Overexpression of Inducible Heat Shock Protein 70 in COS-1 Cells Fails to Protect From Cytotoxicity of Oxidized LDLs

A. Pirillo, G.D. Norata, T. Zanelli, A.L. Catapano

Abstract—Oxidized low density lipoproteins (OxLDLs) are believed to play a central role in atherogenesis and to possess a wide variety of biological properties; among them, OxLDLs are cytotoxic to cultured vascular cells in that they induce necrosis and apoptosis. Moreover, OxLDLs are known to induce the expression of heat shock protein 70 (Hsp70), a protein that protects cells from several cytotoxic stimuli. To determine whether Hsp70 can protect cells against OxLDL-induced cytotoxicity, COS-1 cells were transfected with a construct containing human Hsp70. A number of cell lines permanently expressing Hsp70 were obtained, 1 of which (cos-Hsp70/10, with high Hsp70 expression) was selected for further studies. Hsp70 overexpression protected cells from toxic stimuli, such as H₂O₂, UV irradiation, and heat shock, suggesting that the overexpressed protein was functional. When incubated with OxLDLs, however, the clone overexpressing Hsp70 showed a significant decrease in viability, as determined by the [³H]adenine release assay (319.8 ± 3.16% of control for transfected cells versus 217.6 ± 6.08% for control cells exposed to 100 μg protein/mL of OxLDL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (12.5 ± 0.9% versus 28.9 ± 1.99% of control, respectively), and LDH release (48.4 ± 0.04% versus 15.2 ± 0.06% of control cells). The increased expression of Bax and the decreased expression of Bcl-2 (a proapoptotic and an antiapoptotic protein, respectively) in cos-Hsp70/10 cells and in control cells on incubation with OxLDLs suggested that overexpression of Hsp70 did not confer protection against apoptosis induced by OxLDLs. The analysis of nucleosome content and the nuclear staining with Hoechst 33258 confirmed this finding. These data suggest that overexpression of Hsp70 not only fails to protect COS-1 cells against OxLDL-induced apoptosis but rather confers a higher sensitivity to the cytotoxic action of these lipoproteins. Thus, the Hsp70 response, although induced by OxLDLs, cannot protect cells from lipoprotein toxicity. (Arterioscler Thromb Vasc Biol. 2001;21:348-354.)

Key Words: low density lipoproteins ■ apoptosis ■ necrosis ■ Bcl-2 ■ Bax

When exposed to stresses, such as high temperature or other toxic stimuli, cells react by synthesizing stress proteins, also named heat shock proteins (Hsps).1–6 Hsps include constitutive and inducible forms. Constitutive Hsps play important biological roles, including facilitation of the correct folding of newly synthesized polypeptides7 and their transport across cellular membranes8–10; inducible forms, expressed by cells under stress conditions, are believed to protect cellular proteins from denaturation.11 Hsps show a very high degree of homology among different organisms12; Hsp70, the major Hsp, is the most conserved throughout evolution13 and plays a key role in protecting cells from environmental stresses.14 In fact, overexpression of Hsp70 confers thermotolerance and increases cell survival in the presence of noxious stimuli.15–19 Interestingly, the cellular content of Hsp70 is directly related to the degree of myocardial protection from ischemia in rat hearts20 and in hearts from transgenic mice overexpressing Hsp70.21

Oxidized LDL (OxLDL), a modified lipoprotein that is toxic to the cells of the arterial wall,22,23 probably because it induces apoptosis,24,25 increases the expression of Hsp70 in human endothelial cells26 and in smooth muscle cells,27 suggesting the possibility that Hsp70 may represent a cellular defense against OxLDL toxicity.28 These observations raise the question of whether high levels of Hsp70 expression could protect cells from OxLDL cytotoxicity. To address this issue, we have produced stable transfectants overexpressing the inducible form of the human Hsp70 and investigated whether high intracellular levels of Hsp70 may protect cells from the apoptosis induced by OxLDL.

Methods

Cells
The COS-1 cell line29 was grown at 37°C in a humidified atmosphere of 5% CO₂ in MEM (Sigma Chemical Co) supplemented with 10% FCS (Sigma), 100 U/mL penicillin, 0.1 mg/mL streptomycin,
20 mMol/L tricine buffer, and 1% (vol/vol) nonessential amino acid solution.

Cell Transfection
Hsp70 (plasmid pH 2.3, kindly provided by Dr R. Morimoto) was subcloned into the pBK-CMV vector (Stratagene), which allows eukaryotic and prokaryotic expression. In the former case, expression is driven by the cytomegalovirus (CMV) immediate-early promoter, and transfected cells are selected in the presence of G418 (neomycin, 800 μg/mL, Life Technologies Italia). COS-1 cells were transfected by the calcium coprecipitation method. Cells were seeded at 5 × 10⁴ cells per 10-cm Petri dishes in 10 mL of complete medium the day before transfection. Plasmid DNA (20 μg) was diluted to 1 μg/mL with TE buffer (10 mMol/L Tris and 1 mMol/L EDTA, pH 8.0). The DNA solution was mixed with 500 μL of 0.25 mol/L CaCl₂ and then 500 μL of 2 M N,N-bis(2-hydroxyethyl)-2-aminethanesulfonic acid (pH 6.95) was added, followed by a 20-minute incubation at room temperature. The calcium-phosphate-DNA solution was added dropwise to the medium in the dishes. Cells were incubated for 24 hours at 35°C in 5% CO₂. At the end of the incubation, cells were washed twice in PBS (pH 7.4) and incubated overnight in complete medium at 37°C in 5% CO₂. Cells were then divided 1:10 and permanently transfected cells were selected by growth in 800 μg/mL of the antibiotic G418, resistance to which is dependent on the neomycin resistance gene on the plasmid vector. Neomycin (400 μg/mL) was used to maintain the clones in culture. Cells transfected by use of a plasmid DNA in which the Hsp70 gene was lacking were used as controls.

[³H]Thymidine Incorporation
The growth of transfected cells was evaluated by the [³H]thymidine incorporation assay. Cells were plated in 35-mm wells at different densities (3125 and 6250 cells/cm²) in MEM +10% FCS. After 20 hours, 1 μCi/mL of [³H]thymidine in fresh medium was added, and the incubation was carried out for 3 hours. At the end of the incubation, the media were removed, and the cells were washed 3 times with PBS and incubated with 10% trichloroacetic acid (TCA) for 5 minutes. Free [³H]thymidine (non–TCA precipitable) was removed by washing the cell layer once with 10% TCA and once with distilled H₂O. After the washing, 0.75 mL of 0.1N NaOH was added to each well. Aliquots (200 μL) were used to measure the cell-associated radioactivity, and the remaining part of the sample was used to measure the protein content. The cell-associated radioactivity was normalized for cellular protein content (counts per minute per microgram protein).

Lipoproteins
Human LDL (1.019 to 1.063 g/mL) was isolated by sequential ultracentrifugation from freshly isolated human plasma from normolipidemic volunteers by use of a Beckman 50.2 Ti rotor at 4°C, dialyzed against 0.15 mol/L NaCl and 0.01% EDTA, and sterilily filtered by passage through a 0.45-μm filter (Corning). Before oxidation, EDTA was removed from LDL by a passage on a Sephadex G25 column (PD-10, Pharmacia) in PBS. LDLs were oxidized under sterile conditions at a concentration of 0.2 mg protein/mL with 20 μmol/L CuSO₄ at 37°C for 24 hours. The oxidation was blocked by the addition of 40 μmol/L butylated hydroxytoluene. Under these conditions, we have previously shown that LDL oxidation does not proceed further at 4°C. LDLs were used within 2 days from preparation because the experiments were strongly oxidized, as shown by the level of thiobarbituric acid–reactive substances (0.99 versus 0.043 μg MDA/μg protein of native LDL), and had an electrophoretic mobility relative to native LDL of 2.4.

Immunoblotting
To verify the overexpression of Hsp70, cells were plated in 6-well plates for 24 hours and then lysed with the use of a Tris-glycine buffer (0.25 mol/L Tris and 0.173 mol/L glycine) containing 3% SDS and 1 mmol/L phenylmethylsulfonyl fluoride, and the protein content was evaluated by the method of Lowry et al, with BSA used as a standard. Aliquots of the samples (10 μg) were diluted in a 2% β-mercaptoethanol buffer containing glycerol and bromophenol blue and electrophoresed on 8% SDS-PAGE and then transferred onto a nitrocellulose membrane with use of a Trans Blot Cell (Hoefer Scientific Instrument). The membrane was saturated at 25°C in PBS containing 3% BSA for 1 hour, washed with PBS containing 0.1% Tween 20, and then incubated for 1 hour at 25°C with a mixture of mouse monoclonal antibody specific for the inducible form of Hsp70 (Hsp72, 1:1500, C92F3A-5, StressGen) and anti–β-actin antibody (1:5000, Sigma). An anti-mouse IgG peroxidase conjugate (1:5000, Sigma) was used as a secondary antibody, enhanced chemiluminescence (Amersham) and autoradiography (X-OMAT, Kodak) followed. The bands were quantified by a computer-assisted system for image analysis (ISF Image 1.47), and the expression of Hsp70 was evaluated as the ratio of Hsp70 to β-actin to correct for cell number.

To verify the expression of Bax and Bcl-2, cellular proteins were subjected to Western blotting as described above. The membrane was saturated with 3% BSA/PBS and then incubated with a polyclonal antibody specific for Bax (1:200, Amersham) or a polyclonal antibody specific for Bcl-2 (1:200, Amersham) and with an anti–β-actin antibody (1:5000, Sigma) for 1 hour. As a second antibody, an anti-rabbit or anti-mouse IgG peroxidase conjugate (Bio-Rad) was used; enhanced chemiluminescence and autoradiography followed.

Cell Viability
Cell viability was evaluated after exposure to toxic agents by a [³H]adenine release assay, 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, 37 and LDH release assay. For [³H]adenine release, cells were seeded in 12-well plates (6 × 10⁵ cells per well) in MEM containing 10% FCS; after 24 hours, the cells were incubated with [³H]adenine (1 μCi/mL) in adenine-free medium for 3 hours, washed 3 times with PBS, and incubated overnight in 1 mL of serum-free medium containing OxLDL (up to 100 μg of LDL protein/mL). The incubation medium was collected and centrifuged at 800 rpm, and 200-μL aliquots were counted in a β-counter (Packard) to quantify the radioactivity released from the cells into the medium. One milliliter of 0.25N NaOH was added to the cell monolayers for 3 hours at room temperature, and 200-μL aliquots were used to determine the cell-associated radioactivity. The [³H]adenine release was calculated as the ratio of radioactivity released in the medium to the medium plus cell (total) radioactivity.

For the MTT assay, cells in 12-well plates (6 × 10⁵ cells per well) were exposed to different stresses. At the end of the experiment, the media were removed, and the cells were incubated for a further 3 hours in RPMI 1640 medium (red phenol free, Sigma) containing MTT (1 mg/mL, Sigma). Isopropanol was used to dissolve the formazan salt crystals, and the optical density at 550 nm was read by an automatic plate reader (Titerette Multiscan 2, Bio-Rad). Cell viability was determined by the tetrazolium salt dye reduction assay with a polyclonal antibody specific for the inducible form of Hsp70 (Hsp72, 1:5000, StressGen) or a polyclonal antibody specific for Bax (1:200, Amersham) and with an anti–β-actin antibody (1:5000, Sigma) for 1 hour. As a second antibody, an anti-rabbit or anti-mouse IgG peroxidase conjugate (Bio-Rad) was used; enhanced chemiluminescence and autoradiography followed.

Immunocytochemistry
Cells were cultured on coverslips in 24-well plates (1.5 × 10⁵ cells per well). After incubation with OxLDL, the media were removed, the cells were fixed with 3% paraformaldehyde for 20 minutes and washed twice with PBS, and a solution of 0.2% Triton X-100 was added for 2 minutes at 4°C. After they were washed twice with PBS, the fixed cells were incubated with a polyclonal antibody for Bax or...
Bcl-2 (1:20) for 1 hour at room temperature. As a second antibody, an anti-rabbit IgG biotin conjugate (1:200, Sigma) was used for 1 hour; incubation with streptavidin-FITC (1:200, Sigma) followed for 30 minutes. After they were washed, the coverslips were mounted with Vectashield (Vector Laboratories Inc) and analyzed with a Zeiss Axioscop microscope at 3400 magnification and photographed with 400 ASA Kodak Elite film.

Nuclear morphology was analyzed with the fluorescent dye bis-benzimide (Hoechst 33258). Cells cultured on coverslips were incubated with medium control and OxLDL, the media were then removed, and the cells were fixed with 3% paraformaldehyde for 20 minutes. The fixed cells were incubated with a 1:10,000 solution of Hoechst 33258 for 30 minutes and washed twice with PBS. The coverslips were mounted with a solution of water and glycerol (1:1), analyzed under UV light with a Zeiss Axioscop microscope at 3400 and 31000 magnifications, and photographed with 400 ASA Kodak Elite film.

Nucleosome ELISA

The nucleosome ELISA (Oncogene) allows the quantification of apoptotic cells in vitro by DNA affinity-mediated capture of free nucleosomes, followed by their antihistone-facilitated detection. At the end of the incubation with OxLDL, cells were lysed and frozen for 18 hours at -20°C. After appropriate dilutions, aliquots of the samples were added to each well and incubated at room temperature for 3 hours. The wells were washed 3 times with wash buffer and incubated for 1 hour at room temperature with a biotinylated polyclonal anti–histone 3 antibody, followed by a 30-minute incubation with peroxidase streptavidin conjugate and a 30-minute incubation with a chromogenic substrate. After addition of the stop solution, the absorbance was measured by using a spectrophotometric plate reader at dual wavelengths of 450/595 nm.

Statistical Analysis

Data presented in the text and figures are mean±SD and are representative of 4 different experiments. Statistical analysis was performed by ANOVA with use of the Statsoft Statistica Package.

Results

Expression of Hsp70 in transfected cells was determined by immunoblotting, and clones were chosen on the basis of their relative content in Hsp70 normalized for β-actin content. Ten clones permanently transfected with Hsp70 were obtained; among them, 1 was chosen as a clone with the highest expression (cos-Hsp70/10, 0.639±0.19 Hsp70/β-actin) as determined by immunocytochemistry and immunoblotting (Figure 1). The expression of Hsp70 remained constant with culture time and passages (up to 15).
Control cells (pBk2, 0.345 ± 0.11 Hsp70/β-actin) were obtained by transfection of COS-1 cells with the same plasmid devoid of the Hsp70 gene; the transfection with the pBk2 plasmid did not result in a higher expression of Hsp70 compared with that in nontransfected cells (not shown).

To verify that the overexpression of Hsp70 could protect cells from the classic stimuli known to induce the expression of this stress protein, transfected cells were exposed to toxic agents such as H2O2, heat, and UV irradiation. Compared with pBk2 cells, cos-Hsp70/10 cells were protected against the toxicity of H2O2 as determined by the MTT test (Figure 2a). Cos-Hsp70/10 cells were also protected from UV irradiation (Figure 2b) and from heat shock (Figure 2c).

Because OxLDLs are toxic to cells in culture and because Hsp70 plays a protective role against a number of toxic stimuli, we addressed the question of whether Hsp70 overexpression could protect cells from OxLDL-mediated toxicity. In a preliminary set of experiments, transfected COS-1 cells (pBk2 and cos-Hsp70/10 clones) were incubated in the presence of increasing concentrations of native LDL, CuSO4, and butylated hydroxytoluene in the same conditions used for the experiments with OxLDLs. As shown in Figure 3, we demonstrated that under these experimental conditions, there was no LDH release, and only a minor decrease of the cell number (MTT test), suggesting a very limited, if any, toxicity. Moreover, under these experimental conditions, the expression of Bax and Bcl-2 was not affected (Figure 3). The same results were obtained with control cells (pBk2, data not shown).

In cells overexpressing Hsp70, the incubation with OxLDLs induced a significant increase of [3H]adenine release (Figure 4a). The MTT test and LDH release assay confirmed this finding: after incubation with OxLDLs, cos-Hsp70/10 cells were less viable than were the control cells (Figure 4b and 4c). These data suggest not only that the overexpression of Hsp70 fails to protect cells against OxLDL toxicity but that it may confer a higher sensitivity to the cytotoxic action of OxLDLs.

We have previously reported that OxLDLs are cytotoxic to proliferating cells but not to quiescent cells; therefore, we sought to determine whether Hsp70 overexpression affected cell proliferation, as determined by the [3H]thymidine incorporation assay. Growth rates for all clones were similar to the control condition (data not shown). Therefore, different proliferation rates may not explain differences of sensitivity of the different clones to the OxLDL cytotoxicity. We have previously reported that OxLDLs induce apoptosis in cells of the vascular wall; for this reason, we sought to determine whether the cos-Hsp70/10 cell line undergoes apoptosis while incubated in the presence of OxLDLs. Several lines of evidence suggest that this is the case: (1) For cell morphology, cells incubated with OxLDL (80 µg/mL) showed shrinkage and blebs, characteristic alterations of apoptotic cells.

Figure 5. Cos-Hsp70/10 cells were incubated for 18 hours in control medium (A, C, and E) or in medium containing OxLDLs (80 µg protein/mL; B, D, and F). At the end of the experiment, the morphology of the cells (A and B) and of the nuclei (C through F), evaluated by Hoechst 33258 staining, was studied. The results are representative of 5 different experiments. Original magnification ×400 (A through D) and ×1000 (E and F).
For nuclei morphology, the staining with Hoechst 33258 showed, in cells incubated with OxLDLs (80 μg/mL), condensed nuclei and the appearance of an apoptotic body containing DNA fragments (Figure 5C through 5F), with no differences between pBk2 and cos-Hsp70/10 cells. (3) To confirm these findings, we studied the expression of Bax and Bcl-2, 2 proteins involved in the apoptotic pathway. In cos-Hsp70/10 cells incubated with OxLDLs (80 μg/mL), an increased expression of Bax, a proapoptotic protein, was detected (Figure 6A and 6B), whereas Bcl-2 expression, an antiapoptotic protein, decreased (Figure 6D and 6E) compared with that in control cells. This finding was confirmed by immunoblotting analysis of cell protein extracts (Figure 6C and 6F), with a clear increase of Bax in both clones but a more evident decrease of Bcl-2 in the transfected cells. In pBk2 cells incubated with 80 μg OxLDL/mL, the ratio of Bax to Bcl-2 in cells increased from 0.22 (basal value) to 0.99 and from 0.32 to 1.91 in cos-Hsp70/10 cells, further stressing the proapoptotic state in these conditions. (4) The analysis of the nucleosome content, an index of apoptotic death, showed a larger and significant increase in nucleosomes in cos-Hsp70/10 cells incubated with OxLDL (60 μg/mL) compared with pBk2 cells under the same experimental conditions (Table, P<0.005).

Figure 6. Cos-Hsp70/10 cells were incubated for 18 hours in control medium (A and D) or in medium containing OxLDLs (80 μg protein/mL, B and E). At the end of the incubation, cells were processed for immunostaining of Bax (A and B) or Bcl-2 (D and E). Cellular proteins of pBk2 and cos-Hsp70/10 were subjected to SDS-PAGE and immunoblotting with anti-Bax (C) and anti–Bcl-2 (F). Bcl-2 typically presents with 2 bands. The results are representative of 5 different experiments. Original magnification ×400.
Nucleosome Cellular Content in pBk2 and Cos-Hsp70/10 Cells Before and After Incubation With OxLDL

<table>
<thead>
<tr>
<th>OxLDL, μg/mL</th>
<th>pBk2</th>
<th>Cos-Hsp70/10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>202±3.5</td>
<td>210±12.7</td>
</tr>
<tr>
<td>60</td>
<td>350±5.6</td>
<td>529±13.4*</td>
</tr>
</tbody>
</table>

Values are mean±SD of 3 separate experiments. Absorbance was 450 nm. *P<0.0005 vs relative pBk2 value and basal conditions.

**Discussion**

The inducible form of Hsp70 is produced by cells in the presence of toxic stimuli, such as oxidative injury, and Hsp70 overexpression elicits a cytoprotective function in response to several stresses. OxLDLs represent an oxidative stress to cells, and this effect could account for their ability to induce Hsp70 in cultured cells. Because OxLDLs are cytotoxic to several cell types, we speculated that the Hsp response to OxLDLs could represent a protective response. To address this issue, we permanently transfected COS-1 cells with Hsp70. COS-1 cells are widely used in transfection experiments, including transfection with Hsp. From our transfectants, 1 clone with a high expression of Hsp70 was selected. This clone, as expected, was protected against toxic stimuli such as H₂O₂, heat shock, and UV irradiation, in agreement with in vitro and in vivo data on the role of this protein in protecting from cellular damage. However, when transfected cells were incubated with OxLDL, overexpression of Hsp70 not only failed to protect against OxLDL cytotoxicity but rather conferred a higher sensitivity. This effect is specific for OxLDLs (inasmuch as transfected cells are protected from other toxic stimuli) and appears to be related to the ability of OxLDLs to induce apoptotic death in vascular wall cells.

Why Hsp70 overexpression confers hypersensitivity to OxLDL cytotoxicity is unclear. In vivo, the Hsp70 inducible form has been detected in areas of the arterial wall exposed to OxLDL-induced necrosis. Therefore, it appears that stable overexpression of Hsp70 may protect cells from cytotoxic stimuli leading to necrosis but not to apoptosis driven by Fas. Sat and Walsh have recently suggested that OxLDLs activate the Fas-mediated apoptotic pathway by sensitizing endothelial cells to the death signal from the Fas receptor; perhaps in cells overexpressing Hsp 70 this pathway is further upregulated. It is tempting to speculate that during LDL oxidation substances that interact with the Fas receptors are formed. Whatever the mechanism involved, several hallmarks of apoptosis are present in COS-1 cells exposed to OxLDLs, including nuclear condensation and a prevalence of Bax over Bcl-2. Increased apoptosis is also suggested by the increase of nucleosome cellular content. Whether these findings bear in vivo relevance remains to be addressed in further studies.

In summary, we have shown that the overexpression of Hsp70 fails to protect transfected COS-1 cells from OxLDL cytotoxicity but that it confers a higher sensitivity. This effect is specific for OxLDLs (inasmuch as transfected cells are protected from other toxic stimuli) and appears to be related to the ability of OxLDLs to induce apoptosis.

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**References**


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