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Identification of 30 protein species involved in replicative senescence and stress-induced premature senescence

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Abstract **Exposure of human proliferative cells to subcytotoxic stress triggers stress-induced premature senescence (SIPS) which is characterized by many biomarkers of replicative senescence. Proteomic comparison of replicative senescence and stress-induced premature senescence indicates that, at the level of protein expression, stress-induced premature senescence and replicative senescence are different phenotypes sharing however similarities. In this study, we identified 30 proteins showing changes of expression level specific or common to replicative senescence and/or stress-induced premature senescence. These changes affect different cell functions, including energy metabolism, defense systems, maintenance of the redox potential, cell morphology and transduction pathways.**

Key words: Stress; Replicative senescence; Proteomics; Mass spectrometry; Molecular scars; In vitro toxicology

1. Introduction

Human diploid fibroblasts (HDFs), endothelial cells, melanocytes, etc. exhibit a stress-induced prematurely senescent (SIPS) phenotype after several days of exposure to subcytotoxic stress like UV-irradiation, hyperoxia, H_2O_2, *tert*-butylhydroperoxide (t-BHP), ethanol, etc. (for reviews, see [1,2]). Many biomarkers of replicative senescence (RS) are observed in SIPS, including a senescence-like cell morphology, irreversible cell cycle arrest in G1/S, telomere shortening, senescence-associated β-galactosidase (SA β-gal) activity, deletions of mitochondrial DNA, etc. [3–5]. The level of transcript of many genes is altered in senescent cells when compared to cells at early cumulative population doublings (CPDs), as shown by Northern blot [6], semi-quantitative RT-PCR [5], subtractive hybridization [7], differential display [8] or DNA microarray [9]. Other techniques restricted to a limited set of genes undergoing expression changes in RS show similar changes of expression level in SIPS induced by hyperoxia, H_2O_2 or t-BHP (for a review, see [2]). However, these studies do not consider the weakness of the correlation between a level of transcripts and the abundance of the corresponding proteins [10].

Successful proteomic studies include biological problems as diverse as the comparison of protein expression in normal human luminal and myoepithelial breast cells purified from mammoplasties [11] and the identification of a new anti-proliferative protein in capillary endothelial cells cultivated in vitro [12]. As concerns RS, a recent proteomic analysis using high resolution two-dimensional gel electrophoreses (2DGE) and mass spectrometry (MS) reveals new biomakers of replicatively senescent rat embryo fibroblasts [13]. Another study compares several lines of conditionally immortalized rat embryo fibroblasts and identifies new proteins involved in growth arrest [14]. Another multi-differential proteomic study based on high resolution 2DGE compares the proteomes of WI-38 fetal lung HDFs undergoing RS or SIPS induced by t-BHP or ethanol [15]. This study shows differences between RS and SIPS at the level of protein expression. HDFs in SIPS display differences at the level of protein expression which are either common with RS, specific to the type of stress or to SIPS (regardless of the type of the stress). The differences at the level of protein expression which are specific to the types of stress or to SIPS are defined as 'molecular scars' of subcytotoxic stress [1].

In this work we identified as many as possible of the proteins undergoing changes of expression level in RS and/or SIPS. One-dimensional (1D) Western blot analyses were performed to detect proteins for which several isoforms were identified or suspected. Semi-quantitative real-time RT-PCRs were undertaken to check whether differences at the protein level correlate with differences of the level of the corresponding transcript.

2. Materials and methods

2.1. Cell culture and induction of SIPS

WI-38 HDFs (American Type Culture Collection, Rockville, USA) were routinely cultivated as previously described until RS [16]. The medium+10% fetal calf serum (FCS) of slowly growing cultures was changed every four days. Confluent HDFs at early CPDs (under 60% of their proliferative life span) were exposed five times for 1 h to 30 μM t-BHP (Merck, Darmstadt, Germany) or five times for 2 h to 5% ethanol (v/v; Merck, Darmstadt, Germany). The stressors were diluted in culture medium+10% FCS, with one stress/day for five days. After each stress, the HDFs were rinsed with PBS buffer and incubated in culture medium+10% FCS.

2.2. Preparative 2DGE, protein extraction and MS for protein identification

At 48 h after the last stress the HDFs were washed twice with HBSS buffer and lysed with a 9.5 mol/l urea buffer (Pharmacia, Upp-

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sala, Sweden), 2% (w/v) Nonidet P-40 (Sigma, St. Louis, MO, USA), 5% (v/v) β-mercaptoethanol (Fluka, Buchs, Switzerland) and 2% (v/v) ampholytes pH 7–9 buffer (Pharmacia, Uppsala, Sweden). Preparative IEF and NEpHGE 2DGE were performed similarly to the analytical ones, as described in [15]. The gels were loaded with 200 μg/gel of non-labelled sample mixed with a quantity of labelled sample corresponding to $2{\cdot}10^6$ cpm. These gels were neither fixed nor amplified before exposure to X-ray films (AGFA Curix RP2, Agfa-Gevaert, Morsel, Belgium) for five days. Protein spots were excised from the gel before in-gel tryptic digestion performed as previously [17]. The resulting peptides were purified and concentrated on home-made nano-scale reversed phase disposable columns prior to MS analysis.

The proteins were identified on the basis of their 'peptide map' [18] acquired with a Bruker Reflex II (Bruker Daltonics, Bremen, Germany) matrix-assisted laser desorption/ionization (MALDI) instrument. Peptide Search software was used to search the nrdb sequence database. When a protein was not identified on the basis of its peptide map, peptides of the protein were micro-sequenced using a Q-tof instrument (Micromass, Manchester, UK). The sequence elements obtained were used to perform a 'peptide sequence tag' search in nrdb [19] or to carry out a BLAST search and compare the obtained sequences against the available sequence databases.

2.3. 1D Western blot and semi-quantitative real-time RT-PCR analysis

Cell lysates for immunoblot were prepared in lysis buffer (SDS 2%, glycerol 10%, DTT 50 mM, Bromophenol Blue 0.1% in Tris–HCl, pH 6.8, 62.5 mM) at 72 h after the last stress. Western blots were performed as described [5]. We used anti-HSP27 (Biotech, Santa Cruz, CA, USA), anti-Prx VI (kind gift of Dr. A.B. Fisher, Institute for Environmental Medicine, University of Pennsylvania, Philadelphia, PA, USA), anti-alpha-tubulin (Innogenex, San Ramon, CA, USA) antibodies. The level of tubulin was used as reference level. Semi-quantification was obtained with the ImageMaster TotalLab software (Pharmacia, Uppsala, Sweden).

Total RNA was extracted (total RNA extraction kit, Promega, Madison, USA) at 72 h after the last stress. mRNA was retrotranscribed, starting from 2 μg total RNA (Superscript RII reverse transcriptase, Invitrogen, Carlsbad, USA). The sequences of the primers were determined (Primer Express 5.1 software, Applied Biosystems, Foster City, CA, USA) (Table 1). cDNA and primers concentrations were experimentally determined for the PCRs to remain in the exponential zone of amplification for each gene. Real-time PCRs were performed using SYBR Green as intercalating agent (ABI Prism 7000 instrument, Applied Biosystems). The conditions of PCR were 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. The steady-state level of the mRNA of the 23-kDA highly basic protein was used as reference for semi-quantification with the $\Delta\Delta C_T$ method [20]. The specificity of amplification was checked by performing a dissociation curve (gradient from 60°C to 95°C) and electrophoresis of the amplification products.

3. Results

The aim of this work was to identify as many as possible of the proteins undergoing changes in expression level in RS and/or SIPS. SIPS was induced by repeated subcytotoxic exposures to t-BHP or ethanol [15]. The experimental conditions were: HDFs at early CPDs ('young'), senescent HDFs, HDFs in SIPS induced by t-BHP or ethanol, and control HDFs. Control HDFs were treated in the same conditions as the stressed HDFs but were not exposed to the stressor. Six independent extracts of labelled proteins were prepared. Protein labelling started at the fiftieth hour after the last stress, i.e. when SIPS appeared. From each extract of proteins more than 1800 protein species were visualized on 2DGE. Statistical analysis revealed 50 spots with a significant difference of integrated intensity (I.I.) between HDFs at early CPDs and senescent HDFs. Thirty-eight changes were senescence-specific, whereas the 12 other changes were also found in SIPS induced by t-BHP and/or ethanol. Thirteen and 25 spots had different I.I. in SIPS induced by t-BHP and ethanol, respectively, when compared to both HDFs at early CPDs and control HDFs [15]. Fig. 1 summarizes these results and

a)

Phenotype	Number of spots with changed I.I.	Ratio of I.I. (early CPDs=1)
RS	increased I.I. : 34 decreased I.I. : 16 **Total : 50**	1.3 to 23.8 0.2 to 0.7
t-BHP-induced SIPS	increased I.I. : 11 decreased I.I. : 2 **Total : 13**	1.3 to 2.3 0.5 and 0.7
ethanol-induced SIPS	increased I.I. : 18 decreased I.I. : 7 **Total : 25**	1.3 to 2.5 0.5 to 0.7

b)

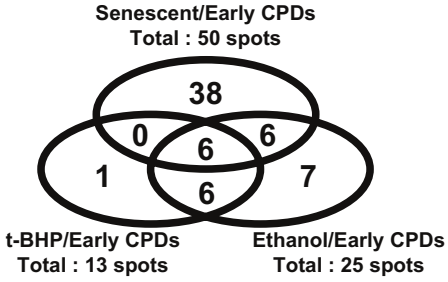

Fig. 1. Summary of the results of the proteomic analysis comparing RS and SIPS in WI-38 HDFs. a: Table presenting the number and ratio of I.I. of spots with increased or decreased I.I. in RS when compared to HDFs at early CPDs, and with increased or decreased I.I. in SIPS induced by t-BHP and ethanol when compared to control HDFs+HDFs at early CPDs. Numbers in bold: total number of changes found in each phenotype. b: Pattern of the number of significant differences in I.I. observed according to the experimental conditions. The experimental conditions are senescent HDFs (compared to HDFs at early CPDs), HDFs in SIPS induced by t-BHP (compared to early CPDs+control HDFs) and HDFs in SIPS induced by ethanol (compared to HDFs at early CPDs+control HDFs).

Table 1
Sequences and melting temperatures (T_m) of the primers

Names	Sequences	T_m (°C)	Size amplicons (bp)	T_m amplicons (°C)
HSP27 forward	TCCCTGGATGTCAACCACTTC	58	84	85
HSP27 reverse	CTCGTGCTTGCCGGTGAT	59		
Prx VI forward	GGACGTGGCTCCCAACTTT	59	99	84
Prx VI reverse	CGAGGGTGGGAGAAGAGAATG	60		
23-kDa HBP forward	GCCTACAAGAAAGTTTGCCTATCTG	60	134	82
23-kDa HBP reverse	TGAGCTGTTTCTTCTTCCGGTAGT	60		

Sizes and T_m of the sequences amplified by real-time PCR.

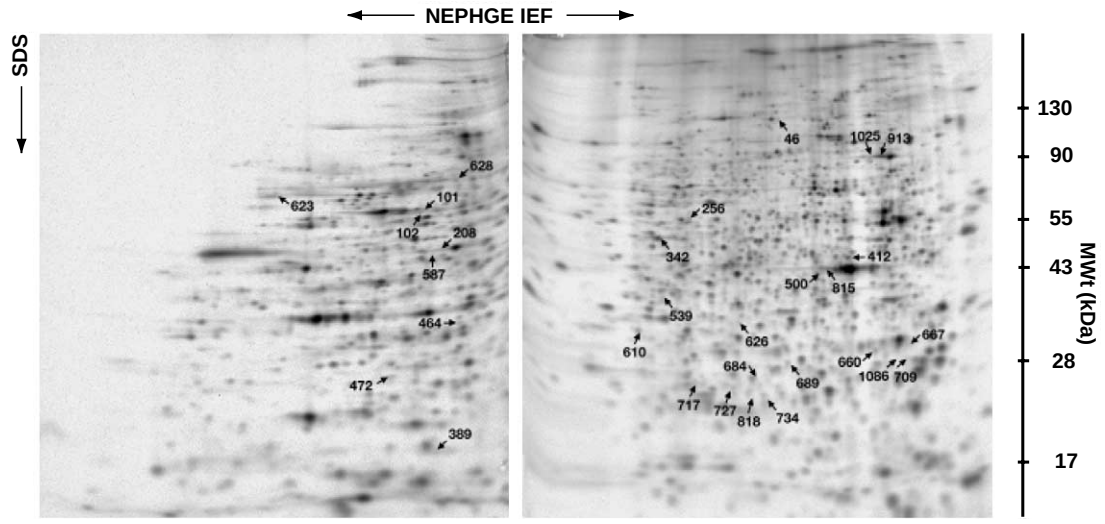

Fig. 2. Typical 2DGE pattern obtained from WI-38 HDFs. For each of the 30 samples produced in this work, two types of gels were run: IEF (from isoelectric point 3.5 to 7.0) and NEpHGE (from isoelectric point 6.5 to 11.0). The same 1819 spots were located on each of the 30 pairs of NEpHGE and IEF gels. The proteins present in the spots indicated by the arrows were identified by MS in this study. Their corresponding changes in I.I. are given in Table 2.

gives the number of spots with different I.I. obtained in the different experimental condition(s).

3.1. Protein identification

Preparative 2DGE and subsequent MS analyses led to the identification of 30 proteins corresponding to spots selected in the multi-differential proteomic comparison described above. These spots were pinpointed on 2DGE images (Fig. 2). Table 2 gives the names of the 30 proteins identified and their variations of I.I. in the different experimental conditions. Nine of the proteins identified were involved in the energy metabolism. Seven of them were overexpressed in RS and/or SIPS. Particularly, pyruvate kinase M 2 (PKM) was overexpressed in RS and in SIPS induced by t-BHP or ethanol (spot N 101). An increased PKM protein content in senescent HDFs could explain the increase of the glycolytic activity and the subsequent higher production of lactate observed in these cells [21]. PKM is a key regulatory enzyme of glycolysis. Indeed, the reaction it catalyzes is thermodynamically irreversible and favors an increase of the flux of metabolites through glycolysis. Pyruvate is produced by PKM and does not seem to be totally consumed by the cycle of tricarboxylic acids in senescent HDFs since lactate accumulates in these cells [21]. Another enzyme involved in glycolysis, alpha-enolase (spots N 208 and N 587), was among the proteins found to be overexpressed in senescent HDFs. An increase in the amount of several key glycolytic enzymes, among which PKM, was previously described in the skeletal muscles of aged rats [22]. An increase of the activity of glycolysis could counteract at least partially the age-associated decrease in the mitochondrial capability of ATP regeneration due to accumulation of damage in the mitochondrial membranes and the mitochondrial DNA. Along this line of evidence, the common 4977-bp deletion of mitochondrial DNA was previously detected WI-38 HDFs, whether in RS or t-BHP-induced SIPS [4]. Ethanol is known to destabilize the biological membranes [23]. It also decreases the respiratory activity of the mitochondria [24]. In addition, pyruvate and D-glucose were found to be key survival factors in various cell types exposed to a variety of stress at cytotoxic doses. Pyruvate and D-glucose also protect WI-38 HDFs against the appearance of the features of SIPS triggered by exposures to subcytotoxic doses of t-BHP and ethanol (for review, see [25]).

It is noteworthy that we observed a decrease of 35% in the amount of glucose-6-phosphate-1-dehydrogenase (G6PDH; spot I 342) in RS. A senescent-like phenotype has been observed in HDFs deficient in G6PDH [26]. Textbooks of biochemistry tell that a decrease in G6PDH content favors the consumption of glucose-6-phosphate by glycolysis since it becomes less consumed by the pentose phosphate pathway. Indeed G6PDH is the key regulator of the pentose phosphate pathway, which is the main producer of cellular NADPH and ribose-5-phosphate [27]. Glutathione reductase consumes NADPH to reduce the thiol group of glutathione. A decrease of 35% in G6PDH content could explain the decrease of the concentration of reduced glutathione observed in senescent HDFs [28]. Selenium-dependent glutathione peroxidase (Se-GPx) oxidizes glutathione when detoxifying hydroperoxides. Se-GPx is a major enzymatic component of the cellular antioxidant system (for a review, see [29]). A shortage of NADPH leads to a decrease of the activity of Se-GPx. This could participate in the increase of the oxidant status taking place during the in vitro ageing of HDFs [30]. Lastly, it is known that ribose-5-phosphate is a precursor of the synthesis of the bases of DNA. A shortage of these bases could be linked to the growth arrest observed in RS.

Peroxiredoxin VI (Prx VI) has selenium-independent GPx and phospholipase A2 activities [31]. Prx VI was identified on our 2DGE in spots I 717, I 727 and I 818. These spots have approximately the same molecular weight and have different isoelectric points. Given that Prx VI has several putative phosphorylation sites [32], these spots could represent different phosphorylated forms of this protein. Spot I 818 was the most acidic spot and was more intense in SIPS induced by t-BHP and ethanol, whereas its intensity decreased in RS. Spot I 727 and I 717 were more intense in RS and in SIPS induced by ethanol (Table 2), respectively. Western blots and semi-quantitative real-time RT-PCRs were performed to

check whether the differences in spot intensity would reflect differences of the total amount of the protein and differences of the steady-state level of mRNA (Fig. 3). A good correlation was found between the differences of the steady-state level of mRNA and total protein level of Prx VI in the different experimental conditions. However, the total protein level of Prx VI detected by Western blots (Fig. 3a) did not correlate with the sum of the values of I.I. found for the three spots identified as Prx VI (Fig. 4). The reason for these discrepancies could be that one or several other isoforms of Prx VI were not detected on the 2DGE, whereas they were detected by Western blot. Fig. 4 indicates that the proportion between the different isoforms of Prx VI identified in this study varied between the different experimental conditions. We suggest that such differential post-translational modifications could influence the activity of this enzyme.

We identified two proteins with differences of expression level in SIPS induced by t-BHP and by ethanol and not in RS. Firstly, the spots I 709 and I 1086 were identified as 14-3-3 tau protein. It has been shown previously that this protein can block the cell cycle [33,34]. It could play a role in the cell cycle arrest observed in SIPS [3,4]. Secondly, spot I 667 corresponded to a 30–35-kDa isoform of actin. Fragments of actin are known to play a role in the reorganization of the cytoskeleton in conditions generating stress fibers such as SIPS [35]. The isoform of actin identified in this work could participate in the reorganization of actin into stress fibers observed in SIPS [36]. These proteins could be considered as molecular scars of the long-term effects of subcytotoxic stresses [1].

Among the other proteins overexpressed in RS, we identified creatine kinase BC (spots I 500 and I 815) and HSP27 (spot I 684) (Table 2). It was found previously that the level of their respective mRNAs is increased in replicatively senescent HDFs and in skeletal muscle of aged mouse [9,37]. It is known that HSP27 can be phosphorylated at different sites. This could lead to the appearance of different spots on 2DGE [38]. In this study Western blots showed that the level of HSP27 was increased in replicatively senescent HDFs when compared to HDFs at early CPDs. When compared to control HDFs (i.e. HDFs at early CPDs treated as the stressed HDFs but not exposed to the stressor), HDFs in SIPS induced by ethanol or t-BHP also contained a higher level of HSP27 (Fig. 5a). In addition, we determined whether these discrepancies existed between the level of protein and the level of transcript of HSP27. Semi-quantitative real-time RT-PCRs revealed an increase in the level of HSP27 mRNA in RS, and in SIPS induced by t-BHP or in ethanol (Fig. 5). As proposed for Prx VI, it could be that not all the isoforms of HSP27 have undergone changes in expression level that were detectable by 2DGE.

4. Discussion

We identified 30 proteins undergoing changes of expression level in RS and/or SIPS. Some of the proteins identified support an increase in glycolytic activity, whereas other proteins identified in this work can explain how a senescent(-like) phenotype appears. Western blots and 2DGE gave complemen-

Table 2
Proteins identified in the proteome analysis of RS and SIPS

Spot #	Identification	DB #	MW	pI	Ratio RS	Ratio t-BHP	Ratio EtOH
N 101	pyruvate kinase M	P14618	*58*	*7.6*	**1.5** ± *0.3*	**1.7** ± *0.4*	**1.4** ± *0.2*
I 818	PeroxiredoxinVI	P30041	*25*	*6.3*	**0.5** ± *0.1*	**1.4** ± *0.3*	**1.6** ± *0.4*
I 709	14-3-3 protein tau	P27348	*28*	*4.7*		**new spot**	**new spot**
I 1086	14-3-3 protein tau	P27348	*28*	*4.7*		**new spot**	**new spot**
I 667	actin isoform (between 30 and 35 kDa)	P53493	*42*	*5.3*		**1.7** ± *0.5*	**2.5** ± *0.6*
I 684	HSP27	P04792	*24*	*7.8*	**1.3** ± *0.1*		
I 660	chloride intracellular channel protein 1	O00299	*27*	*4.9*	**1.5** ± *0.2*		
N 472	electron transfer flavoprotein beta-subunit	P38117	*28*	*8.2*	**1.5** ± *0.2*		
I 734	BAG-family molecular chaperone regulator	O95816	*24*	*6.2*	**1.6** ± *0.2*		
N 389	alpha-actinin isoform (around 20 kDa)	P12814	*103*	*5.2*	**1.7** ± *0.7*		
I 727	Peroxiredoxin VI	P30041	*25*	*6.3*	**1.8** ± *0.3*		
N 628	lamin C	P02546	*65*	*6.8*	**1.9** ± *0.3*		
I 539	alcohol dehydrogenase	P14550	*36*	*6.3*	**2.0** ± *0.5*		
I 815	creatin kinase BC	P12277	*43*	*5.4*	**2.0** ± *0.6*		
I 500	creatin kinase BC	P12277	*43*	*5.4*	**2.1** ± *0.2*		
N 208	alpha-enolase	P06733	*47*	*7.4*	**2.0** ± *0.6*		
N 587	alpha-enolase	P06733	*47*	*7.4*	**5.5** ± *1.5*		
I 626	annexin 1	P04083	*39*	*6.6*	**0.5** ± *0.2*		
N 623	calmodulin-binding protein	O88482	*67*	*11.3*	**0.6** ± *0.1*		
I 256	lamin C	P02546	*65*	*6.8*	**0.6** ± *0.2*		
N 464	NADH-cytochrome B5 reductase	P00387	*34*	*7.6*	**0.6** ± *0.3*		
I 412	actin	P53493	*42*	*5.3*	**0.6** ± *0.0*		
I 342	glucose-6-phosphate-1-dehydrogenase	P11413	*59*	*6.4*	**0.7** ± *0.1*		
I 717	Peroxiredoxin VI	P30041	*25*	*6.3*			**1.5** ± *0.3*
N 102	pyruvate kinase M	P14618	*58*	*7.6*			**1.5** ± *0.2*
I 610	esterase D	P10768	*31*	*7.0*			**1.4** ± *0.3*
I 689	guanidinoacetate N methyltransferase	Q14353	*26*	*5.7*			**1.3** ± *0.1*
I 1025	HSP90	P08238	*83*	*4.8*			**0.6** ± *0.3*
I 913	HSP90	P08238	*83*	*4.8*			**0.7** ± *0.2*
I 46	glucosidase II precursor	Q14679	*107*	*6.0*		**1.7** ± *0.3*	

Gel system with 'N' stands for NEpHGE and 'I' for IEF+spot number (spot #). SWISS-PROT or TrEMBL accession number (DB#), theoretical molecular weight in kDa (MW) and theoretical isoelectric point (pI) are given. The ratio ± standard deviation of the I.I. of corresponding spot between the different situations studied and HDFs at early CPDs is mentioned. 'RS' stands for replicatively senescent HDFs, 't-BHP' for HDFs in SIPS induced by t-BHP, and 'EtOH' for HDFs in SIPS induced by ethanol.

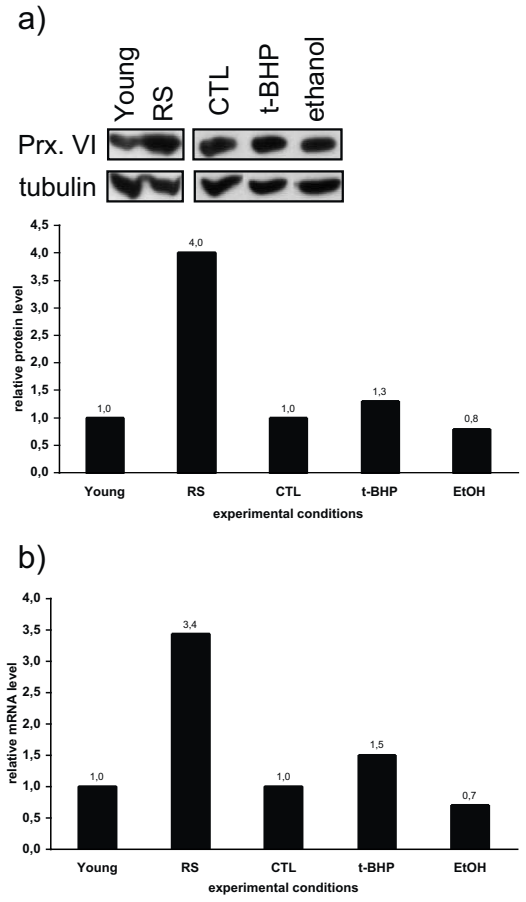

Fig. 3. a: Relative levels of Prx VI protein determined by Western blots. Alpha-tubulin was used as reference for semi-quantification. b: Relative levels of Prx VI transcripts determined by real-time semi-quantitative RT-PCR. The mRNA level of 23-kDa HBP was used as reference for semi-quantification. 'Young' stands for HDFs at early CPDs, 'RS' for replicatively senescent HDFs, 't-BHP' and 'ethanol' for HDFs in SIPS induced by t-BHP and ethanol, respectively. 'CTL' are HDFs at early CPDs treated like the stressed HDFs but with the stressor omitted.

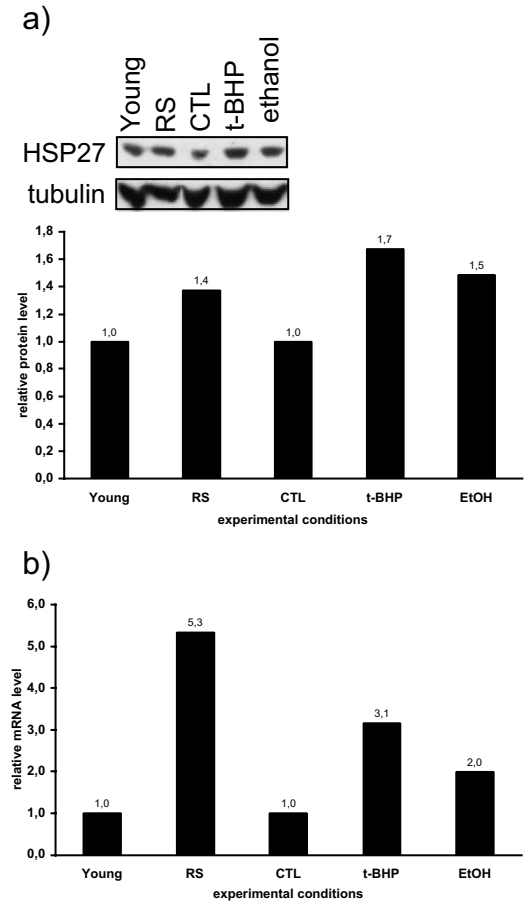

Fig. 5. a: Relative levels of HSP27 protein determined by Western blot. Alpha-tubulin was used as reference for semi-quantification. b: Relative levels of HSP27 transcripts determined by real-time semi-quantitative RT-PCRs. The mRNA level of the 23-kDa HBP was used as reference for semi-quantification. 'Young' stands for HDFs at early CPDs, 'RS' for replicatively senescent HDFs, 't-BHP' and 'ethanol' for HDFs in SIPS induced by t-BHP and ethanol, respectively. 'CTL' are HDFs at early CPDs treated like the stressed HDFs but with the stressor omitted.

tary information on the total level of Prx VI and HSP27 and on the level of some of their various post-translationally modified forms. Semi-quantitative real-time RT-PCRs were also valuable for revealing post-transcriptional regulations.

The stress-induced changes of the level of expression of specific proteins appearing several days after stress, whenever stress-specific or common to several kinds of stress, can be considered 'molecular scars' [1]. Molecular scars might also occur in vitro and in vivo at several days or weeks after exposure to different stressors like H_2O_2, UV, paraquat, ozone, hyperoxia, inflammation, ischemia-reperfusion, ethanol abuse, etc. Finding some of the molecular scars appearing in these conditions could help in understanding the pathophysiological consequences of such conditions. Examples are the study of gastric ulcer and leg venous insufficiency where fibroblasts bearing several biomarkers of SIPS have been found [39]. Lastly, the combination of proteomics and SIPS in vitro represents a valuable toxicoproteomic tool for detecting the effects of subcytotoxic concentrations of molecules in R&D. This kind of screening in vitro should be carried out before in vivo testing. Indubitably this approach would decrease the budgetary and ethical burden of in vivo toxicology.

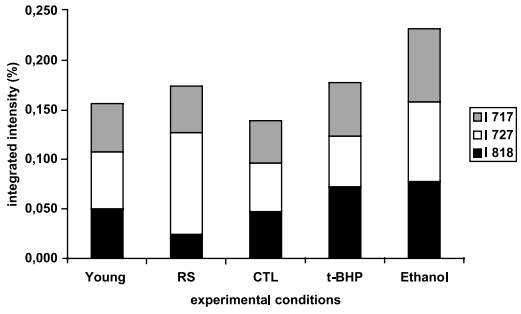

Fig. 4. Representation of the proportion of I.I. of the three different spots (I 717, I 727 and I 818) corresponding to Prx VI identified in the different experimental conditions. The I.I.s of each isoform have been summed in each experimental condition.

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References

[1] Brack, C., Lithgow, G.J., Osiewacz, H.D. and Toussaint, O. (2000) EMBO J. 19, 1929–1934.
[2] Toussaint, O., Medrano, E. and von Zglinicki, T. (2000) Exp. Gerontol. 35, 927–945.
[3] Chen, Q.N., Bartholomew, J.C., Campisi, J., Acosta, M., Reagan, J.D. and Ames, B.N. (1998) Biochem. J. 332, 43–50.
[4] Dumont, P., Burton, M., Chen, Q.M., Gonos, E.S., Frippiat, C., Mazarati, J.-B., Eliaers, F., Remacle, J. and Toussaint, O. (2000) Free Radical Biol. Med. 28, 361–373.
[5] Frippiat, C., Chen, Q.M., Zdanov, S., Magalhaes, J., Remacle, J. and Toussaint, O. (2001) J. Biol. Chem. 276, 2531–2537.
[6] Doggett, D.L., Rotenberg, M.O., Pignolo, R.J., Phillips, P.D. and Cristofalo, V.J. (1992) Mech. Ageing Dev. 65, 239–255.
[7] Hara, E., Yamaguchi, T., Tahara, H., Tsuyama, N., Tsurui, H., Ide, T. and Oda, K. (1993) Anal. Biochem. 214, 58–64.
[8] Linskens, M.H.K., Feng, J., Andrews, W.H., Enloiw, B.E., Saati, S.M., Tonkin, L.A., Funk, W.D. and Villeponteau, B. (1995) Nucleic Acids Res. 23, 3244–3251.
[9] Shelton, D.N., Chang, E., Whittier, P.S., Choi, D. and Funk, W.D. (1999) Curr. Biol. 9, 939–945.
[10] Gygi, S.P., Rochon, Y., Franza, B.R. and Aebersold, R. (1999) Mol. Cell. Biol. 19, 1720–1730.
[11] Page, M.J., Amess, B., Townsend, R.R., Parekh, R., Herath, A., Brusten, L., Zvelebil, M.J., Stein, R.C., Waterfield, M.D., Davies, S.C. and O'Hare, M.J. (1999) Proc. Natl. Acad. Sci. USA 96, 12589–12594.
[12] Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T. and Mann, M. (1996) Nature 379, 466–469.
[13] Benvenuti, S., Cramer, R., Quinn, C.C., Bruce, J., Zvelebil, M., Corless, S., Bond, J., Yang, A., Hockfield, S., Burlingame, A.L., Waterfield, M.D. and Jat, P.S. (2002) Mol. Cell. Proteomics 1, 280–292.
[14] Benvenuti, S., Cramer, R., Bruce, J., Waterfield, M.D. and Jat, P.S. (2002) Oncogene 21, 4403–4413.
[15] Dierick, J.-F., Eliaers, F., Remacle, J., Raes, M., Fey, S.J., Mose Larsen, P. and Toussaint, O. (2002) Biochem. Pharmacol. 64, 1011–1017.
[16] Hayflick, L. and Moorhead, P.S. (1961) Exp. Cell Res. 25, 585–621.
[17] Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Anal. Chem. 68, 850–858.
[18] Mann, M., Hojrup, P. and Roepstorff, P. (1993) Biol. Mass Spectrom. 22, 338–345.
[19] Mann, M. and Wilm, M. (1994) Anal. Chem. 66, 4390–4399.
[20] Pfaffl, M.W. (2001) Nucleic Acids Res. 29, 2002–2007.
[21] Goldstein, S., Ballantyne, S.R., Robson, A.L. and Moerman, E.J. (1982) J. Cell. Physiol. 112, 419–424.
[22] Kopsidas, G., Kovalenko, S.A., Heffernan, D.R., Yarovaya, N., Kramarova, L., Stojanovski, D., Borg, J., Islam, M.M., Caragounis, A. and Linnane, A.W. (2000) Ann. New York Acad. Sci. 908, 226–243.
[23] Sanchez-Amate, M.C., Carrasco, M.P., Zurera, J.M., Segovia, J.L. and Marco, C. (1995) Eur. J. Pharmacol. 292, 215–221.
[24] Marcinkeviciute, A., Mildaziene, V., Crumm, S., Demin, O., Hoek, J.B. and Kholodenko, B. (2000) Biochem. J. 349, 519–526.
[25] Toussaint, O. and Remacle, J. (1996) in: Molecular Gerontology, pp. 87–109 (Rattan, S.I.S. and Toussaint, O., Eds.), Plenum Press, New York.
[26] Ho, H.Y., Cheng, M.L., Lu, F.J., Chou, Y.H., Stern, A., Liang, C.M. and Chiu, D.T. (2000) Free Radical Biol. Med. 29, 156–169.
[27] Kletzien, R.F., Harris, P.K. and Foellmi, L.A. (1994) FASEB J. 8, 174–181.
[28] Yuan, H., Kaneko, T. and Matsuo, M. (1996) Exp. Gerontol. 31, 465–474.
[29] Michiels, C., Raes, M., Toussaint, O. and Remacle, J. (1994) Free Radical Biol. Med. 17, 235–248.
[30] Houben, A., Raes, M., Houbion, A. and Remacle, J. (1984) Mech. Ageing Dev. 25, 23–34.
[31] Chen, J.W., Dodia, C., Feinstein, S.I., Jain, M.K. and Fisher, A.B. (2000) J. Biol. Chem. 275, 28421–28427.
[32] Phelan, S.A. (1999) Antioxidant Redox Signal. 1, 571–584.
[33] Aitken, A. (1995) Trends Biochem. Sci. 20, 95–97.
[34] Sladeczek, F., Camonis, J.H., Burnol, A.F. and Le Bouffant, F. (1997) Mol. Gen. Genet. 254, 571–577.
[35] Mashima, T., Naito, M. and Tsuruo, T. (1999) Oncogene 18, 2423–2430.
[36] Chen, Q.M., Tu, V.C., Catania, J., Burton, M., Toussaint, O. and Dilley, T. (2000) J. Cell Sci. 116, 4087–4097.
[37] Lee, C.-L., Klopp, R.G., Weindruch, R. and Prolla, T.A. (1999) Science 285, 1390–1393.
[38] Faucher, C., Capdevielle, J., Canal, I., Ferrara, P., Mazarguil, H., McGuire, W.L. and Darbon, J.-M. (1993) J. Biol. Chem. 268, 15168–15173.
[39] Mendez, M.V., Stanley, A., Park, H.Y., Shon, K., Phillips, T. and Menzoian, J.O. (1998) J. Vasc. Surg. 28, 876–883.