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Involvement of caspase-10 in advanced glycation end-product-induced apoptosis of bovine retinal pericytes in culture

Marc Lecomte*, Ulriche Denis, Daniel Ruggiero, Michel Lagarde, Nicolas Wiernsperger

Diabetic Microangiopathy Unit, MERCK Santé-INSERM U352, INSA-Lyon, Building Louis Pasteur, 11 Av. J. Capelle, F-69621 Villeurbanne Cedex, France

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Abstract

Apoptosis appears to be the death mechanism of pericyte loss observed in diabetic retinopathy. We have previously shown that advanced glycation end-products (AGE-MGX) induce apoptosis of retinal pericytes in culture associated with diacylglycerol (DAG)/ceramide production. In the present study, we investigated possible caspase involvement in this process. Bovine retinal pericytes (BRP) were cultured with AGE-MGX and apoptosis examined after annexin V staining. Effects of peptidic inhibitors of caspases were determined on DAG/ceramide production and apoptosis. Pan-caspase inhibitor z-VAD-fmk (50 μM) was able to inhibit both DAG/ceramide production and apoptosis, whereas caspase-3-like inhibitor z-DEVD-fmk (50 μM) or caspase-9 inhibitor z-LEHD-fmk (50 μM) was only active on apoptosis. This differential effect strongly suggests involvement of initiator caspase(s) upstream and effector caspase(s) downstream DAG/ceramide production in AGE-mediated apoptosis. Pericyte treatment with caspase-8 inhibitor z-IETD-fmk (50 μM) did not protect cells against AGE-induced apoptosis and we failed to detect caspase-8 in pericytes by immunoblotting assay. Interestingly, one inhibitor of caspase-10 and related caspases z-AEVD-fmk (50 μM) inhibited both AGE-MGX-induced apoptosis and DAG/ceramide formation in pericytes. Cleavage of caspase-10 precursor into its active subunits was demonstrated by immunoblotting assay in pericytes incubated with AGE-MGX. These results strongly suggest that caspase-10, but not caspase-8, might be involved in the early phase of AGE-induced pericyte apoptosis, in contrast to caspase-9 and -3-like enzymes involved after DAG/ceramide production. This finding may provide new therapeutic perspectives for early treatment in diabetic retinopathy.

Keywords: Advanced glycation end-product; Apoptosis; Caspase-10; Pericyte; Diabetic retinopathy

1. Introduction

One of the earliest histopathological changes observed in retinal microvessels during background diabetic retinopathy is the selective disappearance of intramural pericytes [1]. This cellular alteration precedes other pathologic vascular lesions such as microaneurysms, acellular capillaries formation [2], microthrombi, endothelial proliferation and angiogenesis, which ultimately lead to blindness [3]. Apoptosis seems to be the mechanism of death leading to early pericyte loss observed in the course of retinopathy. Supporting this, pericytes stained for markers of apoptosis have been detected in situ in microvessels from retina of diabetic patients recovered post-mortem [2,4,5]. One recent study also showed that anti-pericyte autoantibodies are present in type 2 diabetic patients and are associated with early stage retinopathy, suggesting new antigen expression by ''activated'' pericytes [6]. Despite this, mechanisms underlying retinal pericyte apoptosis during diabetes are still not completely understood.

Abbreviations: AGE-MGX, advanced glycation end-products made with methylglyoxal; A-SMase, acidic sphingomyelinase; BRP, bovine retinal pericytes; BSA, bovine serum albumin; DAG, diacylglycerol; fmk, fluoromethylketone; OMe, oxymethyl; PC-PLC, phosphatidylcholine-phospholipase C; z, benzyloxycarbonyl; z-AEVD-fmk, z-alanine-glutamate(OMe)-valine-aspartate(OMe)-fmk; z-DEVD-fmk, z-aspartate (OMe)-glutamate(OMe)-valine-aspartate(OMe)-fmk; z-IETD-fmk, z-isoleucine-glutamate(OMe)-threonine-aspartate(OMe)-fmk; z-LEHD-fmk, z-leucine-glutamate(OMe)-histidine-aspartate(OMe)-fmk; z-VAD-fmk, z-valine-alanine-aspartate(OMe)-fmk

* Corresponding author. Tel.: +33-4-3791-3150; fax: +33-4-7268-3099.
E-mail address: marc.lecomte@merck.fr (M. Lecomte).

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One possible mechanism could be the accelerated formation of advanced glycation end-products (AGE) observed during diabetes and their cytotoxic effects on retinal pericyte. AGE are formed after a cascade of reactions starting with a nonenzymatic reaction between ketones or aldehydes and free amino groups located on proteins for the most part, but also on nucleic acids or phospholipids. During the course of diabetes, AGE accumulate mainly on long-lived proteins and originate not only from the reaction with glucose but also from other reactive dicarbonyls derived from glucose, such as methylglyoxal [7], which is increased in plasma of diabetic patients [8]. Retinal pericytes are embedded in the basement membrane of microvessels, composed of structural long-lived proteins shown to form and accumulate Amadori products [9] leading to AGE [10] in diabetic patients. Also, RAGE [11,12] and other AGE receptors like p60 and p90 [13,14] have been reported to be present in pericyte plasma membrane. The possible effect of AGE on pericyte loss has been established by studies using different glycation inhibitors, such as aminoguanidine or pyridoxamine. Treatment of diabetic rats with pyridoxamine during 29 weeks protected against pericyte loss measured by acellular capillaries formation and prevented formation of AGE (N(ε)-(carboxymethyl)lysine) in retinal microvessels [15]. Also, diabetic rats treated for 26 weeks with aminoguanidine showed reduced AGE accumulation in retinal microvessels and slower signs of diabetic retinopathy progression measured as pericyte loss, microaneurysms or acellular capillary formation [16]. Another line of evidence for toxic effect of AGE on pericytes came from experiments where infusion of preformed AGE into nondiabetic rats showed 25% reduction of pericyte number after 2-week treatment [17] together with AGE accumulation within or around pericytes [13]. Recent in vitro studies by us [18] and others [19] have shown cytotoxic effects of AGE on retinal pericytes leading to apoptosis together with VEGF production in the later study. Interestingly also, angiogenesis induced by AGE leads to new vessels which are devoid of pericytes, suggesting that AGE impair recruitment or differentiation of mural cell precursors into pericytes [20].

Caspases are a family of cysteine proteases which cleave subtrates at aspartic residues and which are crucial in the initiation and the execution of the apoptotic program [21]. These proteases are present within the cell as inactive precursors which require internal processing to become active. Sequential cleavages at conserved Asp residues in the caspase precursor form lead to liberation of large and small subunits which rearrange to form a double (large + small) dimer active form [22]. Initiator caspase-2, -8 and -10 are at the apex of the signaling cascade induced by apoptotic stimuli such as Fas or TNFα [23,24] and caspase-9 is activated in response to agents leading to cytochrome *c* release from the mitochondria [25]. Initiator caspases activate either directly or indirectly effector caspases including caspase-3, -6 or -7 [26,27], which are the executioners of the apoptotic program leading to cellular disintegration.

Our own study [18] showed that AGE-induced apoptosis of pericytes has been associated with an intracellular formation of diacylglycerol (DAG) and ceramide, produced after activation of phosphatidylcholine-phospholipase C (PC-PLC) and acidic sphingomyelinase (A-SMase), respectively. Growing evidence indicates that ceramide production during apoptosis can be under the control of initiator caspase activation [28–30]. Ceramides produced during apoptosis can further activate a variety of signaling pathways [31,32] leading to effector caspase activation after mitochondrial damage [28,33]. In the present study, we examined the relation between DAG/ceramide generation and caspase activation in apoptotic pericytes and we focused particularly on the nature of the initiator caspase involved in the AGE-induced signaling pathway leading to retinal pericyte apoptosis.

2. Materials and methods

2.1. Materials

All products used for pericyte culture were from Sigma (L'Isle d'Abeau, France) with the exception of fetal calf serum (Invitrogen, Cergy-Pontoise, France) and collagenase/dispase (Roche, Meylan, France). The caspase inhibitor peptides, z-AEVD-fmk, z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk and z-VAD-fmk, were obtained from Calbiochem (Nottingham, UK). DAG kinase, ceramides and DAG (1-palmitoyl-3-stearoyl-rac-glycerol) were obtained from Biomol (Le Perray-en-Yvelines, France). [γ^{32}P]adenosine-5′ trisphosphate (6 Ci/μmol) was obtained from Perkin Elmer-NEN (Paris, France). Precoated TLC plates (silica gel 60 without fluorescent indicator) were from Merck (Fontenay-sous-Bois, France). The Annexin-V-FLUOS staining kit was purchased from Roche. Nitrocellulose membranes (BA 85, 0.45-μm porosity) used for protein determinations were obtained from Whatman (Maidstone, England). Sterile filtration was performed with acrodisc filter (low protein binding, 0.2 μm) from Gelman Laboratory (Ann Arbor, MI, USA). PD-10 gel filtration columns (G-25 M Sephadex) were from Amersham Pharmacia Biotech (Orsay, France). SDS-polyacrylamide minigels (ready gel Tris–HCl 12%), nitrocellulose membranes (0.45 μM) and protein precision standards (broad range) were from Bio-Rad (Marnes-La-Coquette, France). Nitrocellulose membrane blocking agent was either bovine serum albumin (BSA) (Fraction V, 96% minimum) from Sigma or non-fat dry milk (Régilait, Saint-Martin-Belle-Roche, France) from a local general store. Rabbit polyclonal antibody against human caspase-10 (large subunit, sc-7955) and goat polyclonal antibody against human caspase-8 (large subunit, sc-6134), blocking peptide of caspase-8 antibody, active recombinant caspases used as

positive control and secondary polyclonal antibodies anti-rabbit (sc-2313) and anti-goat (sc-2020) were purchased from Biomol.

2.2. AGE-MGX preparation

AGE-MGX were prepared after incubation of 7.2 mg/ml BSA (Fraction V, low endotoxin, fatty acid free) with 100 mM methylglyoxal in 100 mM phosphate buffer, pH 7.4, for 50 h at 37 °C in sterile conditions. Control preparation of BSA was prepared as above but without methylglyoxal. After incubation, AGE-MGX (or control-BSA) preparations were eluted on PD-10 columns with Dulbecco's modified Eagle's medium (DMEM) to remove salts and excess methylglyoxal, further sterilized by filtration and kept at −20 °C until used [18]. AGE-MGX (or control-BSA) concentration was expressed as the BSA protein concentration added in the culture medium.

2.3. Culture of bovine retinal pericytes (BRP) and incubation with peptidic caspase inhibitors

BRP were cultured from bovine retinal microvessels as previously described [34]. Purity of BRP is almost 100% pure as assessed after staining for α_1-smooth muscle actin and a pericyte specific glycolipid antigen (3G5) [35] and confirmed after negative staining for von Willebrand's factor (Factor VIII-related antigen) [34] expressed in endothelial cells. Briefly, microvessel fragments were isolated from fresh bovine retinas (2 retinas/60-mm culture dish) after Dounce homogenization and collagenase-dispase digestion. Digested microvessel fragments were collected on a sieve (70-μm mesh) before seeding on a fibronectin-coated (4 μg/cm^2) culture dish. BRP in primary culture were grown in 3-ml DMEM supplemented with 5 mM glucose, 10% fetal calf serum, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C and culture medium changed every 2 days. When confluent, BRP were trypsinized (trypsin 0.5%, EDTA 0.2%), replated on fibronectin-coated dishes (1:3, P1) and grown in the same medium supplemented with 10 μM unesterified docosahexaenoic acid (DHA) in order to restore the DHA proportion of BRP total lipids close to the original value observed in intact retinal microvessels [34]. AGE-MGX or control-BSA (3 μM final concentration) with or without caspase inhibitor peptides (stock solutions prepared in dimethylsulfoxide, not exceeding 0.2% (v/v) final concentration) were added directly to the supplemented culture medium and cells were incubated chronically with these various agents. P1 cells were split (1:3, P2) and cultured in the same conditions as before for a total of 15 days (P1 + P2) on average. For each independent experiment, effects of AGE-MGX (or BSA-control) with or without inhibitor peptides were analyzed on a same batch of cells coming from the same primary culture.

2.4. Apoptosis detection in cultured pericytes by FITC-labeled annexin V staining

FITC-labeled annexin V binds to phosphatidylserine exposed to the outer leaflet of membrane during apoptosis and necrosis. Propidium iodide is used to differentiate between apoptotic and necrotic cells. This DNA intercalating agent is able to enter the nucleus during necrosis, staining nucleus of necrotic cells in red. Analysis of phosphatidylserine exposure to the outer leaflet of apoptotic pericyte membranes was performed using an Annexin-V-FLUOS staining kit, as previously described [18]. Every step described in the procedure was done at 37 °C. After culture (P1 + P2) for an average of 15 days with AGE-MGX (or control-BSA) with or without peptidic caspase inhibitors, pericytes (35-mm culture dish) were washed with PBS and incubated for 10 min with annexin V-FITC (10 μl) and propidium iodide (10 μl). Pericytes were further observed under an Axioplan fluorescence microscope (Carl Zeiss with filter no. 9, 450 nm < λ excitation < 500 nm and 515 nm < λ detection < 565 nm). Necrotic and apoptotic cells were counted on 10 microscopic fields (× 200), representing a total cell count comprised from 300 to 800 cells. Both categories of cells were expressed as percentage of the total cell count.

2.5. Ceramide and DAG determinations

Total ceramides and DAG were quantified as previously described [18] according to method of Preiss et al. [36] with modifications. At the end of culture (P1 + P2, 15 days on average) with AGE-MGX (or control-BSA) with or without peptidic caspase inhibitors, pericytes (2×10^6 cells) were washed three times with PBS (4 °C) and scraped on ice with a rubber policeman in 2-ml water. Total cellular lipids were then extracted by the method of Bligh and Dyer [37] and dried under nitrogen. Briefly, ceramides and DAG in the sample were metabolized to ceramides 1-[^{32}P]phosphate and 1-[^{32}P]phosphatidates by DAG kinase (*E. coli*) with [γ^{32}P]adenosine-5′ trisphosphate for 1 h. Known amounts of ceramide and DAG standards were treated in parallel. Labeled lipids were extracted and spotted on TLC plates (silica gel 60) developed in chloroform/methanol/acetic acid (65:15:5 v/v/v). Following autoradiography, TLC spots comigrating with ceramide-1-phosphate and phosphatidic acid standards were scraped. Silicagel was humidified with 0.4-ml water/methanol (50:50 v/v) and Pico-Fluor (10 ml) (Perkin Elmer-Packard, Rungis, France). Radioactivity contained in all samples was quantified by liquid scintillation counting. Results were corrected for the protein content of each sample. Proteins were determined by the method of Schaffner and Weissmann [38].

DAG standard used in this study is made of saturated fatty acids (16:0, 18:0) to increase stability during storage. DAG-kinase from *E. coli* has a poor enzymatic activity for these saturated molecular species of DAG, leading to one overestimation of DAG amounts measured in whole lipids

of pericytes as mentionned in Figure legends. Therefore, results were expressed as percentage of control-BSA to represent relative increase of intracellular levels of DAG induced by AGE-MGX in pericytes.

2.6. Immunoblotting of caspases

After culture with AGE-MGX (or control-BSA) for a total of 15 days on average, pericytes (2×10^6 cells) were washed with PBS (4 °C) and scraped on ice with a rubber policeman in 0.5 ml of 0.1 M Tris–HCl pH 8.0 containing protease inhibitors (4.4 μM pepstatin, 6 μM leupeptin and 0.6 mM PMSF) and 10 mM EDTA. After centrifugation, cell pellet was lysed in 100 μl of electrophoresis sample loading buffer (0.15 M Tris–HCl pH 6.8, 5% SDS, 12.5% glycerol, 12.5% β-mercaptoethanol, 1% bromophenol blue) and boiled in one water heating bath for 3 min. Protein amount for each sample was determined by the method of Schaffner and Weissmann [38]. Aliquots (20 μg total proteins) from each sample, human recombinant caspase-8 (1 μg) as standard and molecular weight standards were analysed by SDS-PAGE on a 12% SDS-polyacrylamide gel and a 4% stacking gel with the discontinuous buffer system of Laemmli [39]. Electrophoresis was performed on mini-gels (mini-protean II cell, Bio-Rad) at 200 V in electrophoresis buffer (25 mM Tris, 192 mM glycine, pH 8.3, SDS 0.1%), until bromophenol blue reached the bottom of the gel. Proteins were electrophoretically transferred (mini-transblot cell, Bio-Rad) to nitrocellulose membranes (45 min, 100V, 4 °C) in blotting buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol). After blotting, membranes were stained with Ponceau red to check for protein transfer of samples and further destained (2 times) in Tris buffered saline (TBS) (10 mM Tris–HCl, pH 8.0, 150 mM NaCl). Nitrocellulose membranes were saturated with non-antigenic proteins, after overnight incubation (4 °C) with either 10% (w/v) powdered nonfat milk in TBS with 0.05% Tween 20 (TTBS) for western blots using rabbit polyclonal caspase-10 antibody or with both 5% (w/v) BSA and 5% (w/v) powdered nonfat milk in TTBS for Western blots using goat polyclonal caspase-8 antibody.

After extensive washing with TTBS (15, 5, 5 min), membranes were incubated for 2 h with primary antibodies (raised against the large subunits of caspase-8 or -10) diluted in TTBS (1/2000 for rabbit caspase-10 antibody and 1/1000 for goat caspase-8 antibody). Membranes were washed with TTBS as above, incubated for 90 min with horseradish peroxidase-coupled secondary antibodies diluted in TTBS (1/6000 for anti-rabbit antibody and 1/3000 for anti-goat antibody), washed again with TTBS and once in TBS (5 min), before detection of antigen by enhanced chemiluminescence (ECL) assay. For caspase-8, in order to check specificity of antibody recognition, caspase-8 antibody was preincubated with blocking peptide in excess (1:5, w/w) in a small PBS volume (1/2 dilution) overnight (4 °C) and used in conditions described above for caspase-8 antibody. Blocking peptide for caspase-10 antibody was not available. Caspase-10 western blots were analyzed after densitometry scanning (GS-700 densitometer and Molecular Analyst software from Bio-Rad).

2.7. Statistical analysis

Data are expressed as means ± S.D. of n independent experiments. For each independent experiment, cells from the same primary culture (P0) were cultured (P1 + P2) either in control conditions (BSA-control with or without caspase inhibitors) or in the presence of AGE-MGX (with or without agents), allowing comparison of paired data.

The Wilcoxon signed-ranks test was used to define the significance of the difference between (a) mean values of each group (AGE-MGX/control-BSA) and (b) mean percent inhibition observed with caspase inhibitors tested as compared to the control group without inhibitors (0% inhibition). $P < 0.05$ was considered as statistically significant.

3. Results

3.1. z-VAD-fmk, z-DEVD-fmk and z-LEHD-fmk have differential effects on AGE-induced apoptosis and DAG/ceramide production

Apoptosis of BRP induced by AGE-MGX (3 μM) was assessed by annexin V-FITC labeling (Fig. 1A) and expressed as the percentage of apoptotic cells in the total cell population after culture during two passages (15 days on average), as described previously [18]. Apoptosis rate increased three times in pericytes cultured with AGE-MGX (3 μM) as compared to cells cultured with control-BSA only ($7.40 \pm 0.62\%$ vs. $2.64 \pm 0.60\%$, mean ± S.D., $n = 6$, $P < 0.05$) (Fig. 1B). Apoptosis induced by AGE-MGX was dose-dependent [18] and evaluated also after oligonucleosome quantitation with same results as described above [18]. Percentage of necrotic cells in the cell population was always below 0.1% and this percentage was not affected by the type of treatment (not shown). In parallel to apoptosis, lipids (DAG and ceramides) (Fig. 1C) involved in the apoptotic signaling pathway induced by AGE ($135 \pm 7\%$ and $145 \pm 11\%$ of control-BSA for DAG and ceramides ($P < 0.05$), respectively) were also measured [18] in order to investigate possible involvement of caspases on the production of these lipid mediators.

One peptidic inhibitor of caspases, substituted at NH_2 and COOH termini, z-VAD-fmk (a pan-caspase inhibitor) [40,41], was first tested in order to determine whether caspases could be involved in the apoptotic pathway activated by AGE-MGX in BRP. z-VAD-fmk inhibited completely BRP apoptosis induced by AGE-MGX (Fig. 1B) which returned to the basal level observed with control-BSA alone ($P < 0.05$ as compared to AGE-MGX without z-VAD-fmk). In addition, z-VAD-fmk was able to completely block

DAG/ceramide production induced by AGE-MGX in BRP (Fig. 1C) ($P<0.05$ as compared to AGE-MGX alone), suggesting involvement of caspase(s) upstream of DAG/ceramide production.

We further tested two substituted inhibitors z-DEVD-fmk (a caspase-3-like (caspase-3, -6, -7, -8 and -10) inhibitor) [40 41] and z-LEHD-fmk (a caspase-9 inhibitor) [42] to investigate possible sequential activation of caspase-9 and -3, -6 or -7 in this apoptotic cascade. Both z-DEVD-fmk and z-LEHD-fmk strongly inhibited (100% and 75%, respectively, $P<0.05$ as compared to AGE-MGX alone) pericyte apoptosis induced by AGE-MGX (Fig. 2A) without inhibiting DAG/ceramide production induced by AGE-MGX in BRP (Fig. 2B and C, respectively).

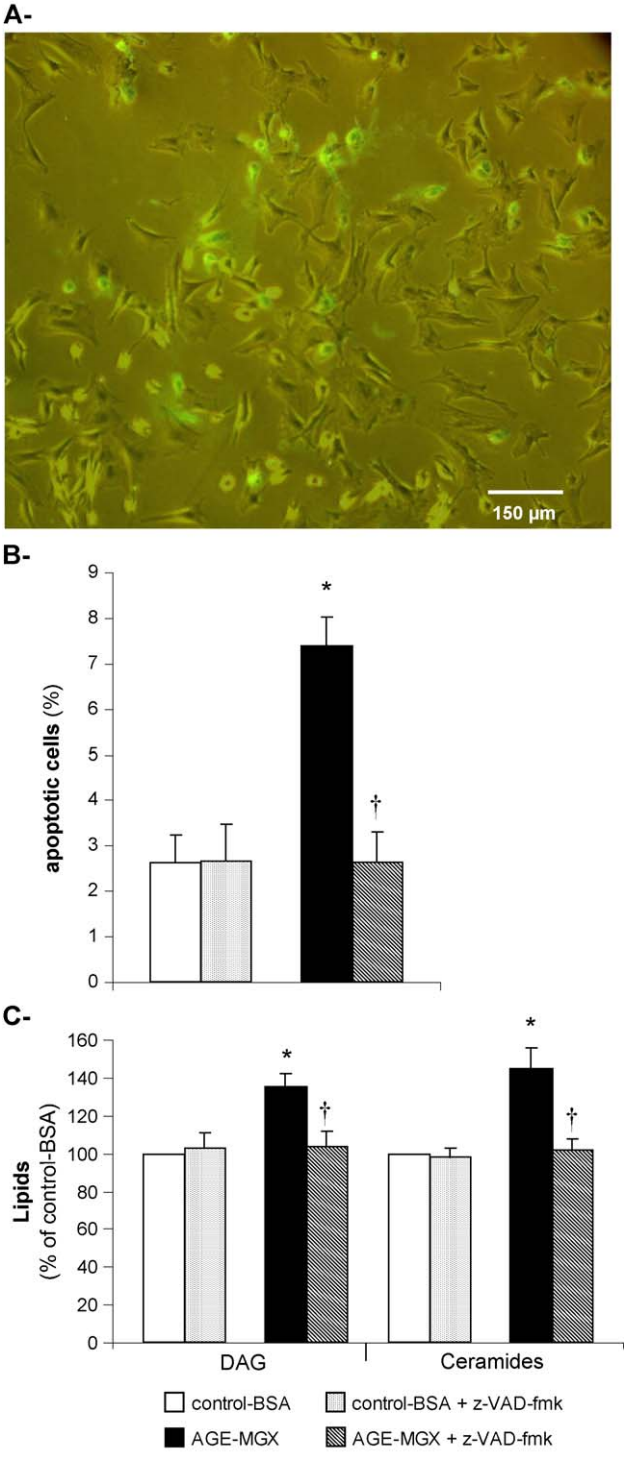

Fig. 1. Effect of z-VAD-fmk (pan caspase inhibitor) on AGE-induced pericyte apoptosis and cellular DAG/ceramide levels. Cells were cultured throughout two passages (15 days on average) with AGE-MGX or control-BSA (3 µM) with or without inhibitor peptide z-VAD-fmk (50 µM). Apoptosis was detected (A) and quantified (B) by the annexin V-FITC method, as described in Materials and methods. Panel A is a surperimposed photomicrograph of pericytes cultured with AGE-MGX (day 15) which represents the same field (original magnification ×100) in phase-contrast and in fluorescence (FITC), showing annexin V-positive cells in green. Results are expressed as percentage of apoptotic cells (B) and are mean ± S.D. of six independent experiments. DAG and ceramides levels in cells treated with z-VAD-fmk (C) were assayed by the DAG kinase method, as described in Materials and methods. Results, expressed as percentage of control-BSA, are mean ± S.D. of five independent experiments. Amount of ceramides measured in cells cultured with control-BSA (100%) for experiments with z-VAD-fmk was 720 ± 140 ng ceramides/mg proteins. DAG amounts are probably overestimated as discussed in Materials and methods. *: $P<0.05$ vs. control-BSA; †: $P<0.05$ vs. AGE-MGX without agents. (For color see online version).

3.2. Caspase-8 is not involved in BRP apoptosis induced by AGE-MGX

We investigated the possible involvement of initiator caspase-8 in the signaling pathway leading to BRP apoptosis induced by AGE-MGX. Incubation of cells (P1 + P2) with AGE-MGX for 15 days on average in the presence of z-IETD-fmk (50 µM), a caspase-8 inhibitor [40], did not affect AGE-induced apoptosis (Fig. 3A). We further studied caspase-8 zymogen expression in BRP and activation in AGE-treated BRP after immunoblotting using one polyclonal antibody raised against the large subunit of human caspase-8 (Fig. 4). Proteins from pericytes incubated with AGE-MGX (or control-BSA) were separated after electrophoresis and caspase-8 was detected after Western blot with caspase-8 antibody. The active form of human caspase-8 was used as standard. Western blot was done with caspase-8 antibody incubated (Fig. 4, lanes 5 to 7) or not (lanes 1 to 4) with one antibody blocking peptide. Caspase-8 antibody recognized only one major band of (Mr) 45 000 approximately either before (Fig. 4, lanes 2 and 3) or after (Fig. 4, lanes 6 and 7) incubation with the blocking peptide. Blocking peptide was able to abolish specific antigenic recognition of caspase-8, as shown in Fig. 4, where detection of intermediary fragment (p30), large (p18) and small (p12) subunits of human caspase-8 is almost abolished after incubation with the blocking peptide (compare lanes 5 and 4). These results obtained with one blocking peptide on caspase-8 recognition support that protein band of (Mr) 45 000 is nonspecific and suggest that caspase-8 is not detected in bovine pericytes incubated with AGE-MGX or control-BSA.

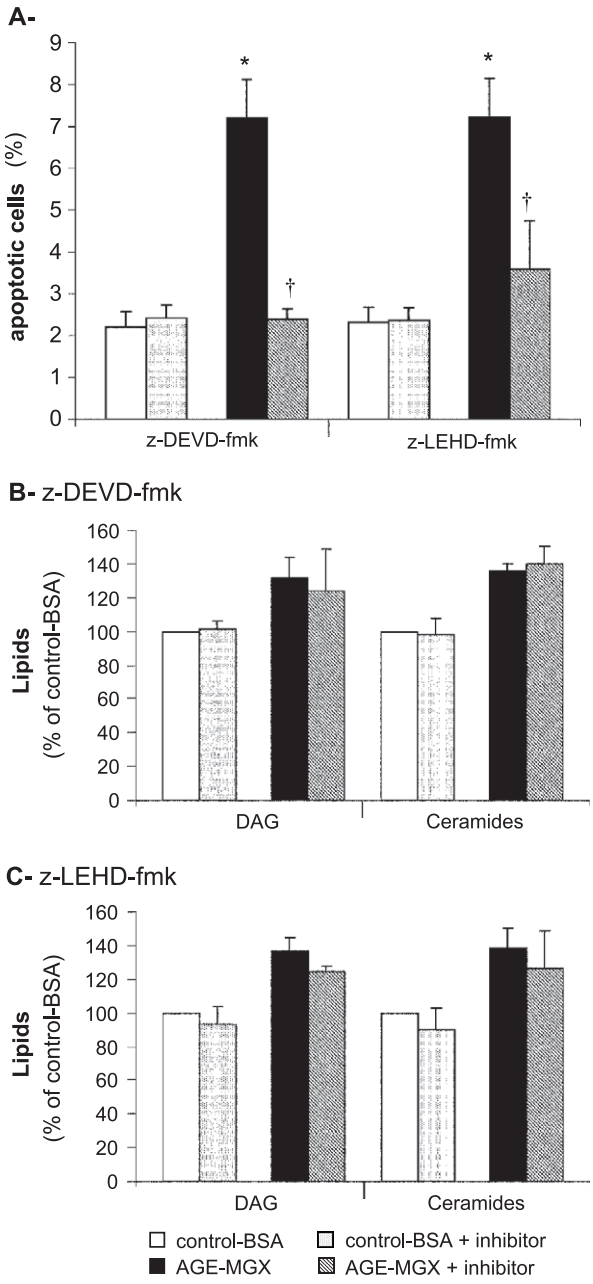

Fig. 2. Effects of z-DEVD-fmk (caspase-3-like enzyme inhibitor) and z-LEHD-fmk (caspase-9 inhibitor) on AGE-induced apoptosis and cellular DAG/ceramide levels. Cells were cultured throughout two passages (15 days on average) with AGE-MGX or control-BSA (3 μM) with or without caspase inhibitory peptide (50 μM). (A) Apoptosis was detected and quantified by the annexin V-FITC method, as described in Materials and methods. Results are expressed as percentage of apoptotic cells and are mean ± S.D. of six or five independent experiments for z-DEVD-fmk and z-LEHD-fmk, respectively. DAG and ceramide levels in cells treated with z-DEVD-fmk (B) or z-LEHD-fmk (C) were assayed by the DAG kinase method, as described in Materials and methods. Results, expressed as percentage of control-BSA, are mean ± S.D. of three independent experiments for both inhibitory peptides. Amount of ceramides measured in cells cultured with control-BSA (100%) for experiments with z-DEVD-fmk was 725 ± 114 ng ceramides/mg proteins and 689 ± 99 ng ceramides/mg proteins for experiments with z-LEHD-fmk. DAG amounts are probably overestimated as discussed in Materials and methods. *: $P<0.05$ vs. control-BSA, †: $P<0.05$ vs. AGE-MGX without agents.

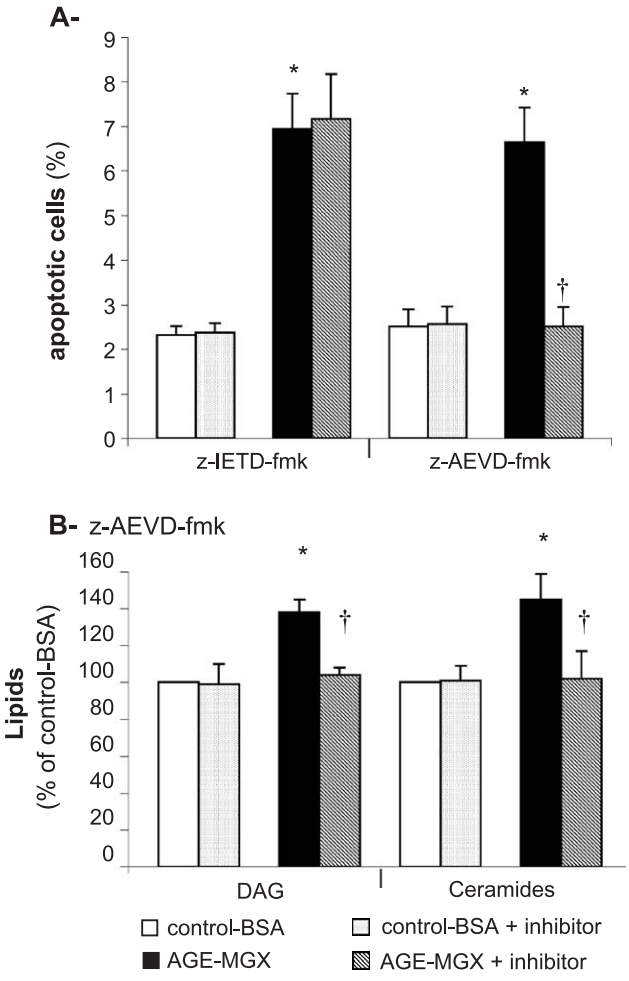

Fig. 3. Effects of z-IETD-fmk (caspase-8 inhibitor) and z-AEVD-fmk (caspase-10, -6, -8 and -9 inhibitor) on AGE-induced pericyte apoptosis and DAG/ceramide levels. Cells were cultured throughout two passages (15 days on average) with AGE-MGX or control-BSA (3 μM) with or without caspase inhibitory peptide (50 μM). (A) Apoptosis was detected and quantified by the annexin V-FITC method, as described in Materials and methods. Results are expressed as percentage of apoptotic cells and are mean ± S.D. of three or six independent experiments for z-IETD-fmk and z-AEVD-fmk, respectively. (B) DAG and ceramides levels in cells treated with z-AEVD-fmk were assayed by the DAG kinase method, as described in Materials and methods. Results, expressed as percentage of control-BSA, are mean ± S.D. of six independent experiments. Amount of ceramides measured in cells cultured with control-BSA (100%) for experiments with z-AEVD-fmk was 712 ± 132 ng ceramides/mg proteins. DAG amounts are probably overestimated as discussed in Materials and methods. *: $P<0.05$ vs. control-BSA, †: $P<0.05$ vs. AGE-MGX without agents.

3.3. Caspase-10 is involved in BRP apoptosis induced by AGE-MGX

We further investigated the possible involvement of initiator caspase-10 in the apoptotic pathway induced by AGE-MGX in pericytes. The effect of z-AEVD-fmk, an inhibitor of caspase-10 and related caspase-6, -8 and -9 [41], was tested on apoptosis and DAG/ceramide production in BRP cultured chronically (15 days) with AGE-MGX. z-

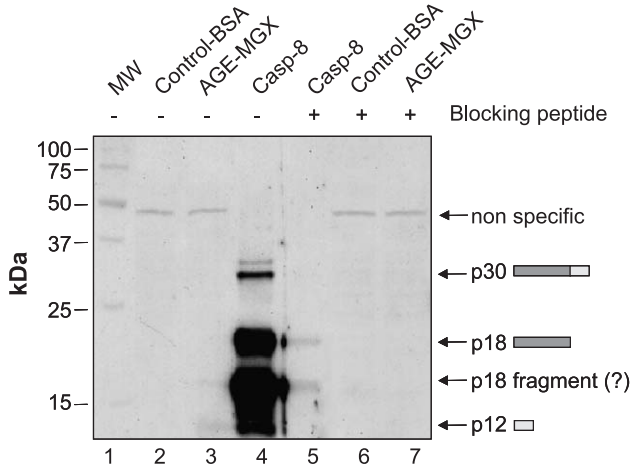

Fig. 4. Caspase-8 is not detected in BRP treated with AGE-MGX or control-BSA. Cells were cultured throughout two passages (15 days on average) with AGE-MGX or control-BSA (3 μM). Molecular weight standards (1 μl), total proteins from cellular lysates (20 μg) or human caspase-8 (1 μg) were analyzed by immunoblotting as described in Materials and methods. Samples were revealed with primary anti-caspase-8 antibody preincubated (lanes 5, 6, 7) or not (lanes 2, 3, 4) with blocking peptide as described in Materials and methods. Relative molecular mass of detected proteins was determined after comparison with proteins of known molecular mass (MW). This caspase-8 Western blot is representative of four independent experiments coming from different primary cultures.

AEVD-fmk (50 μM) was able to completely inhibit both apoptosis (Fig. 3A) and DAG/ceramide formation (Fig. 3B) in pericytes incubated with AGE-MGX. This simultaneous effect of z-AEVD-fmk on apoptosis and DAG/ceramide is different from the one observed with a specific caspase-8 inhibitor (no effect on apoptosis, see Fig. 3A) or with either a specific caspase-9 or a caspase-3-like (including caspase-6) inhibitor (no effect on DAG/ceramide production, see Fig. 2C and B, respectively). We further looked at possible caspase-10 expression and activation in BRP incubated chronically (P1 + P2) with AGE-MGX for an average duration of 15 days, after Western blotting of total proteins from cellular homogenates (Fig. 5A). Caspase-10 antibody used in these experiments is a polyclonal antibody raised against the large human caspase-10 subunit allowing recognition of both precursor zymogen and activated caspase (large subunit). Immunodetection with caspase-10 antibody shows a major band of (Mr) 64 000 (precursor) in control cells (control-BSA) (Fig. 5A, lane 2) whose intensity is decreased in AGE-MGX treated cells, together with a parallel increase in intensity of a protein band of (Mr) 27 000 (large subunit) (Fig. 5A, lane 3). From the whole data set of blots analysed by densitometric scanning (Fig. 5B), we observed a significant decrease (40 ± 13%, $P < 0.05$) of (Mr) 64 000 band together with a significant increase of (Mr) 27 000 band (54 ± 30%, $P < 0.05$). Relative molecular masses (Mr) as reported above were determined after comparison of migration distances with known molecular weight standards in seven independent experiments. In control experiments using recombinant human caspase-10, we checked the specificity of our antibody against the large subunit of caspase-10 (not shown).

4. Discussion

We have previously shown that AGE-MGX are able to induce pericyte apoptosis associated to sequential activation of PC-PLC coupled to A-SMase, leading to intracellular production of DAG and ceramides [18]. To our knowledge, the results reported here are the first to document involvement of initiator and effector caspases in apoptosis of retinal pericytes induced by AGE. This work further identifies caspase-10 as one initiator caspase acting in the early phase of AGE-induced apoptosis of pericytes based on experiments done with caspase peptidic inhibitors but also with specific antibodies used in Western blots.

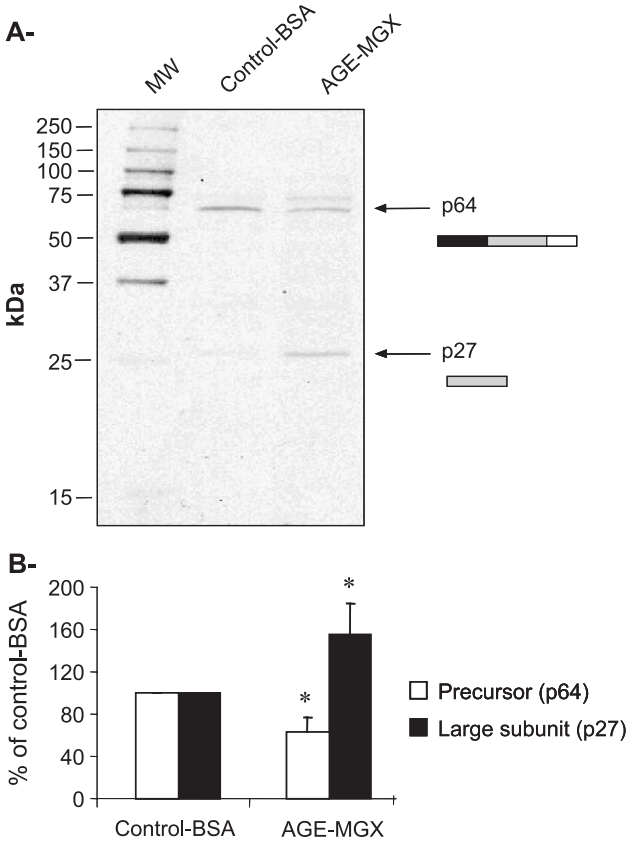

Fig. 5. AGE-induced activation of caspase-10 in BRP. Cells were cultured throughout two passages (15 days on average) with AGE-MGX or control-BSA (3 μM) (A) Molecular weight standards (1 μl) and total proteins from cellular lysates (20 μg) were analyzed by immunoblotting as described in Materials and methods. Polyclonal caspase-10 antibody used was raised against the large subunit of active caspase 10 and recognizes both the precursor form and the active caspase. Relative molecular mass of detected proteins was determined after comparison with proteins of known molecular mass (MW). This caspase-10 immunoblot is representative of seven independent experiments. (B) p64 and p27 bands detected in lanes 2 and 3 were quantified by densitometric scanning. Results, expressed as percentage of BSA-control, are mean ± S.D. of seven independent experiments. *$P < 0.05$ vs. control-BSA.

Results obtained with a nonspecific inhibitor of caspases, z-VAD-fmk, show that this peptide was able to completely inhibit AGE-induced apoptosis of pericytes but also DAG and ceramide accumulation in these cells, pointing to possible implication of caspases upstream of DAG and ceramide generation in the signaling cascade induced by AGE. Initiator caspases have been described in some apoptotic models to control A-SMase activation, reported to be dependent [28] or not [43,44] on PC-PLC activation. A-SMase activation has also been shown to be dependent on FADD [44], an adaptator molecule involved only in caspase-8 and -10 death receptor recruitment and activation [23,45], suggesting that these caspases might be involved in A-SMase activation during AGE-induced apoptotic process in retinal pericytes.

Looking at the exact nature of initiator caspase activated upstream of DAG/ceramide generation in AGE-induced apoptosis, we first investigated possible caspase-8 activation. Caspase-8 inhibitor z-IETD-fmk did not protect pericytes against AGE-induced apoptosis and we did not detect caspase-8 in retinal pericytes in our western blot experiments. These results strongly suggest that caspase-8 is not involved in the AGE-induced signaling pathway leading to pericyte death.

We further focused on caspase-10. z-AEVD-fmk, an inhibitor of caspase-10 and related caspase-6, -8 and-9, inhibited both AGE-induced apoptosis and DAG/ceramide formation in pericytes. This result is different from the one observed with a specific caspase-8 inhibitor which did not inhibit apoptosis or with either a specific caspase-9 inhibitor or a caspase-3-like (including caspase-6) inhibitor which did not affect DAG/ceramide formation. Taken together, these results strongly suggest that caspase-10 was involved upstream of DAG/ceramide formation in AGE-induced apoptosis of pericytes. Involvement of caspase-10 in AGE-induced apoptosis was further strengthened after immunoblotting experiments using an antibody raised against the large subunit of caspase-10 human enzyme. Western blots of proteins coming from pericytes incubated with AGE-MGX show a protein of (Mr) 64 000 approximately which intensity is decreased as compared to control cells (BSA-control), and a protein of (Mr) 27 000 approximately which is almost absent in control cells. This profile is in agreement with the one observed for caspase activation, the decrease of one caspase precursor band in parallel with increased intensity of the large subunit band only due to our antibody specificity. Relative molecular weights of bovine caspase-10 precursor (p64) and large subunit (p27) reported here are a little bigger (about 4 kDa) than the ones reported for long isoforms of human caspase-10/b (p59/p23) [46] and -10/d (p59/p23) [47]. This could reflect species differences regarding protein sequences, as observed for example for the bovine receptor RAGE for AGE which is bigger than its human counterpart [48].

Caspase-10 is a receptor-proximal caspase involved in death receptor-mediated apoptosis which has been described only recently. Proteolytic cleavage of pro-caspase-10 into active caspase-10 requires ligand-induced oligomerisation of death receptor (Fas or TNF-R) and recruitment of adaptator molecule FADD to the cytoplasmic death domain of the receptor [45]. Caspase-10 activation in retinal pericytes submitted to AGE-MGX suggests the involvement of transmembrane receptor in AGE-induced apoptosis. Apoptotic effects of AGE-MGX in retinal pericytes could be mediated either directly by AGE-MGX interaction with specific receptor(s) and induction of the apoptotic cascade, or indirectly after synthesis of one apoptosis mediator acting as one autocrine signal for pericyte apoptosis. Upon activation, RAGE receptor for AGE has been shown to induce secretion of TNFα in several cell lines like macrophages [49] or microvascular endothelial cells through NF-κB activation [50]. Pericytes in hyperglycemia are also able to secrete TNFα [51] through NF-κB. The other possibility is that binding of AGE to the AGE-receptor complex (p60, p90 and galectin 3) [52] found in cholesterol-rich lipid raft could activate caspase-10 by a mechanism which remains to be elucidated. One speculation could be that some protein like CARMA1 [53], containing a caspase recruitment motif and found constitutively associated with lipid rafts, could segregate with AGE receptor complex and activate caspase-10.

We have also examined the effects of z-LEHD-fmk, a specific inhibitor for caspase-9 [42], and z-DEVD-fmk, a specific inhibitor for caspase-3 and -7 and less active on caspase-6, -8 and -10 [41], on AGE-induced apoptosis and DAG/ceramide production. Interestingly, either z-LEHD-fmk or z-DEVD-fmk was able to inhibit apoptosis of pericytes induced by AGE-MGX, without affecting DAG and ceramide production in these cells. Taken together, these results suggest that caspase-9 and caspase-3, -6 or -7 act downstream of DAG and ceramide in the cascade induced by AGE-MGX. Activation of caspase-9 requires apoptosome formation dependent on mitochondrial leakage of cytochrome *c* [25]. One recent study on mesangial cells suggests a mechanism by which AGE can lead to mitochondrial alteration and caspase-9 activation: mesangial cell apoptosis induced by AGE leads to Bax overexpression [54], which in turn is able to induce mitochondrial cytochrome *c* leakage [55]. Supporting this, Bax overexpression in apoptotic pericytes has already been documented in retinas obtained postmortem from diabetic patients [5]. Activation of effector caspase-3, -6 or -7 in pericyte apoptosis, as suggested here, is supported by studies made by Li et al. [4] where overexpression of caspase-3 gene has been demonstrated in apoptotic pericytes purified from retinas obtained from diabetic patients.

In addition to this, activation of a large panel (not including caspase-10) of caspases in whole retinas of diabetic patients or mice in the course of diabetic retinopathy has recently been demonstrated [56]. Time-course analysis of caspase activation in retinal homogenates of diabetic mice revealed that caspase-3 induction was associ-

ated with diabetes duration at which microvascular cells undergo apoptosis [56], in accordance with our in vitro data on pericyte apoptosis induced by AGE. This study also showed increase of caspase-3 and -6 activities in homogenates of retinas obtained from diabetic type 2 patients.

In conclusion, our study shows involvement of caspases in the apoptotic cascade induced by AGE-MGX in BRP. AGE-induced apoptosis of pericytes seems to trigger one caspase that initiates the apoptotic signaling cascade (initiator caspase-10) but also caspases that execute the apoptotic program such as caspase-3, -6 or -7 and caspase-9. Accelerated apoptosis of pericytes was recently found to precede the characteristic diabetic vascular damages (microaneurysms, acellular capillaries) characteristic of diabetic retinopathy [2] and may therefore be a key event in the early phase development of this ocular complication of diabetes. Identification of caspases such as initiator caspase-10 involved in AGE-induced apoptosis of retinal pericytes may provide new therapeutic perspectives in early diabetic retinopathy.

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