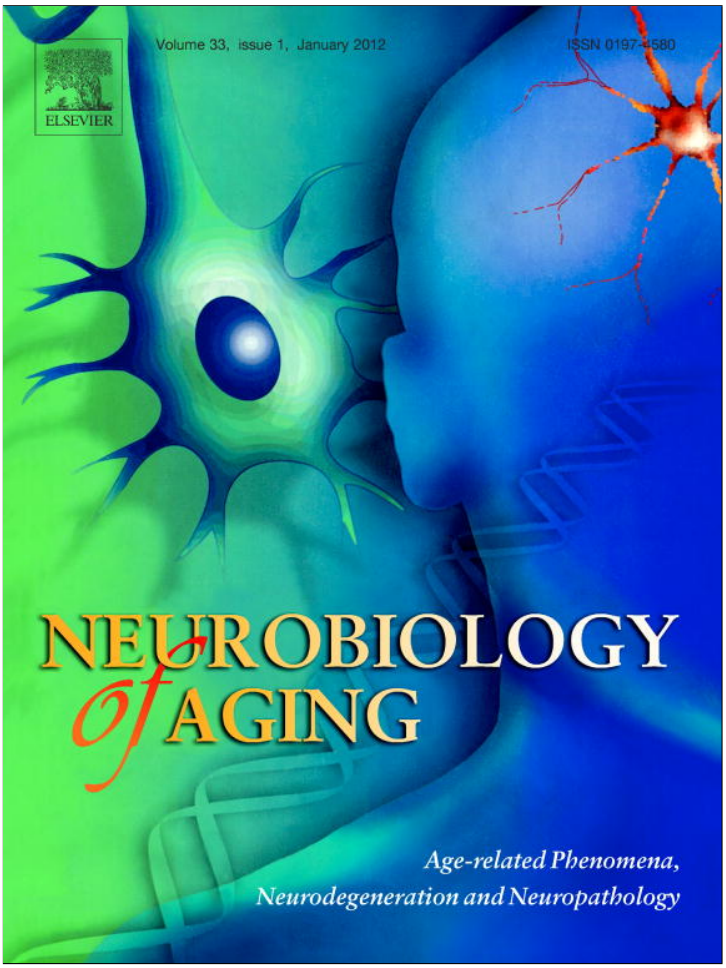

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AGEs/RAGE complex upregulates BACE1 via NF-κB pathway activation

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Abstract

Although the pathogenesis of sporadic Alzheimer disease (AD) is not clearly understood, it is likely dependent on several age-related factors. Diabetes is a risk factor for AD, and multiple mechanisms connecting the 2 diseases have been proposed. Hyperglycemia enhances the formation of advanced glycation end products (AGEs) that result from the auto-oxidation of glucose and fructose. The interaction of AGEs with their receptor, named RAGE, elicits the formation of reactive oxygen species that are also believed to be an early event in AD pathology. To investigate a functional link between the disorders diabetes and AD, the effect of 2 AGEs, pentosidine and glyceraldehydes-derived pyridinium (GLAP), was studied on BACE1 expression both in vivo, in streptozotocin treated rats, and in vitro in differentiated neuroblastoma cells. We showed that pentosidine and GLAP were able to upregulate BACE1 expression through their binding with RAGE and the consequent activation of NF-κB. In addition, both pentosidine and GLAP were found to be increased in the brain in sporadic AD patients. Our findings demonstrate that activation of the AGEs/RAGE axis, by upregulating the key enzyme for amyloid-β production, provides a pathologic link between diabetes mellitus and AD.

Keywords: Advanced glycation end products; RAGE; BACE1; Alzheimer disease; Diabetes; NF-κB pathway

1. Introduction

The central pathological event in Alzheimer disease (AD) is the progressive accumulation of amyloid-β (Aβ) in the brain. Aβ derives from sequential cleavage of the β-amyloid precursor protein (APP) by the β- and the γ-secretase. The β-secretase (β-site amyloid precursor protein cleaving enzyme, BACE1) cleaves the ectodomain of APP, producing an APP C-terminal fragment. This fragment is further cleaved within the transmembrane domain by the γ-secretase, resulting in the release of a family of Aβ peptides with different C-terminal variants, predominantly Aβ40 and Aβ42 (Selkoe, 2001).

AD can be classified in 2 major forms: sporadic AD accounting for ~ 99% of cases and familial AD comprised of rare early-onset forms in which gene mutations have been identified that foster the production or the aggregation of Aβ. Although the pathogenesis of sporadic AD is not clearly understood, it is likely the consequence of several,

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different, age-related factors. One of these risk factors, type 2 diabetes, has been associated with AD, and multiple hypotheses connecting the 2 diseases have been proposed (Granic et al., 2009; Jones et al., 2009; Kojro and Postina, 2009; Reddy et al., 2009).

Hyperglycemia, a consequence of diabetes, enhances the formation of advanced glycation end products (AGEs), senescent protein derivatives that result from the auto-oxidation of glucose and fructose (Bucala and Cerami, 1992). AGEs are, in fact, linked to the cytoskeletal changes occurring in AD (Dukic-Stefanovic et al., 2001; Kuhla et al., 2007). Moreover, glycation of Aβ is reported to enhance its aggregation and subsequent formation of senile plaques (Sasaki et al., 1998; Smith et al., 1994; Vitek et al., 1994). In addition, the interaction of AGEs with their receptor, RAGE, elicits the production of reactive oxygen species (ROS) that are also believed to occur early in AD pathology (Lue et al., 2001; Schmidt et al., 2001). Oxidative stress and Aβ are linked in a spiraling relationship: Aβ induces oxidative stress (Harkany et al., 2000; Mattson, 2004; Murray et al., 2007; Zhu et al., 2004) and pro-oxidant agents promote Aβ production (Atwood et al., 2003; Misonou et al., 2000; Paola et al., 2000; Zhang et al., 2009). We, and others, have shown that the activity of BACE1 and γ-secretase, key enzymes for Aβ production, are increased by oxidants (Chen et al., 2008; Guglielmotto et al., 2009; Kao et al., 2004; Tamagno et al., 2002, 2005, 2008; Tong et al., 2005), and that there is correlation between BACE1 activity and oxidative markers in sporadic AD (Borghi et al., 2007).

Recently, different types of AGEs have been characterized, depending on the molecule from which they originate (Takeuchi et al., 2007). One in particular, identified as AGE-2, is a glyceraldehyde-derived pyridinium (GLAP) AGE, a toxic molecule.

To demonstrate a novel functional link between diabetes and AD, we studied the effect of 2 AGEs, pentosidine and GLAP, on BACE1 expression both in vivo, in streptozotocin-treated rats, and in vitro, in differentiated SK-N-BE neuroblastoma cells. An additional line of evidence confirming our results is the correlation of the AGEs and BACE1 activity in the cortex in AD cases.

2. Methods

2.1. Tissues

We used frozen cerebral cortex (superior frontal gyrus) from 2 groups of cases: (1) 26 control cases, free of amyloid plaques as determined by immunocytochemistry with the monoclonal antibody 4G8, which recognizes residues 17–24 in Aβ. The mean age at death was 73 years $\pm$ 9 and the postmortem delay 9.2 hours; and (2) 32 cases with late-onset sporadic AD with a clinical history of disease and pathological diagnosis according to Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria, provided by the brain bank of the Case Western Reserve University, Cleveland, OH. The mean age at death was 78 years $\pm$ 10 and postmortem delay 10.2 hours.

2.2. Animal model of diabetes

Male Wistar rats (Harlan-Italy, Udine, Italy) weighing 200–220 g were cared for in compliance with the Italian Ministry of Health Guidelines (no. 86/609/EEC) and with the Principles of Laboratory Animal Care (NIH no. 85-23, revised 1985). The scientific project, including animal care, was reviewed and approved by the local committee on ethics. Animals were provided with Piccioni pellet diet (no. 48, Gessate Milanese, Italy) and water ad libitum. Hyperglycemia was induced through a single injection of streptozotocin (STZ) (50 mg/kg body wt) in the tail vein. Three days later glycemia was measured with the Accu-Check Compact kit (Roche Diagnostics, GmbH, Mannheim, Germany) on blood collected from the tail vein.

Only rats with blood glucose levels above 18 mmol/L were used in the experiments; normoglycemic rats were used as controls. After 6 weeks, control and hyperglycemic rats (n = 12–14 per group) were anesthetized with 20 mg/kg body wt of Zoletil 100 (Tiletamine-Zolazepam, Virbac, Carros, France) and euthanized by decapitation after aortic exsanguination. Blood was collected and the plasma isolated. The brain was removed, weighed, and a 100 mg portion was homogenized. Glycemia was evaluated as described above. The plasma insulin level was measured using enzyme-linked immunosorbent assay (ELISA) kits (Rat Insulin ELISA, Sylveniusgaten, Sweden).

2.3. Cell culture differentiation and treatments

SK-N-BE neuroblastoma cells were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium containing 2 mmol/L glutamine and supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and a 1% antibiotic mixture (penicillin-streptomycin-amphotericin) (all Sigma Chemical Co, CA, USA), in a humidified incubator at 37 °C with 5% CO_2. For differentiation, 2×10^6 cells were plated in 75 cm^2 culture flasks (Costar, Lowell, MA) and treated with 10 μM retinoic acid for 10 days. Pentosidine and GLAP were added to 1 μM for 72 hours. Eighteen μM NF-κB p50 or NF-κB p65 inhibitors (Santa Cruz Biotechnology) or a negative control peptide (Santa Cruz Biotechnology) were added immediately before the incubation with pentosidine or GLAP.

2.4. Tissue and cell extracts

Cytosolic and nuclear extracts were prepared using the method of Meldrum et al. (1997). Briefly, a 10% (wt/vol) rat brain homogenate was prepared using a Potter Elvehjem homogenizer (Wheaton, NJ) in a homogenization buffer containing 20 mM 4(2-hydroxyethyl) acid 1-piperazineethanesulfonic (HEPES), pH 7.9, 1 mM $MgCl_2$, 0.5 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT),

0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL aprotinin, and 2.5 μg/mL leupeptin. Homogenates were cleared by centrifugation at 1000*g* for 5 minutes at 4 °C. Supernatants were removed and centrifuged at 15,000*g* at 4 °C for 40 minutes to obtain a cytosolic fraction. The pelleted nuclei were resuspended in an extraction buffer containing 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% (wt/vol) glycerol, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, and 2.5 μg/mL leupeptin. The suspensions were incubated on ice for 30 minutes for high-salt extraction followed by centrifugation at 15,000*g* for 20 minutes at 4 °C. The resulting supernatants containing nuclear proteins were carefully removed and the protein concentration was determined using the Bradford assay (Bradford, 1976). Samples were stored at −80 °C until use. To obtain total extract, tissue samples were homogenized at 10% (wt/vol) directly with extraction buffer and centrifuged at 1000*g* for 5 minutes at 4 °C. Supernatants were stored at −80 °C. Preparation of cell lysates and nuclear extracts from tissue culture cells was performed as previously described (Tamagno et al., 2002, 2005).

2.5. Cell viability

Lactate dehydrogenase (LDH) activity was determined in culture media using a photometric assay based on the conversion of pyruvic acid to lactic acid by the enzyme LDH as previously described (Tamagno et al., 2003). Values for control and treated cells were expressed as percentage of the total LDH activity released by untreated cells lysed with Triton X-100.

2.6. Oxidative stress determinations

Hydrogen peroxide production by the cytosolic fractions of brain or cells was assessed by monitoring the generation of hydrogen peroxide (H_2O_2). The reaction was performed in a cuvette containing (1 mL final volume): 7.4 IU horseradish peroxidase, 40 μM acetylated ferrocytochrome-c, 5 μM p-hydroxyphenilacetic acid, and 100 μL of the cytosolic fractions. H_2O_2 release was expressed as the increase in the rate of ferrocytochrome-c acetylation, as a function of the 550 nm value minus the 540 nm value and an absorption coefficient of 19.9 mmol/L × cm-1, as described by Zoccarato et al. (1993).

Catalase activity was evaluated following the method of Aebi (1984). The reaction mixture (final volume 3 mL) was prepared directly in a cuvette: 1 mL of 30 mM H_2O_2 diluted in 50 mM sodium phosphate buffer, pH 7.0 and 2 mL of cytosol (diluted 1:100) were combined and monitored at 240 nm for 1 minute. A blank was prepared for each sample.

Glutathione-peroxidase Se-dependent (GSH-PX) activity was assayed by the method of Flohe and Gunzler (1984). A mixture (final volume of 1 mL) containing 0.6 M EDTA diluted in 6 mM K-phosphate buffer, pH 7.0, 100 μL of cytosol (diluted 1:100), 1.37 IU glutathione-reductase (GSH-reductase), 1 mM GSH and 0.1 mM Na-azide was incubated for 10 minutes at 37 °C. After incubation, 0.15 mM nicotinamide adenine dinucleotide phosphate reduced form (NADPH) diluted in 0.1% (wt/vol) $NaHCO_3$ was added to the mixture; 0.15 mM hydrogen peroxide, used as substrate, was then added to initiate the reaction. A kinetic analysis at 340 nm was performed and monitored for 3 minutes. Reduced and oxidized glutathione content was evaluated in cytosolic fractions following Owens's method (Owens and Belcher, 1965). The difference between total glutathione and reduced GSH content represents the GSSG content (expressed as μg/mg protein).

2.7. Synthesis of AGEs

Pentosidine and GLAP (glyceraldehyde derived pyridinium) were obtained by microwave-assisted synthesis starting from butoxy carbonyl (BOC) protected amino acids (data not shown) and characterized as described (Słowik-Zyłka et al., 2004; Usui et al., 2007).

2.8. Analyses of pentosidine and GLAP by mass spectrometry

Fractions of total extract (200 μL) were treated with 0.6 M hydrochloric acid for 2 hours at 40 °C and then centrifuged at 6000*g*. Twenty μL of supernatant were injected. A liquid chromatography-Fourier transform mass spectrometry (LC-FTMS) instrument was used. Chromatographic separations were achieved using a Dionex Ultimate 3000 system composed of a degasser, pump, autosampler, and column oven. Mass spectrometric analyses were performed using an LTQ-Orbitrap (Thermo, Rodano, Italy) spectrometer, with an electrospray interface and an ion trap as a high resolving power mass analyzer.

The chromatographic separations were run on a Phenomenex Synergi C18 column at 40 °C (150 × 2 mm, 3-μm particle size; Phenomenex, Torrance, CA). The flow rate was 200 μL/minute. A reverse phase mobile gradient was used: 95/5 to 0/60 v/v water/acetonitrile in 5 mM heptafluorobutanoic acid in 13 minutes.

The LC column eluent was delivered to the ion source, using nitrogen as sheath and auxiliary gas. The source voltage was set at 4.5 kV in the positive mode. The heated capillary was maintained at 265 °C. The acquisition method used was optimized in the tuning sections for pentosidine quasi-molecular ion (capillary, magnetic lenses, and collimating octapole voltages) to obtain maximum sensitivity. The collision energy (CE) was chosen to maintain about 10% of the precursor ion. The main tuning parameters adopted for electrospray ionization (ESI) source were capillary voltage 13.00 V, tube lens 70 V. Mass accuracy of recorded ions (vs calculated) was ± 15 ppm (without internal calibration). Mass spectra were collected in tandem mass spectrometry (MS) mode: MS^2 of (+) 379 pentosidine or 255 GLAP m/*z* with 30% collision energy in the range 100–400 m/*z*.

2.9. Real-time reverse transcription polymerase chain reaction

DNase was added to ribonucleic acid (RNA) to remove genomic DNA. One microgram of total RNA was reverse-

transcribed using the iScript cDNA synthesis kit. Primers were designed using the Beacon Designer 5.0 program and applying the parameters outlined in the Bio-Rad iCycler manual. The specificity of the primers was confirmed by Basic Local Alignment Search Tool (BLAST) analysis. The following primers were used: BACE-1 forward, 5′-TTCGCCGTCTCACAGTCATCC, and reverse, 5′-CTGCCGCCGTCCTGAACTC; β-actin forward, 5′-CCACACCCGCCACCAGTTC, and reverse, 5′-GACCCATACCCACCATCACACC; β2-microglobulin forward, 5′-TCTTTCTGGTGCTTGTCTCTCTGG, and reverse, 5′-CTATCTGAGGT GGGTGGA ACT GAG; L13A forward, 5′-AGGTGGTGGTTGTACGCTGTG, and reverse, 5′-GGTTGG TGTTCATCCGCTTTCG. Real-time polymerase chain reaction (PCR) was performed using a Bio-Rad iQ iCycler detection system with SYBR green fluorophore. Reactions were run in a total volume of 25 μL, including 12.5 μL of IQ SYBR Green Supermix, 0.5 μL of each primer at 10-μM concentration, and 5 μL of the previously reverse-transcribed complementary DNA (cDNA) template. The protocol for the BACE1 primer set was optimized using serial 5 × dilutions of template cDNA obtained from the brains of control rats. The protocol used was denaturation (95 °C for 5 minutes) amplification repeated 40 times (95 °C for 15 seconds, 60 °C for 1 minute) and extension at 72 °C. A melting curve analysis was made after each run to ensure a single amplified product for every reaction. All reactions were run in triplicate, at least, for each sample. Results were normalized using β-actin, β2-microglobulin, and L13A as internal controls.

2.10. Antibodies and immunoblot analysis

The following antibodies were used: polyclonal anti-BACE1 (Chemicon, Temecula, CA); monoclonal anti-β-actin (Sigma Chemical Company, Beverly, MA); polyclonal anti-RAGE, polyclonal anti-lamin A, and monoclonal anti-NF-κBp65 (Santa Cruz Biotechnology, Santa Cruz, CA). Cell lysates and nuclear and cytosolic fractions of tissue extracts were separated on 9.3% sodium dodecyl sulfate-polyacrlamide gels using the mini-PROTEAN II electrophoresis cell (Bio-Rad). Proteins were transferred onto nitrocellulose membranes (Hybond-C Extra, Amersham Life Science, Arlington Heights, IL). Nonspecific binding was blocked with 5% nonfat dry milk in 50 mmol/L Tween 20 (Tris-buffered saline Tween). The blots were incubated with different primary antibodies, followed by incubation with a secondary antibody; either peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in Tris-buffered saline Tween containing 2% nonfat milk. Immunoreactions were visualized using an enhanced chemiluminescence system according to the manufacturer's protocol (Amersham-Pharmacia Biotech Italia, Cologno Monzese, Italy).

Specific bands were quantified by densitometry using analytic software (Bio-Rad, Multi-Analyst, München, Germany). β-actin, using an anti-β-actin antibody, served as loading control for cytosolic and total protein extracts. Lamin A, using a Lamin A specific antibody, served as loading control for nuclear extract.

2.11. RNA interference

RNA interference experiments to knock down RAGE expression were performed using RNai (Santa Cruz Biotechnology, Santa Cruz, CA). The short interfering RNA (siRNA) (1 μg) were transfected into cells using a siRNA transfection reagent in RPMI (Roswell Park Memorial Institute) medium for 48 hours. Transfected cells in fresh medium were exposed for 3, 6, or 12 hours to pentosidine or GLAP and then harvested for sample preparation.

2.12. NF-κB activity

The activity of NF-κB was determined using a commercially available kit (Active Motif, Rixensart, Belgium), with a 96-well plate on which has been immobilized an oligonucleotide containing an NF-κB consensus binding site. NF-κB contained in the nuclear extracts specifically binds to this oligonucleotide. The primary antibodies used in the kit recognize accessible epitopes on p65, p50, p52, c-Rel, and Rel b proteins upon DNA binding.

2.13. Statistical analysis

All results are presented as means ± SD for 10–12 rats. A "T" test was performed using the GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA); p values < 0.05 were considered significant.

3. Results

3.1. Induced diabetes in STZ-rats results in increased brain CNS oxidative stress and AGEs

After 3 weeks of streptozotocin (STZ) treatment, rats were monitored to assess induction of a diabetic condition. As shown in Table 1, STZ-rats showed a significantly lower body weight compared with controls (−30%), paralleled by a strong increase in glycemia (+270%) and a decrease in insulin levels (−80%). In Table 2, various parameters of oxidative stress were evaluated. As shown, STZ-rats showed a significant brain increase of hydrogen peroxide (136%) and of the ratio GSSG/GSH (70%).

The H_2O_2 increase was followed by a significant increase in catalase (+55%); likewise, the decrease in GSH was

Table 1
Body weight, glycemia and insulin levels of control and STZ rats

	Control	STZ
Body weight (g)	305 ± 7	217 ± 32 [a]
Glycemia (mg/dL)	104 ± 18	388 ± 26 [b]
Insulin (μg/mL)	0.92 ± 0.10	0.18 ± 0.04 [a]

Data are means ± SD of 8–10 rats per group.
Key: STZ, streptozotocin-treated.
[a] $p < 0.05$ versus control.
[b] $p < 0.005$ versus control.

Table 2
Hydrogen peroxide, GSSG/GSH ratio, catalase, and GSH-peroxidase activities in cytosolic fractions from the brain of controls and STZ rats

	Control	STZ
H_2O_2 (nmol/min/mg protein)	0.328 ± 0.068	0.777 ± 0.432[a]
GSSG/GSH (ratio)	0.0944 ± 0.0497	0.1612 ± 0.0399[a]
Catalase (pmol/mg protein)	39.44 ± 13.52	61.16 ± 15.83[a]
GSH peroxidase (μmol/NADPH/min/mg protein)	9.95 ± 1.06	5.52 ± 0.88[b]

Data are means ± SD of 8–10 rats per group.
Key: GSH, reduced glutathione; GSSG, oxidized glutathione; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; STZ, streptozotocin-treated.
[a] $p < 0.05$ versus control.
[b] $p < 0.01$ versus control.

related to a significant decrease in the activity of the antioxidant enzyme, GSH-peroxidase (-55%) that requires reduced GSH for activity.

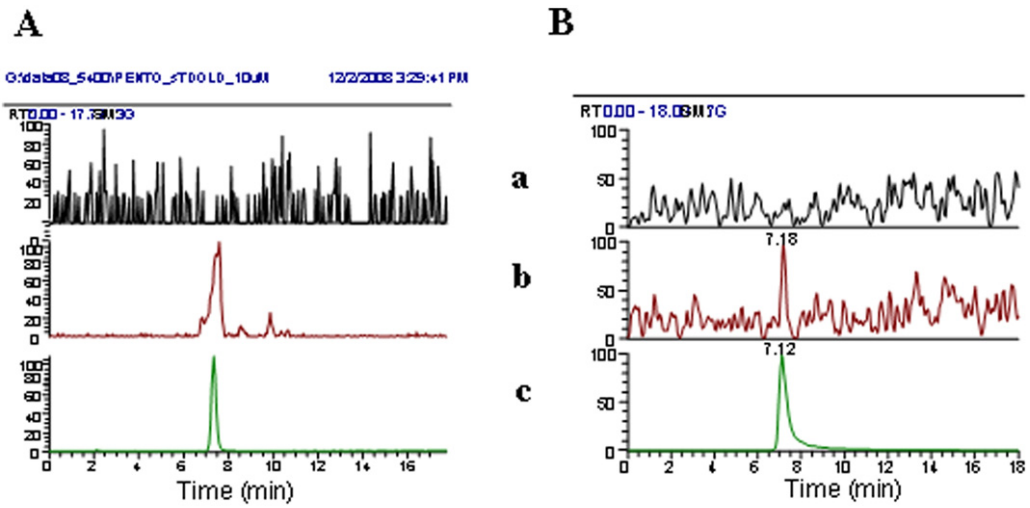
Diabetes was accompanied by a significant increase in different classes of AGEs. Among the different types of AGEs found, we concentrated on pentosidine and GLAP.

Pentosidine is significantly increased in serum from patients with AD and is considered an important indicator that is useful for the diagnosis of AD (Meli et al., 2002). GLAP is a recently described glyceraldehydes-derived AGE (Glycer-AGE) containing a piridinium moiety (Hayase et al., 2005; Takeuchi and Yamagishi, 2009), a predominant feature of toxic AGEs (TAGE).

Pentosidine (Fig. 1A) or GLAP (Fig. 1B) is greatly increased in brain samples derived from STZ-ras (b) compared with control animals (a). Samples were compared with standard control (c). Values normalized against protein content are reported in Fig. 1C. As shown, pentosidine increased greater than 2-fold in STZ rats compared with controls, reaching a concentration of 1 μM and GLAP, which was very low in control rats (0.02 μM), was strongly increased in diabetic rats reaching a concentration of 5 μM.

3.2. Diabetes in STZ-rats induces RAGE followed by an increase of BACE1 expression

As shown in Fig. 2, STZ rats showed a significant increase of BACE1 messenger RNA (mRNA) expression (1.5 fold increase; Fig. 2A) as well as a significant increase in BACE1 protein level (~2-fold increase; Fig. 2B). Because AGEs toxicity is mediated through interaction with their receptor RAGE (Qin et al., 2008), we determined the



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	CONTROL	STZ
PENTOSIDINE (μM)	0.430 ± 0.138	0.977 ± 0.444*
GLAP (μM)	0.02 ± 0.01	5.84 ± 0.89***

Fig. 1. Pentosidine and glyceraldehydes-derived pyridinium (GLAP) levels were increased in brains of control rats compared with streptozotocin (STZ)-treated rats. (A) Representative chromatogram of pentosidine in a control rat (a) or in an STZ rat (b), and a pentosidine standard solution (c). (B) Representative chromatogram of GLAP in control rat (a) or in an STZ rat (b), and a GLAP standard solution (c). (C) Values of pentosidine or GLAP normalized against the protein content and expressed as μM concentration in total brain homogenates from control and STZ rats. Advanced glycation end products (AGEs) are significantly higher in STZ rats compared with control rats. Data are means ± SD of 8–10 rats per group. Statistical significance: *$p <$ 0.05 versus control; ***$p < 0.005$ versus control.

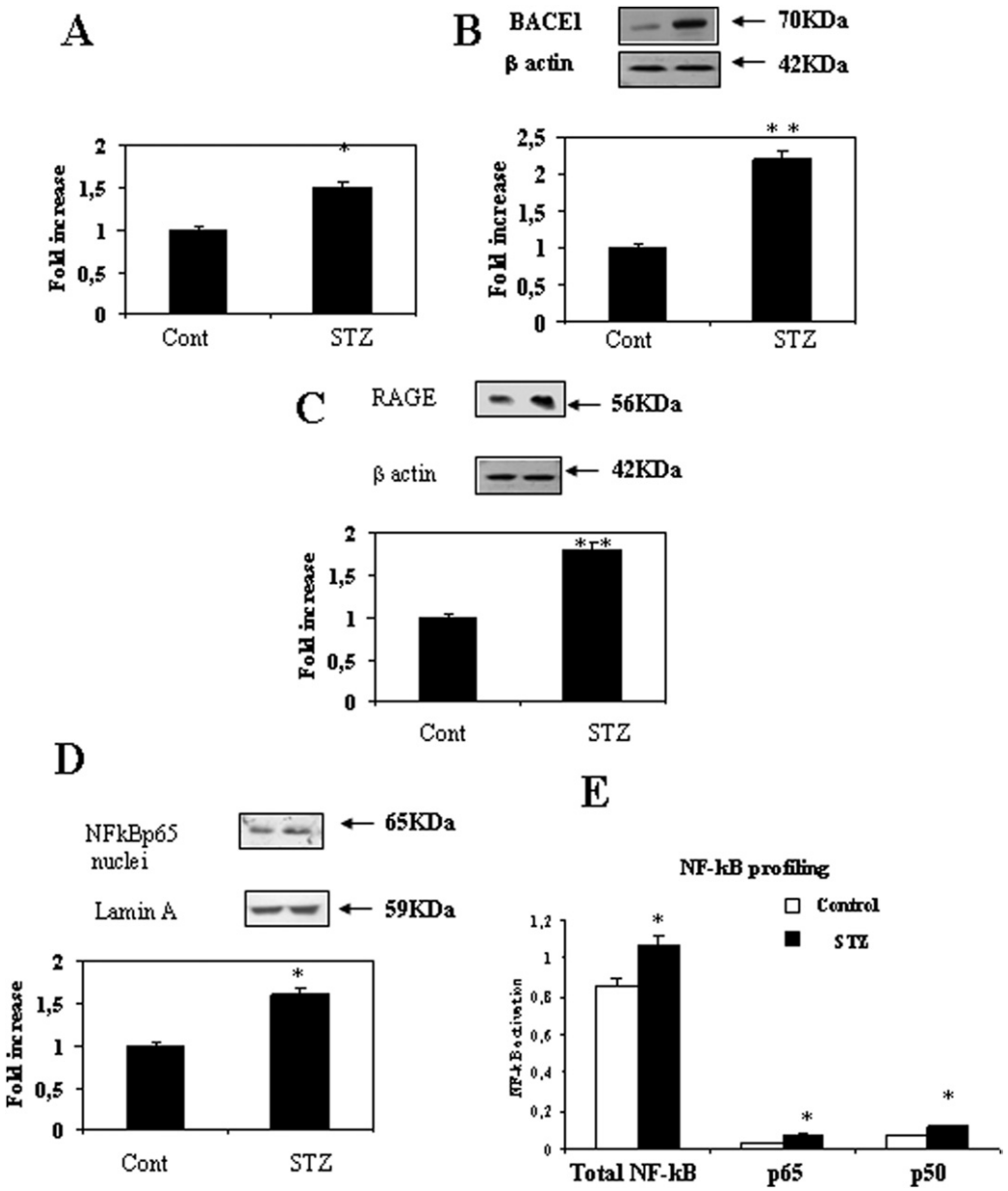
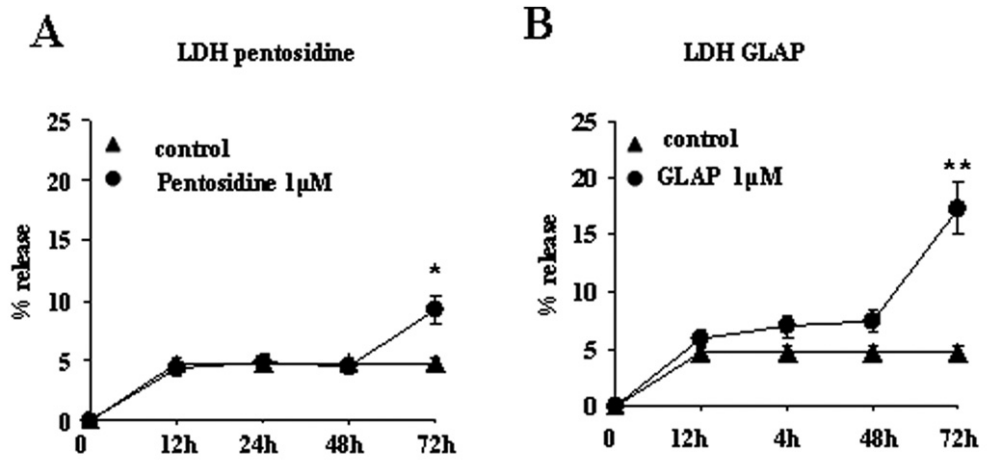


Fig. 2. BACE1 mRNA and protein levels, as well as RAGE and total NF-κB and p65 and p50 subunits, are significantly increased in streptozotocin (STZ)-treated rats. BACE1 messenger RNA (mRNA) (A), as determined by real time polymerase chain reaction (PCR), as well as BACE1 protein levels (B), as revealed by immunoblot and densitometric analysis, are significantly increased in STZ rats compared with controls. (C) RAGE, (D) NF-κB p65 protein levels, as determined by immunoblot and densitometric analysis and (E) total NF-κB, together with p65 and p50, as determined by enzyme-linked immunosorbent assay (ELISA), parallel the advanced glycation end products (AGEs)-mediated increase of BACE1. Data are means ± SD of 8–10 rats per group. Statistical significance: $^{*}p < 0.05$ versus control; $^{**}p < 0.01$ versus control.

protein levels of RAGE in control and STZ rats. As expected, RAGE protein levels were also significantly increased (1.7 fold increase) in STZ rats compared with controls (Fig. 2C).

The upregulation of RAGE is followed by the activation of the NF-κB pathway (Lander et al., 1997); NF-κB mediates upregulation of BACE1 (Buggia-Prevot et al., 2008). We confirmed that NF-κB was activated in our studies by assessing the movement of p65 from the cytosol to the nucleus. As shown in Fig. 2D, p65 protein levels were significantly increased (+65%) in the brain-derived nuclear extracts from STZ rats compared with control. In parallel, a similar decrease (−50%) of p65 protein levels was observed in the cytosol (data not shown). To further confirm that there was an activation of NF-κB pathway we evaluated the amount of the transcription factor with an ELISA kit. The STZ-treated rats showed increased levels of total NF-κB (+25%) as well as of p65 and p50 subunits (Fig. 2E).



C

Pentosidine	CONTROL	1h	3h	6h
H_2O_2 (nmol/min/mg prot)	0.336 ± 0.028	0.406 ± 0.033	0.456 ± 0.012*	0.778 ± 0.092**
GSSG/GSH (ratio)	0.805 ± 0.147	0.851 ± 0.231	0.956 ± 0.071*	1.053 ± 0.199*
CATALASE (µmol/mg protein)	43.50 ± 9.22	50.00 ± 11.98	106.59 ± 21.99**	185.55 ± 25.33**
GSH PEROXIDASE µmol NADPH/min/mg prot	0.185 ± 0.011	0.145 ± 0.023*	0.137 ± 0.012*	0.186 ± 0.041

D

GLAP	CONTROL	1h	3h	6h
H_2O_2 (nmol/min/mg prot)	0.338 ± 0.032	0.434 ± 0.043	0.680 ± 0.062**	0.578 ± 0.072**
GSSG/GSH (ratio)	0.745 ± 0.123	0.841 ± 0.171	0.860 ± 0.051*	0.960 ± 0.139*
CATALASE (µmol/mg protein)	34.54 ± 8.43	38.00 ± 9.98	108.65 ± 17.99**	207.25 ± 32.11**
GSH PEROXIDASE µmol NADPH/min/mg prot	0.288 ± 0.042	0.192 ± 0.033*	0.168 ± 0.031**	0.206 ± 0.031*

Fig. 3. Incubation of differentiated SK-N-BE with 1 μM pentosidine or glyceraldehydes-derived pyridinium (GLAP) is followed by a small induction of cell death and a significant increase in oxidative stress. (A and B) Incubation of cells with pentosidine or GLAP mediate a minor increase in cell death, determined by lactate dehydrogenase (LDH) release, that is only significant after 72 hours. (C and D) Cells incubated with pentosidine and GLAP exhibit a significant increase in oxidative stress, as revealed by the increase of H_2O_2 production, of GSSG/GSH ratio and of catalase activity as well as by a decrease in GSH-peroxidase activity. Experiments were performed in triplicate. Statistical significance: *$p < 0.05$ versus control; **$p < 0.01$ versus control.

3.3. Pentosidine and GLAP mediate oxidative stress and overexpression of BACE1 in differentiated SK-N-BE neuroblastoma cells

To confirm that the upregulation of BACE1 observed in diabetic STZ rats was induced by AGEs, differentiated SK-N-BE neuroblastoma cells were treated with pentosidine or GLAP up to 12 hours. Pentosidine and GLAP were added to a final concentration of 1 μM, an amount comparable to that found in brain of STZ rats (see Fig. 2C). To exclude the possibility that streptozotocin had a direct toxic effect in the observed BACE1 induction cells were treated with 1 μM streptozotocin for 3 and 6 hours; BACE1 protein levels were unchanged (data not shown).

Differentiated SK-N-BE cells exhibited no change in viability after exposure to pentosidine or GLAP for up to 48 hours (Fig. 3A and B); a modest increase in cell death was observed at 72 hours of incubation (+10% with pentosidine and 18% with GLAP). Subsequently, oxidative stress in SK-N-BE cells exposed to AGEs was measured (Fig. 3C and D). After 6 hours of treatment with pentosidine, a significant increase in hydrogen peroxide (+130%), oxidized glutathione/reduced glutathione (GSSG/GSH) ratio

(+30%), and catalase activity (+300%), as well as a significant decrease in GSH-peroxidase activity (−20%) was observed (Fig. 3C).

Similar results were obtained after cells were treated with GLAP; incubation of cells with GLAP was followed by an increase in hydrogen peroxide (+100%), GSSG/GSH ratio (+30%), and catalase activity (+500%). Moreover, the activity of GSH-peroxidase was decreased by 40% (Fig. 3D).

3.4. Pentosidine and GLAP mediate BACE1 overexpression through RAGE activation

The results in vitro paralleled those obtained in STZ rats. Cells treated with pentosidine showed a significant increase in BACE1 mRNA expression after 3 hours of incubation that persisted up to 6 hours (Fig. 4A). GLAP treatment induced a significant increase in BACE1 mRNA expression after 1 hour of incubation (Fig. 4B). BACE1 protein levels were also induced by treatment with AGEs (Fig. 4C and D). As shown, both pentosidine (Fig. 4C) and GLAP (Fig. 4D) significantly increased BACE1 protein (+100% and 150%, respectively). The role of RAGE in upregulating BACE1 in response to AGEs was further confirmed in differentiated SK-N-BE cells (Fig. 5). Treatment with pentosidine was followed by a strong and early increase of RAGE that peaked after 1 hour of incubation and persisted for up to 6 hours (+150%; Fig. 5A). Treatment with GLAP was followed by a more gradual increase that was significant after 1 hour but peaked between 3 and 6 hours of incubation (+50% to +100%; Fig. 5B).

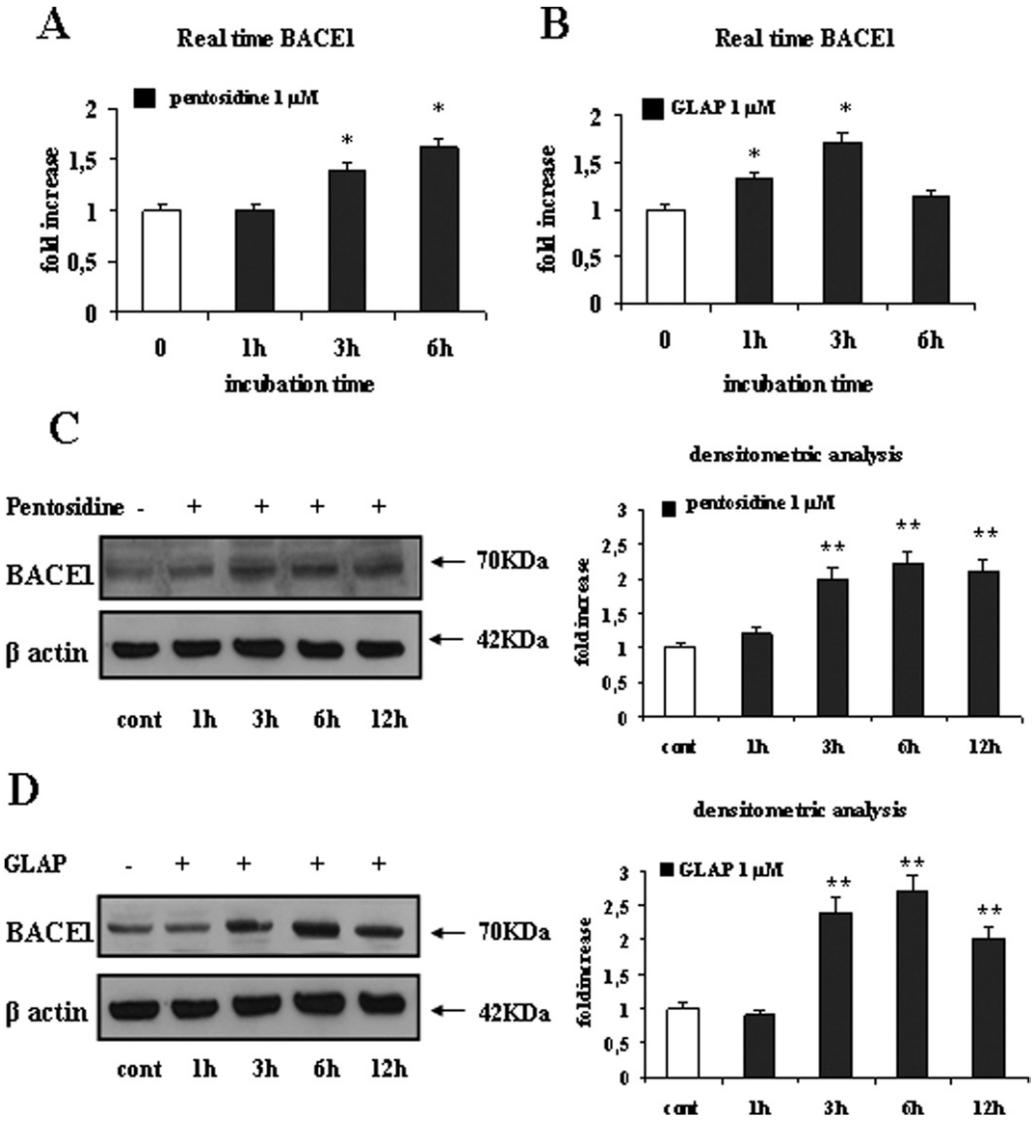


Fig. 4. Incubation of differentiated SK-N-BE with 1 μM pentosidine or glyceraldehydes-derived pyridinium (GLAP) is followed by an increase in BACE1 expression. (A and B) BACE1 messenger RNA (mRNA) is significantly increased after exposure to pentosidine or GLAP up to 6 hours of incubation. (C and D) BACE1 protein levels increase after 3 hours and up to 12 hours of incubation of differentiated SK-N-BE cells with pentosidine or GLAP, as determined by immunonoblot and densitometric analysis. Experiments were performed in triplicate. Statistical significance: *$p < 0.05$ versus control; **$p < 0.01$ versus control.

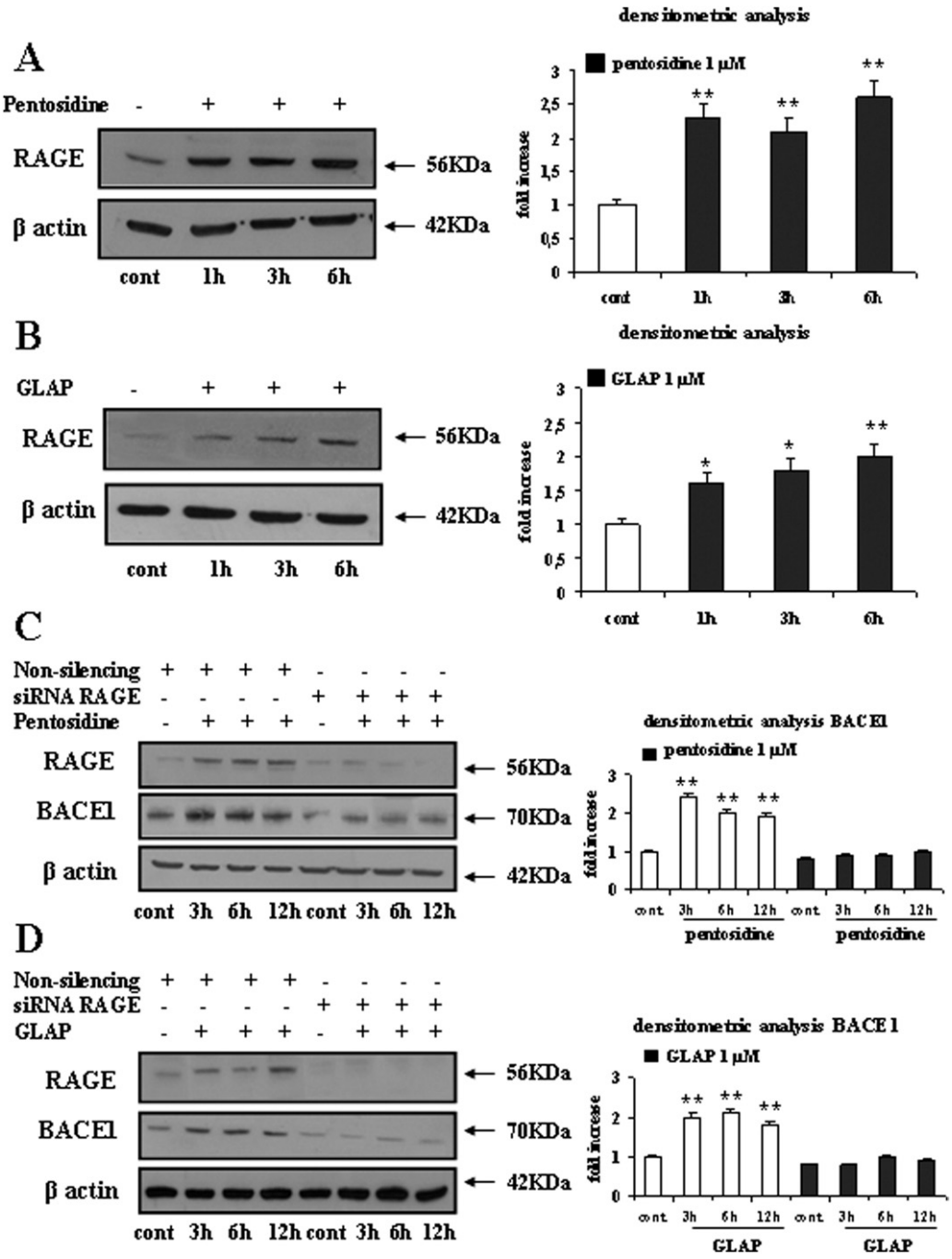


Fig. 5. Advanced glycation end products (AGEs)-mediated BACE1 upregulation requires RAGE induction. (A and B) RAGE protein levels results are significantly increased after 1 hour up to 6 hours of incubation of cells with pentosidine and glyceraldehydes-derived pyridinium (GLAP); (C and D) Western blot analysis of BACE1 in SK-N-BE differentiated cells with or without silencing of RAGE for 12 hours. In cells treated with pentosidine or GLAP, silencing RAGE completely prevents the upregulation of BACE1 induced by pentosidine or GLAP. Experiments were performed in triplicate. Statistical significance: $*p < 0.05$ versus control; $**p < 0.01$ versus control.

The involvement of RAGE was directly confirmed by silencing RAGE using RNA interference (Fig. 5C and D). Blocking RAGE induction, as expected, almost completely blocked upregulation of BACE1 by pentosidine (Fig. 5C) and GLAP (Fig. 5D).

3.5. The NF-κB pathway is involved in AGEs-mediated BACE1 overexpression

As demonstrated in Fig. 6A and B, both pentosidine and GLAP induced nuclear activation of total NF-κB (+29% and

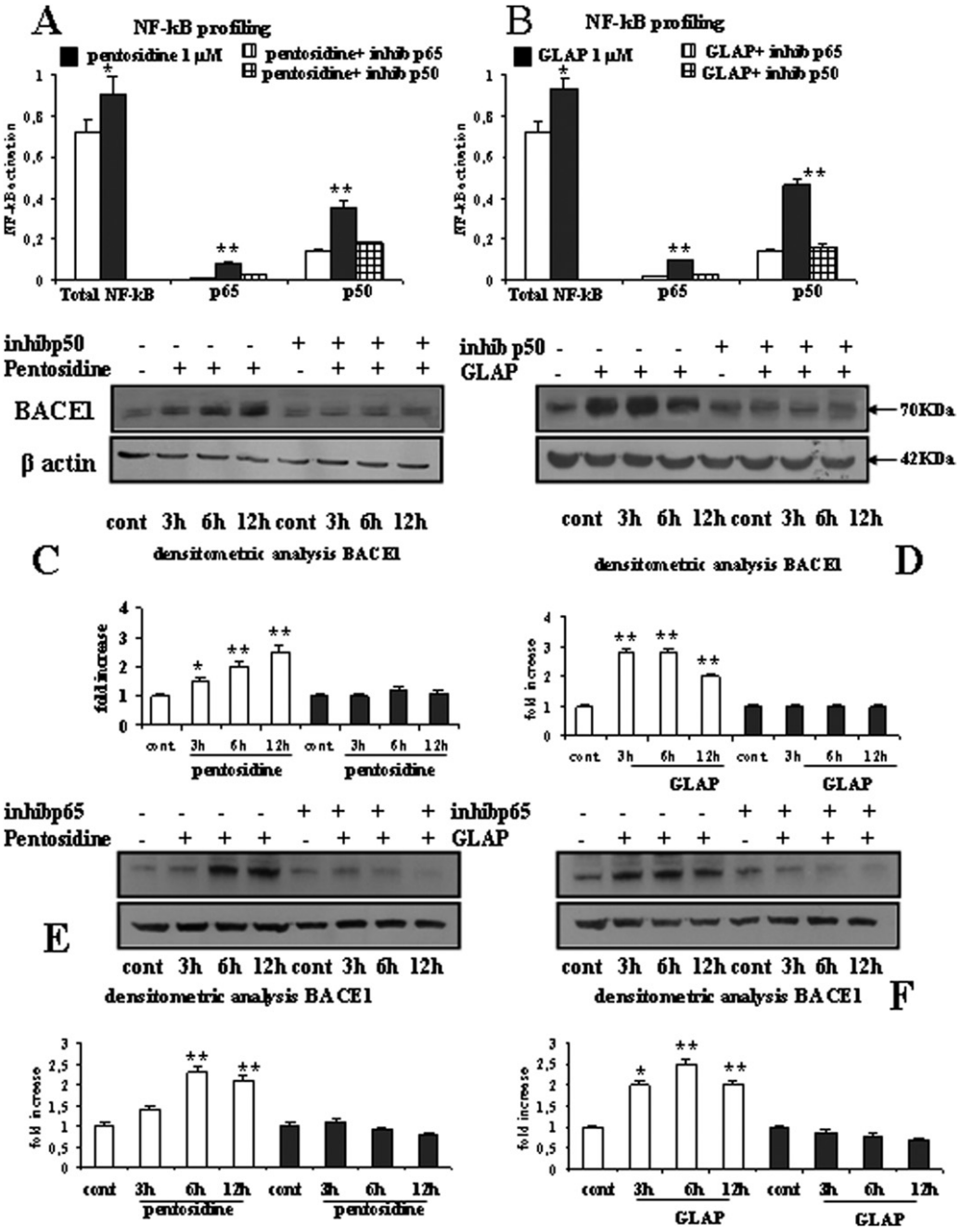
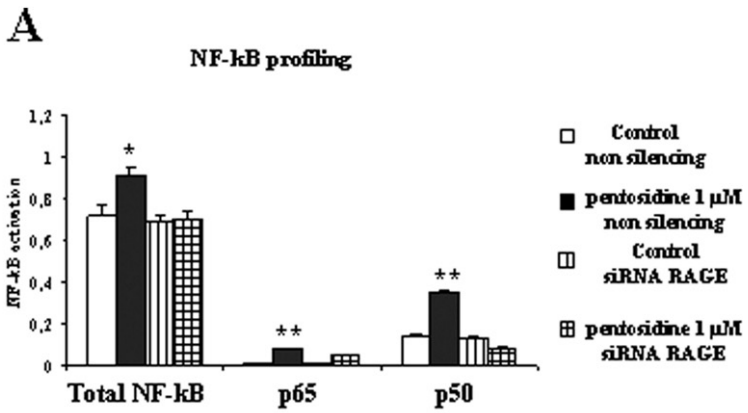
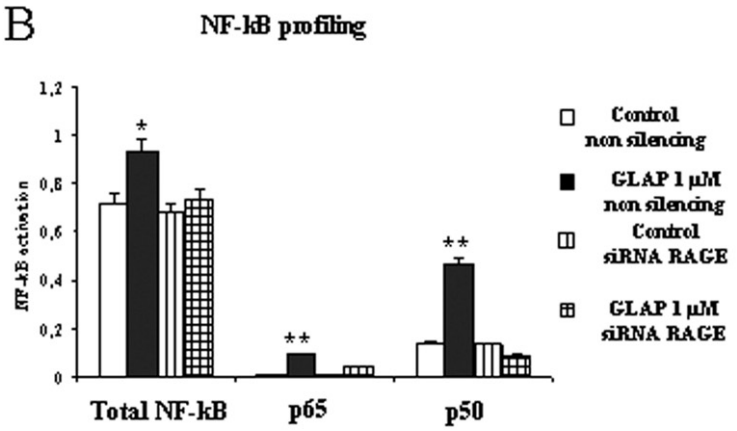


Fig. 6. Interaction of RAGE/advanced glycation end products (AGEs) mediates BACE1 upregulation through the activation of NF-κB pathway. (A and B) Detection of nuclear activation of total NF-κB together with p65 and p50 by enzyme-linked immunosorbent assay (ELISA) analysis in differentiated SK-N-BE exposed to pentosidine or glyceraldehydes-derived pyridinium (GLAP) is reported; addition of pharmacological inhibitors for p65 and p50 block their nuclear translocation of 70%. (C and D) Pretreatment of SK-N-BE cells with a pharmacological inhibitor for p50 protects the upregulation of BACE1 mediated by pentosidine or GLAP. (E and F) Pretreatment of SK-N-BE cells with a pharmacological inhibitor for p65 protects the upregulation of BACE1 mediated by pentosidine or GLAP. Experiments were performed in triplicate. Statistical significance: $^{*}p < 0.05$ versus control; $^{**}p < 0.01$ versus control.

+26%, respectively) as well as a significant increase in nuclear translocation of p65 and p50 subunits, after 1 hour of incubation. Pretreatment of cells with pharmacological NF-κB inhibitors that block the nuclear translocation of p65 or p50, the NF-κB transactivating subunits, failed to increase the BACE1 protein levels after treatment with pentosidine (Fig. 6C and E) or GLAP (Fig. 6D and F). Moreover the inhibitors blocked the nuclear translocation of NF-κB subunits of approximately 70%.

We then investigated the connection between RAGE that has been demonstrated implicated in BACE1 upregulation, with NF-κB activation. As shown in Fig. 7, the inhibition of RAGE





C

Pentosidine	Control non silencing	Pentosidine non silencing	Control siRNA RAGE	Pentosidine siRNA RAGE
H_2O_2 (nmol/min/mg prot)	0.310 ± 0.034	0.640 ± 0.076 **	0.356 ± 0.022	0.420 ± 0.084
CATALASE (pmol/mg protein)	40.51 ± 11.63	145.77 ± 21.98 **	30.55 ± 17.54	57.54 ± 15.43

D

GLAP	Control non silencing	GLAP non silencing	Control siRNA RAGE	GLAP siRNA RAGE
H_2O_2 (nmol/min/mg prot)	0.200 ± 0.034	0.670 ± 0.053 **	0.320 ± 0.032	0.390 ± 0.044
CATALASE (pmol/mg protein)	41.23 ± 10.54	192.507 ± 31.11 **	37.51 ± 15.22	77.43 ± 20.41

Fig. 7. RAGE is required for NF-κB activation and oxidative stress induction. (A and B) Detection of nuclear activation of total NF-κB together with p65 and p50 by enzyme-linked immunosorbent assay (ELISA) analysis in differentiated SK-N-BE silenced or not for RAGE and exposed to pentosidine or glyceraldehydes-derived pyridinium (GLAP) is reported. (C and D) Cells incubated with pentosidine and GLAP exhibit a significant increase in oxidative stress, as revealed by the increase of H_2O_2 production and of catalase activity; silencing of RAGE almost completely prevents oxidative stress induction. Experiments were performed in triplicate. Statistical significance: $*p < 0.05$ versus control; $**p < 0.01$ versus control.

expression, prevented the activation of NF-κB pathway mediated by pentosidine (Fig. 7A) and GLAP (Fig. 7B). Finally we showed the role of ROS, elicited by RAGE, in the BACE1 upregulation. In Fig. 7C and D are reported the levels of hydrogen peroxide and catalase activity after 6 hours of exposure to AGEs with or without silencing of RAGE. Silencing of RAGE is followed to an almost complete prevention of oxidative stress (Fig. 7C and D).

3.6. Pentosidine and GLAP are increased in cerebral cortex of AD patients

As shown in Fig. 8, the level of pentosidine in AD was 2 μM (Fig. 8A), whereas GLAP content was 10 μM (Fig. 8B); these levels are comparable to those found in the STZ rats. AGEs were almost undetectable in control tissues,

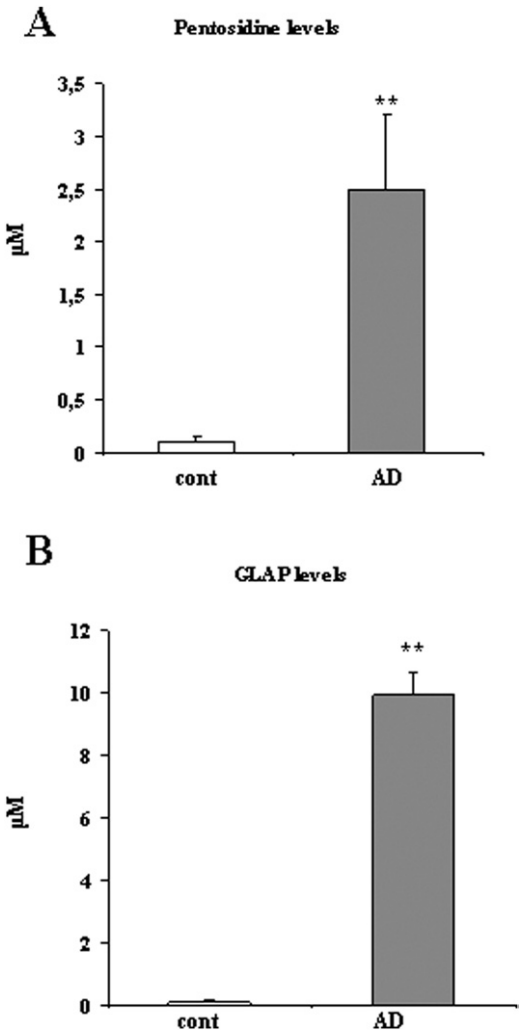


Fig. 8. Pentosidine and glyceraldehydes-derived pyridinium (GLAP) are increased in cortical brain samples of Azheimer's disease (AD) patients. (A and B) detectable pentosidine and GLAP are observed in brain cortex homogenates of AD patients; in normal aging controls advanced glycation end products (AGEs) levels are almost undetectable, ranging values lower than 0.1 μM. The concentration of pentosidine reaches approximately 2.5 μM and the GLAP concentration approximately 10 μM. Experiments were performed in triplicate. Statistical significance: $^{**}p < 0.01$ versus normal aging.

ranging concentration of approximately 0.1 μM for pentosidine and 0.05 μM for GLAP.

4. Discussion

AGEs are a heterogeneous group of molecules formed by the Maillard reaction, a nonenzymatic reaction between the aldehyde group of glucose and the amino group of proteins to form reversible Schiff bases and then Amadori products (Bucala and Cerami, 1992; Takeuchi and Makita, 2001). These products undergo further rearrangement to form the irreversible compounds known as AGEs (Brownlee et al., 1988).

The formation and accumulation of AGEs in various tissues are known to occur in normal aging, and, at an extremely accelerated rate, in diabetes mellitus and renal failure (Ahmed and Thornalley, 2007; Goh and Cooper, 2008; Jerums et al., 2003). AGEs have been detected in vascular walls, lipoproteins, and lipid constituents where they lead to macro- and microangiopathy and amyloidosis (Gasser and Forbes, 2008; Schmidt et al., 1999). The involvement of AGEs in brain aging and in AD was reported more than 10 years ago, in studies that demonstrated that the microtubule associated tau protein as well as Aβ are substrates for glycation (Ledesma et al., 1994; Smith et al., 1994; Vitek et al., 1994; Yan et al., 1994, 1995). The tau protein is preferentially glycated at its tubulin binding site, suggesting that glycation may be 1 of the modifications able to hamper the binding of tau to tubulin (Ledesma et al., 1994). Increased extracellular AGEs formation has been demonstrated in amyloid plaques in different cortical areas (Salkovic-Petrisic and Hoyer, 2007). Moreover, formation of AGEs accelerates the conversion of Aβ from monomeric to oligomeric or higher molecular weight forms (Loske et al., 2000).

In addition to producing posttranslational modification of proteins, AGEs have other pathologic effects at the cellular and molecular levels. One of the proposed mechanisms of AGEs-mediated damage are through reactive oxygen species (ROS), particularly superoxide and hydrogen peroxide (Carubelli et al., 1995; Muscat et al., 2007; Ortwerth et al., 1998). Indeed, glycated proteins increase the rate of free radical production compared with native proteins (Mullarkey et al., 1990). Another mechanism through which AGEs mediate the production of oxidative stress is through with the activation of rage, a multiligand receptor in the immunoglobulin superfamily of cell surface molecules (Neeper et al., 1992; Qin et al., 2008; Schmidt et al., 1992).

In the present study, we demonstrated a novel pathologic mechanism of AGEs through its contribution to Aβ accumulation. In STZ rats, as well as in SK-N-BE differentiated neuroblastoma cells, 2 different AGEs, pentosidine and GLAP, were able to upregulate BACE1 expression through their binding with RAGE. The role of RAGE in the pathogenesis of AD has been extensively studied, because it also binds Aβ (Yan et al., 1996), causing an increase in Aβ influx into the brain across the blood-brain barrier (Arancio et al., 2004; Takuma et al., 2009). To determine the molecular mechanism of RAGE-mediated BACE1 overexpression, we focused on the NF-κB pathway, which is a representative transcription factor activated by RAGE-ligand interactions (Granic et al., 2009; Lander et al., 1997). As expected, activation of this pathway was observed in vitro as well as in vivo, and its role in BACE1 upregulation was confirmed using pharmacological inhibitors of NF-κB that prevented the nuclear translocation of p50 or p65 and the consequent dimerization that is required for NF-κB signaling.

Another mechanism by which RAGE could mediate BACE1 upregulation is through the ROS production ob-

served in both experimental models. We and others have shown that the expression and activity of BACE1 is increased by oxidant agents and by the lipid peroxidation product 4-hydroxynonenal (HNE) (Tamagno et al., 2005), and that oxidative stress-mediated BACE1 upregulation requires γ-secretase activity (Tamagno et al., 2008).

The role of RAGE on BACE1 upregulation was recently reported in an AD animal model and in cultured cells (Cho et al., 2009). BACE1 upregulation in cells overexpressing RAGE and in RAGE-injected brains of Tg2576 mice harboring a human APP transgene with the Swedish mutation, was reported. The present study confirmed and extended this finding by showing that natural ligands of RAGE mediate BACE1 upregulation in a diabetic animal model as well as in a cell culture model.

Thus, activation of the AGEs/RAGE axis, as a result of hyperglycemia, driving the upregulation of the key enzyme for Aβ production, provides a mechanistic link between diabetes mellitus and AD.

Disclosure statement

The authors declare no actual or potential conflicts of interest, including any financial, personal or other relationships with other people or organizations within 3 years of beginning the work submitted that could inappropriately influence (bias) their work.

The animals were cared for in compliance with the Italian Ministry of Health Guidelines (no. 86/609/EEC) and with the Principles of Laboratory Animal Care (NIH no. 85-23, revised 1985). The scientific project, including animal care, was reviewed and approved by the local committee on ethics.

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