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Internalization of the Receptor for Advanced Glycation End Products (RAGE) is Required to Mediate Intracellular Responses

Natalia Sevillano[1], María D. Girón[1], Mercedes Salido[2], Alberto M. Vargas[1], José Vilches[2] and Rafael Salto[1,*]

[1]Department of Biochemistry and Molecular Biology II, School of Pharmacy, University of Granada, Campus de Cartuja sn, E-18071 Granada, Spain; and [2]Department of Histology, School of Medicine, University of Cádiz. Confocal Microscopy and Cell Culture Unit, Central Research Services in Health Sciences, Edificio Policlínico, C/Dr Marañon 3, E-11002 Cádiz, Spain

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To dissect the rat receptor for advanced glycation end products (RAGE) subcellular distribution and trafficking in eukaryotic cells, an expression system coding for a fusion protein between the RAGE and an enhanced green fluorescent protein (EGFP) has been used. The RAGE–EGFP protein is expressed at the plasma membrane of CHO-k1 and Neuro-2a (N2a) cells and retains the capacity to bind Texas Red-labelled advanced glycation end products (AGEs). AGEs addition to the cell cultures induced a change in the subcellular distribution of the fluorescent RAGE–EGFP protein compatible with an internalization of the AGEs–RAGE complex. Furthermore, while N2a cells expressing the RAGE–EGFP showed an increase in ERK1/2 phosphorylation and NF-κB DNA binding in response to AGEs, pre-incubation with dansyl-cadaverine or phenylarsine oxide, inhibitors of receptors internalization, blocked the activation of ERKs and other intracellular responses mediated by AGEs. These results suggest that internalization plays a key role in the signal transduction mediated by RAGE.

Key words: enhanced green fluorescent protein, ERK1/2, NF-κB, receptor for advanced glycation end products, receptor internalization.

Abbreviations: AGEs, advanced glycation end products; CHO-k1, Chinese hamster ovary cell line; DAPI, 4′-6-Diamidino-2-phenylindole; DC, dansyl-cadaverine; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; N2a, neuro-2a neuroblastoma cell line; PAO, phenylarsine oxide; PBS, phosphate-buffered saline; RAGE, receptor for advanced glycation end products.

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules (*1*, *2*). It has an extracellular region containing three immunoglobulin domains, one V-type and two C-type domains, that confers the ligand-binding properties. This region is followed by a hydrophobic transmembrane-spanning domain and a short cytosolic tail essential for the RAGE-mediated cellular effects (*1*, *3*, *4*).

In different tissues, RAGE interacts with numerous ligands such as advanced glycation end products (AGEs), S100/calgranulins, amphoterin and amyloid fibrils, that are involved in several pathophysiological processes, e.g. immune/inflammatory disorders, Alzheimer's disease, tumourigenesis and abnormalities associated with diabetes (*5–9*). Binding of the different ligands to RAGE does not accelerate their clearance or degradation but rather induces expression of the receptor itself and amplifies a pro-inflammatory response leading to cellular activation and tissue dysfunction by a receptor-dependent mechanism (*3*, *6*, *10*). RAGE activates a range of signal transduction pathways including Ras, Rac/Cdc42 and Jak/Stat pathways. Many of these signalling cascades have been shown to result in the activation of the downstream transcription factor NF-κB and to produce cellular activation including an increase in RAGE promoter activity (*4*, *10–13*).

There is little known about the intracellular traffic of the receptor and it is not even known if its internalization is necessary to produce intracellular responses. Therefore, it could be relevant to elucidate the subcellular trafficking of the RAGE as a target for treatment of diabetes and other diseases related with the AGE–RAGE system.

In this work, we have developed a novel system for monitoring RAGE trafficking in real time in living cells by the design of an expression system that codes for a fusion protein between the rat RAGE and an enhanced green fluorescent protein (EGFP). This construct takes advantage of the capabilities of EGFP to be detected *in vivo* (*14*, *15*). The fusion protein behaves like the native receptor and is a useful tool to study RAGE intracellular trafficking. Using this system, we have demonstrated that the internalization of the receptor–ligand is essential to produce a cellular activation in response to AGEs.

*To whom correspondence should be addressed. Tel: +34-958-246363, Fax: +34-958-248960, E-mail: rsalto@ugr.es

MATERIALS AND METHODS

Materials—Dansyl-cadaverine (DC), Phenylarsine oxide (PAO), monoclonal anti-GFP antibody and horseradish peroxidase (HRP) conjugated secondary antibodies were from Sigma (St Louis, MO, USA). Tissue culture media and supplements were from Sigma and Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Cultek (Madrid, Spain). ERK1/2 polyclonal antibody was from Upstate (Waltham, MA, USA) and phosphorylated ERK1/2 monoclonal antibody was from Cell Signaling (Beverly, MA, USA). AGEs were obtained in our laboratory using bovine serum albumin (BSA) as described (*16*). BSA–AGEs were conjugated with Texas Red sulfonyl chloride following manufacturer's instructions (Sigma).

Design of the Expression Constructions—The cDNA corresponding to the rat RAGE gene was retrotranscripted using total RNA from rat lung with a *Not*I-oligo(dT) primer. NH_2-Green 5′-AGATCTGCC*ATG* CCAACGGGGACAG-3′ and COOH-Green 5′-GTCGACA GGTCCCCCTGCAC-3′ primers with introduced *Bgl*II and *Sal*I restriction endonuclease sites (underlined), respectively, were used to amplify by PCR, the coding sequence of the rat receptor from the AUG to the last amino acid, without the stop codon. DNA fragment was *Bgl*II-*Sal*I digested and cloned upstream from the EGFP coding region into *Bgl*II-*Sal*I digested pEGFP-N3 vector (Clontech, Palo Alto, CA, USA). The sequence of the construction was verified by automated DNA sequencing and named pRAGE–EGFP.

The construction of the RAGE promoter-luciferase (pGL3-RAGE) was made by cloning a genomic DNA fragment encompassing positions −2635 to +1 of the rat RAGE gene into the pGL3-Basic vector (Promega, Madison, WI, USA), which codes for firefly luciferase.

Cell Culture and DNA Transfection Assays—Wild type Chinese Hamster Ovary (CHO-k1; ATCC No. CCL-61) and Neuro-2a (N2a; ATCC No. CCL-131) cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 2 mM glutamine plus 100 U/ml penicillin and 0.1 μg/ml streptomycin. For transfection experiments, cells were used at 80–90% confluence. Transfection was performed using LipofectAMINE 2000 (Invitrogen) following manufacturer's instructions. Stably transfected cells were selected by addition of 700 μg/ml geneticin to normal growth medium 48 h after transfection.

To study ligand binding and ligand-induced internalization, transfected cells were grown to 50–60% confluence, fasted for 12 h and then incubated in the absence or presence of Texas Red-labelled AGEs–BSA (50 μg/ml) or BSA–AGEs (100 μg/ml) in DMEM supplemented with 3% BSA for the indicated time at 37°C. Ligands were removed and cells were washed with cold phosphate-buffered saline (PBS).

RAGE–EGFP Protein Analyses—Cell extracts (30 μg protein) were electrophoresed in 12% SDS–polyacrylamide gels and processed for western blot with a polyclonal anti-RAGE antibody raised in our laboratory using as antigen a rat RAGE fragment comprising the last 174 amino acids of the protein or a monoclonal anti-GFP antibody. The antigen–antibody complexes were detected with HRP-conjugated secondary antibodies and visualized by chemiluminiscence.

Confocal Microscopy—For confocal microscopy, cells were grown on coverslips to 50–60% confluency and treated as described above. Cells were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and coverslips were mounted on glass slides using Vectashield mounting media (Vector Laboratories, Inc., Burlingame, CA, USA).

Confocal microscopy was performed either on a Leica TCS-SL or a Leica DMI6000 confocal microscope. To prevent cross-talk of EGFP and Texas Red chromophores, distinct excitation laser lanes and non-overlapping detection channels were selected for their detection: EGFP was excited using the 488 nm line of a krypton/argon laser and the emitted fluorescence was detected with a 515–540 nm channel. Texas Red was detected using the 514 nm excitation line and a 590–685 nm channel. For dual-colour analysis, images were collected separately in single channel mode using a sequential acquisition mode. Under these conditions, no signals were detected in cells expressing pRAGE–EGFP in the absence of labelled ligand using the Texas Red-detection parameters.

All samples were exposed to laser for a time interval not >5 min to avoid photobleaching. The laser was set to the lowest power able to produce a fluorescent signal. Maximum voltage of photomultipliers was used to decrease the required laser power as much as possible. A pinhole of 1 Airy unit was used. Images were acquired at a resolution of 1024 × 1024. Series were acquired in the xyz mode.

AGE-mediated Phosphorylation of Extracellular Signal-regulated Kinases—Cells were grown to confluence, deprived of FBS for 24 h and then incubated in the absence or presence of BSA–AGEs (100 μg/ml) for 30 min. For internalization inhibition, DC (100 μM) or PAO (1 μM) were added 30 min prior to BSA–AGEs addition. In all cases, plates were processed as described in (*17*). Proteins were separated by SDS–PAGE and immunoblotted with a polyclonal antibody to total ERK1/2 or a monoclonal antibody to phosphorylated ERK1/2.

Electrophoretic Mobility Shift Assays—Nuclear extracts from transfected cells were prepared as described in (*18*). Electrophoretic mobility shift assays (EMSAs) were performed using a double-stranded oligonucleotide probe corresponding to a functional NF-κB binding site (5′-AGTTGAGGGGACTTTCCCAGGC-3′) that was end-labelled using T4 polynucleotide kinase and γ-[^{32}P]-ATP (GE Healthcare Life, Uppsala, Sweden). Binding reactions of the probe (200 fmol) were performed with 10 μg protein from nuclear extracts as described (*18*). Competitive binding assays were performed under the same conditions, with the addition of 100-fold molar excess of unlabelled oligonucleotides.

Luciferase Assay—To study the promoter activity, N2a cells were transfected as above, using a mixture of the pGL3-RAGE and pRL-TK reference plasmids (1 : 5) as an internal control to normalize transfection efficiencies. Forty-eight hours after transfection, cells were deprived of FBS for 8 h and incubated in the absence or presence

of BSA–AGEs (100 μg/ml) for 3 h. For internalization inhibition studies, DC (100 μM) was added 30 min prior to BSA–AGEs incubation. Luciferase activities were measured using a Dual-Luciferase reporter assay system (Promega).

Statistical Methods—Results are expressed as means ± SEM for the number of experiments indicated. The statistical significance of variations was evaluated using a one-way ANOVA. When a significant effect was found, *posthoc* comparisons of the means were done using the *t*-adjusted Tukey test. A P-value < 0.05 was considered significant.

RESULTS

Expression of RAGE–EGFP in Eukaryotic Cells—A pRAGE–EGFP plasmid that codifies for a fusion protein with the EGFP was designed to study the sub-cellular distribution and functionality of the RAGE. The expression of the fusion protein was assayed in CHO-k1 and N2a cells. Analysis by western blot using anti-GFP or anti-RAGE antibodies (Fig. 1) or by RT-PCR, using pairs of oligonucleotides that hybridize either in the RAGE or EGFP coding sequences (ESM, Fig. 1) confirmed the over-expression of the fusion protein in these cell lines.

CHO-k1 or N2a cells expressing EGFP or RAGE–EGFP proteins were visualized using confocal microscopy. In CHO-k1 cells transfected with the control plasmid pEGFP-N3, the green fluorescence is distributed inside the cell with a preferential accumulation in the nucleus (Fig. 2A) as has been described for the EGFP protein (*19*). On the contrary, the RAGE–EGFP fusion protein was detected predominantly on the cell plasma membrane, while some intracellular fluorescence was localized in the perinuclear region (Fig. 2B). Since the fusion protein was completely excluded from the nucleus, this suggests that the junction between the two proteins was not susceptible to generate native EGFP.

In the N2a differentiated cells, the RAGE–EGFP fusion protein was also mainly located in the plasma membrane and the outgrowth neurite plasma membranes were clearly marked with green fluorescence (Fig. 2C). An animation of the series obtained in the confocal microscopy is shown in ESM animation 1. In both cells lines, the over-expression of RAGE–EGFP protein induced the formation of vesicles on the plasma membrane in a significant number of transfected cells (arrows in Fig. 2B, C and D). Although this cell morphology could suggest an apoptotic process, cell viability remained unchanged (data not shown). Furthermore, when the nuclei of transfected cells were stained with 4′-6-Diamidino-2-phenylindole (DAPI), transfected cells showing plasma membrane vesicles did not have an apoptotic nuclear morphology (Fig. 2D).

RAGE–EGFP Ligand Binding and AGEs–RAGE Complex Internalization—To study the binding of ligands to the fusion protein, transient CHO-k1 and N2a transfected cells were incubated for 3 h with Texas Red-labelled BSA–AGEs and analysed by confocal microscopy using detection conditions designed to prevent the cross-talk of chromophores.

As shown in Fig. 3A, in the CHO-k1 and N2a transfected cells, green (receptor) and red (ligand) fluorescences co-localized at the plasma membrane. In this figure, it could also be noted that the binding capacity of Texas Red BSA–AGEs by the untransfected N2a cells is significantly lower than in the transfected cells, but not null (as in CHO-k1 untransfected cells that do not constitutively express the RAGE gene, data not shown), probably due to the endogenous RAGE expressed by N2a cell line.

Moreover, in CHO-k1 and N2a transfected cells incubated for 3 h with the labelled ligand, a significant amount of green and red fluorescences co-localized in the cytoplasm (Fig. 3A). These results suggest that the binding of the ligand to the RAGE–EGFP protein induces an internalization of the ligand–receptor complex.

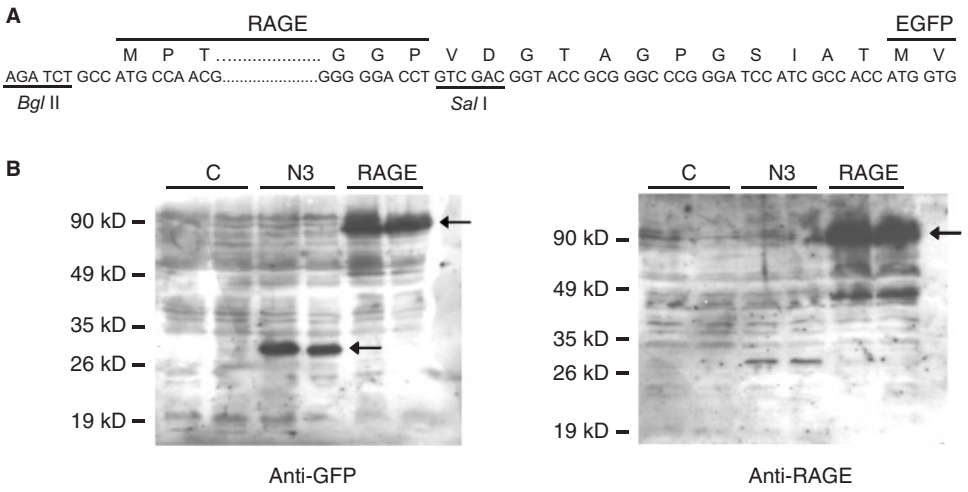

Fig. 1. **RAGE–EGFP expression.** (A) Schematic representation of the construct pRAGE–EGFP. The deduced amino acids sequence of the flanking region between the RAGE and EGFP proteins is depicted. (B) Western blot. Total cellular extracts from CHO-k1 untransfected cells (C) and transfected with the plasmid pEGFP-N3 (N3) or the pRAGE–EGFP construction (RAGE) were analysed by immunoblotting using anti-GFP or anti-RAGE antibodies. The arrows mark the expected band sizes.

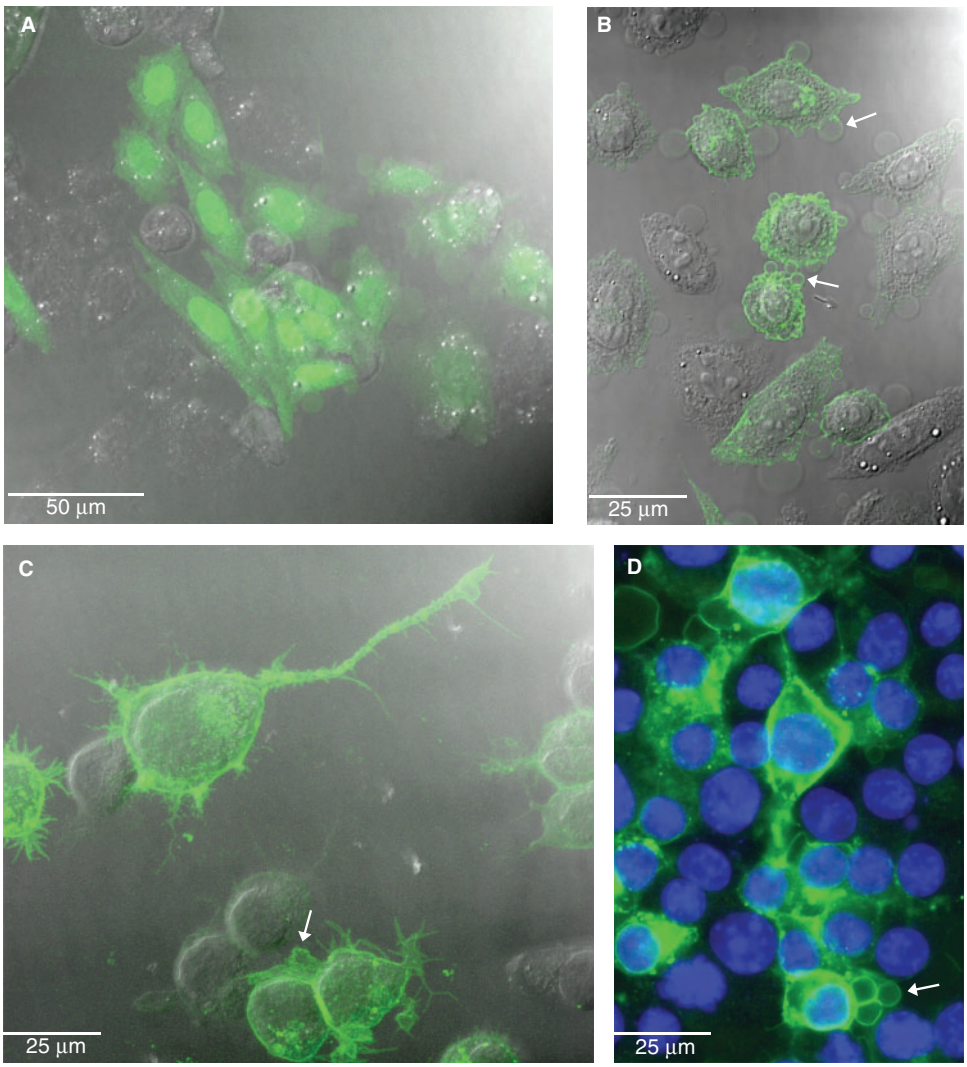

Fig. 2. **Confocal fluorescence micrographs of CHO-K1 and N2a transfected cells.** Merged GFP and differential interference contrast (Nomarski) images are shown. GFP was excited at 488 nm. (A) CHO-k1 cells transfected with the plasmid pEGFP-N3. (B) CHO-k1 transfected cells expressing the fusion protein RAGE–EGFP. (C) N2a transfected cells expressing the fusion protein RAGE–EGFP. (D) Fluorescent micrograph of N2a cells transfected with the pRAGE–EGFP plasmid. Nuclei have been stained with DAPI. The arrows indicate the vesicles in plasma membrane in response to the over-expression of RAGE–EGFP protein.

To confirm it, and to rule out any artifacts due to the Texas Red labelling of ligands, experiments in N2a cells were carried out using unlabelled AGEs and the green fluorescence associated to the receptor was monitored by confocal microscopy. As expected, in N2a transfected cells incubated with unlabelled BSA–AGEs for 3 h, a redistribution of the green fluorescence from the plasma membrane to cytoplasm was observed, since an increase in punctuated green fluorescence was detected in the cytoplasm (Fig. 4A). Furthermore, Z-sections of transfected cells in the absence or presence of AGEs showed a fluorescent distribution at the plasma membrane or inside the cell compatible with an internalization process.

Role of the Ligand-induced Internalization in the Generation of Intracellular Responses—To study the relevance of the ligand-induced receptor internalization in the signal transduction mediated by RAGE, first we have assessed the capability of the RAGE–EGFP fusion protein to mediate intracellular responses. For this purpose, the activation of the ERK1/2 and the increase in NF-κB DNA binding were assayed in isolated stably transfected N2a cells incubated with BSA–AGEs.

As shown in Fig. 5A, N2a cells, either transfected with the pEGFP-N3 empty vector or the pRAGE–EGFP plasmid, showed similar basal ERK1/2 phosphorylation levels. However, the incubation with BSA–AGEs increased significantly ERK1/2 phosphorylation only in the cells over-expressing the RAGE–EGFP protein. No changes in the total amount of ERK1/2 were observed for any condition. A time course of the effects of the BSA–AGEs addition to pRAGE–EGFP transfected N2a cells on ERK1/2 phosphorylation is shown in Fig. 5B. ERK phosphorylation peaked at 10–15 min and

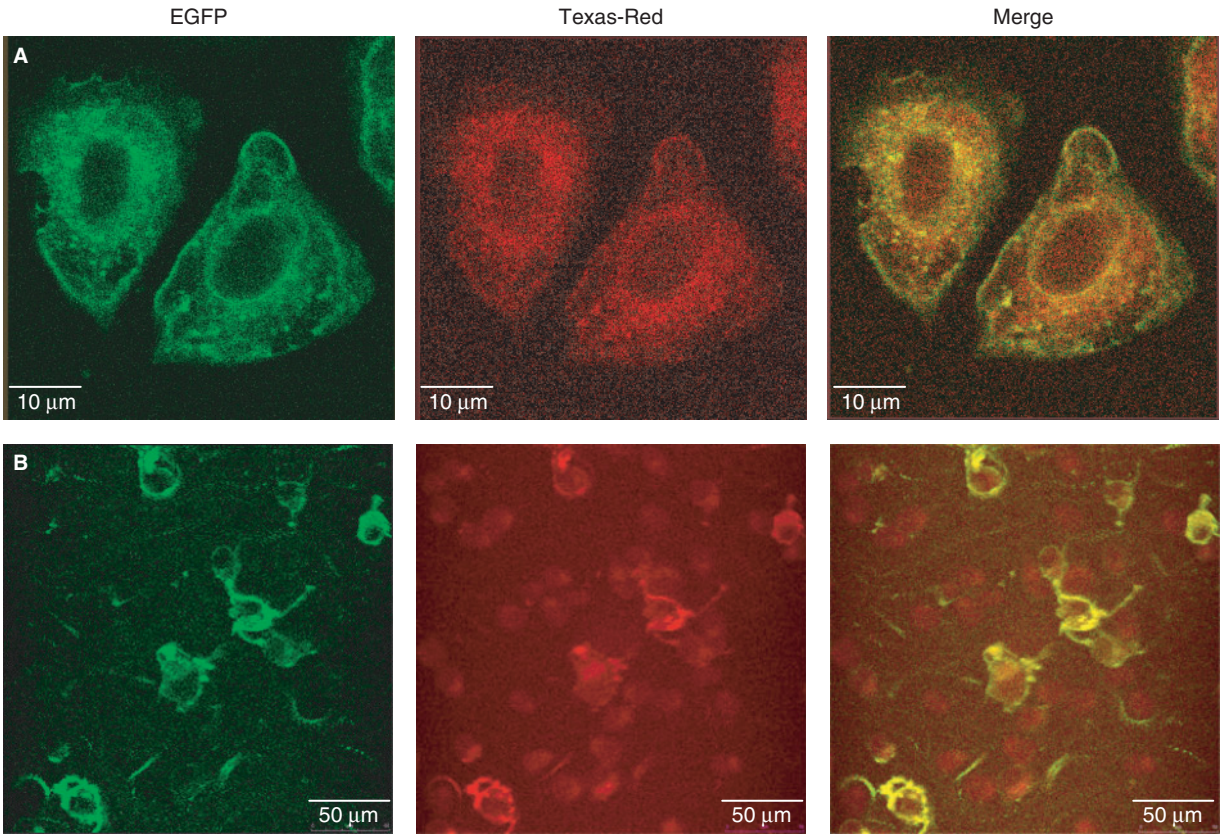

Fig. 3. **AGEs binding to RAGE–EGFP.** CHO-k1 (A) and N2a (B) transfected cells were incubated with Texas Red-labelled AGEs–BSA (50 μg/ml) and visualized with confocal microscopy using excitation and emission wavelengths specific to EGFP or Texas Red. Merged micrographs of RAGE–EGFP and ligands are shown. The co-localization of RAGE and ligands is shown in yellow in the merged micrographs.

remained elevated up to 60 min after the addition of the ligand.

Also, we studied the NF-κB activation in response to BSA–AGEs addition in N2a cells. For this, NF-κB DNA binding was analysed by EMSA (Fig. 5C). While basal NF-κB binding capacity in the pRAGE–EGFP stably transfected cells was similar to the untransfected cells, the incubation with AGEs increased the NF-κB binding activity only in the nuclear extracts of transfected cells. This binding was specifically inhibited by an excess of unlabelled competitor DNA.

Once validated the RAGE–EGFP fusion protein as a tool to study the intracellular signalling mediated by RAGE, we have used it to determinate the relevance of the ligand-induced receptor internalization in the generation of these intracellular responses. For this purpose, experiments in the presence of DC and PAO, specific inhibitors of the internalization of receptors (*20*, *21*), were carried out.

First, we assayed the effects of the pre-incubation for 30 min with these two inhibitors (DC, 100 μM and PAO, 1 μM) on the AGEs-induced internalization of the RAGE–EGFP fusion protein using confocal microscopy (Fig. 4B). As expected, in presence of AGEs–BSA the fusion protein was located predominantly inside the cells, while the pre-incubation with DC or PAO blocked the internalization process.

Next, N2a cells expressing RAGE–EGFP fusion protein were pre-treated with DC or PAO for 30 min before the addition of BSA–AGEs and the ERKs phosphorylation was analysed. As shown in Fig. 6A, the treatment with DC (100 μM) or PAO (1 μM) suppressed the AGE-mediated ERKs phosphorylation. To demonstrate that DC and PAO are suitable to study the effective suppression of receptor internalization without affecting the ERK signalling pathway by other mechanisms, we have assayed the effects of sodium tungstate, an ERKs phosphorylation activator (*17*, *22*), in the presence or absence of DC and PAO (ESM, Fig. 2). Under the same experimental conditions, DC and PAO have no effects on the tungstate-mediated ERK phosphorylation.

Finally, to confirm the inhibitory effects of DC on the generation of intracellular responses mediated by RAGE, we studied in N2a untransfected cells the effects of the BSA–AGE addition on the RAGE promoter activity. For this purpose, N2a cells were transfected with a plasmid encoding for luciferase under the control of the rat RAGE promoter. Our results show that the addition of BSA–AGEs to these cells increased significantly the promoter activity. This increase in the RAGE promoter, driven by the endogenously RAGE expressed by the N2a cells, was completely blocked by a pre-treatment with DC (Fig. 6B).

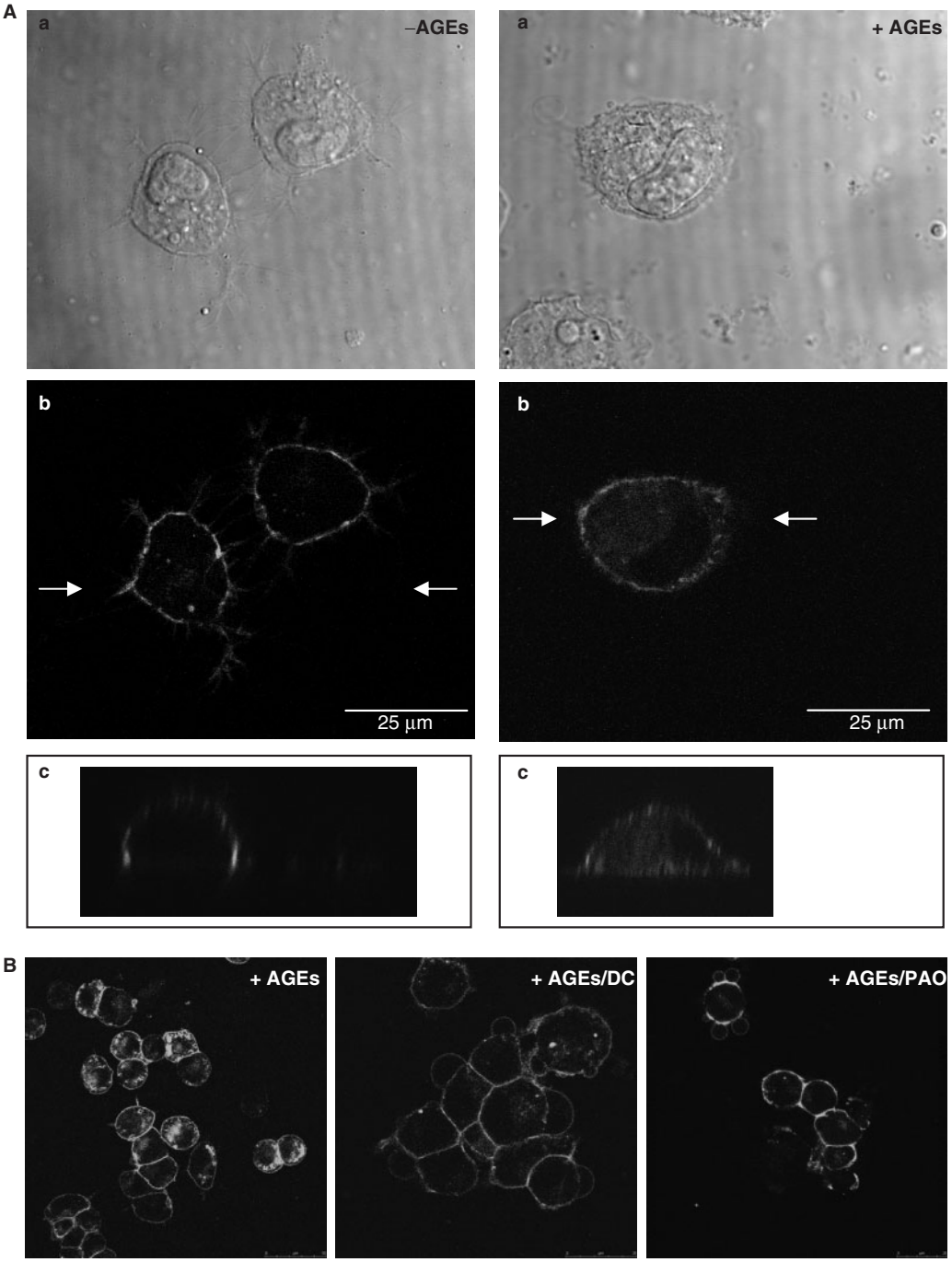

Fig. 4. **AGEs–RAGE complex internalization.** (A) N2a pRAGE–EGFP transfected cells were incubated in the presence or absence of BSA–AGEs (100 μg/ml) for 3 h and analysed by confocal fluorescence microscopy. (a) Differential interference contrast (Nomarski) images, (b) EGFP fluorescence images, (c) EGFP fluorescence images of Z-sections of the cells through the lines marked with arrows shown in (b). (B) N2a pRAGE–EGFP transfected cells were pre-incubated or not for 30 min with DC (100 μM) or PAO (1 μM) and then incubated with BSA–AGEs (100 μg/ml) for 3 h. Cells were analysed for EGFP by confocal fluorescence microscopy.

DISCUSSION

The RAGE is expressed in a wide range of cell types: vascular smooth muscle cells, endothelial cells, mononuclear phagocytes, mesangial cells, neurons and lung alveolar epithelial cells (*5*). The receptor, through the interaction with its ligands, is involved in numerous pathological processes as immune/inflammatory disorders, Alzheimer's disease, tumourigenesis and long-term complications associated with diabetes (*5–9*, *23*).

Binding of the ligands to the receptor induces expression of the receptor itself and amplifies a

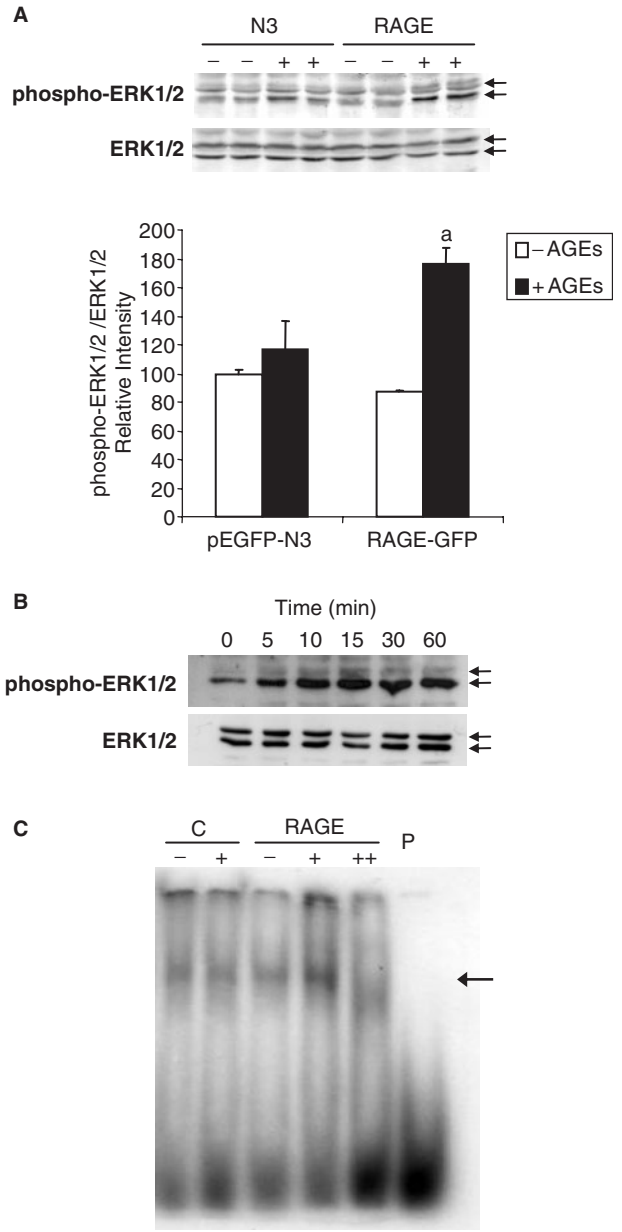

Fig. 5. **RAGE–EGFP-mediated intracellular responses.** (A) AGE-mediated phosphorylation of ERK1/2. N2a isolated clones transfected with pEGFP-N3 or pRAGE–EGFP were incubated in absence (–) or presence (+) of BSA–AGEs (100 μg/ml) for 30 min and subjected to SDS–PAGE and western blotting using ERK1/2 or phospho-ERK1/2 antibodies. Results are expressed as means ± SEM of four independent experiments. a: $P < 0.001$ compared with control cells. (B) Time course of AGE-mediated ERK phosphorylation. (C) AGE-mediated NF-κB activation. N2a cells were incubated in absence (–) or presence (+) of BSA–AGEs (100 μg/ml) for 24 h and the activation of NF-κB was determined by an EMSA. C: untransfected cells, RAGE: cells transfected with pRAGE–EGFP, P: free probe, '++': competition with 100-fold unlabelled NF-kB probe.

pro-inflammatory response (*3*, *6*, *10*) through the activation of several signal transduction pathways as Ras, Rac/Cdc42 and Jak/Stat. Although the involvement of RAGE in pathological processes is well studied, little is known of the molecular mechanisms that connect the receptor with the different signalling pathways. For example, a direct interaction between the cytosolic tail of RAGE and ERKs (*24*) has been reported, but the molecule able to bring together the receptor and the ERKs is unkown. Also, little is known about the receptor intracellular trafficking and it is not even known if its internalization is necessary to produce intracellular responses.

To study the rat RAGE intracellular trafficking and to facilitate *in cellula* studies of the receptor functionality, we have generated a construct that codifies for a fusion protein between rat RAGE and EGFP to facilitate the receptor visualization (*25*). A 12 amino acids (VDGTAGPGSIAT) linker was placed in the RAGE–EGFP boundary. For this linker, molecular modelling (*26*) predicts a non-defined secondary structure and therefore the adoption of an extended flexible conformation. We have tested the usefulness of the fusion protein to study the receptor for AGEs functionality. Our results indicate that the fusion protein between the RAGE and EGFP is not toxic for the cell and it behaves like the native RAGE, since the fusion protein (i) is located predominantly at the plasma membrane (Fig. 2), (ii) retains the capability to bind AGEs (Fig. 3) and (iii) is able to activate signal transduction pathways as ERK1/2-mediated signalling that leads to a cellular activation measured as an increase in NF-κB DNA binding (Fig. 5). This is the first time that the strategy of fusing the RAGE to the EGFP is shown to be an useful tool to characterize the receptor.

Using the RAGE–EGFP protein, we have studied the intracellular trafficking of the receptor after ligands binding. By confocal microscopy with Texas Red BSA–AGEs as ligand, we observed a co-localization of the green and red fluorescence inside the cell (Fig. 3). Also, upon incubation with unlabelled AGEs, we observed that the green fluorescence localized in the plasma membrane is dispersed as granules in the cytoplasm of cells (Fig. 4). These results suggest an internalization of the receptor after ligand binding.

Since the intracellular signals triggered by the binding between ligands and RAGE are important in pathological processes, we have determined whether the internalization of the AGEs–RAGE complex is needed for the cellular activation. For this purpose, we have used two specific inhibitors of receptor internalization DC and PAO (*20*, *21*). First, we have shown that pre-incubation with these two inhibitors prevents the AGE-induced RAGE–EGFP subcellular relocalization (Fig. 4B). As the activation of ERK1/2 has been described as a key step in the signalling mediated by the receptor (*4*, *11*, *13*), we have measured the effects of the BSA–AGEs addition on the phosphorylation of ERK1/2 in the absence or presence of DC and PAO. Both compounds have been previously used as specific inhibitors of receptors internalization that involve the ERKs signalling pathway (*21*). Furthermore, to confirm that these compounds are not acting as unspecific ERKs phosphorylation inhibitors, we have assayed the phosphorylation of ERKs induced by sodium tungstate (*17*, *22*) in the presence of DC and PAO (ESM Fig. 2). Neither DC nor PAO has any effects on the ERKs phosphorylation mediated by tungstate.

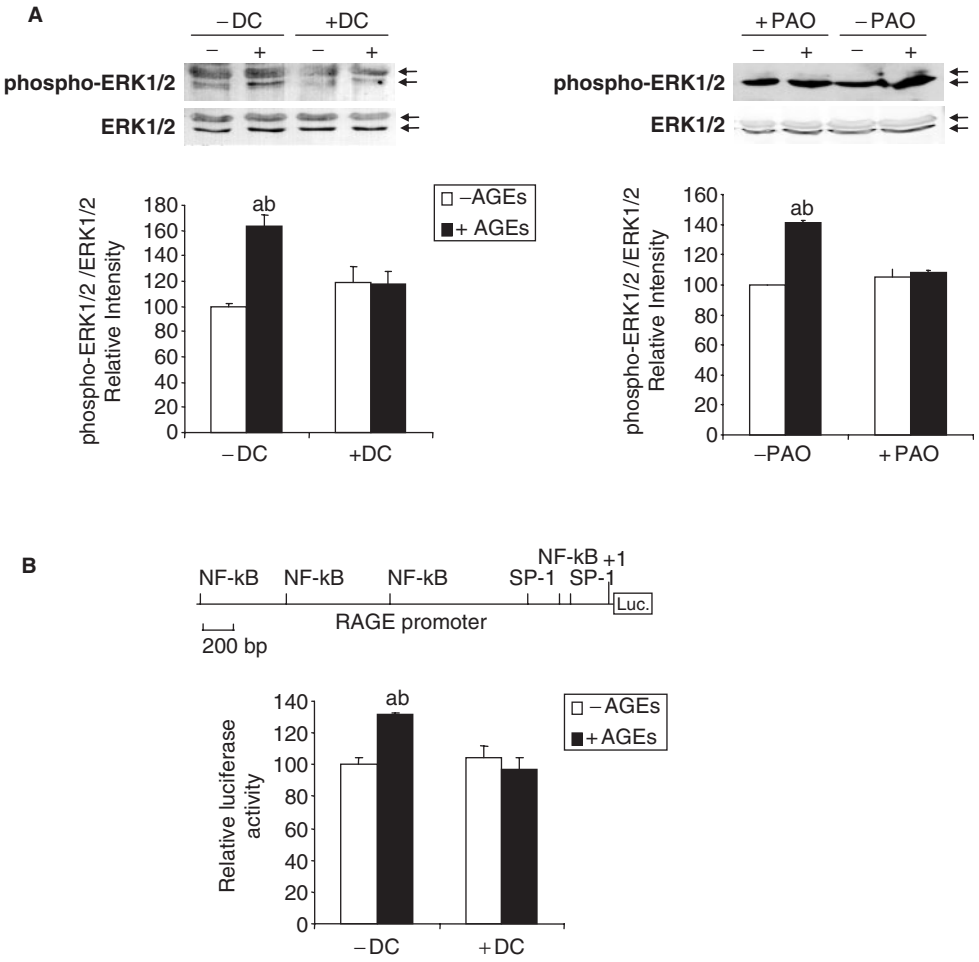

Fig. 6. **Role of the ligand-induced internalization in the generation of intracellular responses.** (A) Suppression of AGE-mediated phosphorylation of ERK1/2 by inhibition of RAGE internalization. N2a cells expressing RAGE–EGFP were pre-incubated in the absence or presence of DC or PAO for 30 min and then incubated for 30 min with BSA–AGEs (100 μg/ml). Cell lysates were immuno-blotted with ERK1/2 and phospho-ERK1/2 antibodies. Results are expressed as means ± SEM of four independent experiments. a: $P < 0.01$ compared with cells without BSA–AGEs, b: $P < 0.05$ compared with cells incubated with BSA–AGEs and with DC or PAO. (B) Luciferase activity of cells transfected with pGL3-RAGE. A scheme of the rat RAGE promoter region studied is shown. The relative NF-kB binding sites in the promoter are indicated. N2a cells transfected with the pGL3-RAGE construction were pre-incubated in the absence or presence of DC for 30 min and then incubated for 3 h with BSA–AGEs (100 μg/ml). Results are expressed as means ± SEM of five independent experiments. a: $P < 0.01$ compared with cells without BSA–AGEs, b: $P < 0.01$ compared with cells incubated with BSA–AGEs and with DC.

Once validated the use of DC and PAO as receptors internalization inhibitors, we have used them to study the effects of RAGE internalization on the ERKs signalling pathway. In the N2a pRAGE–EGFP transfected cells, the addition of AGEs increased significantly the ERK1/2 phosphorylation compared to the pEGFP-N3 transfected cells (Fig. 5A and B), while pre-incubation with DC or PAO blocked the AGE-mediated phosphorylation of ERK in N2a cells expressing RAGE–EGFP (Fig. 6A).

Additionally, to rule out artifacts in the internalization due to the fusion to the EGFP, we have assayed the effects of DC in a downstream step of the activation of the signalling pathways mediated by RAGE in the untransfected N2a. The AGEs augment the RAGE promoter activity through an increase in the DNA binding capacity of NF-κB mediated by the RAGE (*27*) as shown in Fig. 4C. Since the N2a cells express the RAGE constitutively, we have measured the increase in the RAGE promoter activity due to AGEs in the presence or absence of DC. Our results show that while in the untransfected cells the addition of AGEs significantly increases the promoter activity, pre-incubation with DC inhibits the response to AGEs (Fig. 6B).

In their totality, our results support the idea that the RAGE internalizes upon binding to AGEs and that the internalization is needed for the generation of intracellular responses. Several approaches have been proposed to block the generation of intracellular responses mediated by AGEs. These methods are based in blocking the binding of the AGEs to RAGEs. For example, the use of a recombinant protein corresponding to the extracellular domain of the receptor *in vivo* has shown to be able to block the generation of intracellular responses (*28*).

Also, transfection of cells with an isoform of the receptor lacking the intracellular domain has been shown in cultures to decrease the signal transduction mediated by the native receptor (*29*). Here, we demonstrate a new mechanism able to block the signal transduction mediated by the AGEs–RAGE interaction. The fact that the internalization of receptor is needed for the generation of intracellular responses could be important in the therapy of long-term complications of diabetes, since blocking specifically the internalization of the RAGE, it would be possible to prevent some of these complications.

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CONFLICT OF INTEREST

None declared.

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