Proteasome-dependent degradation of oxidized proteins in MRC-5 fibroblasts

Nicolle Sitte, Katrin Merker, Tilman Grune*

Clinics of Physical Medicine and Rehabilitation, Medical Faculty (Charité), Humboldt University Berlin, Schumannstr. 20/21, D-10098 Berlin, Germany

Received 14 October 1998; received in revised form 29 October 1998

Abstract Fibroblasts were exposed to various concentrations of hydrogen peroxide and the removal of oxidized proteins was followed by determining protein-bound carbonyls. Fibroblasts are able to increase the turnover of metabolically radiolabeled proteins after treatment with hydrogen peroxide. It was demonstrated for the first time, that the increased protein turnover was accompanied by a removal of protein-bound carbonyl groups. The proteasome-specific inhibitor lactacystin was able to inhibit the elimination of protein-bound carbonyl groups. Therefore, the key role of the proteasome in the degradation of oxidized proteins in fibroblasts could be demonstrated.

Key words: Protein oxidation; Fibroblast; Proteasome; Lactacystin

1. Introduction

Protein oxidation in vivo is a natural consequence of aerobic life. The selective removal of the oxidized proteins by the 20S proteasome is an essential part of antioxidant defenses against oxidative stress. In recent years many investigations about proteolysis after oxidative stress were published [1–4]. Most of the studies were focused on the degradation of isolated oxidized model proteins [5–9]. Due to the great variety of oxidative changes in the primary and other structures of proteins various markers of protein oxidation were employed, including dityrosine and methionine sulfoxide formation [9,10], protein surface hydrophobicity [6,7] and the protein carbonyl content [11]. The question whether proteases are able to recognize these oxidatively protein modifications specifically remains still unsolved, although several attempts show a correlation between surface hydrophobicity and proteolytic susceptibility. Unfortunately there are no quantitative data regarding the question of physiological relevance of protein modification [12]. To our knowledge the degradation of oxidized proteins in living cells was not demonstrated yet by removal of protein oxidation markers.

Since it was shown by us [1,2,5] and others [3,4,6–9] that the proteasome is able to recognize and specifically degrade oxidized proteins in isolated systems a key role of this enzyme in the degradation of oxidized proteins in living cells was proposed [12]. However, several authors seriously doubted the physiological relevance of the removal of oxidized proteins at all [13] or the role of the proteasome in this process [14].

Recently the group of Winterbourn [15] developed a new ELISA-based assay for determining protein carbonyls, this method became easily applicable for tissue culture.

Therefore, we demonstrate here the degradation of oxidized proteins in dividing human fibroblasts after hydrogen peroxide treatment. Cells are able to increase proteolysis rates after treatment with the oxidant. For the first time we can demonstrate using the carbonyl ELISA method published recently that oxidized proteins were removed. By using the proteasome specific inhibitor lactacystin we are able to show that the removal of oxidized proteins is proteasome dependent.

2. Material and methods

2.1. Cell culture

MRC-5-fibroblasts (human fetal lung) were obtained from the European Collection of Cell Cultures (Salisbury, UK) at passage 18. The cell line was cultured in Dulbecco's minimal essential medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (US origin; Seromed, Berlin, Germany) under normoxia (air plus 5% CO_2). Cells were subcultivated before reaching confluence using a seeding density of 0.5×10^4 cells/cm^2. The medium was changed once a week. Cell viability was measured by trypan blue exclusion.

2.2. H_2O_2 treatment and proteolysis measurements

The measurement of degradation of metabolically radiolabeled proteins in confluent fibroblasts was performed after a 16-h labeling procedure. During the labeling procedure cells were incubated with [^{35}S]methionine in methionine-free minimal essential medium. After 16 h of incubation at 37°C the labeling mixture was removed and the cells were washed twice with phosphate-buffered saline. Afterwards, the fibroblasts were treated for 30 min at 37°C with H_2O_2 in phosphate-buffered saline (pH 7.4) in the following concentrations: 0.1, 0.2, 0.4, 0.6 and 1.0 mM. After this treatment the H_2O_2 solution was removed and the cells were cultured in normal medium for up to 48 h. The degradation of metabolically labeled proteins was quantified following addition of an equal volume of 20% trichloroacetic acid and scintillation counting was performed of the acid-soluble counts in the supernatant after centrifugation at $14\,000\times g$ for 10 min.

The degradation of the fluoropeptide suc-LLVY-AMC was measured after addition of the substrate to unlabeled cell lysates. Fibroblasts were treated with H_2O_2 as described above. Cells were washed twice with PBS and then lysed in 1 mM dithiothreitol during vigorous shaking for 1 h at 4°C. Non-lysed cells, membranes, and nuclei were removed by centrifugation at $14\,000\times g$ for 30 min. The supernatant was incubated in a buffer containing 50 mM Tris-HCl (pH 7.8), 20 mM KCl, 0.5 mM Mg-acetate, and 1 mM dithiothreitol. After a 1-h incubation with 200 μM of the fluorogenic peptide suc-LLVY-AMC the proteolysis was terminated by addition of an equal volume of ice-cold ethanol. Measurements were performed at 380 nm excitation and 440 nm emission after addition of 0.125 M sodium borate (pH 9.0) using free AMC as standard.

2.3. Protein carbonyl measurement

The protein carbonyl content was determined in cell lysates (4 mg/ml) by the ELISA of Buss et al. [15] except for using another detection system. The primary antibody used was an anti-dinitrophenyl-rabbit-IgG-antiserum (Sigma, Deisenhofen, Germany), the secondary antibody was a monoclonal anti-rabbit-IgG-antibody peroxidase conju-

*Corresponding author. Fax: (49) (30) 2093-7204.
E-mail: grune@rz.charite.hu-berlin.de

PII: S0014-5793(98)01495-1

gated (Sigma, Deisenhofen, Germany) developed with *o*-phenylenediamine.

3. Results and discussion

3.1. Turnover of metabolically radiolabeled proteins after oxidative stress

In order to measure the turnover of proteins in living cells we metabolically radiolabeled cellular proteins. After removal of the non-incorporated label, the liberation of acid-soluble counts was taken as a measure for the proteolytic degradation of proteins. In Fig. 1A the time dependence of the degradation of proteins in non-treated cells is shown. The liberation of acid-soluble counts after treatment of cells with various concentrations of hydrogen peroxide is demonstrated in Fig. 1B. As one can see MRC-5 fibroblasts are able to increase the protein turnover in response to oxidative stress. However, after a maximal increase of protein degradation in response to treatment with 0.4 mM hydrogen peroxide a higher oxidant concentration is not accompanied by increased proteolysis

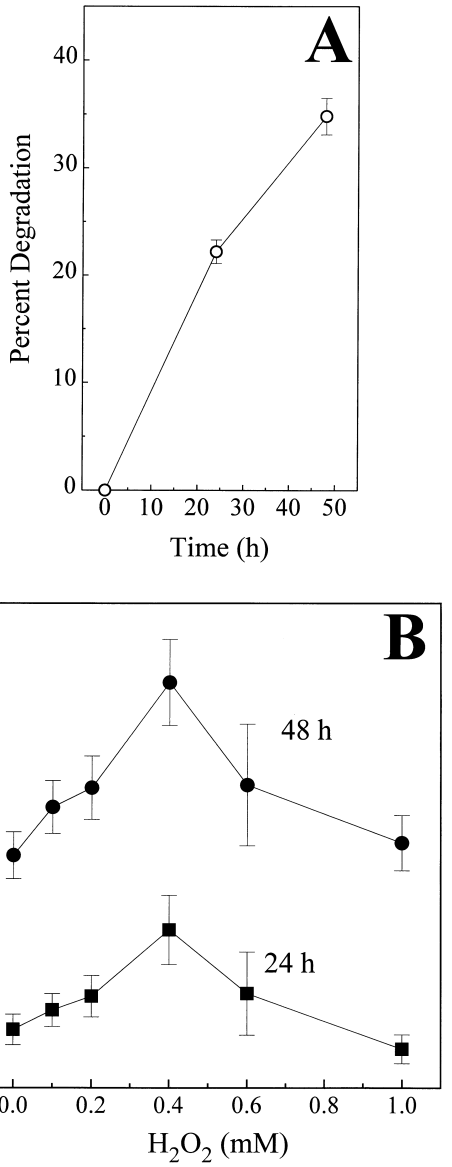

Fig. 1. Degradation of cellular proteins in MRC-5 fibroblasts after hydrogen peroxide treatment. Cells were cultivated as described in Section 2. The labeling was performed for a 16-h period using [^{35}S]methionine. After removal of the non-incorporated label the release of radioactivity from the protein pool was measured as the acid-soluble counts. Percent degradation was calculated as: (acid-soluble counts−background)/(total radioactivity−background)×100. The values represent the mean of four independent measurements from three experiments with S.D. always smaller than 15% of the value. Panel A demonstrates the time dependence of protein turnover in non-treated cells. Panel B shows the results of hydrogen peroxide treatment on the protein degradation after 24 h and 48 h.

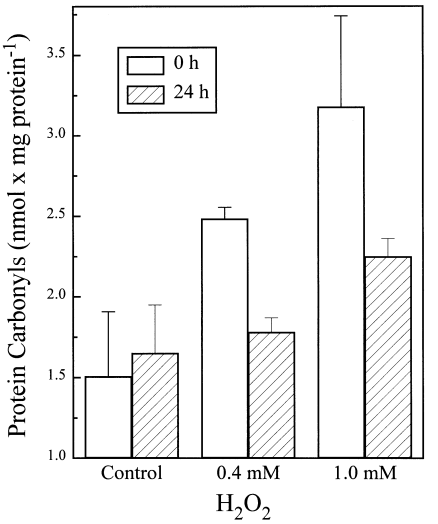

Fig. 2. Protein carbonyl content in MRC-5 fibroblasts after hydrogen peroxide treatment. Cells were cultivated, treated and harvested as described in Section 2. The cells were either harvested directly after the treatment (0 h) or 24 h later. The ELISA was performed as described in Section 2. The values represent the mean ± S.D. of four independent measurements.

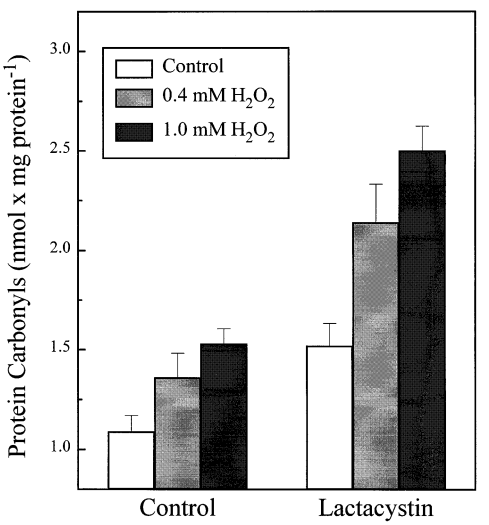

Fig. 3. Protein carbonyl content in MRC-5 fibroblast lysates 24 h after hydrogen peroxide treatment. Cells were cultivated and treated as described earlier. Protein carbonyl determination is described in Section 2. After treatment of cells with hydrogen peroxide the cells were cultivated for another 24 h under normal conditions (Control) or in the presence of the proteasome specific inhibitor lactacystin (Lactacystin). Lactacystin was added to the cell culture medium at a final concentration of 5 μM. The values represent the mean ± S.D. of three independent measurements.

Table 1
Proteolytic activity of the proteasome in cell lysates of MRC-5 fibroblasts after hydrogen peroxide treatment

	Proteolytic activity (nmol/mg/min)		
	Before H_2O_2 treatment	Directly after H_2O_2 treatment	After 24 h
Control	11.38 ± 0.80	12.27 ± 0.47	10.94 ± 2.20
0.4 mM H_2O_2	11.38 ± 0.80	9.32 ± 0.81	10.44 ± 0.91
1.0 mM H_2O_2	11.38 ± 0.80	6.62 ± 0.76	10.83 ± 1.02

Cells were cultivated, treated with hydrogen peroxide and harvested as described in Section 2. The cells were lysed and centrifuged. The centrifuged lysates were incubated with the fluorogenic peptide suc-LLVY-AMC for 60 min at 37°C. The proteolytic activity was determined by measuring the fluorescence at 380/440 nm. For details see Section 2. The data are given as the mean ± S.D. of four independent measurements of two experiments.

rates. After treatment with 0.6 mM and 1.0 mM hydrogen peroxide the proteolysis rates decline again to levels comparable to those without treatment. This concentration dependence could be demonstrated for both investigated time points: 24 h and 48 h (Fig. 1B). Interestingly, there was no dramatic decrease in cell viability, even if the cells were treated with 1 mM hydrogen peroxide (10% as measured by trypan blue exclusion).

The increase of intracellular protein degradation at moderate concentrations of hydrogen peroxide and the decline at high concentrations were already documented for other cell lines [1,2]. However, these studies could never directly demonstrate the degradation of oxidized proteins. Therefore, we used a novel ELISA-based method to measure the protein carbonyl content.

3.2. Carbonyl content after hydrogen peroxide treatment

We measured a protein carbonyl content of 1.3 nmol × mg protein^{-1} in MRC-5 cells. The carbonyl content after treatment of MRC-5 fibroblasts with hydrogen peroxide is demonstrated in Fig. 2. As one can see the carbonyl content in fibroblasts is increasing in a dose-dependent manner up to 2-fold (3.2 nmol × mg protein^{-1}) for 1 mM hydrogen peroxide. In order to investigate the ability of cells to degrade the carbonyl-containing oxidized proteins we studied the protein carbonyl content 24 h after treatment of MRC-5 cells with hydrogen peroxide. As demonstrated in Fig. 2 the protein carbonyl content in MRC-5 cells drastically declined. In the case of cellular treatment with 0.4 mM hydrogen peroxide 24 h after exposure to the oxidant the protein carbonyls did not differ from the control. Therefore, we were able to show that oxidant treated cells can degrade oxidatively modified proteins. This function of cells was already proposed several times [1–9]. However, it is not finally clear that the proteasomal system is responsible for the degradation of oxidized proteins, although a number of authors suggested this as one of the physiological functions of the proteasomal system [6–9,16]. Therefore, it was of interest whether the proteasome is involved in the removal of oxidized proteins in MRC-5 fibroblasts.

3.3. Role of the proteasome in the degradation of oxidized proteins

To test the possible role of the proteasome in the degradation of oxidatively modified proteins we first checked for the proteolytic activity of the proteasome in order to test whether the protease itself is resistant to oxidative stress. As demonstrated in Table 1 after treatment with 1 mM hydrogen peroxide a 40% decrease of the proteasomal activity occurs. The activity of the proteasome is completely restored after 24 h, indicating that the proteasome itself is resistant to oxidative stress applied on the MRC-5 cells. This is in agreement with our earlier findings [17] and those from Strack et al. [18]. However, it seems to be clear that the relative resistance of the proteasomal activity towards oxidative stress gives only the possibility of the involvement of this protease in the degradation of oxidized proteins. Since knockout mutations of the proteasome are lethal to cells [19,20] our group earlier employed antisense oligodeoxynucleotides against one of the proteasome subunits to deplete cells from the protease [1,2]. More recently a number of more or less specific inhibitors of the proteasome are described [21,22]. We used lactacystin, a specific proteasome inhibitor [22] to demonstrate the involvement of this protease in the degradation of oxidized proteins. Lactacystin is a protease inhibitor specific for the proteasome, without documented inhibitory actions towards other proteases. As demonstrated in Fig. 3 the inhibition of the proteasome is accompanied by a loss of the ability of MRC-5 cells to degrade oxidatively modified proteins. The protein carbonyl content in MRC-5 fibroblasts in the presence of the proteasome specific inhibitor lactacystin is not declining in a time dependent manner, indicating the key role of the proteasome in the removal of oxidized proteins.

We therefore demonstrated in this study the increase of cellular protein turnover after oxidative stress and furthermore that this is accompanied by the specific removal of oxidized proteins. Since the proteasome specific inhibitor lactacystin is able to prevent the degradation of oxidized proteins we concluded that the proteasome is the major protease involved in this process. Therefore, we were able to demonstrate that the specific degradation of oxidized proteins is proteasome-dependent.

Acknowledgements: This work was supported by the 'Stiftung für Verhalten und Umwelt'. Lactacystin was a kind gift of Prof. S. Omura (Tokyo, Japan).

References

[1] Grune, T., Reinheckel, T., Joshi, M. and Davies, K.J.A. (1995) J. Biol. Chem. 270, 2344–2351.
[2] Grune, T., Reinheckel, T. and Davies, K.J.A. (1996) J. Biol. Chem. 271, 15504–15509.
[3] Rivett, A.J. (1985) J. Biol. Chem. 260, 300–305.
[4] Rivett, A.J. (1985) J. Biol. Chem. 260, 12600–12606.
[5] Grune, T., Blasig, I.E., Sitte, N., Roloff, B., Haseloff, R. and Davies, K.J.A. (1998) J. Biol. Chem. 273, 10857–10862.
[6] Pacifici, R.E., Kono, Y. and Davies, K.J.A. (1993) J. Biol. Chem. 268, 15405–15411.
[7] Guilivi, C., Pacifici, R.E. and Davies, K.J.A. (1994) Arch. Biochem. Biophys. 311, 329–341.
[8] Friguet, B., Szweda, L.I. and Stadtman, E.R. (1994) Arch. Biochem. Biophys. 311, 168–173.
[9] Levine, R.L., Mosoni, L., Berlett, B.S. and Stadtman, E.R. (1996) Proc. Natl. Acad. Sci. USA 93, 15036–15040.

[10] Heinecke, J.W., Li, W., Daehnke, H.L. and Goldstein, J.A. (1993) J. Biol. Chem. 268, 4069–4077.
[11] Shacter, E., Williams, J.A., Lim, M. and Levine, R.L. (1994) Free Radic. Biol. Med. 17, 429–437.
[12] Beckman, K.B. and Ames, B.N. (1998) Physiol. Rev. 78, 547–581.
[13] Dean, R.T., Geiseg, S. and Davies, M.J. (1993) Trends Biochem. Sci. 18, 437–441.
[14] Matthews, W., Tanaka, K., Driscoll, J., Ichihara, A. and Goldberg, A.L. (1989) Proc. Natl. Acad. Sci. USA 86, 2597–2601.
[15] Buss, H., Chan, T.P., Sluis, K.B., Domigan, N.M. and Winterbourn, C.C. (1997) Free Radic. Biol. Med. 23, 361–366.
[16] Grune, T., Reinheckel, T. and Davies, K.J.A. (1997) FASEB J. 11, 526–534.
[17] Reinheckel, T., Sitte, N., Ullrich, O., Kuckelkorn, U., Davies, K.J.A. and Grune, T. (1998) Biochem. J., in press.
[18] Strack, P.R., Waxman, L. and Fagan, J.M. (1996) Biochemistry 35, 7142–7149.
[19] Fujiwara, T., Tanaka, K., Orino, E., Yoshimura, T., Kumatori, A., Tamura, T., Chung, C.H., Nakai, T., Yamaguchi, K., Shin, S., Kakizuka, A., Nakanishi, S. and Ichihara, A. (1990) J. Biol. Chem. 265, 16604–16613.
[20] Ghislain, M., Udvardy, A. and Mann, C. (1993) Nature 366, 358–360.
[21] Vinitski, A., Cardozo, C., Sepp-Lorenzino, L., Michaud, C. and Orlowski, M. (1994) J. Biol. Chem. 269, 29860–29866.
[22] Fenteany, G. and Schreiber, S.L. (1998) J. Biol. Chem. 273, 8545–8548.