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Proteomic analysis of the cellular proteins induced by adaptive concentrations of hydrogen peroxide in human U937 cells

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Abbreviations: 2-DE, two-dimensional polyacrylamide gel electrophoresis; MALDI-TOF, matrix associated laser desorption/ionization time-of-flight

Abstract

When cells are first exposed to low levels of oxidative stress, they develop a resistance to a subsequent challenge of the same stress, even at higher levels. Although some protein(s) induced by oxidative stress likely mediated this adaptive response, the nature of these proteins is unknown. In this study, the total proteins extracted from human U937 leukemia cells exposed to 50 μM H_2O_2 for 24 h to induce an optimal protective response were analyzed by two-dimensional polyacrylamide gel electrophoresis. H_2O_2 treatment induced elevation of level of 34 protein spots. An analysis of these spots by a matrix associated laser desorption/ionization time-of-flight mass spectrometry identified 28 of the H_2O_2-induced proteins. These include proteins involved in energy metabolism, translation and RNA processing, chaperoning or mediating protein folding, cellular signaling, and redox regulation, as well as a mitochondrial channel component, and an actin-bundling protein. Therefore, it appears that the cellular adaptation to oxidative stress is a complex process, and is accompanied by a modulation of diverse cellular functions.

Keywords: biological adaptation, cell death, hydrogen peroxide, oxidative stress, proteomics

Introduction

Depending on its intensity, oxidative stress can regulate cellular viability in two opposing way. While a relatively high intensity kills cells, an adaptive response is induced by lower intensity. For example, when cells are first exposed to low H_2O_2 concentrations, they develop a resistance to a subsequent challenge with higher concentrations of the same agent that would otherwise be lethal (Farr and Kogoma, 1991; Davies *et al*., 1995; Wiese *et al*., 1995; Lee and Um, 1999). In the case of human U937 leukemia cells and hamster HA-1 fibroblasts, induction of the protective response to optimal levels requires a time lag of approximately 18-24 h (Wiese *et al*. 1995; Lee and Um, 1999), which may reflect the need for protein synthesis prior to establishing the protective effect of H_2O_2. This possibility was supported by the observation that cycloheximide, an inhibitor of protein synthesis, abolished the protective response in HA-1 cells (Wiese *et al*., 1995), or rendered U937 cells hypersensitive to the lethal action of H_2O_2 (Lee and Um, 1999). Moreover, two-dimensional polyacrylamide gel electrophoreses (2-DE) showed that H_2O_2 (150-400 μM) can induce the synthesis of 20-25 new proteins in vascular endothelial and fibroblast cells (Lu *et al*., 1993; Wiese *et al*., 1995). Although the nature of these proteins is largely unknown, it was reported that H_2O_2 could elevate the cellular levels and the activities of H_2O_2-degrading enzymes such as catalase and glutathione peroxidase (Shull *et al*., 1991; Lu *et al*., 1993; Lee and Um, 1999). Therefore, it is believed that cells adapt to H_2O_2, at least in part, by enhancing their ability to degrade H_2O_2.

In recent years, evidence for an alternative mechanism of H_2O_2 adaptation has been accumulating. For instance, H_2O_2 pretreatment conferred U937 cells with a cross-resistance to C_2-ceramide (Lee and Um, 1999). Interestingly, C_2-ceramide killed U937 cells in a manner independent of H_2O_2 in the absence of such pretreatment. Lethal concentrations of C_2-ceramide did not elevate the cellular levels of H_2O_2, and the cell death induced by C_2-ceramide was not suppressed by the addition of antioxidants (Lee and Um, 1999). Therefore, the enhanced cellular capacity to degrade H_2O_2 does not explain the H_2O_2-induced

resistance of the U937 cells to C_2-ceramide. Moreover, the adaptation of U937 and HA-1 cells to H_2O_2 was not always accompanied by an increase in the H_2O_2-degrading enzyme levels and by an enhancement in the cellular capacity to degrade H_2O_2 (Wiese *et al.*, 1995; Lee and Um, 1999). These observations suggest that H_2O_2 can enhance the survival ability of cells in a manner independent of the H_2O_2-degrading activity. This alternative mechanism was induced selectively when the U937 cells were exposed to 50 μM H_2O_2, whereas an increase in the H_2O_2-degrading activity of the same cells required 250 μM H_2O_2 (Lee and Um, 1999). Therefore, the former mechanism appears to be more sensitive to H_2O_2 than the latter; at least in U937 cells. However, there is little information on the cellular factors associated with the antioxidant-independent adaptation.

In this study, we have characterized the proteins induced during the antioxidant-independent adaptation by using methods available in proteomics. Human U937 cells were used as the model, because the adaptive conditions in these cells are well defined (Lee and Um, 1999). The results show for the first time a list of the H_2O_2-responsive proteins, which appears to be important for a further analysis of H_2O_2 adaptation.

Materials and Methods

Cell culture and treatments

U937 cells were cultured in a RPMI 1640 medium supplemented with 10% heat-inactivated FBS and gentamicin (50 μg/ml) at 37°C and 5% CO_2. To induce the adaptive responses, cells were exposed to 50 μM H_2O_2 at the density of 3×10^5/ml for 24 h as described previously (Lee and Um, 1999).

Cell lysis and 2-DE

The treated and untreated control cells (1.2×10^7) were lysed in 400 μl of a buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 2% Pharmalyte (pH 3-10) (Amersham Pharmacia, Upsala, Sweden), and 100 mM dithioerythritol. The cell debris was removed by a centrifugation (48,000 *g*, 4°C, 1 h). 2-DE was performed as described previously (Steiner *et al.*, 2000). Briefly, 50 μl of the supernatant was diluted in 400 μl of the buffer, which was applied to the immobilized nonlinear gradient strips at a pH 3 to 10 (Amersham Pharmacia). The first dimensional isoelectric focusing was carried out using the following conditions; (1) 30 V for 3 h; (2) 100 V for 1 h; (3) 200 V for 1 h; (3) 500 V for 1 h; (4) 1,000 V for 1 h; and (4) 8,000 V for 11 h. The strips were equilibrated in 6 M urea containing 20% glycerol, 2% SDS and 0.01% bromophenol blue with 10 mM tributyl phosphine. The second dimensional SDS-PAGE was performed using 8-18% linear gradient acrylamide gels in an Ettan Dalt system (Amersham Pharmacia). Proteins were visualized by the staining with Coomassie blue G-250.

Identification of protein spots

The stained gels were scanned on a GS800 densitometer (Bio-Rad, Richmond, CA) and converted into data files, which were then analyzed with the Melanie III computer software (GenBio, Geneva, Switzerland). The spots where the staining intensities were elevated after the H_2O_2 treatment were excised, and digested using 12.5 μg/ml trypsin (Shevchenko *et al.*, 1996). The supernatant peptide mixtures were loaded onto a Poros R2 column (Applied Biosystems, Framingham, MA), which had been washed with the following solutions: (1) 70% acetonotrile in 5% formic acid, (2) 100% acetonotrile, and (3) 5% formic acid. The peptides were eluted using 2 μl of 10 mg/ml α-cyano-4-hydroxycinnamic acid, and analyzed by a matrix associated laser desorption/ionization time- of-flight (MALDI-TOF) mass spectrometer (Voyager DE-PRO, Applied Biosystems) (Erdjument-Bromage *et al.*, 1998). The database searches were carried out using MS-Fit, which has access to the World Wide Web at either http://kr.expasy.org or http: //www.ncbi.nlm.nih.gov. All the searches were performed in 50 ppm mass tolerance, miss cleavage 1.

Results and Discussion

The U937 cells were exposed to 50 μM H_2O_2 for 24 h in order to identify the proteins induced by adaptive concentrations of H_2O_2. This treatment was shown to be optimal for inducing the antioxidant-independent adaptation (Lee and Um, 1999). Total cellular proteins were prepared from the untreated control and treated adapted cells, and separated by a 2-DE. Commassie blue staining revealed approximately 900-1,000 and 700-800 protein spots in the sample obtained from the control and adapted cells, respectively. Figure 1 shows a result from the experiments repeated three times. H_2O_2 treatment consistently enhanced the staining intensity of 34 spots (Figure 1A and B). Two examples of such spots are magnified in Figure 1C. Densitometric analyses showed that the H_2O_2 treatment enhanced the intensity of 29 spots approximately 2-13 fold (Table 1). The rest 5 spots were detected only in the treated, but not untreated control, samples.

These spots were analyzed by MALDI-TOF mass spectrometry, and their identity was searched using the MS-Fit database. This allowed the identification of

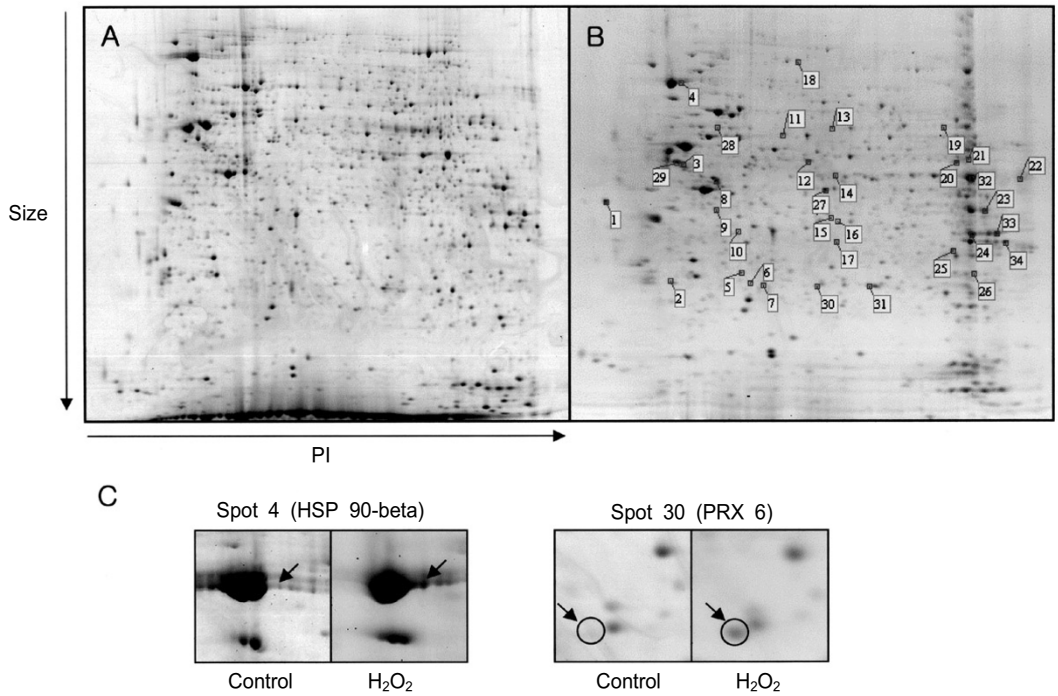

Figure 1. 2-DE maps of U937 cell proteins. U937 cells were treated with 50 μM H_2O_2 for 24 h. Total cellular proteins were prepared from the untreated control (A) and treated adapted cells (B), and were analyzed by 2-DE. The gels were stained with Coomassie G-250. Numbered are the spots of which intensities were elevated by the H_2O_2 treatment. Two of such spots (spot No 4 and 30) are magnified in (C). The arrows indicate that the intensity of spot No 4 was elevated by H_2O_2 treatment, whereas the spot No 30 was detected only in the treated, but not untreated control sample.

Table 1. Levels of the H_2O_2-induced proteins. Staining intensity of the indicated spots was analysis on densitometer. The numbers represent % volume of the spots.

Spot No	Control	H_2O_2	Fold increase	Spot No	Control	H_2O_2	Fold increase
1	0.1026	0.4165	4.0581	18	0.0094	0.0936	9.8811
2	0.0419	0.1736	4.1429	19	0.0145	0.1523	10.4595
3	0.0000	0.0967	new spot	20	0.0948	0.3839	4.0490
4	0.0196	0.1084	5.5320	21	0.0360	0.1762	4.8869
5	0.0938	0.1957	2.0856	22	0.0320	0.1331	4.1610
6	0.0728	0.1508	2.0714	23	0.0375	0.0322	8.5757
7	0.0536	0.1204	2.2436	24	0.3942	0.7928	2.0113
8	0.0326	0.3181	9.7449	25	0.0507	0.2075	4.0886
9	0.0352	0.1484	4.2104	26	0.0127	0.1249	9.7996
10	0.0131	0.0868	6.6318	27	0.0867	0.4544	5.2410
11	0.0473	0.1016	2.1468	28	0.0127	0.1704	13.3972
12	0.1174	0.2483	2.1136	29	0.4480	0.9025	2.0145
13	0.0406	0.0969	2.3875	30	0.0000	0.1880	new spot
14	0.0209	0.1689	8.0816	31	0.0000	0.1901	new spot
15	0.0061	0.0466	7.5697	32	0.0000	2.0570	new spot
16	0.0045	0.0426	9.4765	33	0.0385	0.4905	12.7255
17	0.0197	0.1149	5.8254	34	0.0000	0.3107	new spot

28 proteins. As shown in Table 2, the identified proteins were sorted into the following groups based on their known functions; proteins affecting energy metabolism (8 spots), proteins functioning in translation and RNA processing (6 spots), chaperones or mediators of protein folding (5 spots), proteins that can influence cellular signaling (3 spots), redox regulators (2 spots), a mitochondrial channel component (1 spot), an actin-bundling protein (1 spot), and poorly characterized miscellaneous proteins (2 spots). These results supported the view that the cellular adaptation to oxidative stress is a complex process, which is accompanied by the modulation of diverse cellular functions.

We previously reported that 50 μM H_2O_2 does not enhance the capacity of U937 cells to degrade H_2O_2, as analyzed using the oxidation-sensitive fluorescent probe, 2',7'-dichlorofluorescein diacetate (Lee and Um,

Table 2. Identification of the H_2O_2-induced proteins

Function	Proteins identified	Spot No.	Accession No	Mr (kDa)	PI	Sequence coverage (%)
Energy metabolism	NADH-ubiquinone oxidoreductase, 30 kDa subunit	6	O75489	30.2	6.98	36
	Pyruvate dehydrogenase E1, beta subunit	10	P11177	39.2	6.21	37
	APT synthease, alpha chain	20	P25705	59.7	9.16	28
	Fructose-bisphosphate aldolase A	23	P04075	39.4	8.3	28
	Glyceraldehydes 3-phosphate dehydrogenase, liver	24	P04406	36.0	8.57	23
	ATP synthease, beta chain	29	P06576	56.5	5.26	31
	Alpha enolase	32	P06733	47.1	7.01	52
	Glyceraldehydes 3-phosphate dehydrogenase, liver	33	P04406	36.0	8.57	25
Translation and RNA processing	Eukaryotic initiation factor 4A-1	8	P04765	46.1	5.32	35
	Eukaryotic initiation factor 2B, beta subunit	15	P49770	38.9	5.77	24
	hnRNP H	12	P31943	49.2	5.89	34
	hnRNP L	19	P14866	60.1	6.65	15
	hnRNP K	28	Q07244	50.9	5.39	31
	hnRNP A2/B1	34	P22626	37.4	8.97	24
Protein folding	Protein disulfide isomerase A6 precusor	3	Q15084	48.1	4.95	29
	Heat shock portein 90-beta	4	P08238	83.2	4.97	31
	T-complex protein 1, alpha subunit	11	P17987	60.3	5.8	29
	T-complex protein 1, alpha subunit	13	P49368	60.3	6.23	31
	Prohibitin	5	P35235	29.8	5.57	49
Cell signaling	Set protein	1	Q01105	32.1	4.12	18
	Calcium-dependent protease, small subunit	2	P04632	28.3	5.05	30
	PKCq-interacting protein PICOT	9	6840947	37.4	5.38	38
Redox regulators	Peroxiredoxin 4	7	Q13162	30.5	5.86	55
	Peroxiredoxin 6	30	P30041	25.0	6.00	38
Channel components	Voltage-dependent anion-selective channel protein 2	25	P45880	38.0	6.32	45
Cell structure	Fascin	21	Q16658	54.5	6.84	46
Miscellaneous	Proliferation associated protein 2G4	14	Q9UQ80	43.7	6.13	31
	Putative	18	577295	106.7	5.71	21
Unidentified		16, 17, 22, 26, 27, 31				

1999). Given that 50 μM H_2O_2 enhances the cellular levels of peroxidases that can degrade H_2O_2 to water (Figure 1C and Table 2), it is possible that the probe was not sufficiently sensitive for detecting the H_2O_2-degrading activity of the induced peroxidases. For this reason, the possibility that the induced peroxidases contribute to the resistance of the adapted cells to H_2O_2 by enhancing their capacity to degrade H_2O_2 cannot be excluded. However, it should be noted that the cells pretreated with 50 μM H_2O_2 can also withstand a subsequent challenge from C_2-ceramide (Lee and Um, 1999). As this stimulus kills the unadapted control U937 cells in a manner independent of H_2O_2, peroxidase induction does not appear to be responsible for the H_2O_2-induced resistance to C_2-ceramide. Therefore, it is likely that relatively low H_2O_2 concentrations induce an additional cytoprotective component(s) that does not act as an antioxidant.

The list shown in Table 2 shows that HSP90 is a potential candidate for such a cytoprotector. The ability of H_2O_2 to induce heat shock proteins was also reported using other cell types (Marini *et al*., 1996). Importantly, HSP90 overexpression inhibited the U937 cell death induced by staurosporine (Pandey *et al*., 2000). The cytoprotective role of HSP90 against 3-hydroxykynurenine and other cytotoxic agents in neuronal and leukemia cells was also reported (Blagosklonny *et al*., 2001; Lee *et al*., 2001). Moreover, it was reported that HSP90 could directly bind to Apaf-1 and inhibit its oligomerization and further recruitment of pro-caspase-9 (Pandey *et al*., 2000; Garrido *et al*., 2001). Therefore, HSP90 might act as a mediator of H_2O_2-induced cell survival. This possibility is currently under investigation.

In conclusion, the nature of the proteins induced by adaptive concentrations of H_2O_2 was determined for the first time. This information will be useful for a further analysis of the cellular responses to oxidative stress.

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