Metformin Reverts Deleterious Effects of Advanced Glycation End-Products (AGEs) on Osteoblastic Cells

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Key words
- metformin
- advanced glycation end product
- osteoblasts
- oxidative stress
- RAGE

Abstract

Advanced glycation endproducts (AGEs) are implicated in the complications of diabetes and ageing, affecting several tissues, including bone. Metformin, an insulin-sensitizer drug, reduces the risk of life-threatening macrovascular complications. We have evaluated the hypothesis that metformin can abrogate AGE-induced deleterious effects in osteoblastic cells in culture. In two osteoblast-like cell lines (UMR106 and MC3T3E1), AGE-modified albumin induced cell death, caspase-3 activity, altered intracellular oxidative stress and inhibited alkaline phosphatase activity. Metformin-treatment of osteoblastic cells prevented these AGE-induced alterations. We also assessed the expression of AGE receptors as a possible mechanism by which metformin could modulate the action of AGEs. AGEs-treatment of osteoblast-like cells enhanced RAGE protein expression, and this up-regulation was prevented in the presence of metformin. Although the precise mechanisms involved in metformin signaling are still elusive, our data implicate the AGE-RAGE interaction in the modulation of growth and differentiation of osteoblastic cells.

received 09.08.2007
first decision 09.10.2007
accepted 15.10.2007

Bibliography
DOI 10.1055/s-2007-992786
Published online: February 14, 2008
Exp Clin Endocrinol Diabetes 2008; 116: 333–340

ISSN 0947-7349

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Introduction

Several studies have demonstrated the deleterious effects of diabetes on bone (Bouillon, 1991; Carnevale et al., 2004; Schvartz, 2003; Vestergaard, 2007). Diabetic patients exhibit an increased risk of fracture when compared to the non diabetic population. Patients with type 1 diabetes have low bone mass caused by reduced bone formation and impaired fracture healing, with reduced number and function of osteoblasts. In type 2 diabetes, which is associated with an increased bone mineral density, there is evidence of increased risk of fracture and impaired bone healing (Schwartz et al., 2001; Strotmeyer et al., 2005; Vestergaard et al., 2005). Other authors have proposed that diabetes is associated with a decreased osteoblastic recruitment and function and as a consequence with low bone turn-over that retards age-related bone loss. This would lead to an accumulation of microarchitectural bone damage, which could explain the increase in bone fractures observed in these patients (Krakauer et al., 1995). In addition, it has recently been reported that diabetes enhances the apoptosis of fibroblasts and osteoblasts mainly by activating caspase-8, -9 and -3 expression and activity, which thus impairs bone healing (Al-Mashat et al., 2006).

Advanced glycation endproducts (AGEs) are implicated in the complications of diabetes and ageing (Brownlee, 2005). AGEs can arise from the non-enzymatic reaction of free amino groups of proteins, lipids or nucleic acids with reducing sugars to initially form unstable Schiff bases, and then rearrange to form Amadori products that finally undergo oxidative and non-oxidative modifications. Long lived proteins such as collagen are targets for AGEs formation. AGEs-mediated collagen overcrosslinking can cause loss of flexibility and elasticity and increased tissue brittleness (Ulrich and Cerami, 2001).

We have shown that soluble and matrix-associated AGEs can modulate osteoblastic growth and differentiation (McCarthy et al., 1997; McCarthy et al., 2001). Although initially AGEs induce a stimulation of osteoblastic growth, long-term exposition to AGEs-modified proteins elicit an inhibition of proliferation, differentiation and mineralization of osteoblastic cultures. These effects are probably mediated by an increase in intracellular oxidative stress via receptors specific for AGEs proteins (Cortizo et al., 2003; McCarthy et al., 1999; Mercer et al., 2004; Mercer et al., 2007). We have reported

that osteoblastic cell lines express detectable levels of RAGE and AGE-R3/galectin-3. These receptors for AGEs are regulated in a time- and dose-dependent manner by AGEs in osteoblasts in culture. It has recently been reported that AGEs stimulate osteoblast apoptosis through its interaction with RAGE, via the MAP kinase signaling pathway and caspase-8 activation of caspase-3 (Alikhani et al., 2006).

Metformin is an insulin-sensitizer drug widely used in conditions associated with insulin-resistance such as type 2 diabetes. The UK Prospective Diabetes Study (UKPDS) showed that metformin treatment reduces the risk of life-threatening macrovascular complications compared to other anti-hyperglycaemic agents (UKPDS 34, 1998). A cardioprotective effect has also been suggested for the HMG-CoA reductase inhibitor cerivastatin, since it lowers the serum levels of carboxymethyl-lysine, and established AGE structure (Scharnagl et al., 2007). Metformin was shown to inhibit cytosolic and mitochondrial reactive oxygen species production induced by AGEs in endothelial and smooth muscle cells (Bellin et al., 2006). We have recently shown that metformin induces a dose-dependent increase in cell proliferation, differentiation and mineralization in two osteoblast cell lines (UMR106 and MC3T3E1), probably mediated by activation and redistribution of ERK 1/2 and induction of eNOS and iNOS (Cortizo et al., 2006).

In the present study we have evaluated the hypothesis that metformin can abrogate AGE-induced deleterious effects in osteoblasts in culture. To address this issue we investigated the effects of AGEs with or without metformin on osteoblastic differentiation, apoptosis and oxidative stress in two osteoblastic cell lines. We also assessed the expression of AGE receptors as a possible mechanism by which metformin could modulate the action of AGEs.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen, Buenos Aires, Argentina). Tissue culture disposable material was from Nunc (Tecnolab, Buenos Aires, Argentina). Centricon 10 kDa cutoff filter cartridges were purchased from Amicon Inc. (Beverly, Mass., USA). Goat polyclonal anti-RAGE was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The fluorogenic caspase-3 substrate Ac-DEVD-AFC was purchased from BIOMOL International (L.P., PA, USA). Di-aminobencidine (DAB), bovine serum albumin (BSA), ribose and Triton X-100 were obtained from Sigma-Aldrich (Buenos Aires, Argentina). Dihydro-rhodamine 123 (DHR), annexin V-FITC and propidium iodide (PI) were from Molecular Probes (Eugene, OR). Metformin was donated by Quimica Montpellier (Buenos Aires, Argentina). All other chemicals and reagents were purchased from commercial sources and were of analytical grade.

Preparation of AGEs

AGEs-BSA was produced by incubation of 10 mg/ml BSA with 100 mM ribose in 150 mM phosphate-buffered saline (PBS), pH 7.4 at 37 °C for 3 weeks (McCarthy et al., 1997). Ribose was used as the glycating sugar instead of glucose to speed up non-enzymatic glycosylation. Control BSA was incubated in the same conditions without sugar. Unbound sugar was removed by centrifugation/filtration with Centricon filter cartridges. The formation of AGEs was assessed by their characteristic fluorescence-emission maximum at 420 nm upon excitation at 340 nm. The estimated levels of AGEs-BSA obtained in this *in vitro* incubation were 18.5 % relative fluorescence intensity/mg protein, in contrast to 3.2 % for control-BSA.

Cell culture and incubations

UMR106 rat osteosarcoma cells and MC3T3E1 mouse calvaria-derived cells were grown in DMEM containing 10 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5 % CO_2 atmosphere (McCarthy et al., 1997). Cells were seeded on 75 cm^2 flasks, sub-cultured using trypsin-EDTA and replated on multi-well plates. The UMR106 cell line has been shown to conserve certain characteristics of differentiated osteoblastic phenotype (Partridge et al., 1983). In the case of non-transformed MC3T3E1 cells, previous studies have demonstrated that expression of osteoblastic markers begins after culturing the cells with medium supplemented by 5 mM β-glycerol-phosphate (βGP) and 25 μg/ml ascorbic acid (AA) (Quarles et al., 1992). Under these culture conditions, alkaline phosphatase activity (ALP) begins to be expressed after 1 week and reaches a maximum after 2 weeks, while mineralization is achieved after extending the culture to 3 weeks. However, the cells only undergo active replication during the first 5 days of incubation. For apoptosis and oxidative stress experiments in both cell lines, and differentiation experiments with UMR106 osteoblasts, cells seeded on multi-well plates were incubated in DMEM medium with different doses of BSA or AGEs-BSA with or without metformin, during the periods of time indicated in the legends of figures. For ALP expression experiments with MC3T3E1 osteoblasts, cells were cultured for 2 weeks in DMEM/FBS supplemented with β-glycerol-phosphate and ascorbic acid changing the medium every 2 days, after which they were serum-starved and incubated in DMEM with or without different doses of BSA or AGEs-BSA with or without metformin, for an additional 72 hours.

Assays for osteoblast differentiation

Osteoblastic differentiation was evaluated by measuring alkaline phosphatase activity (ALP). ALP was assayed as we have previously described (Cortizo and Etcheverry, 1995; Molinuevo et al., 2004). Briefly, the cell layer was washed with PBS and solubilized in 0.5 ml 0.1 % Triton X-100. Aliquots of the total cell extract were used for protein determination by the Bradford technique (Bradford, 1976). Measurement of ALP was carried out by spectrophotometric determination of initial rates of hydrolysis of *para*-nitrophenyl-phosphate (p-NPP) to *para*-nitrophenol (p-NP) at 37 °C for 10 min. The production of p-NP was determined by absorbance at 405 nm. Under our experimental conditions p-NP formation was linear for 15 min.

Evaluation of cell death

Apoptosis induced by AGEs-BSA was evaluated using an annexin V-FITC/propidium iodide (PI) assay as we have previously described (Molinuevo et al., 2004). Early apoptotic stages were characterized by annexin V-FITC-positive/PI-negative (V^+/PI^-) staining, while apoptotic/necrotic stages were V^+/PI^+. The percentage of apoptotic and necrotic osteoblasts was determined by counting the cells per field in 20 fields per coverslip. To evaluate possible pathways involved in osteoblastic cell death, caspase-3 activity was measured by determining the degradation of the fluorogenic substrate Ac-DEVD-AFC as previously described (Plotkin et al., 1999). Briefly, after incubation with BSA or AGEs-

Table 1 Effects of AGE and metformin on the apoptosis of osteoblasts. Cells were cultured in DMEM with unmodified BSA or AGEs-BSA with or without 500 μM metformin for 24 hours, and then evaluated using an annexin V-FITC/propidium iodide (PI) assay. Early apoptotic osteoblasts were characterized by annexin V-FITC-positive/PI-negative (V^+/PI^-) staining

Dose [μg/ml]	BSA	BSA + Met	AGE	AGE + Met
MC3T3E1				
100	100 ± 16	178 ± 6	134 ± 15*	109 ± 13
200	100 ± 14	76 ± 6	125 ± 16*	75 ± 12
UMR106				
100	100 ± 11	82 ± 19	144 ± 11*	103 ± 10
200	100 ± 9	109 ± 13	131 ± 10*	75 ± 8

Met : 500 μM metformin

Results are expressed as % of basal (mean ± SEM). *$p < 0.05$

BSA with or without 500 μM Metformin for 24 h, cells were lysed and protein content assessed by the Bradford technique (Bradford, 1976). Lysates (100 μg protein) were incubated with Ac-DEVD-AFC for 1 h at 37 °C. The released fluorescent product AFC was measured with a spectrofluorometer (excitation wavelength 400 nm, emission wavelength 482 nm).

Determination of reactive oxygen species (ROS) formation

Intracellular ROS generation in osteoblastic cells was measured by oxidation of dihydro-rhodamine 123 (DHR) to rhodamine as we have previously described (McCarthy et al., 2001). Osteoblastic cell monolayers growing in 24-well plates were submitted to various culture conditions. Medium was replaced by phenol red-free DMEM with 10 μM DHR and the cells were further incubated for 4 hours. After washing with PBS, the monolayer was lysated in 0.1 % Triton X-100. The oxidised product present in the cell extract (rhodamine), was analysed by measuring fluorescence (excitation wavelength 495 nm, emission wavelength 532 nm).

Western blot analysis of RAGE

We evaluated the expression of RAGE by both cell lines, the effect of osteoblastic exposure to AGEs-modified BSA on their expression of RAGE, as well as the possible effect of metformin. In these experiments, osteoblastic cells grown for different periods of time with control-BSA or AGEs-modified BSA, with or without metformin, were lysated in Laemmli's buffer (Laemmli, 1970), and the protein content was evaluated by the method of Lowry (Lowry et al., 1951). These lysates were heated at 100 °C for 3 min and 30 μg of protein subjected to 12 % SDS-PAGE. The separated proteins were then transferred to PVDF membranes. After washing and blocking, the membranes were incubated with anti-RAGE polyclonal antibodies for 24 h at 4 °C. Blots were developed using DAB-peroxidase staining. The intensity of the specific bands was quantified by densitometry after scanning of the membrane. Images were analysed using the Scion-beta 2 program.

Immunofluorescent evaluation of RAGE

Sub-confluent osteoblasts grown on glass coverslips were washed in PBS and fixed with 4% paraformaldehyde in PBS (10 min at room temperature) (Mercer et al., 2004). Non-specific binding sites were blocked with 1 % BSA in PBS for 2 h. Cells were then incubated with goat polyclonal anti-RAGE antibodies (1:100 in blocking buffer) overnight at 4 °C. After washing, cells were exposed to a rabbit anti-goat IgG-FITC (1:200) for 1 h at room temperature. Cells were mounted in 80 % glycerol in PBS and observed under a Nikon-5000 fluorescence microscope. All fluorescence microscopy evaluations were performed by the same operator, and 20 representative fields were evaluated per coverslip.

Statistical analysis

The results are expressed as the mean ± SEM and were obtained from three separate experiments performed in triplicate. Differences between the groups were assessed by Oneway ANOVA with Tukey post-hoc test. For non normal distributed data, non parametrical Kruskal Wallis with Dunn post-hoc test was performed using GraphPad In Stat version 3.00 (Graph Pad Software, San Diego, California, USA). A p value < 0.05 was considered significant for all statistical analyses.

Results

Effect of AGEs and metformin on osteoblastic death and differentiation

To investigate the effects of AGEs on apoptosis and differentiation of osteoblasts, two osteoblast-like cell lines (MC3T3E1 and UMR106) were used. The effect of different doses of control-BSA or AGEs-modified BSA on apoptosis and alkaline phosphatase activity were measured after 24–72 h in osteoblastic cells.

Early apoptosis (annexin-V^+/PI^-) was estimated by FITC-annexin-V binding to externalized phosphatydyl serine, while cell death by late apoptosis/necrosis (annexin-V^+/PI^+) was evaluated by additional nuclear staining with PI. After 24 h incubation, 100 and 200 μg/ml AGEs-BSA induced an increase in the number of apoptotic (**Table 1**) as well as necrotic cells (**Table 2**) in both osteoblastic lines. This effect was dose-dependent in the case of late apoptosis/necrosis. No effects were observed in the presence of lower (50 μg/ml) doses of AGEs-BSA (data not shown). The co-incubation of 500 μM metformin with BSA for 24 h did not affect the proportion of necrotic osteoblasts; however this drug blunted the AGEs-induced increase in cell death in the osteoblastic cultures.

To confirm these results, and evaluate possible pathways involved in osteoblastic cell death, we performed a fluorogenic assay to measure the activity of caspase-3 in different experimental conditions. As can be seen in **Fig. 1**, AGEs-BSA induced a dose-dependent increase of caspase-3 activity in both cell lines. This pro-apoptotic effect was totally reverted by 500 μM metformin in UMR106 cells and 200 μg/ml AGE-BSA-treated MC3T3E1 cells. Metformin partially reverted the effect of higher doses of AGE-BSA in the MC3T3E1 line.

In order to examine the effect of AGEs and metformin on osteoblast differentiation, alkaline phosphatase activity was evalu-

Table 2 Effects of AGE and metformin on the late apoptosis/necrosis of osteoblastic cells. Cells were cultured in DMEM with unmodified BSA or AGEs-BSA with or without 500 μM metformin for 24 hours, and then evaluated using an annexin V-FITC/propidium iodide (PI) assay. Late apoptotic /necrotic osteoblasts were characterized by annexin V-FITC-positive/PI-positive (V^+/PI^+) staining

Dose [μg/ml]	BSA	BSA + Met	AGE	AGE + Met
MC3T3E1				
100	100 ± 26	51 ± 10	161 ± 37	60 ± 15
200	100 ± 24	82 ± 14	251 ± 33*	73 ± 17
UMR106				
100	100 ± 12	111 ± 31	118 ± 12*	65 ± 8
200	100 ± 15	86 ± 29	213 ± 32*	122 ± 26

Met : 500 μM metformin

Results are expressed as % of basal (mean ± SEM). *$p < 0.05$

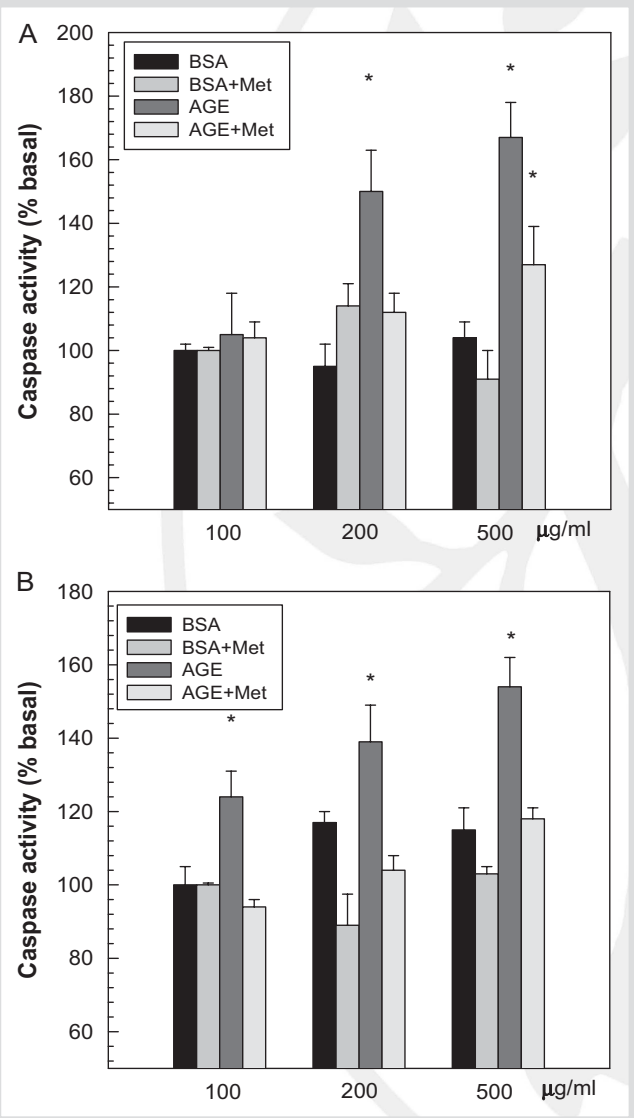

Fig. 1 Effect of AGEs and metformin on osteoblastic Caspase-3 activity. After incubation with BSA or AGEs-BSA with or without 500 μM metformin for 24 h, MC3T3E1 (**A**) or UMR106 (**B**) osteoblastic cells were lysed and protein content determined. Lysates (100 μg protein) were incubated with Ac-DEVD-AFC for 1 h at 37 °C. The released fluorescent product AFC was measured with a spectrofluorometer (excitation wavelength 400 nm, emission wavelength 482 nm). Results are expressed as % of basal. Each bar represents the mean ± SEM; *$p < 0.05$.

ated. Pre-osteoblastic MC3T3E1 cells were differentiated in the presence of ascorbic acid and β-glycerol phosphate for 2 weeks. Then, differentiated MC3T3E1 or UMR106 osteoblastic cells were treated with different doses of control-BSA or AGEs-BSA with or without 500 μM metformin for 72 h. **Fig. 2A** shows that 500 μg/ml AGEs-BSA induced a statistically significant inhibition in the ALP of MC3T3E1 cells. In the case of UMR106 cells, 100 μg/ml AGEs-BSA elicited a significant inhibition of ALP (**Fig. 2B**), while a non-significant tendency to decrease was also observed at 200 and 500 μg/ml AGEs-BSA. The co-incubation of AGEs with 500 μM metformin completely prevented the AGEs-induced decrease in osteoblastic ALP activity, in both cell lines.

Effect of metformin on AGEs-induced oxidative stress in osteoblasts

The induction of oxidative stress in osteoblasts in culture was evaluated by the DHR123 fluorogenic assay. In this assay, DHR taken up by osteoblasts can be oxidized by intracellular ROS to the fluorophore rhodamine.

In the MC3T3E1 cells, 100–200 μg/ml AGEs-BSA significantly inhibited rhodamine-associated fluorescence after 24 h incubation (**Fig 3A**). On the contrary, the same doses of AGEs enhanced intracellular oxidative stress in the osteosarcoma UMR106 cell line (**Fig. 3B**). When cells were incubated in the presence of 100–200 μg/ml AGEs-BSA and 500 μM metformin, this anti-diabetic drug abolished the AGEs-induced effect on intracellular oxidative stress in both osteoblastic lines.

Effect of metformin on AGEs receptor protein expression

The above studies provide evidence that metformin can prevent deleterious effects induced by AGEs in osteoblasts. We next performed studies to investigate the possible involvement of the receptor for AGEs, RAGE. The effects of metformin on RAGE expression in osteoblasts were evaluated by Western immunoblot and by immunofluorescence using specific anti-RAGE antibodies. **Fig. 4** shows that 48 h-treatment with 200 μg/ml AGEs-BSA increased RAGE protein expression as measured by immunoblot, in both MC3T3E1 and UMR106 osteoblasts. However, when cells were co-incubated with 500 μM metformin, the AGEs-induced up-regulation of RAGE was abrogated in both osteoblastic lines (**Fig. 4**). Treatment of osteoblastic cells with metformin in the presence of unmodified-BSA (basal condition), did not affect RAGE expression.

RAGE-associated immunofluorescence was present in both cell lines with a diffuse staining pattern (**Fig. 5A,B**). Both osteoblastic cell lines which had previously been exposed to AGEs-

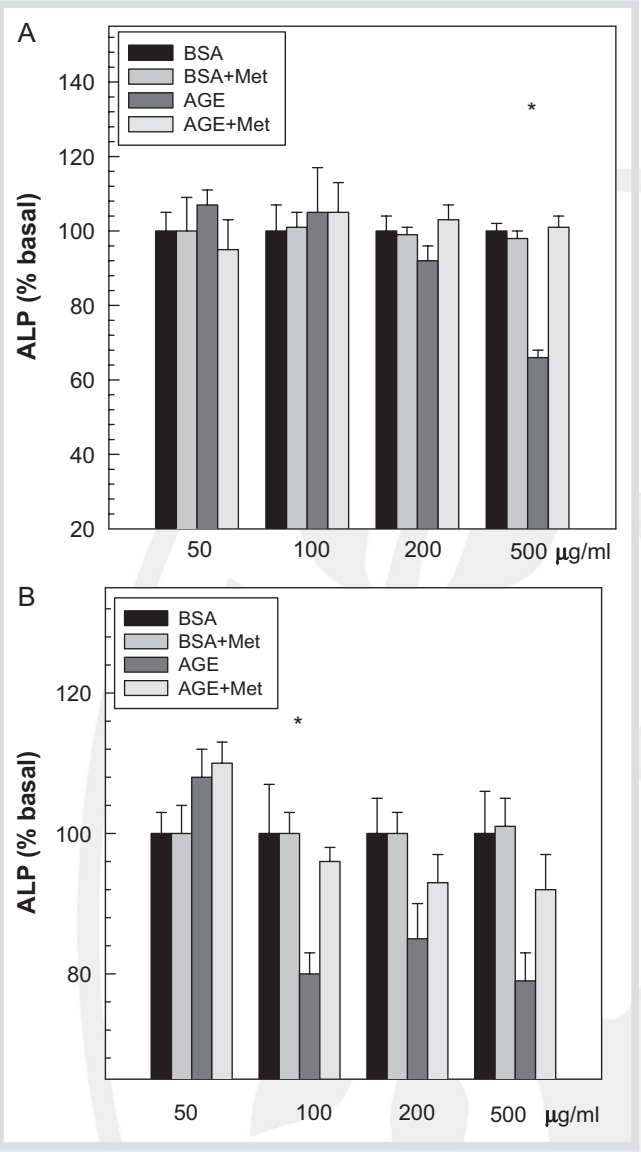

Fig. 2 Effect of AGEs and metformin on osteoblastic differentiation. (**A**) MC3T3E1 cells were grown and differentiated for 15 days in DMEM–10 % FBS, ascorbic acid (25 μg/ml) and β-glycerophosphate (5 mM). Then, cells were treated with different doses of unmodified-BSA or AGE-BSA with or without 500 μM metformin for 72 h. (**B**) UMR106 cells were seeded at 50–60 % density and grown for 24 h in DMEM–10 % FBS. Cells then were incubated in DMEM medium in the presence of different doses of BSA or AGE-BSA with or without 500 μM metformin for 48 h. Alkaline phosphatase specific activity was determined as described in Materials and methods and is expressed as % of basal. Each bar represents the mean ± SEM; $^*p<0.05$.

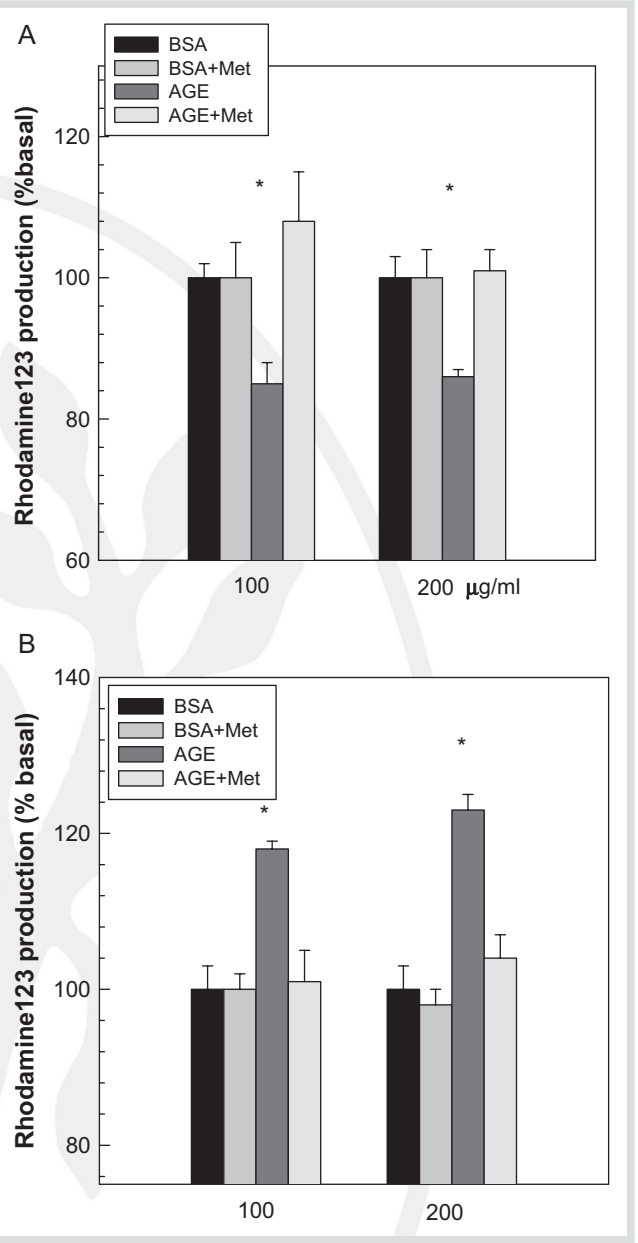

Fig. 3 Effect of AGEs and metformin on intracellular oxidative stress. MC3T3E1 (**A**) or UMR106 (**B**) cells were seeded at 70–80 % density in 24-well plates and grown for 24 h. Cells were then incubated in DMEM medium with different doses of unmodified-BSA or AGEs-BSA with or without 500 μM metformin. After 24 hours, media were replaced by phenol red-free DMEM with 10 μM dihydro-rhodamine, and cells were incubated for an additional 4 hours. At the end of this incubation the cell monolayers were washed with PBS and lysated with 0.1 % Triton X-100, and the levels of the oxidation product rhodamine were measured in the lysates by determination of fluorescence intensity (excitation wavelength 495 nm, emission wavelength 532 nm). Results are expressed as % of basal. Each bar represents the mean ± SEM; $^*p<0.05$.

BSA showed an increase in cell-surface RAGE-associated immunofluorescence, when compared with osteoblasts incubated with BSA alone, BSA plus metformin (data not shown), or AGEs-BSA plus metformin. These observations further support the idea that metformin prevents the up-regulation of RAGE induced by treatment with AGEs.

Discussion

Chronic complications of Diabetes are multifactorial in origin (Forbes et al., 2007). However, excess formation of AGEs is believed to play a predominant role in their pathogenesis. AGEs accumulate in various tissues in patients with diabetes and in aged individuals, and have been implicated in chronic complications, including diminished bone formation and turnover (Brownlee, 2005; Odetti et al., 2006; Hein et al., 2006; Hein et al., 2003). However, the mechanisms by which AGEs affect bone are not completely known. We have previously shown that AGEs can directly affect growth and differentiation of osteoblasts

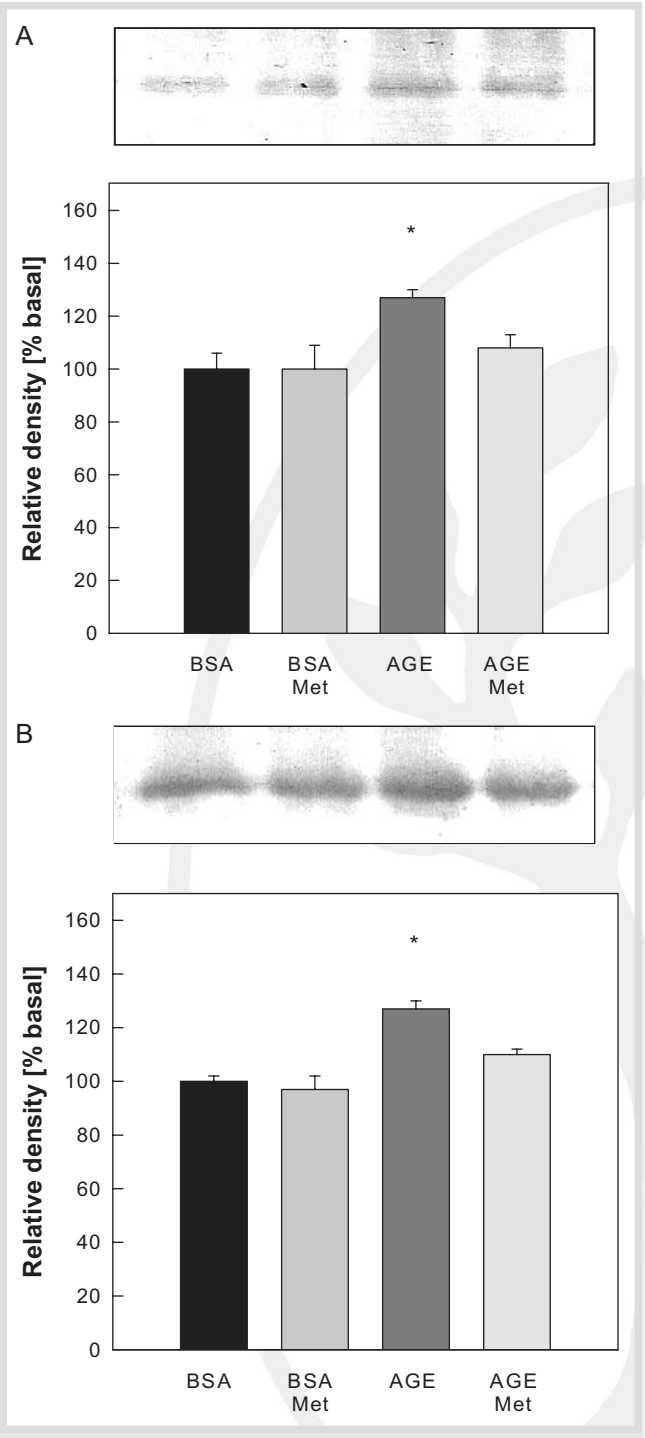

Fig. 4 Effects of metformin on AGE-induced up-regulation of RAGE. MC3T3E1 (**A**) and UMR106 (**B**) cells were seeded in 6-well plate and grown in DMEM–10 % FBS for 24 h. Cells were then cultured in DMEM medium with 200 μg/ml unmodified-BSA or AGE-BSA with or without 500 μM metformin for 48 h. Cells were washed with PBS, proteins extracted with Laemli's buffer and Western blot analysis was performed for RAGE as described in Materials and methods. Figures are representative of three separate experiments. Each bar represents the mean ± SEM; $^{*}p < 0.05$.

(McCarthy et al., 1997; McCarthy et al., 2001). These actions seem to be mediated through the activation of specific receptors for AGEs such as RAGE and galectin-3, which are present in osteoblasts (Cortizo et al., 2003; Mercer et al., 2004; Mercer et al., 2007). Recently, it has been demonstrated that AGEs-modified proteins induce apoptosis in osteoblasts (Alikhani et al., 2006) and mesenchymal stem cells (Kume et al., 2005). It has also been shown that the inhibition of diabetes-associated osteoblastic apoptosis significantly improves new bone formation, suggesting that this mechanism is physiologically relevant (Al-Mashat et al., 2006).

Metformin is an insulin sensitizing agent widely used in patients with diabetes and metabolic syndrome. We have recently shown that this drug exerts direct effects on osteoblasts in culture, enhancing their proliferation, differentiation and mineralization (Cortizo et al., 2006). Thus, we hypothesized that metformin could either block or prevent the deleterious effects of AGEs on bone-derived cells. Data presented here demonstrates that AGEs-modified albumin enhances osteoblast death by both apoptotic and necrotic mechanisms, by pathways which involve caspase-3 activation. Similar doses have been previously shown to inhibit the proliferation and differentiation of osteoblasts and to engage AGEs-binding sites (McCarthy et al., 1997; McCarthy et al., 2001; McCarthy et al., 1999). The present data also shows that metformin is able to prevent AGEs-induced apoptosis and necrosis in osteoblasts in culture. These results agree well with our previous report (Cortizo et al., 2006) that 500 μM metformin stimulates osteoblastic proliferation without inducing cell death. However, although our present results in the case of MC3T3E1 osteoblasts do not show significant differences in apoptosis/necrosis between cells incubated with BSA or BSA plus metformin, there does appear to be a tendency toward an anti-apoptotic effect of metformin. If this were the case, the observed suppression by metformin of the apoptotic/necrotic effects of AGEs might simply be the addition of two independently acting mechanisms.

In this study, we also assessed the effect of AGEs on osteoblastic differentiation by evaluating the specific marker alkaline phosphatase. After 72 h incubation, AGEs inhibited ALP in both cells lines, although a higher dose of AGEs was needed to induce a significant effect in the non transformed MC3T3E1 osteoblasts. This difference in responsiveness probably reflects the differences which exist in ALP expression of transformed versus non-transformed osteoblastic cell lines, since UMR106 osteosarcoma cells greatly over-express alkaline phosphatase in their basal state. Nevertheless, metformin was able to completely abrogate the effect of AGEs in both cells lines. We have previously demonstrated that metformin does not affect ALP in the osteosarcoma UMR106 cells but slightly (20 % over basal) stimulates this marker after 24 h incubation in differentiated MC3T3E1 cells (Cortizo et al., 2006). Altogether, the net effect observed in the present study was a reversion by metformin to the basal levels of ALP activity in both UMR106 and MC3T3E1 cells.

The mechanisms of action involved in the effects of AGEs and metformin are not completely known. AGEs have been shown to bind with specific receptors such as RAGE and galectin-3 (Bierhaus et al., 2005; Bierhaus et al., 2006). AGEs-RAGE interaction can induce activation of MAPK, nuclear translocation of NF-KB transcription factor, and subsequent induction of cell-type specific subsets of NF-KB-responsive genes (Haslbeck et al., 2005). Metformin probably has multiple cellular actions, for example as an antioxidant and as an indirect AMP-activated kinase stimulator. In particular, in the MC3T3E1 and UMR106 osteoblastic cells we have previously found that both AGEs and metformin can independently activate the MAPK pathway (i.e., ERK 1/2 phosphorylation), and also stimulate eNOS and iNOS expression (Cortizo et al., 2003; Cortizo et al., 2006; McCarthy et al., 2001). The fact that different agents with apparently oppo-

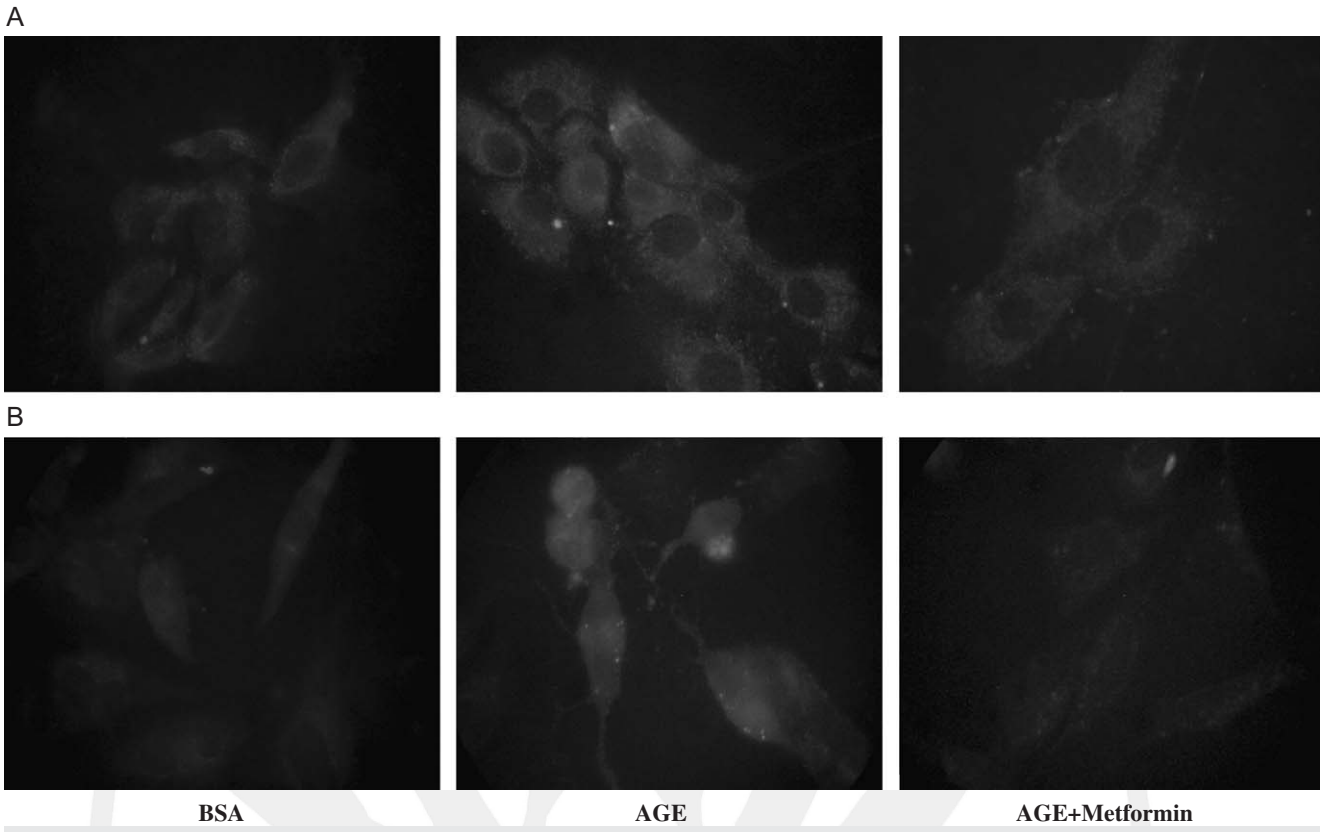

Fig. 5 Effect of metformin and AGEs on RAGE-associated immunofluorescence. MC3T3E1 (**A**) and UMR106 (**B**) osteoblastic cells were cultured in DMEM with 200 μg/ml of unmodified BSA or AGEs-BSA with or without 500 μM metformin for 48 h. Cells were then fixed and stained with an anti-RAGE antibody followed by FITC-conjugated secondary antibody. Specimens were examined by fluorescence microscopy. Obj 100x.

site effects on osteoblastic growth and development have similar effects on cell transduction pathways, can be explained by the pleiotropic nature of these mechanisms, although one cannot discard a divergence in the more downstream phases of these pathways. Unfortunately, the similar effects of AGEs and metformin on osteoblastic MAPK and NOS activation, precludes the design of experiments with specific inhibitors such as PD98059 or aminoguanidine, to demonstrate the involvement of these cell signaling mechanisms.

Our group and other investigators have previously demonstrated that AGEs induce cellular events associated with oxidative stress and generation of ROS. In the present study we have found that 100–200 μM AGEs affect intracellular oxidative stress in both cell lines but in opposite directions. While in non-transformed MC3T3E1 cells AGEs appear to inhibit ROS production, in UMR106 osteosarcoma cells a significant increase in ROS generation was detected by the DHR assay. These results agree with our previous report of ROS production in osteoblastic cell lines (McCarthy et al., 2001) and could be indicating differences in the responsiveness to AGEs of transformed versus non-transformed osteoblastic cells. Importantly, in the present study co-incubation with metformin prevented the alterations in intracellular oxidative stress induced by AGEs in both cell lines.

Considerable evidence has accumulated suggesting that the engagement of AGEs with RAGE triggers several deleterious effects of AGEs (Kaji et al., 2003; Mukherjee et al., 2005). In addition, it has recently been shown that metformin inhibits the expression of RAGE in endothelial cells (Ouslimani et al., 2007). Based on those results, on our previous studies in which we demonstrated that AGEs up-regulate the expression of RAGE in osteoblastic cells (Cortizo et al., 2003; Mercer et al., 2007), and on the present observations of the overall ability of metformin to prevent the effects of AGEs on osteoblasts, we hypothesized that metformin could reduce the deleterious effect of AGEs via a regulation in the expression of RAGE. Our present results indicate that AGEs-treatment of osteoblasts enhances RAGE protein expression (evaluated by Western immunoblot and immunofluorescence techniques), and that this up-regulation is prevented in the presence of metformin. This modulation of RAGE protein is probably due to changes in gene expression, since the RAGE gene promoter possesses an NF-KB-responsive element, and has been shown to be up-regulated by AGEs and other agents such as TNF-α (Marx et al., 2004; Tanaka et al., 2000) in various cell types. However, we cannot exclude the possibility of an increase in RAGE mRNA stability or post-transcriptional mechanisms, and further experiments are in progress in our laboratory to clarify this point. Osteoblasts also express AGER-2/galectin-3, which we have also demonstrated previously to be up-regulated in the presence of AGEs (Mercer et al., 2004; Mercer et al., 2007). In the present study, metformin was not able to block the AGEs-induced up-regulation of galectin-3 in both cell lines, suggesting that this receptor does not play a role in the protective effect of metformin on the AGEs-exposed osteoblasts (data not shown).

In conclusion, our present study demonstrates that metformin is able to prevent the increase in apoptosis, caspase 3 activity, inhibition of ALP and alterations in intracellular oxidative stress induced by AGEs in osteoblastic cells. Although the precise mechanisms involved in metformin signaling are still elusive, our data implicate AGEs-RAGE interaction in this modulation of the growth and differentiation of osteoblasts.

Acknowledgements

We wish to thank Química Montpellier Argentina for their generous donation of metformin and Dr. Claudio Gonzalez for his kind collaboration with the statistical analysis of the data. This work was partially supported by grants from Universidad Nacional de La Plata, Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICPBA), and the Novo Nordisk Laboratory, Argentina. AMC is a member of the Carrera del Investigador, CICPBA.

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