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Experimental Induction of AGEs in Fetal L132 Lung Cells Changes the Level of Intracellular Cathepsin D

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The effect of the carbonyl compound glyoxal on the induction of advanced glycation end products (AGEs) in the fetal epithelial lung cells L132 was investigated using immunohistochemical, immunoelectron microscopic, and biochemical methods. It was found that glyoxal treatment resulted in morphological changes of the cells and in the membranous and cytosolic localization of AGEs such as methyl-glyoxal-derived compounds, *N*-(carboxymethyllysine) (CML) and imidazolone. The formation of AGEs was accompanied with a change in the intracellular expression of cathepsin D and a loss of enzymatic activity. © 1999 Academic Press

Advanced glycation endproducts (AGEs) are reactive derivatives of the Maillard reaction, a nonenzymatic glycation and oxidation between carbohydrate-derived carbonyl compounds and protein amino groups. Usually, glucose and other reducing sugars form in the first step with proteins reversible Schiff bases and, later, Amadori products, which undergo further chemical modifications to become irreversibly crosslinked, heterogeneous fluorescent and protease-resistant derivatives (for review see [1]). AGEs have been implicated as causal factors for vascular complication in several diseases and during normal aging [2–7]. Several reports demonstrated the presence of AGEs in normal and injured tissues as well as in body fluids [8–13]. AGEs are effective modulators of the functional properties of cellular and extracellular proteins and may be toxic for selected cell types such as pericytes in diabetic microangiopathy [14] or neuronal cells in Alzheimer's and other neurodegenerative diseases [15–17]. The underlying molecular mechanism of these effects remain unknown though there is evidence for an AGE-induced activation of intracellular signal transduction pathways leading to the generation of oxidative stress and finally to the damage of cells [1, 6, 18].

AGEs accumulate in alveolar macrophages and bronchial epithelial cells in patients with diffuse alveolar damage and idiopathic pulmonary fibrosis [19]. Since AGE-modified proteins have been shown to generate reactive oxygen species, oxidative stress reactions in epithelial lung cells could also contribute to the development of pulmonary fibrosis.

Recently, Niwa *et al.* [20] provided a culture system of neuronal cells in the presence of 3-deoxyglucosone and glyoxal for the experimental induction of AGEs. This method allows the *in vitro* analysis of cellular changes after AGE formation and to evaluate the role of glycoxidation reaction in neuronal degeneration. To understand the biological role of AGE formation during the development of pulmonary fibrosis, we applied this technique to the study of fetal lung cells L132. The effect of *in vitro* formed AGEs on the expression of cathepsin D, a protease which is involved in lung fibrogenesis [21], was also investigated. Furthermore, lysosomal proteases like cathepsin B and L have been shown to exhibit reduced enzyme activities after *in vitro* incubation with preformed AGEs [22].

MATERIALS AND METHODS

1. Cell culture. L132 embryonic lung cells (ATTC) were grown in DMEM/F12 medium supplemented with 5% fetal calf seum (FCS) and gentamycin (6.4 mg/ml) at 37°C in 5% CO_2.

2. Application of glyoxal. Glyoxal at various concentrations (160 and 320 μMol) was added to the culture medium for 24 hours. Cells that remained viable during incubation were determined by their ability to exclude trypan blue. The viability of untreated and glyoxal-

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TABLE 1

List of Antibodies Used for Immunohistochemistry

Antibody	Specificity	Species	Dilution	Pretreatment	Source
Anti-CML	CML	Rabbit, polyclonal	1:8000	No	Dr. Perry, Cleveland
Anti-MG-derived modifications	Methylglyoxal-derived modifications	Rabbit, polyclonal	1:1000	No	Dr. Nagaraj, Cleveland
AG-1	Imidazolone	Mouse, monoclonal	1:200	Microwave	Dr. Niwa, Nagoya
PU205-UP	Cathepsin D	Rabbit, monoclonal	1:320	No	Biogenex
OS 13A	Cathepsin D	Mouse, monoclonal	1:100	Microwave	Becton Dickinson

treated cells was about 92% and 84%, respectively. The glyoxal experiment was performed in triplicate.

3. Histology and immunohistochemistry. For the following investigations, the cell medium was removed, cells were washed with 0.1 M phosphate buffered saline (PBS), pH 7.4, scraped off, centrifugated (twice, 5 min, 580 g) and transferred into Eppendorf cups. Cultured cells were carefully mixed with 60 μl fibrin glue (Tissuecol Duo S, Immuno GmbH, Heidelberg, Germany). The compound was coagulated with 60 μl of a thrombin solution (Immuno GmbH) and fixed with 0.1 M PBS containing 4% paraformaldehyde for 1 h at 4°C, washed three times for 30 min in PBS, embedded in paraplast, cut (5 μm) and mounted on silane-coated slides. The sections were dewaxed, dried over night and irradiated with microwaves in 0.01 M sodium citrate buffer (pH 6.0), 2 × 5 min at 850 W. After washing in PBS, the sections were treated with 0.3% hydrogen peroxide for 30 min, incubated with respective normal sera and then incubated for 1 h at 37°C with primary antibodies (Table 1). The specificity of cathepsin D-specific antibodies was previously shown by Western blots of human lung tissue homogenates [21]. The characteristics of the AGE-specific antibodies are described in detail [4, 23].

The antibodies were detected with biotinylated secondary antibodies followed by a streptavidin/biotin-peroxidase complex (Vectastain Elite, Vector, Burlingame, CA). The peroxidase activity was visualized with 3′-3′-diaminobenzidine.

4. Antibody inhibition experiment. To test, if the polyclonal antibody against methylglyoxal-derived modifications also recognizes glyoxal-derived modifications, we incubated the protein-containing culture medium of L132 cells with 160 μM glyoxal for 1 h at 37°C, added the polyclonal antibody to the medium for further 30 min at 37°C and used this mixture for immunohistochemistry on paraffin-embedded lung cells (see below). As control we used medium without glyoxal.

5. Transmission and immunoelectron microscopy. Cells were fixed in 0.1 M PBS containing 4% paraformaldehyde and 0.05% glutaraldehyde for 1h at room temperature (RT). After a short rinse in buffer, the specimens were incubated in 1% OsO_4 in PBS for 2 h at RT, dehydrated with a graded alcohol series, contrasted with uranyl acetate *en bloc* in 70% ethanol overnight at 4°C and flat-embeded in Epon 812. Ultrathin sections were mounted on Formvar-coated slot copper grids. After a further rinse in buffer, the sections were stained with 2% aqueous uranyl acetate (8 min) and lead citrate (Reynolds, 2 min).

For immunoelectron microscopy, fixed L132 cells were incubated with 2.3 M sucrose in 0.1 M PBS overnight at 4°C and cryosubstituted with methanol at −80°C for 90 h using the Leica AFS system. The cells were embedded in Lowicryl HM20 (Polysciences Europe; methanol, methanol:Lowicryl 1:1, 1:2, Lowicryl) and polymerised in the Leica AFS in flat embedding moulds with UV irradiation for 48 h at −45°C. Ultrathin sections were mounted on pioloform-coated slot nickel grids and preincubated with 10% normal goat serum in TRIS-buffered saline (TBS, pH 7.6) for 45 min, and incubated with the polyclonal antisera against cathepsin D (dilution 1:4), and against methylgyloxal-derived modifications (dilution 1:50). Washings with buffer (TBS containing 0.2% BSA) were followed by incubation with 10-nm gold-conjugated anti-rabbit IgG (1:50 in TBS) for 1 h. After further washings with buffer, the sections were stained with 2.5% uranyl acetate and 1% lead citrate for 2 min each.

As negative control, we replaced the primary antibodies by TBS. Rat lung tissue containing alveolar macrophages served as positive control for cathepsin D immunostaining [21].

6. Enzymatic assay of cathepsin D. Cathepsin D activity was assayed spectroscopically by measuring the release of tyrosine-containing peptides from acid-denatured hemoglobin [24]. Briefly, cell-free extracts were prepared by sonication and centrifugation. Each 10 μg of total protein was incubated with 10 mg/ml hemoglobin in acetate-buffer (pH 3.5) for 10 min at 37°C. After acid-denaturation (5% TCA) and centrifugation (13000 rpm for 10 min), the peptide concentration in the supernatant was determined at 280 nm. Tyrosine released was calculated from a standard curve prepared with tyrosine. Cathepsin units (μMol tyrosine released per minute and mg protein) were calculated from a standard curve using a purified cathepsin D (Calbiochem, Bad Soden, Germany). To test the specificity of the protease action, the cathepsin D inhibitor pepstatin was added to the assay.

7. RT-PCR. Total RNA was prepared by phenol extraction and column purification using the RNeasy system (Quiagen, Hilden, Germany). Reverse transcription of total RNA (5 μg) was performed with the reverse transcription system provided by Promega (Promega, Madison, U.S.A.). The cathepsin D cDNA (0.1 μg) was amplified by PCR (30 cycles with 60 s at 94°C, 60 s at 54°C and 30 s at 72°C) using the primer pair 5′-CCAGCCCCCAATCCCAACCCCACCTCCAG-3′ and 5′-CACTGAAGCTGGGAGGCAAAGGCTACAAGC-3′ [25] and analyzed by 1% agarose gel electrophoresis.

8. Western blot analysis. Cells were washed with ice-cold PBS and lysed with 400 μl sample buffer for SDS–PAGE [26]. The cell lysate was passed 5 times through a 20G and a 27G syringes. The cell lysate was loaded on a 12.5% SDS–PAGE, stained with Coomassie Brilliant Blue, and total protein concentration was estimated with densitometry. Identical amounts of total protein were loaded on 12.5% SDS–PAGE gels, separated electrophoretically and transferred to nitrocellulose (Sartorius, Göttingen, Germany) with a mini wet blot chamber (Sigma, Deisenhofen, Germany). The blotted proteins were processed for immunolabelling as recently described [27]. The blotted nitrocellulose was probed against AGEs with antibodies against methylglyoxal-derived modifications and CML (Table 1). An alkaline-phosphatase-conjugated anti rabbit IgG served as secondary antibody and was visualized with NBT/BCIP staining solution.

RESULTS

In the present study, we demonstrated that the carbonyl compound glyoxal is able to induce the formation of AGEs in the epithelial lung cell line L132. When sections of paraffin embedded cells from normal and

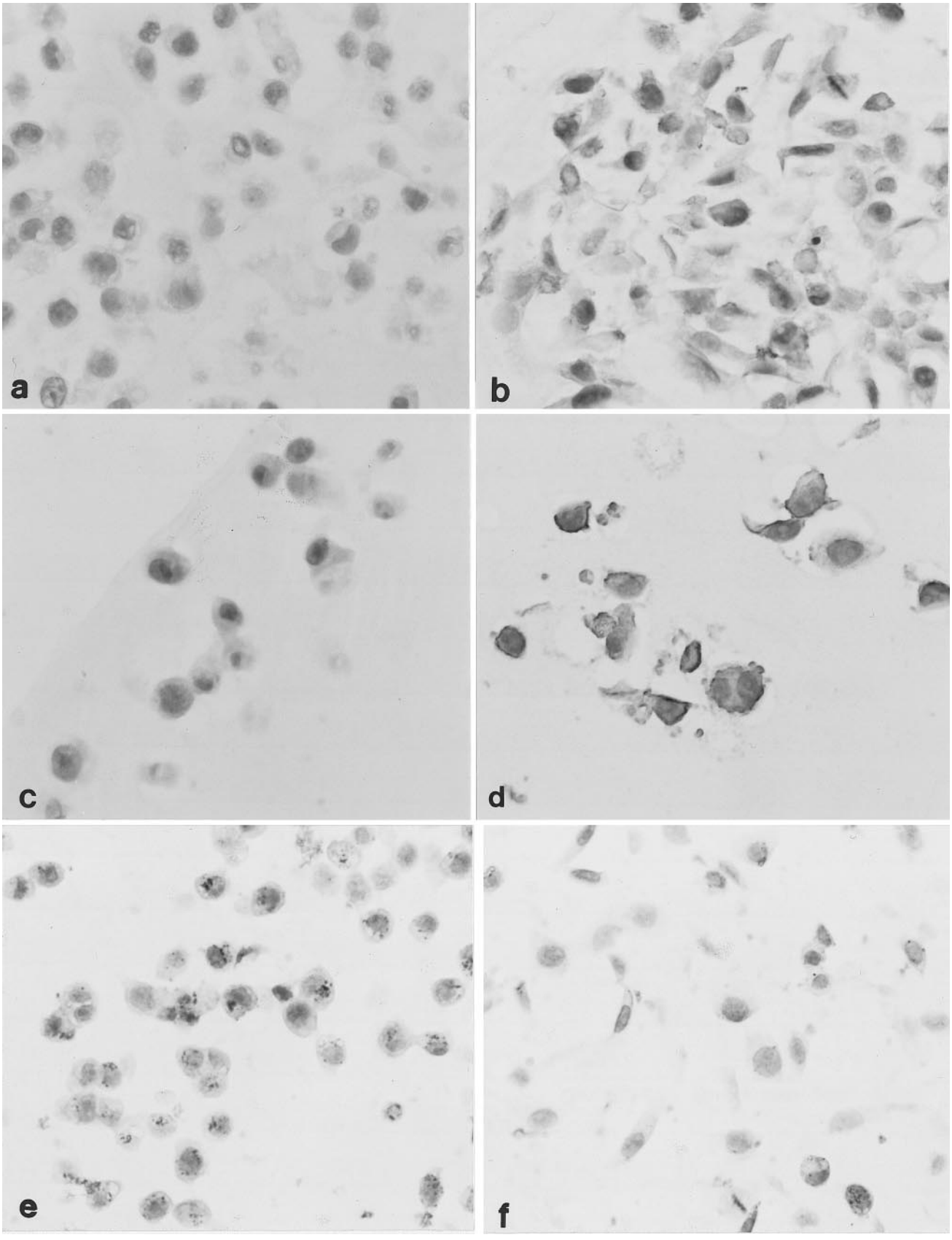

FIG. 1. Paraffin sections of human fetal lung cell line L132. Immunohistochemical demonstration of AGEs in glyoxal-treated cells (b: antibody against imidazolone; d: antibody against methylglyoxal-derived modifications). Untreated cells (a, c) were negative. (e, f) Cathepsin D immunoreactivity in untreated (e) and glyoxal-treated (f) cells. ABC technique, HE counterstain; ×300.

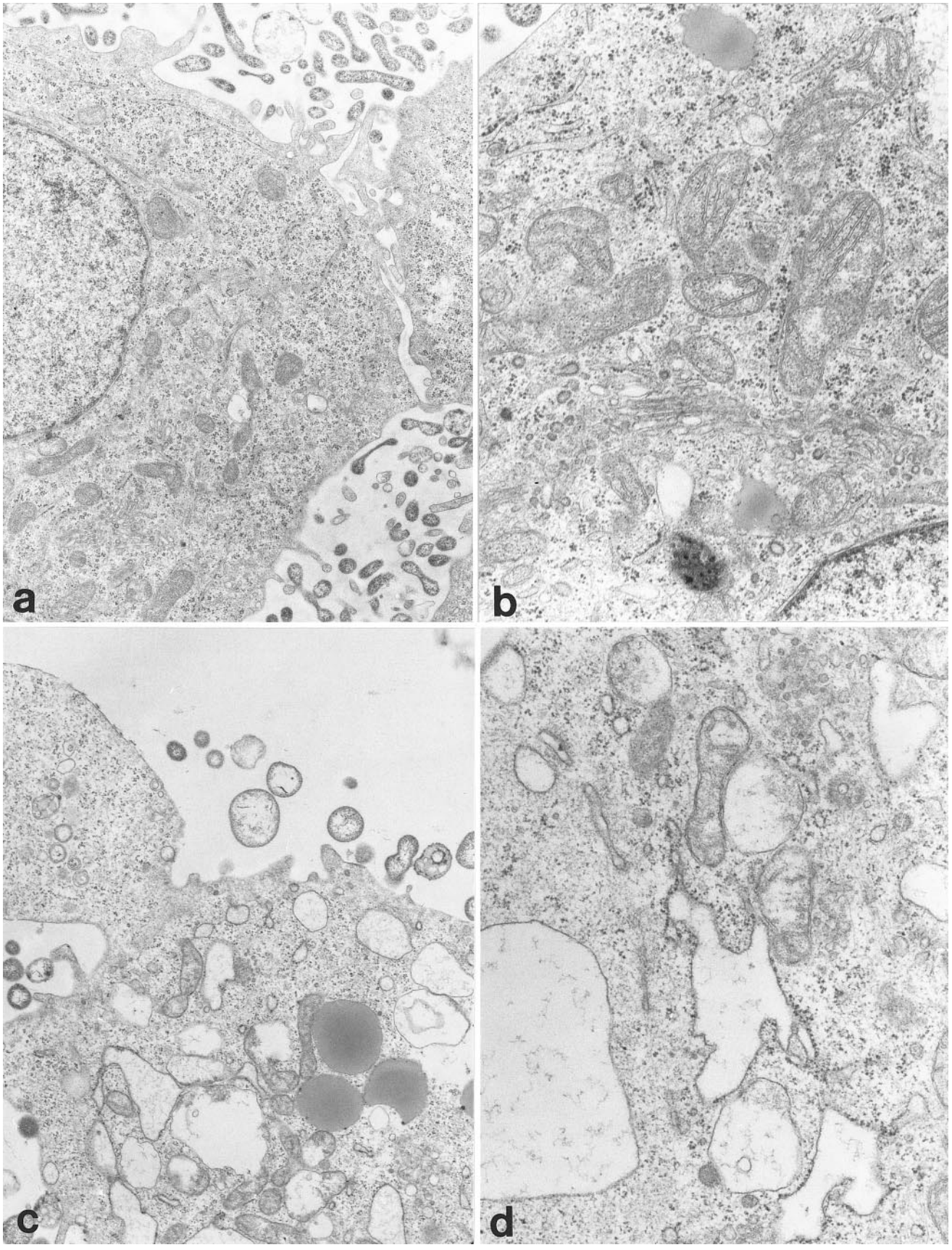

FIG. 2. Conventional transmission electron microscopy of untreated (a, b) and glyoxal-treated (c, d) L132 cells. a, c, ×11600; b, d, ×25000.

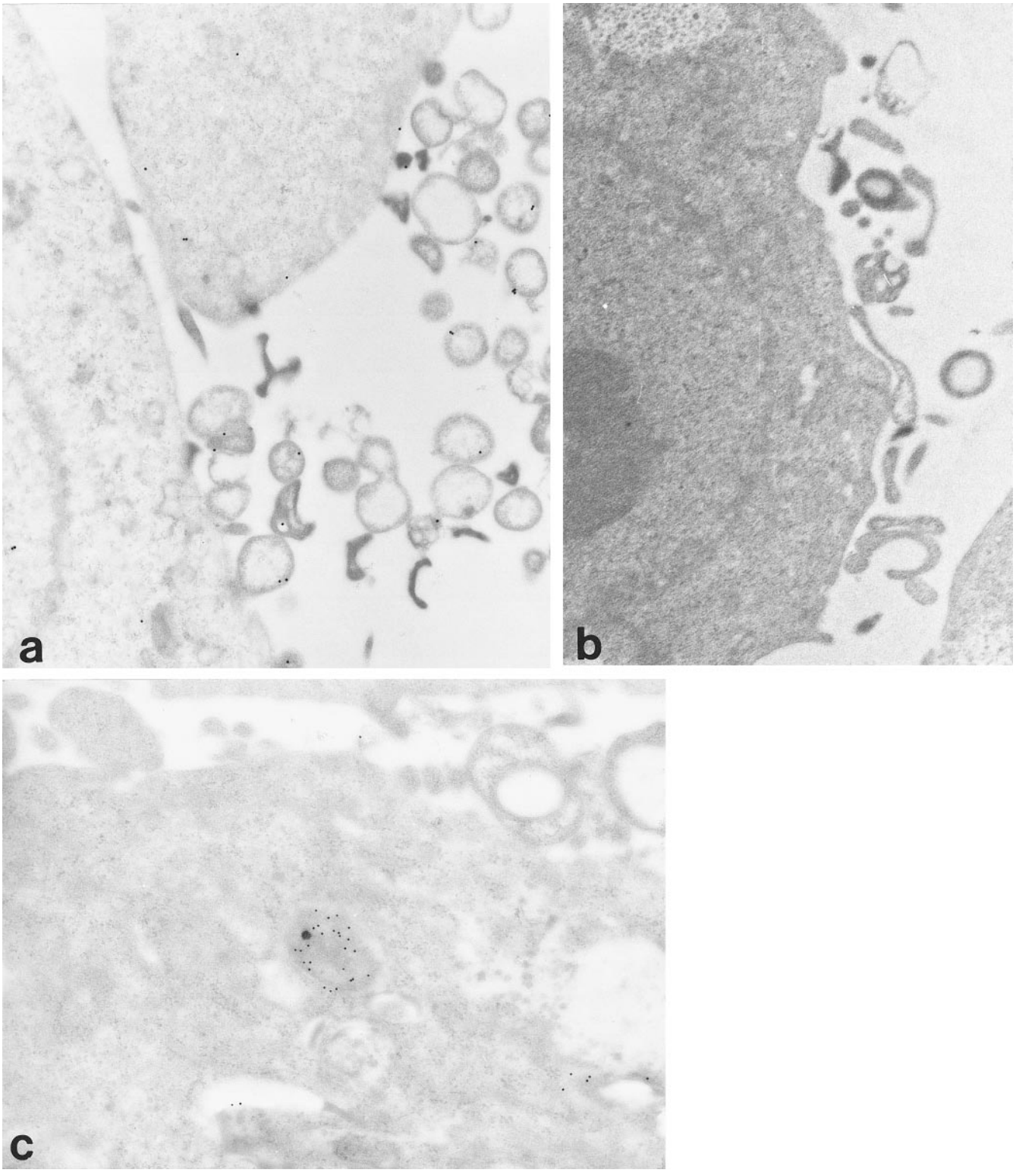

FIG. 3. Immunogold localization of AGEs in glyoxal-treated L132 cells (a; 20 nm gold). Compare the negative immunoreactivity of untreated cells in b. c: Lysosomal localization of cathepsin D in untreated cells (10 nm gold); Lowicryl HM20 embedding. a, b, ×19400; c, ×41700.

glyoxal-treated L 132 cells were examined, the most striking feature was the prominent immunolabelling of glyoxal treated cells with antibodies against the epitopes of early advanced glycation endproducts such as methylglyoxal-derived modifications, imidazolone (Fig. 1) and carboxymethyllysine (CML; not shown).

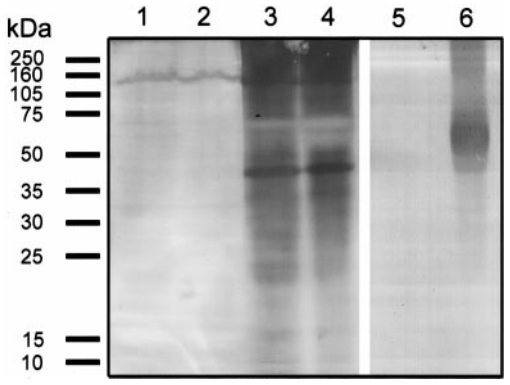

FIG. 4. Western blot of cultured L132 cells incubated wtih 5 μM (lane 2), 160 μM (lane 3) and with 320 μM (lanes 4 and 6) glyoxal for 24 h. Multiple bands are found after incubation with 160 and 320 μM glyoxal using an antibody against CML (lanes 1–4) or against methylglyoxal-derived modifications (lane 6). No bands are found without (lanes 1 and 5) or after incubation with 5 μM glyoxal.

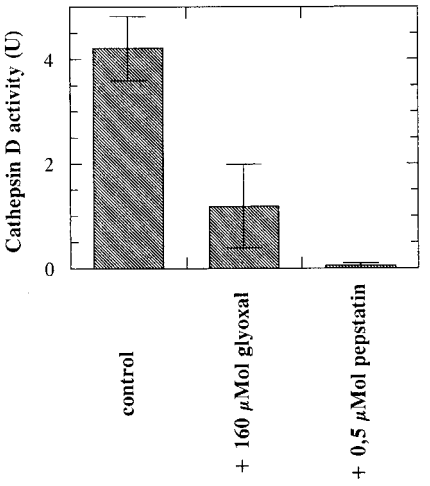

FIG. 5. Cathepsin D activity in cell-free extracts of glyoxal-treated L132 cells. Cathepsin activities of untreated cells (control) and cells incubated in the presence of 160 μM glyoxal are shown. The specificity of the reaction was confirmed by the inhibition of protease activities by the cathepsin D-specific inhibitor pepstatin.

Whereas the imidazolone-specific antibody preferably reacted with cytoplasmic constituents (Fig. 1b), the staining of the polyclonal antiserum against methylglyoxal-derived compounds was mainly confined to the cell membrane and to the nucleus (Fig. 1d). The latter reaction patterns were confirmed by postembedding immunoelectron microscopy (Fig. 3a,b). In the inhibition experiment, the polyclonal antiserum, which probably has been absorbed by AGEs, preformed during incubation of glyoxal with serum proteins of the culture medium, failed to react with the glyoxal-treated L 132 cells (not shown). Western blot analyses of cell homogenates with antibodies against methylglyoxal-derived modifications or CML revealed multiple bands after incubation with 160 μM and with 320 μM glyoxal and no bands after incubation with 5 μM glyoxal (Fig. 4).

In previous experiments, we found that concentrations of glyoxal from 32-320 μM are effective to form AGEs with a cell viability over 80% (own unpublished data). The cells did not show clear signs of morphological damage or an apoptotic phenotype (i.e., cellular shrinkage, nuclear fragmentation and chromatin condensation). However, we observed a change from a round to a long-shaped morphology and further some blebs at the plasma membrane. Conventional electron microscopy revealed subcellular changes such as swelling of mitochondria, presence of new vesicles, protrusions of the cell surface (Fig. 2).

Cathepsin D immunohistochemistry with two different antibodies against cathepsin D revealed a dramatic loss of immunoreactivity after glyoxal treatment (Fig. 1e,f). In a few cells, glyoxal treatment caused an immunohistochemically detectable redistribution of smaller cathepsin D-positive lysosomes to the cell membrane (not shown). At both the light microscopical and electron microscopical levels, cathepsin D was found primarily in lysosomal structures in the untreated lung epithelial cells (Fig. 1e,f; Fig. 3c). Using immunoelectron microscopy, however, cathepsin D was not found in lysosomes and in the cytosol of glyoxal-treated cells.

The loss of immunoreactivity was accompanied by a concomitant, approximately 3-fold loss of enzymatic activities (Fig. 5). Furthermore, determination of cytosolic cathepsin D mRNA by RT-PCR showed an approximately 3-fold reduction of cathepsin D mRNA levels after 12 hours of incubation with 160 μM glyoxal (Fig. 6).

DISCUSSION

The *in vitro* study of AGEs has focused on the effects on living systems, such as, for example, the

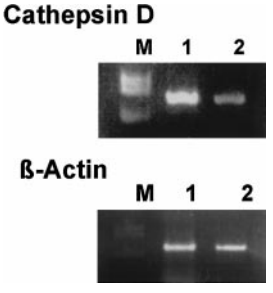

FIG. 6. Effect of glyoxal on cathepsin D mRNA expression measured by RT-PCR. Treatment with glyoxal (160 μM glyoxal for 24 h) causes a decrease in cathepsin D mRNA levels (lane 2) while β-actin mRNA levels are not affected.

expression of adhesion molecules or tissue factor after stimulation of monocytes with AGEs [3, 28], the induction of the expression of VEGF in retinal Müller cells [29], the activation of NF-κB in endothelial cells [6], the modulation of the nitric oxide synthase pathway in retinal vascular endothelial cells [14], the inhibition of protease activities in kidney proximal tubulus cells [22], or the induction of the expression of PDGF in retinal pigment epithelial cells [30]. In all described cases preformed AGEs were used. Very recently, Niwa *et al.* [20] provided first evidence for the experimental induction of AGEs in neuronal cells under serum-free condition using the carbonyl compounds 3-deoxyglucosone and glyoxal. We applied this technique for the investigation of the epithelial lung cell line L132 and found positive immunoreactions with antisera against methylglyoxal-derived modifications, CML and imidazolone. Our immunocytochemical findings of a strong immunoreaction of glyoxal-treated cells for AGEs was further supported by immunoelectron microscopy. Western blot analyses demonstrated the formation of AGEs as indicated by the multiple bands after incubation with glyoxal. We can, however, not exclude the extracellular formation of AGEs during the incubation time of glyoxal with the FCS-containing cell culture medium. AG-1 antibody reacts only with imidazolone derived from 3-deoxyglucosone and arginine, but does not react with other glyoxal-derived imidazolone-like compounds [23].

Searching for other phenotypic changes in the cells after induction of AGEs we observed a remarkable loss of cathepsin D immunoreactivity, cathepsin D mRNA expression as well as enzymatic activity. It is well known that AGE-modified proteins are stimuli for cell activation associated with cell proliferation, apoptosis and oxidative stress (reviewed by Thornalley [1]). Oxidative stress may down-regulate glial lysosomal cathepsins as part of a concerted cellular stress response [31]. Also, Sebekova *et al.* [22] reported impaired protease activities in LLC-PK1 cells after AGE-BSA incubation. Moreover, in cell culture experiments using different cell types such as fibroblasts, myocytes and lymphoma cells, oxidative stress caused a redistribution of cathepsin D from lysosome-like structures to the cytosol [18]. In contrast, increased expression of cathepsin D in neurons of the aged brain and its colocalization with aged proteins has been described [32].

The cellular mechanisms by which AGEs inhibit lysosomal enzyme activities have yet to be determined. Our data of impaired protease activities are in line with previous immunohistochemical findings of a reduced cathepsin D immunoreactivity in desquamated, detached pneumocytes in human pulmonary fibrosis specimens [21]. Whether this finding is related to the *in vivo* formation of AGEs in alveolar epithelial cells during fibrogenesis, has to be proved in future studies.

Furthermore, it has not unequivocally been shown by our experiment whether the carbonyl compound glyoxal is directly responsible for the dramatic cellular alterations described already by previous groups (reviewed by Thornalley [33]), or whether AGEs induced by carbonyl stress exert the biochemical and morphological effects on the fetal lung cells.

In summary, our study establishes that gyloxal-derived products form *in vitro* in epithelial lung cells and strongly reduce the intracellular contents of the lysosomal protease cathepsin D.

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REFERENCES

1. Thornalley, P. J. (1998) *Cell Mol Biol (Noisy-le-grand)* **44,** 1013–1023.
2. Emekli, N. (1996) *J. Marmara Univ. Dent. Fa.c* **2,** 530–534.
3. Kunt, T., Forst, T., Harzer, O., Buchert, G., Pfutzner, A., Lobig, M., Zschabitz, A., Stofft, E., Engelbach, M., and Beyer, J. (1998) *Exp. Clin. Endocrinol. Diabetes* **106,** 183–188.
4. Shamsi, F. A., Lin, K., Sady, C., and Nagaraj, R. H. (1998) *Invest. Ophthalmol. Vis. Sci.* **39,** 2355–2364.
5. Wautier, J. L., and Guillausseau, P. J. (1998) *Vasc. Med.* **3,** 131–7.
6. Bierhaus, A., Hofmann, M. A., Ziegler, R., and Nawroth, P. P. (1998) *Cardiovasc. Res.* **37,** 586–600.
7. Chappey, O., Dosquet, C., Wautier, M. P., and Wautier, J. L. (1997) *Eur. J. Clin. Invest.* **27,** 97–108.
8. Bruel, A., and Oxlund, H. (1996) *Atherosclerosis* **127,** 155–165.
9. Chen, J. R., Takahashi, M., Suzuki, M., Kushida, K., Miyamoto, S., and Inoue, T. (1998) *J. Rheumatol.* **25,** 2440–2444.
10. Degenhardt, T. P., Grass, L., Reddy, S., Thorpe, S. R., Diamandis, E. P., and Baynes, J. W. (1997) *Kidney Int.* **52,** 1064–1967.
11. Miyata, T., Ishiguro, N., Yasuda, Y., Ito, T., Nangaku, M., Iwata, H., and Kurokawa, K. (1998) *Biochem. Biophys. Res. Commun.* **244,** 45–49.
12. Ling, X., Sakashita, N., Takeya, M., Nagai, R., Horiuchi, S., and Takahashi, K. (1998) *Lab. Invest.* **78,** 1591–1606.
13. Stitt, A. W., Moore, J. E., Sharkey, J. A., Murphy, G., Simpson, D. A., Bucala, R., Vlassara, H., and Archer, D. B. (1998) *Invest. Ophthalmol. Vis. Sci.* **39,** 2517–2523.
14. Chakravarthy, U., Hayes, R. G., Stitt, A. W., McAuley, E., and Archer, D. B. (1998) *Diabetes* **47,** 945–952.
15. Munch, G., Thome, J., Foley, P., Schinzel, R., and Riederer, P. (1997) *Brain Res. Brain Res. Rev.* **23,** 134–143.
16. Munch, G., Gerlach, M., Sian, J., Wong, A., and Riederer, P. (1998) *Ann. Neurol.* **44,** S85–S88.
17. Vitek, M. P., Bhattacharya, K., Glendening, J. M., Stopa, E.,

Vlassara, H., Bucala, R., Manogue, K., and Cerami, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4766–4770.

18. Roberg, K., and Ollinger, K. (1998) *Am. J. Pathol.* **152,** 1151–1156.
19. Matsuse, T., Ohga, E., Teramoto, S., Fukayama, M., Nagai, R., Horiuchi, S., and Ouchi, Y. (1998) *J. Clin. Pathol.* **51,** 515–519.
20. Niwa, H., Takeda, A., Wakai, M., Miyata, T., Yasuda, Y., Mitsuma, T., Kurokawa, K., and Sobue, G. (1998) *Biochem. Biophys. Res. Commun.* **248,** 93–97.
21. Kasper, M., Lackie, P., Haase, M., Schuh, D., and Muller, M. (1996) *Virchows Arch.* **428,** 207–215.
22. Sebekova, K., Schinzel, R., Ling, H., Simm, A., Xiang, G., Gekle, M., Munch, G., Vamvakas, S., and Heidland, A. (1998) *Cell Mol. Biol. (Noisy-le-grand)* **44,** 1051–1060.
23. Takayama, F., Aoyama, I., Tsukushi, S., Miyazaki, T., Miyazaki, S., Morita, T., Hirasawa, Y., Shimokata, K. and Niwa, T. (1998) *Cell Mol. Biol. (Noisy-le-grand)* **44,** 1101–1109.
24. Turk, V., Brzin, J., Kotnik, M., Lenarcic, B., Popovic, T., Ritonja, A., Trstenjak, M., Begic-Odobasic, L., and Machleidt, W. (1986) *Biomed. Biochim. Acta* **45,** 1375–1384.
25. Liu, Z., Brattain, M. G., and Appert, H. (1997) *Biochem. Biophys. Res. Commun.* **231,** 283–289.
26. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
27. Hofer, A., Saez, J. C., Chang, C. C., Trosko, J. E., Spray, D. C., and Dermietzel, R. (1996) *J. Neurosci.* **16,** 4311–4321.
28. Khechai, F., Ollivier, V., Bridey, F., Amar, M., Hakim, J., and de Prost, D. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17,** 2885–2890.
29. Hirata, C., Nakano, K., Nakamura, N., Kitagawa, Y., Shigeta, H., Hasegawa, G., Ogata, M., Ikeda, T., Sawa, H., Nakamura, K., Ienaga, K., Obayashi, H., and Kondo, M. (1997) *Biochem. Biophys. Res. Commun.* **236,** 712–715.
30. Handa, J. T., Reiser, K. M., Matsunaga, H., and Hjelmeland, L. M. (1998) *Exp. Eye Res.* **66,** 411–419.
31. Chopra, V. S., Moozar, K. L., Mehindate, K., and Schipper, H. M. (1997) *Exp. Neurol.* **147,** 221–228.
32. Nakanishi, H., Amano, T., Sastradipura, D. F., Yoshimine, Y., Tsukuba, T., Tanabe, K., Hirotsu, I., Ohono, T., and Yamamoto, K. (1997) *J. Neurochem.* **68,** 739–749.
33. Thornalley, P. J. (1996) *Gen. Pharmacol.* **27,** 565–573.