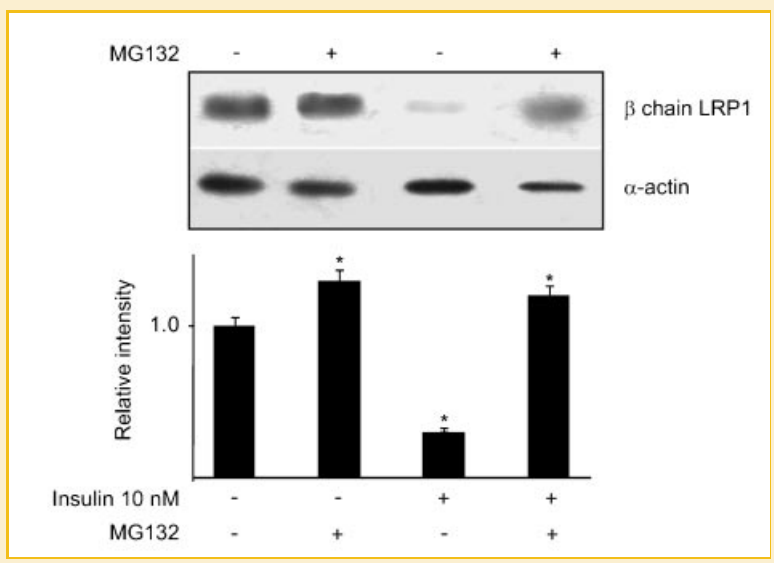

Fig. 4. Analysis of Western blot for LRP1 in J774 cells treated with MG132 proteasomal inhibitor and insulin. Total proteins were extracted from J774 cells treated with 10 nM insulin for 24 h. Previous to insulin stimulation, the cell cultures were incubated with 20 μM MG132 for 30 min in serum-free medium. After electrophoresis and electrotransfer to the nitrocellulose membrane, LRP1 was detected with primary antibody and revealed with secondary horseradish peroxidase-conjugated antibody as described in Materials and Methods Section. The protein loading control of α-actin is also shown. The peroxidase-conjugated antibodies were detected by enhanced chemiluminescence. The bars show the relative intensity of LRP1 with respect to controls, representing the mean ± SE from triplicate experiments. The symbol (*) denotes *P* values (<0.01) significantly different to controls.

LRP1 with ubiquitin in control, MG132-treated, and insulin-treated J774 cells (Fig. 5A, panels a, b, c, respectively). In agreement with previous reports [Melman et al., 2002], MG132 increased the LRP1 level when the J774 cells were cultured in the presence of this proteasomal inhibitor, but without any evident overlapping with ubiquitin (Fig. 5A, panel b). In addition, and in agreement with our previous results, the insulin treatment decreased the LRP1 level (Fig. 5A, panel c). In contrast, panel d of Figure 5A shows that MG132 blocks the protein degradation of LRP1 induced by insulin, with an evident overlapping between LRP1 and ubiquitin. Figure 5B shows eight subsequent confocal sections of 1.1 μm corresponding to Figure 5A-d, where the overlapping images of LRP1 with ubiquitin are evident in each section. In contrast, these analyses of different confocal sections did not show overlapping images between LRP1 and ubiquitin in J774 cells cultured under the experimental conditions indicated above in the panels a, b, and c of Figure 5A (data not shown). Thus, we conclude that insulin induces LRP1 ubiquitination in J774 macrophage-derived cells, which suggests that this ubiquitin protein event is mediating the LRP1 degradation by the proteasomal system.

INSULIN REDUCES THE CELLULAR INTERNALIZATION OF ACTIVATED α2M IN J774 CELLS

Considering that insulin induces protein degradation of LRP1, we then evaluated whether this hormone also affects the internalization

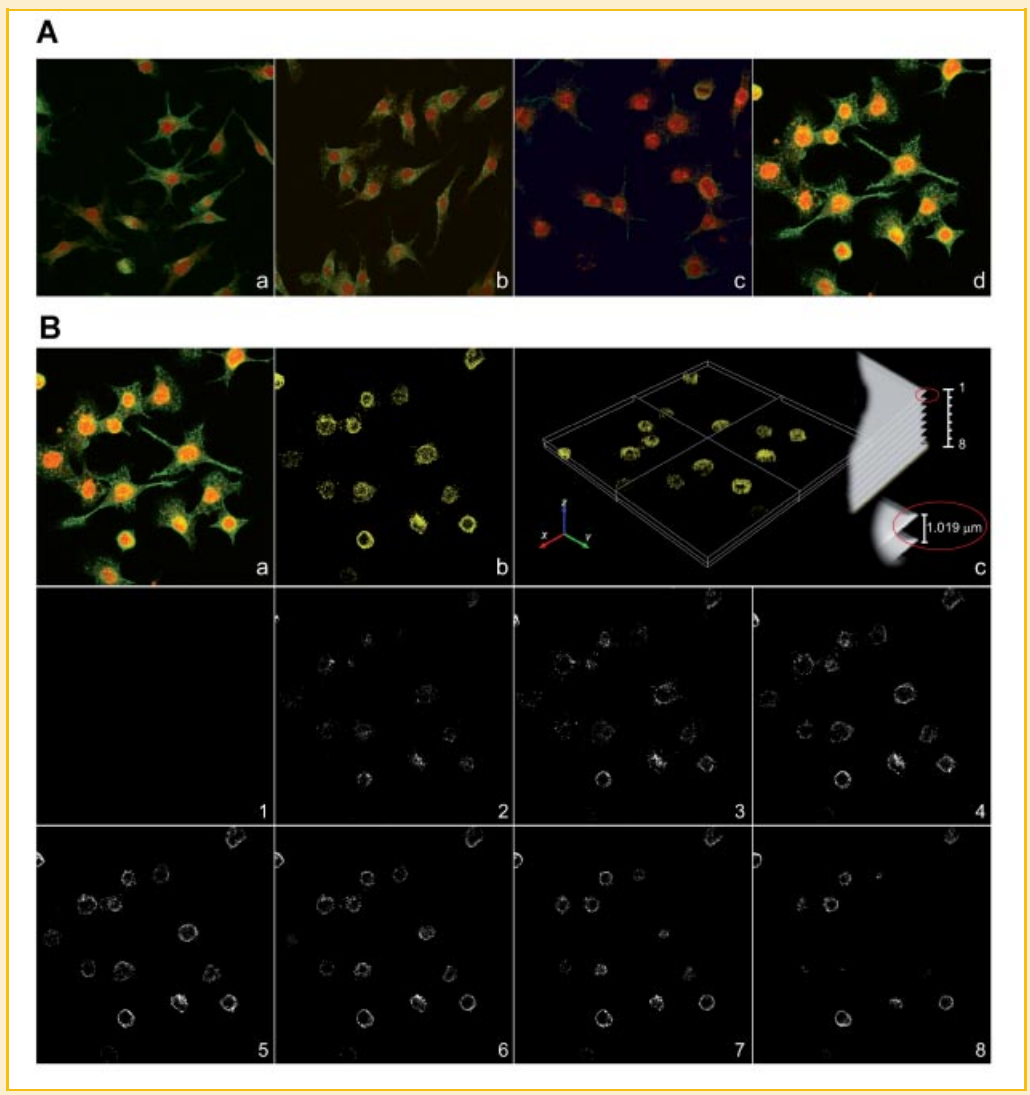

Fig. 5. Confocal microscopy analysis for LRP1 and ubiquitin in insulin-treated J774 cells. The cells were incubated with 10 nM insulin for 24 h in the presence or absence of 20 μM MG132. A: The merge + co-localization images of LRP1 and ubiquitin in control cells (a), MG132-treated cells (b), insulin-treated cells (c), and insulin and MG132-treated cells (d). B: The merge + co-localization (a), co-localization–merge (b), and orthogonal plane (1.019 μm) images (c) of insulin and MG132-treated cells. Eight subsequent optical slices corresponding to co-localization–merge of insulin and MG132-treated cells (panels 1–8, respectively). Experimental details are indicated in Materials and Methods Section. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of specific ligands, such as α_2M^*, in J774 macrophage-derived cells. To carry out this, J774 cells, previously treated with insulin for 24 h, were incubated in the presence of α_2M^*-FITC for 30 min at 4°C to allow binding to LRP1 on the cellular membrane. Then, the internalization of the α_2M^*(FITC)–LRP1 complex was induced for 10 min at 37°C and visualized by epifluorescence microscopy. The average number of granules, representing endocytic vesicles, was counted using an image analyzer. Figure 6 shows that insulin reduced the ability of J774 cells to internalize α_2M^*-FITC, which was determined by the significant decrease in the average number of endocytic vesicles with respect to control. Thus, these results indicate that the protein degradation of LRP1 induced by insulin also affected the functional ability of this receptor to internalize the specific ligand α_2M^* in J774 macrophage-derived cells.

DISCUSSION

In the present work, we have demonstrated that insulin induces protein degradation of LRP1 in J774 macrophage-derived cells mediated by the proteasomal system. This insulin effect on the LRP1 degradation did not affect the mRNA level of LRP1, but was dependent on the PI_3K/Akt signaling pathway activation. The insulin-induced protein degradation of LRP1 was fully blocked by the proteasomal inhibitor, MG132. In addition, we observed that the protein degradation of LRP1 induced by insulin was associated with an enhanced ubiquitin–receptor conjugation. Finally, as a consequence of this protein degradation, we also demonstrated that insulin affected the cellular internalization of specific LRP1 ligands such as α_2M^*.

An important number of publications have reported that insulin modifies the intracellular distribution of LRP1, which is characterized by an increased cell surface presentation of LRP1 in adipocytes [Ko et al., 2001; Hofmann et al., 2007] and hepatocytes [Tamaki et al., 2007]. This effect of insulin on LRP1 is mediated by PI_3K activation, and it follows a protein distribution pattern similar to other insulin-regulated cell surface proteins, including Tfr [Ko et al., 2001]. This increased cell membrane presentation of LRP1 in these types of cells was found to be associated with the lipid metabolism of fat tissues and the hepatic clearance of amyloid β-peptide (1–40) (Aβ(1–40)) from plasma, respectively [Hofmann et al., 2007; Tamaki et al., 2007]. In contrast, by using the J774 macrophage-derived cell line, in the present work, we observed that insulin induced protein degradation of LRP1, without affecting the cellular protein level of Tfr. This effect of insulin was fully blocked by LY294002, an inhibitor of the intracellular PI_3K/Akt signaling pathway. In addition, the insulin-induced protein degradation of LRP1 also produced a significant decrease in the cellular internalization of α_2M^*. Interestingly, we recently demonstrated that the α_2M^*–LRP1 interaction in J774 cells induced intracellular MAPK-ERK1/2 signaling activation and cellular proliferation [Bonacci et al., 2007]. Thus, we suggest that the insulin-induced LRP1 degradation in macrophages could affect at less two essential functions of this receptor: (i) the extracellular proteolytic activity control by a decreased cellular internalization of α_2M–protease complexes and (ii) the macrophage activation by a reduced

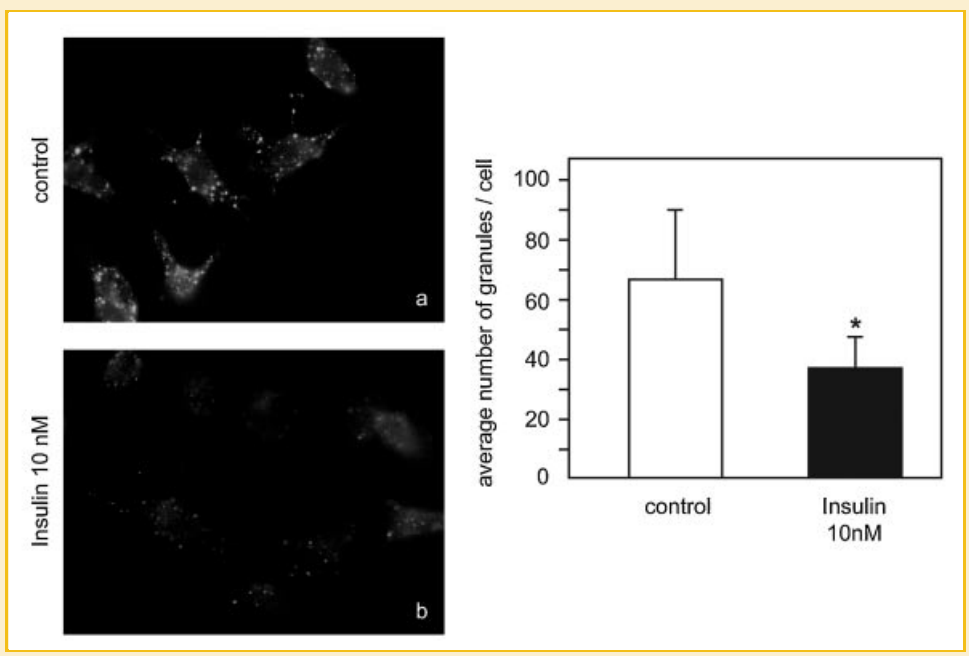

Fig. 6. Analysis of immunofluorescence microscopy of the α2M*–FITC internalization in insulin-treated J774 cells. The cells were nontreated (a) and treated (b) with 10 nM insulin for 24 h at 37°C. After this period, the cells were incubated with 5 nM α2M*–FITC for 30 min at 4°C (binding step) and then immediately incubated for 10 min at 37°C (internalization step). After washing with cold acid solution, the cells were fixed in paraformaldehyde and visualized in a Nikon TE2000-U epifluorescence microscope. The fluorescence intensity of internalized α_2M^*–FITC was represented as the average number of granules per cell (bar graph). Experimental details are indicated in Materials and Methods Section.

ligand–receptor interaction and its subsequent downstream reduction in intracellular signal pathway activation.

It has been previously reported that the proteasomal system plays a key role in the normal protein degradation and protein turnover of LRP1 [Melman et al., 2002], being demonstrated that a short region of the intracellular β-subunit LRP1 tail (residues 60–78) is required for the proteasomal regulation. However, these authors proposed that the regulation of the ubiquitin–proteasome system could be mediated by the ubiquitination of an ancillary protein, which itself may be ubiquitinated and interact with the intracellular LRP1 tail. In another report, it was demonstrated that the extracellular α-subunit LRP1 was able to be ubiquitinated, and mediate its endocytosis, after interacting with certain ligands, such as α_2M^* and *Pseudomonas* exotoxin A [Misra and Pizzo, 2001]. In the present work, we demonstrated that insulin induces protein degradation of LRP1 in J774 cells, which was fully inhibited by MG132, an inhibitor of the proteasomal system. Furthermore, by confocal microscopy we demonstrated that MG132 produced an enhanced signal of co-localization between anti-ubiquitin and anti-β subunit LRP1 antibodies, but only when the J774 cells were treated with insulin, suggesting that the ubiquitin–LRP1 conjugation is induced by insulin. However, further studies are necessary in order to clarify the ubiquitination mechanism of LRP-1 as well as the putative ubiquitin–receptor conjugation site induced by insulin in J774 macrophage-derived cells.

Finally, it is known that macrophages play a key role in the 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]. These modified lipoproteins are then recognized and internalized by scavenger receptors, including SR-A and CD36, whereas non-oxidized and apoE-rich lipoproteins are internalized by LRP-1 [Krieger and Herz, 1994]. The foam cell formation is exacerbated in pathologic conditions such as metabolic syndrome and type II diabetes mellitus, which are associated with insulin resistance in fat tissues [Linton and Fazio, 2003; Amar et al., 2006; Schmitz and Grandl, 2008]. 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., 2006b] atherogenic properties. Related to this, through an in vivo study, using macrophage-specific LRP1-deficient mice on an apoE/LDLR double-deficient background, it was demonstrated that macrophage LRP1 has an atheroprotective potential [Hu et al., 2006b]. Therefore, we propose that an increased degradation of LRP-1 induced by insulin in macrophages could contribute to the development of atherosclerosis. Obviously, further detailed studies are required to gain additional insight into the underlying mechanisms about how insulin affects the LRP1 expression in macrophages, and its role as a key mediator in the pathogenesis of atherosclerosis.

ACKNOWLEDGMENTS

We are grateful to Dr. Paul David Hobson, native speaker, for checking the language of this manuscript. D.G.C. is a postdoctoral fellow of CONICET, and M.C.S. and G.A.C. are members of the Research Career of CONICET.

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