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Proteomic analysis reveals that treatment with tocotrienols reverses the effect of H_2O_2 exposure on peroxiredoxin expression in human lymphocytes from young and old individuals☆

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Abstract

Vitamin E has been suggested to modulate age-associated changes by altering the redox balance resulting in altered gene and/or protein expression. Here we have utilized proteomics to determine whether such regulation in protein expression occurs in human lymphocytes from two different age groups stressed with H_2O_2 and then treated with vitamin E in the form of tocotrienol-rich fraction (TRF). In this study, lymphocytes obtained from young (30–49 years old) and old (>50 years old) volunteers were first challenged with 1 mM H_2O_2. They were then treated by exposure to 50, 100 and 200 μg/ml TRF. Two-dimensional gel electrophoresis followed by MALDI-TOF/TOF (matrix-assisted laser desorption/ionization time-of-flight/time-of-flight) tandem mass spectrometry was then performed on whole-cell protein extracts to identify proteins that have changed in expression. A total of 24 proteins were found to be affected by H_2O_2 and/or TRF treatment. These included proteins that were related to metabolism, antioxidants, structural proteins, protein degradation and signal transduction. Of particular interest was the regulation of a number of proteins involved in stress response—peroxiredoxin-2, peroxiredoxin-3 and peroxiredoxin-6—all of which were shown to be down-regulated with H_2O_2 exposure. The effect was reversed following TRF treatment. The expression of peroxiredoxin-2 and peroxiredoxin-6 was confirmed by quantitative reverse transcriptase polymerase chain reaction. These results suggested that TRF directly influenced the expression dynamics of the peroxiredoxin-2, thus improving the cells ability to resist damage caused by oxidative stress.

Keywords: Tocotrienol-rich fraction; Age-related changes; Oxidative stress; Lymphocyte; Two-dimensional electrophoresis

1. Introduction

Aging is a process associated with physiological and biological changes. These changes begin at the molecular level, eventually leading to changes in proteins and protein expression. It has been proposed that these alterations contribute to aging-related impairments. Such age-related changes have been observed in colon tissue where proteins involved in metabolism, energy production, chaperones, antioxidants, signal transduction, protein folding and apoptosis changed in expression with age [1]. It has also been shown that older individuals are more susceptible to DNA damage [2] and show an increase in protein oxidation [3] and an accumulation of protein carbonyls [4]—all of which may lead to alterations in protein expression.

The accumulation of reactive oxygen species (ROS) and oxidative stress damage is strongly associated with aging [5]. In contrast, the attenuation of oxidative stress is associated with increased longevity. This suggest that the effects of ROS may be countered by antioxidants such as vitamin E. Antioxidants help maintain the homeostatic level of ROS in our body by terminating a chain reaction of oxidizing events [6]. Tocotrienol (α, β, γ, δ), along with tocopherol (α, β, γ, δ) stereoisomers, represents the two naturally occurring subclasses of vitamin E compounds.

Tocotrienols extracted from crude palm oil consist mainly of a mixture of α-, γ- and δ-tocotrienols and a small proportion of α-tocopherols. This is our extract of interest and is referred to as tocotrienol-rich fraction (TRF). Its effects on biological functions especially in humans are not well characterized. However, evidence suggests that tocotrienol exhibit functions that are not shared by α-tocopherol [7]. For example, tocotrienol and not tocopherol

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administration reduces oxidative protein damage and extends the mean life span of *Caenorhabditis elegans* [8]. A separate in vivo study of tocotrienol supplementation showed that it produced a protective effect against DNA damage and protein oxidation [2].

Apart from its antioxidant and protective properties, vitamin E has also been shown to function as a signaling molecule. This suggests that vitamin E may act via alternative molecular pathways in exhibiting its protective effects. It has been shown that vitamin E affect proteins and protein expression through direct interactions with transcription factors or signal transduction enzymes. These include effects on phosphorylation and dephosphorylation enzymes, cell cycle and proliferation related enzymes and lipid signaling enzymes [9–15].

The effects of vitamin E have been shown to be different on individuals from different age groups [2,16] . One possible explanation for this observation is that there may be a difference in the mode of action of vitamin E within young and old individuals. It is likely that it involves a variety of sophisticated pathways associated with a number of different biological processes. This, in turn, would involve the regulation of a number of different proteins. Therefore, one way to understand the mechanisms involved would be to study the effect of vitamin E on protein expression within different age groups.

Proteomics is a study that allows for both the identification and quantification of changes in global protein expression. Sample availability is often a problem in proteomics. In humans, the availability of tissues to be studied ex vivo is often limited. However, cells from blood such as lymphocytes can be easily and repeatedly obtained in sufficient quantities over a long period of time. Lymphocytes exhibit receptors and metabolic pathways in common with other cells [17,18] and thus have been used in medical research and diagnostic applications. Lymphocytes from aged individuals show age-altered characteristics such as increased cell damage from environmental agents [19], impaired cell to cell communication and an apparent decrease in the ability to utilize interleukin-2 and an abnormal cytokine and/or receptor production [20–23]. Their ready availability and the fact that they too undergo age-associated changes make lymphocytes an ideal model with which to study changes in proteins and protein expression in relation to aging and vitamin E.

Using lymphocytes as a model, this study was performed to determine the effect of H_2O_2 exposure followed by TRF treatment on protein expression in lymphocytes from two different age groups.

2. Materials and methods

2.1. Study population and sample collection

Sample collection was performed with approval from the Human Ethics Committee, Universiti Kebangsaan Malaysia, Kuala Lumpur. After obtaining written informed consent, peripheral venous blood (10 ml) was collected between 8 and 10 AM and placed into heparin-containing tubes from young (39–50 years) and old (>50 years) individuals previously determined to be healthy by a full physical checkup followed by hematological and biochemical tests. A total of three young and three old donors were recruited.

We have previously shown that there were different degrees of DNA damage when we divide individuals into two age groups—younger group (35–49 years old) and older group (>50 years old) where DNA damage was shown to be significantly higher in the older age group [2]. Following supplementation with tocotrienol, the older subjects showed reduction in total DNA damage after only 3 months. On the other hand, in the younger group, a lower level of DNA damage was observed only after 6 months of supplementation. The degree of reduction was also greater in the older group. From these results, we believe that there would also be significant differences in the reaction to supplementation in terms of protein expression between these two groups. Thus, to investigate this, experiments were performed on individuals from within these two age groups.

Subjects in this study were recruited from among individuals who have previously been screened in a population study. Information regarding their medical history, ethnicity, religion, marital status, occupation, socioeconomic status, physical activity and diet was obtained via questionnaires. Subjects were also required to undergo a full physical examination, and their biochemical profiles were screened to confirm that all participants were in good health. The following including/excluding criteria were also considered—subjects must be nonsmokers, have no significant clinical disease, are not pregnant, and are not taking medication, alcohol, or supplements.

2.2. Lymphocyte isolation and cell culture conditions

Lymphocytes were isolated by Lymphoprep (AXIS-Shield, Oslo, Norway) density centrifugation as recommended by the manufacturer. The resulting buffy coat was collected, washed and suspended in RPMI-1640 medium containing 10% fetal bovine serum. This yielded mononuclear cells, which were then depleted of monocytes by allowing adhesion to a plastic tissue culture flask surface in a humidified incubator for 1 h at 37°C in 5% CO_2. During and after treatment, the cells were maintained in complete culture medium at 37°C supplemented with 10% inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin in a humidified atmosphere of 5% CO_2.

2.3. MTS viability assay following exposure to H_2O_2 and treatment with TRF

Cell viability was assessed using CellTiter 96* Aqueous Non-Radioactive Cell Proliferation Assay (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; Promega, Madison, WI, USA). This was performed to determine the dose of H_2O_2 that would induce oxidative damage to human lymphocytes and optimum doses of TRF for treatment. The H_2O_2 concentration was determined by exposing lymphocytes (1×10^6 cells/ml) to varying concentrations of H_2O_2 ranging from 0 to 10 mM for time periods ranging from 5 min to 24 h. The IC_{40} (concentration that causes 40% cell death) value for H_2O_2 was used in subsequent experiments (IC_{50} [half maximal inhibitory concentration] cannot be determined as higher concentrations of H_2O_2 did not increase the percentage of cell death). In order to measure the optimum doses of TRF, cells were either pretreated with TRF (50, 100 and 200 μg/ml) for 24 h prior to 1 mM H_2O_2 exposure for 6 h. For posttreatment studies, cells were first exposed to 1 mM H_2O_2 for 6 h before treatment with TRF for 24 h.

MTS assay was performed as recommended by the manufacturer. Briefly, 20 μl MTS solution was added to each well and incubated in a humidified incubator at 37°C in 5% CO_2 for 3 h. Absorbance was then measured at 490 nm with a microtiter plate reader (VeraMax; Molecular Devices, Sunnyvale, CA, USA).

2.4. Protein extraction and quantification

Lymphocytes were harvested by low-speed centrifugation at 1000 rpm for 10 min. It was then washed three times with phosphate-buffered saline. The resulting cell pellet was resuspended and left for 1 h on ice in lysis buffer containing 8 M urea, 4% 3-[(cholamidopropyl) dimethyl ammonio]-1-propanesulfonate, 40 mM dithiothreitol (DTT) and 0.01% protease inhibitor cocktail. Protein extraction was completed with the addition of 2% immobilized pH gradient (IPG) buffer (pH 3–10). Following this procedure, cellular debris was removed by centrifugation at 13,000×*g* for 60 min at 4°C. Supernatant was collected, and this was used as the whole cell extract in two-dimensional gel electrophoresis (2DE) analysis. Protein content was then estimated by Bradford assay. Aliquots of protein samples were stored at-70°C.

2.5. Two-dimensional gel electrophoresis

2.5.1. First-dimension isoelectric focusing

First-dimension isoelectric focusing was performed on an Ettan IPGPhor 3 isoelectric focusing System (GE Healthcare Bio-Sciences, Uppsala, Sweden) according to the manufacturer's recommendations on 24-cm Immobiline DryStrips (pH3-10). Briefly, dry strips were first rehydrated for 16 h with 450 μl of rehydration buffer containing 8 M urea, 2% 3-[(cholamidopropyl) dimethyl ammonio]-1-propanesulfonate, 0.5% IPG buffer (pH 3–10) and trace amounts of bromophenol blue. Protein from cell lysate extracts were then applied by cup loading at the acidic end of the dry strip (100 μg for analytical gels, 200 μg for preparative gels). The dry strips were focused for a total of 45 kVh.

2.5.2. Second-dimension sodium dodecyl polyacrylamide gel electrophoresis

Focused IPG strips were equilibrated for 15 min in equilibration buffer (6 M urea, 75 mM Tris–HCl pH 8.8, 29% glycerol, 2% SDS and trace amounts of bromophenol blue) containing 1% DTT. It was then alkylated in the same buffer containing 2.5% iodoacetamide for a further 15 min. Electrophoresis of reduced and alkylated samples was then performed on 24 cm, 12.5% sodium dodecyl polyacrylamide gel electrophoresis gels on an ETTAN DALT II electrophoresis platform (GE Healthcare). The gels were run at 40 mA per gel until the bromophenol blue tracking dye reached the bottom of the gel.

2.6. Silver staining

Following sodium dodecyl polyacrylamide gel electrophoresis, protein spots were visualized using protocols described in the PlusOne Silver staining kit (GE Healthcare Bio-Sciences). The complete protocol was followed for analytical gels. For preparative gels, the protocol was modified so that glutaraldehyde was omitted from the sensitization step and formaldehyde omitted from the silver reaction step [24]. Silver-stained gels were scanned (UMAX PowerLook 1000 Imaging system), and protein profiles compared (Image Master Platinum software, version 6.0; GE Healthcare Bio-Sciences).

2.7. Image analysis

All experiments were performed in triplicate (three biological and three experimental replicates). A total of nine gels were run per sample (three separate cultures, three gels run per culture) to account for experimental and biological variations. To further select against variations, the following steps were performed. First, gels were compared within their respective groups, and a representative gel was generated. For the generation of the representative gel, only spots present across all gels within the groups were considered. This was performed to eliminate differences due to interindividual variations. The representative gels were then compared between the different samples to identify differentially expressed proteins. Only spots that were found to be differentially expressed by more than twofold were accepted for further consideration. Statistical analysis of differentially expressed spots was then performed by Student's *t* test. A *P* value of <.05 was considered as statistically significant.

2.8. In-Gel tryptic digestion

Protein spots were excised, and in-gel was digested with trypsin (Promega) for mass spectrometric analysis according to published protocols [25–27]. Briefly, excised spots were first destained in destaining solution [15 mM potassium ferricyanide/50 mM sodium thiosulphate, 1:1 (vol/vol)]. The spots were then reduced in a solution containing 10 mM DTT/100 mM ammonium bicarbonate for 30 min at 60 °C and alkylated in 55 mM iodoacetamide/100 mM ammonium bicarbonate for 20 min in the dark. The gel pieces were then washed (3×20 min) in 50% acetonitrile/100 mM ammonium bicarbonate. This was followed by dehydration of the gel pieces with 100% acetonitrile and drying in a vacuum centrifudge (SpeedVac, Thermo Scientific, Savant DNA 120). Subsequently, the dried gel pieces were rehydrated with 25 μl of 7 ng/μl trypsin (Promega trypsin gold) in 50 mM ammonium bicarbonate buffer and digested at 37°C for 18–20 h. Tryptic peptides were then extracted using 50% acetonitrile for 15 min, followed by 100% acetonitrile for 15 min. The extracted solutions were then pooled into a single tube and dried in a SpeedVac concentrator and solubilized with 10 μl of 10% acetonitrile/40 mM ammonium bicarbonate.

2.9. MALDI-TOF/TOF mass spectrometry analysis and database searching

Extracted peptides were first desalted using ZipTip C18 (Millipore, Billerica, MA, USA) according to protocols described by the manufacturer. The final elution volume following ZipTip cleanup was 1.5 μl. The peptide samples were then mixed (1:1) with a matrix consisting of a saturated solution of CHCA (α-cyano-4-hydroxycinnamic acid; Sigma) prepared in 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). Aliquots of samples (0.7 μl) were spotted onto stainless-steel sample target plates. Peptide mass spectra were obtained on a MALDI-TOF/TOF (matrix-assisted laser desorption/ionization time-of-flight/time-of-flight) mass spectrometer (ABI 4800 plus; Applied Biosystems) in the positive ion reflector mode. For precursor ion selection, all fractions were measured in single mass spectrometry (MS) before MS/MS was performed. For MS/MS spectra, the peaks were calibrated by default. The 20 most abundant precursor ions *per* sample were selected for subsequent fragmentation by high-energy collision-induced dissociation (CID). The collision energy was set to 1 keV, and the air was used as the collision gas. The criterion for precursor selection was a minimum S/N of 5. The mass accuracy was within 50 ppm for the mass measurement and within 0.1 Da for CID experiments. The other parameters for searching were of trypsin, one missed cleavage, variable modification of carbamidomethyl and oxidation of methionine, peptide charge of 1+ and monoisotopic. For database searches, known contamination peaks such as keratin and autoproteolysis peaks for trypsin were removed before searching. Spectra were processed and analyzed by the Global Protein Server Explorer 3.6 software (Applied Biosystems). This uses an internal MASCOT (Matrix Science, London, UK) program for matching MS and MS/MS data against database information. The data obtained were screened against human databases downloaded from the Swiss-Prot/TrEMBL homepage (http://www.expasy.ch/sprot).

2.10. Classification of identified proteins

Identified proteins were classified according to their known biological functions by database searching and cross-referencing with published reports.

2.11. Quantitative real-time reverse transcriptase polymerase chain reaction analysis

Total RNA was extracted from lymphocytes using TRIzol reagent according to the manufacturer's instruction (Invitrogen, Paisley, UK). Complement DNA was synthesized by reverse transcribing 3 μg of total RNA with iScript One-Step RT-PCR Kit With SYBR Green (BioRad). All primers were designed for SYBR Green probes with Primer3 software. The primers used are shown in Table 1. All samples were analyzed within-run and in duplicates, and they were averaged where differences between the two values were less than 5%. The expression level of each target gene was normalized against the expression of β-actin. Changes in expression were statistically analyzed using a paired *t* test and one-way analysis of variance where significance was defined as $P<.05$.

Among 24 proteins that were affected by H_2O_2 and/or TRF treatment, the expression dynamics of five proteins were validated using real-time reverse transcriptase polymerase chain reaction (RT-PCR). These proteins were cofilin-1 (structural protein), peroxiredoxin 2 and peroxiredoxin 6 (stress-related proteins), triosephosphate isomerase (TIM; metabolic enzyme) and proteosome activator complex subunit 1 (degradation-related protein). Validation was performed with samples from lymphocytes exposed to 1 mM H_2O_2 and treated with TRF (100 μg/ml for the younger group and 200 μg/ml for the older group). These five proteins were selected due the marked difference in their expression dynamics after H_2O_2 and/or TRF treatment in both age groups. They also represented proteins from various functional categories, all of which may potentially play a role in the effects observed following tocotrienol treatment.

Table 1
Primers used in quantitative RT-PCR analysis of proteins identified by 2-DE

Protein	Accession no.	Primer sequence 5′-3′
COF1	NM_005507	F: acaagaagaacatcatcctggag
		R: tcttgctctccttggtctcatag
PRDX2	NM_181738	F: tcctcttttttctaccctctggac
		R: cttagctgcaacctccctctt
PRDX6	NM_004905	F: ggtgtttgtttttggtcctgata
		R: tcagctggagagagatgactacc
PA28	NM_006263	F: ctatttccccaagaagatttctga
		R: ctcttttctctttctccttgactgg
TIM	NM_000365	F: aagtgactaatggggctttactg
		R: ctttcatctagcttctccccaat
β-Actin	NM_001101	F: catccacgaaactaccttcaact
		R: aggagcaatgatcttgatcttca

3. Results

3.1. Determination of H_2O_2 and TRF treatment conditions

Following 6 h of exposure to H_2O_2 (at 1 mM up to 4 mM), a significant decrease in the viability of human lymphocytes was observed (40% cell death, IC_{40}; *$P<.05$; Fig. 1A). Concentrations above 1 mM did not increase the percentage of cell death; therefore; this concentration was used to induce oxidative stress in further experiments.

We then determined the effects of TRF pretreatment and posttreatment on H_2O_2-induced oxidative stress. TRF pretreatment provided no protective effect (against cell death) at doses <400 μg/ml. At 400–1000 μg/ml, a 12% reduction in cell viability was observed (Fig. 1B). TRF posttreatment, on the other hand, increased cell viability in a dose-dependent manner.

TRF concentrations of 50, 100 and 200 μg/ml were used in further experiments as it reflected normal circulating levels of plasma vitamin E (total tocopherol and tocotrienols), which is in the range of 80 to 100 μg/ml.

3.2. Resolving the lymphocyte proteome by 2DE

A total of 1000±148 individual protein spots were resolved on silver-stained 2DE gels. A representative gel is shown in Fig. 2. In total, 26 protein spots exhibited significant change in expression ($P<.05$) following the different treatments. We were able to identify 24 of these spots by MALDI-TOF/TOF (Table 2) . Two protein spots could not be identified due to low abundance resulting in a database search score that was not sufficient to yield an unambiguous result. The identified proteins were classified into seven functional categories — proteins involved in metabolism, stress-related proteins, structural proteins, proteins involved in protein degradation, regulatory proteins, translation initiation factors and others. The index numbers in Table 2 correspond to the numbers in Fig. 2, and they indicate the location of the proteins on 2DE gels.

3.3. The effect of oxidative stress and TRF treatment on protein expression in lymphocytes from different age groups

Following exposure to H_2O_2, comparative analysis of 2DE gels identified 13 proteins that changed in expression. Proteins that

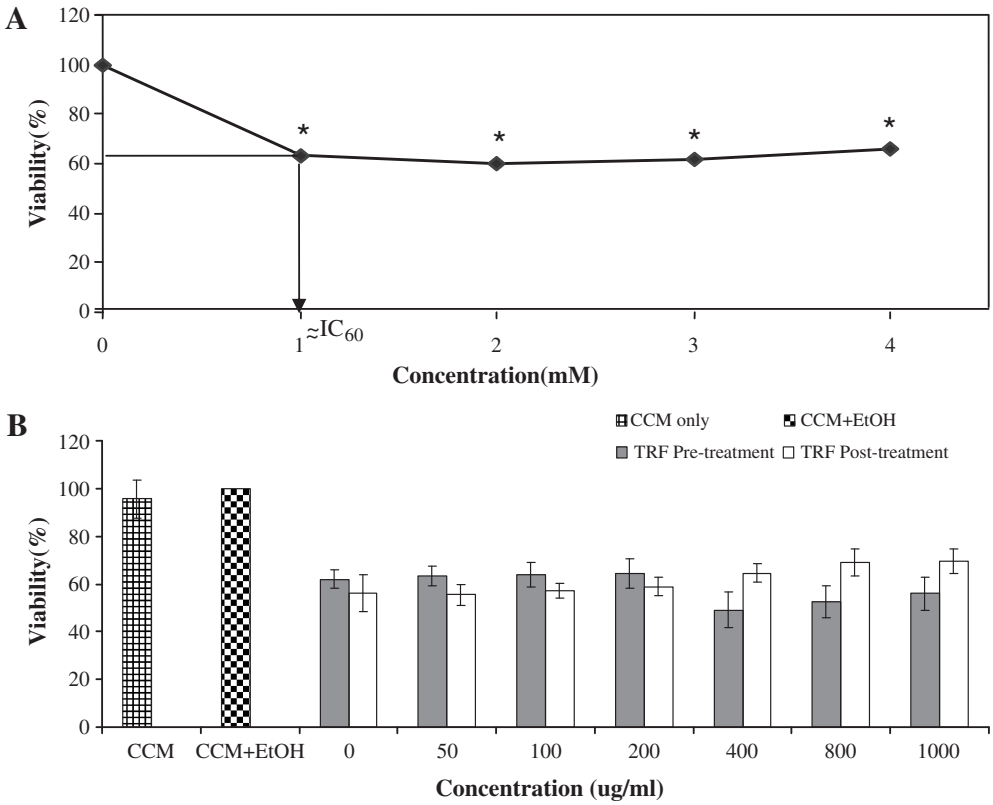

Fig. 1. Effects of different concentrations of H_2O_2 and TRF treatment on cell viability. (A) There was a significant decrease in viability of human lymphocytes after 6-h exposure to various concentrations of H_2O_2 (*P<.05). Maximum percentage of cell death (40%, IC_{40}) was achieved at 1 mM H_2O_2. Concentrations >1 mM did not increase the percentage of cell death. Thus, the concentration of 1 mM H_2O_2 was chosen for further experiments. (B) TRF pretreatment significantly reduced lymphocyte viability at concentrations >400 μg/ml (*P<.05). In contrast, TRF posttreatment increased cell viability in a dose-dependent manner (P<.05). Data are expressed as mean±S.D. CCM, complete culture media.

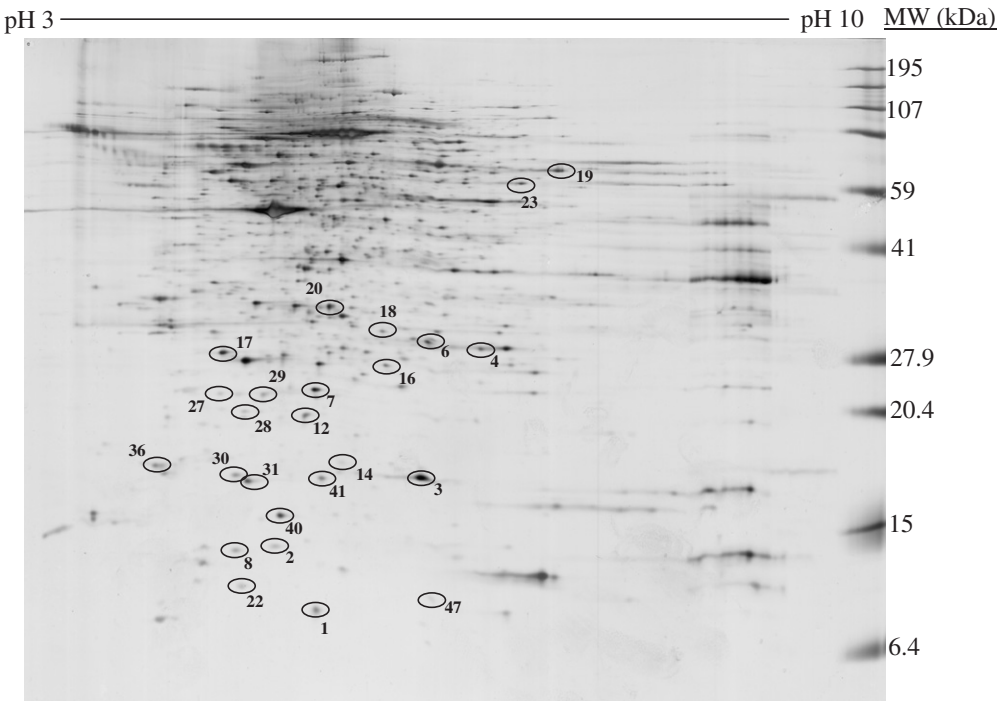

Fig. 2. Representative 2DE gel of human lymphocytes. Proteins from whole cell lysate were extracted and resolved by 2DE. The resulting gels were visualized by silver staining. Circles indicate protein spots that changed in volume following exposure to H_2O_2 and treatment with TRF. The numbers correspond to proteins in Fig. 3.

Table 2
List of proteins identified as being differentially expressed following H_2O_2 exposure and TRF treatment

Index no.	Protein name	Abbreviation	Swiss-Prot accession no.	MW/pI		Protein score	Peptides (%cov)
				Experimental	Theoretical		
Protein involved in metabolism							
4	Triosephosphate isomerase (EC 5.3.1.1)	TIM	P60174	29/7	26.6/6.45	923	25 (73)
12	Adenine phosphoribosyltransferase (EC 2.4.2.7)	APRT	P07741	20/5.5	19.6/5.78	399	12 (38)
18	Enoyl Co-A hydratase (EC 4.2.1.17)	ECH	P30084	31.2/6.2	31.4/8.34	429	16 (31)
23	Glutamate dehydrogenase (EC 1.4.1.3)	GDH	P00367	65/7.33	61.4/7.66	762	25 (40)
29	ATP synthase D chain	ATP5	P30049	22.7/5.11	18.5/5.21	288	10 (30)
Antioxidant and stress-related protein							
6	Peroxiredoxin-6 (EC 1.11.1.15)	PRDX6	P30041	30.1/5.55	25/6.00	233	13 (37)
7	Peroxiredoxin-2 (EC 1.11.1.15)	PRDX2	P32119	23.9/5.76	21.9/5.66	409	14 (35)
14	Superoxide dismutase (SOD[Cu-Zn]) (EC 1.15.1.1)	SODC	P04179	17.7/6.22	15.9/5.70	123	4 (24)
16	Peroxiredoxin-3 (EC 1.11.1.15)	PRDX3	P30048	26.2/6.72	27.7/7.67	233	8 (26)
27	Lactoylglutathione lyase (EC 4.4.1.5)	LGUL	Q04760	22.7/4.83	20.7/5.12	206	6 (23)
Structural/structure-related protein							
3	Cofilin-1	COF1	P23528	17.3/6.5	18.5/8.22	137	9 (34)
19	Adenylyl cyclase associated protein-1	CAP1	Q01518	77/7.61	51.8/8.27	420	17 (27)
30	Glia maturation factor gamma	GMFG	O60234	17.3/4.94	16.8/5.18	160	6 (25)
40	Coactosin-like protein	COTL1	Q14019	15.9/5.25	15.9/5.54	277	8 (43)
41	Actin-related protein 2/3 complex subunit 5	ARPC5	O15511	17.3/5.64	16.3/5.47	236	9 (29)
Proteins involved in degradation							
17	Proteosome activator complex subunit 1	PA28 alpha	Q06323	29/4.73	28.7/5.78	310	16 (36)
Regulatory proteins							
1	Protein S100-A4	S100A4	P26447	8.8/5.61	11.7/5.85	74	5 (19)
8	Galectin-1	LEG1	P09382	13.1/4.87	14.7/5.34	180	10 (52)
22	Myotrophin	MTPN	P58546	10.7/4.91	12.9/5.27	73	3 (27)
28	Sorcin	SORCN	P30626	20.4/4.92	21.7/5.32	321	11 (44)
36	Myosin regulatory side chain	MLRM	P19105	17.7/4.17	19.8/4.67	299	10 (40)
Initiation factor							
31	Eukaryotic translation initiation factor 5A-1	IF5A1	P63241	16.8/4.94	16.8/5.08	245	11 (42)
Others							
2	SH3 domain-binding glutamic-acid-rich-like protein	SH3L1	O75368	13.7/5.22	12.8/5.22	185	9 (58)
47	β-2 Microglobulin	B2MG	P61769	10/7.22	13.7/6.06	57	2 (18)

The proteins were grouped into seven functional categories — proteins involved in metabolism, antioxidant and stress-related proteins, structural/structure-related proteins, protein involved degradation, regulatory proteins, initiation factor and others. The accession number (Swiss-Prot), pI, molecular weight (MW), protein score, number of matched peptides and percentage coverage (%cov) are shown in the table.

exhibited altered expression profiles were found to be different between the two age groups. In the younger group, a total of 11 proteins changed in their expression, with one showing up-regulated expression and 10 proteins showing down-regulated expression. In the older group, seven proteins were found to be down-regulated in their expression.

We then proceeded with TRF treatment. It was generally found that increasing the concentration of TRF increased the number of proteins that changed in expression. Although the proteins that were altered by TRF treatment all changed in the same direction, no clear dose–response relationship was evident. However, for the younger group (age <50 years), treatment with 200 μg/ml TRF reduced the number of proteins that changed in expression. Proteins that changed in expression following H_2O_2 exposure and TRF treatment and their expression dynamics are shown in Fig. 3A–E. The change in expression of a number of proteins following H_2O_2 exposure was reversed with TRF treatment.

These differentially expressed proteins were classified into different functional categories. Of particular interest were proteins involved in metabolism, stress-related proteins, structural proteins and proteins involved in protein degradation (Fig. 3A–C).

3.4. Proteins involved in metabolism

TIM, ATP synthase D chain (ATP5), glutamate dehydrogenase (GDH), enoyl Co-A hydratase (ECH) and adenine phosphoribosyltransferase (APRT) were regulated following exposure to H_2O_2 and/or treatment with TRF.

TIM was down-regulated in both the old and young groups following exposure to H_2O_2 (Fig. 3A). Treatment with TRF reversed this effect in the younger group (at 100 μg/ml TRF). It was also shown to cause the up-regulation of a further three proteins related to metabolism — ATP5, GDH and APRT. ATP5 and GDH were up-regulated only in the younger group (at 100 μg/ml TRF), whereas APRT was up-regulated only in the older group (at 100 μg/ml TRF). ECH was up-regulated only in the younger group with exposure to H_2O_2.

Quantitative real-time RT-PCR was performed for TIM, but the results did not show a similar expression profile with that observed in 2DE. On the contrary, for the younger group, the relative expression observed was the reverse of that observed in 2DE (Fig. 4E).

3.5. Stress-related proteins

Peroxiredoxin-2 (PRDX2), peroxiredoxin-3 (PRDX3), peroxiredoxin-6 (PRDX6), superoxide dismutase (SODC) and lactoylglutathione lyase (LGUL) changed in expression following exposure to H_2O_2 and/or treatment with TRF.

PRDX2, PRDX3 and PRDX6 were markedly down-regulated in both age groups following H_2O_2 exposure (Fig. 3B). TRF treatment reversed the change in expression of PRDX2 and PRDX6. The expressions of these proteins were markedly increased at all concentrations of TRF except for 200 μg/ml in the younger group (Fig. 3B). A further two stress-related proteins — SODC and LGUL — were also up-regulated with TRF treatment at 100 μg/ml in the younger age group and 50 μg/ml in the older age group (Fig. 3B).

Quantitative RT-PCR showed that the relative gene expression profiles of PRDX2 and PRDX6 were similar to the protein expression observed in 2DE. In the younger group, there was significant up-regulation with H_2O_2 exposure and a further up-regulation with TRF treatment of both PRDX2 and PRDX6 relative expressions (Fig. 4B and C). In the older group, PRDX2 showed up-regulation with exposure to H_2O_2 and a further up-regulation with TRF treatment. PRDX6, on the

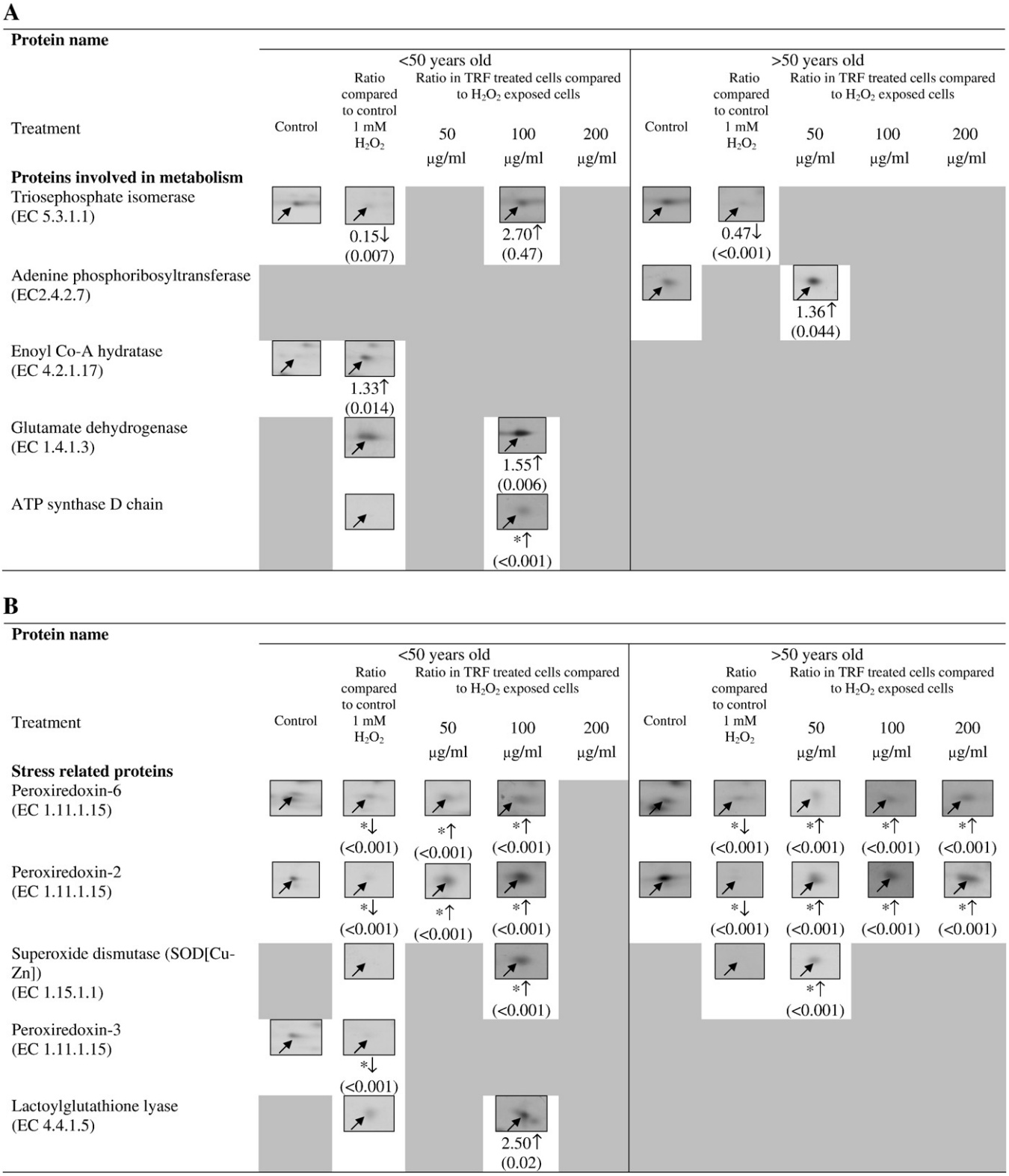

Fig. 3. Summary of protein spots with expression changes in lymphocytes exposed to H_2O_2 (1 mM) and treated with different concentrations of TRF (50, 100 and 200 µg/ml). Proteins were classified according to their roles in biological processes. Proteins involved in metabolism (A), stress-related proteins (B), structural/structure-related proteins and protein involved degradation (C), regulatory proteins (D), and initiation factor and others (E). Representative spots and their expression ratios are shown for proteins that show statistically significant change ($P<.05$, P value given in brackets). *Ratio=∞.

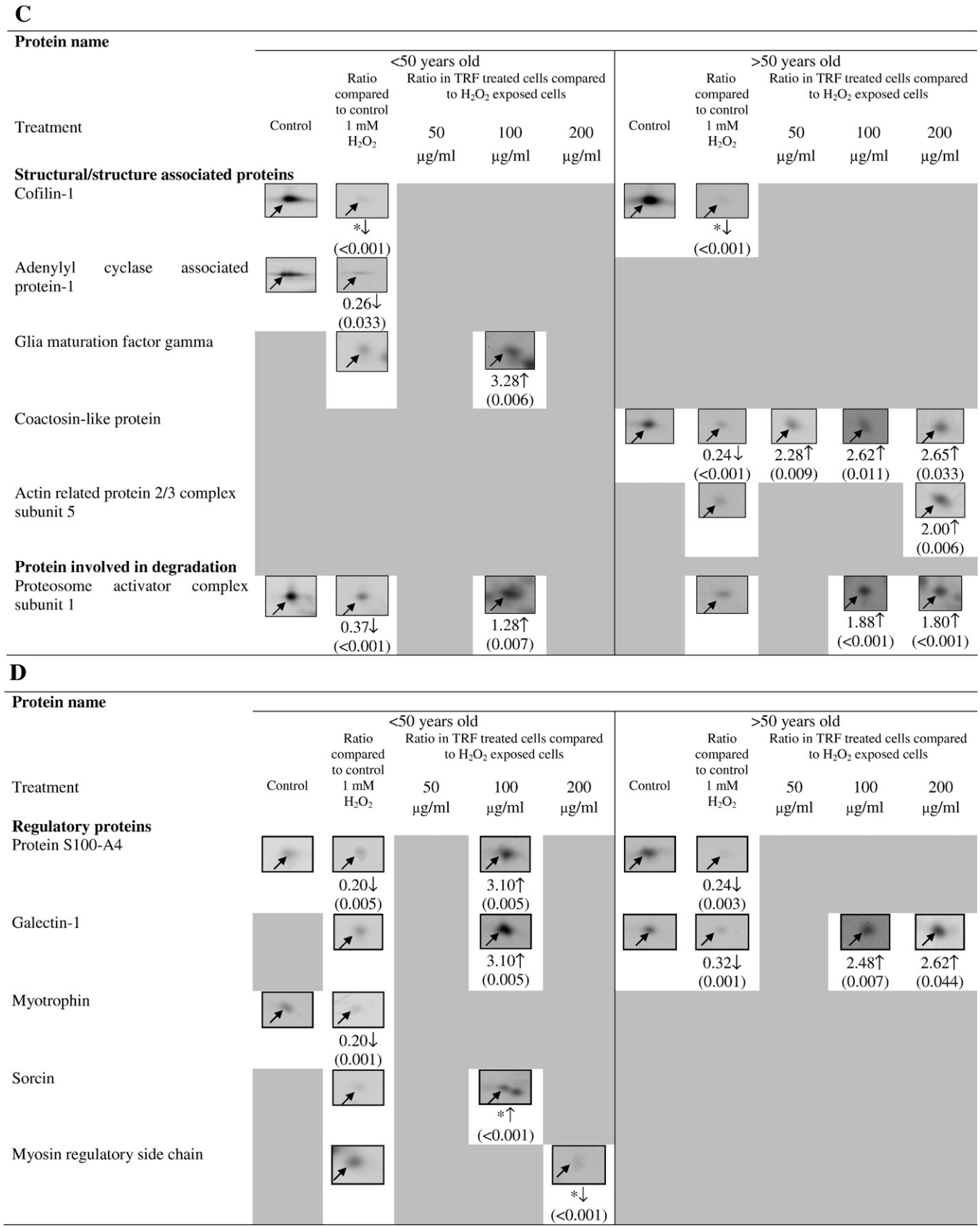

Fig. 3. (continued).

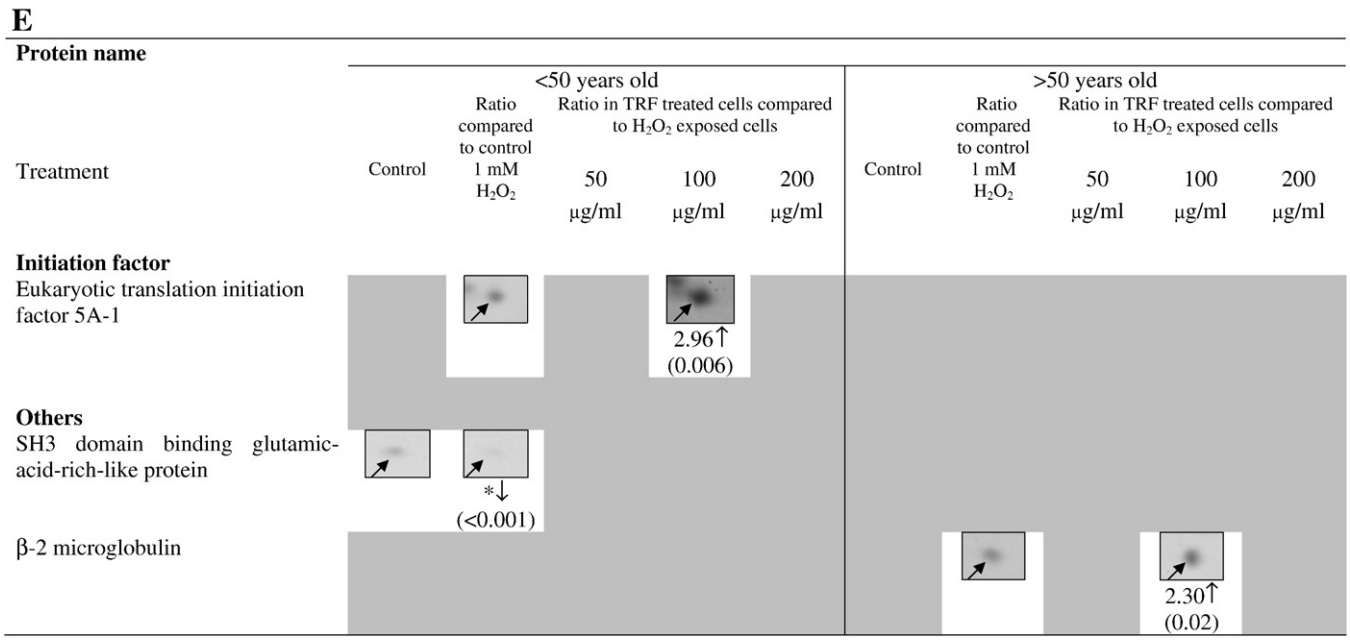

Fig. 3. (continued).

other hand, showed slight down-regulation with H_2O_2 exposure but was up-regulated with TRF treatment (Fig. 4B and C).

3.6. Structural and structure-related proteins

Cofilin 1 (COF1), adenylyl cyclase associated protein-1 (CAP1) and coactosin-like protein (COTL1) were all down-regulated following exposure to H_2O_2 (Fig. 3C). Following treatment with TRF, the down-regulation of COTL1 was reversed in the older group at all three concentrations of TRF. A further two proteins — glia maturation factor gamma (GMFG) and actin-related protein 2/3 complex subunit 5 (ARPC5) — were up-regulated with TRF treatment. GMFG was up-regulated only in the younger group (at 100 µg/ml TRF), whereas ARPC5 was up-regulated only in the older group (200 µg/ml TRF) (Fig. 3C).

Quantitative RT-PCR was performed to confirm the expression of COF-1. However, the resulting relative expression profile of COF-1 (Fig. 4A) was not in agreement with what was observed in 2DE.

3.7. Proteins involved in protein degradation

H_2O_2 exposure down-regulated the expression of proteosome activator complex subunit 1 (PA28 alpha) in the younger group (Fig. 3C). Following treatment with TRF, PA28 alpha was up-regulated in both the younger group (at 100 µg/ml TRF) and the older group (100 and 200 µg/ml TRF). However, results from quantitative RT-PCR did not show any statistically significant difference in relative gene expression of PA28 alpha between the different treatments (Fig. 4D).

3.8. Other proteins that change in expression

3.8.1. Regulatory proteins and initiation factors

Protein S100-A4 (S100A4), galectin (LEG1), myotrophin (MTPN), sorcin (SORCN) and myosin regulatory side chain (MLRM) were regulated following exposure to H_2O_2 and treatment with TRF (Fig. 3D).

Exposure to H_2O_2 down-regulated the expression of S100A4 in both groups. LEG1 was only down-regulated in the older group, whereas MTPN was down-regulated in the younger group. Treatment with TRF reversed the effects of H_2O_2 exposure on the expression of S100A4 in the younger group (at 100 µg/ml TRF) but not in the older group. The expression of LEG1 was up-regulated in both the young (at 100 µg/ml TRF) and old (at 100 and 200 µg/ml TRF) groups. There was no change in the expression of MTPN with TRF treatment. The expression of SORCN and MLRM was affected by TRF treatment only in the younger group where SORCN was up-regulated (at 100 µg/ml TRF) and MLRM was down-regulated (at 200 µg/ml TRF).

The expression of eukaryotic translation initiation factor 5A-1 (IF5A1) remained unchanged with exposure to H_2O_2 but was up-regulated with TRF treatment in the younger group (at 100 µg/ml TRF; Fig. 3E).

3.9. Others

The expression of SH3 domain-binding glutamic-acid-rich-like protein was down-regulated with exposure to H_2O_2 (Fig. 3E). Treatment with TRF did not affect its expression. Following exposure to H_2O_2, the expression of β-2 microglobulin (B2MG) remained unchanged. TRF treatment up-regulated B2MG expression in the older group (at 100 and 200 µg/ml TRF).

4. Discussions

The presence/absence of a protein and/or its isoforms may represent events occurring within a cell, which, in turn, correspond to cellular functions. Thus, identifying proteins whose expressions were affected by H_2O_2 within the two different age groups and how they changed with TRF treatment may give us insights into the events that lead to the manifestations of the effects of H_2O_2 and the protective effects of TRF.

In terms of change in protein expression, H_2O_2 exposure affected the older group less when compared to the younger group. This suggested a decreased sensitivity or susceptibility to oxidative stress-induced events. A similar observation has previously been reported by Monti et al. [28]. They showed that oxidative stress-induced apoptosis in peripheral blood mononuclear cells occurred less readily in samples from healthy older subjects. It is possible that this was a result of adaptation to a lifelong exposure to ROS.

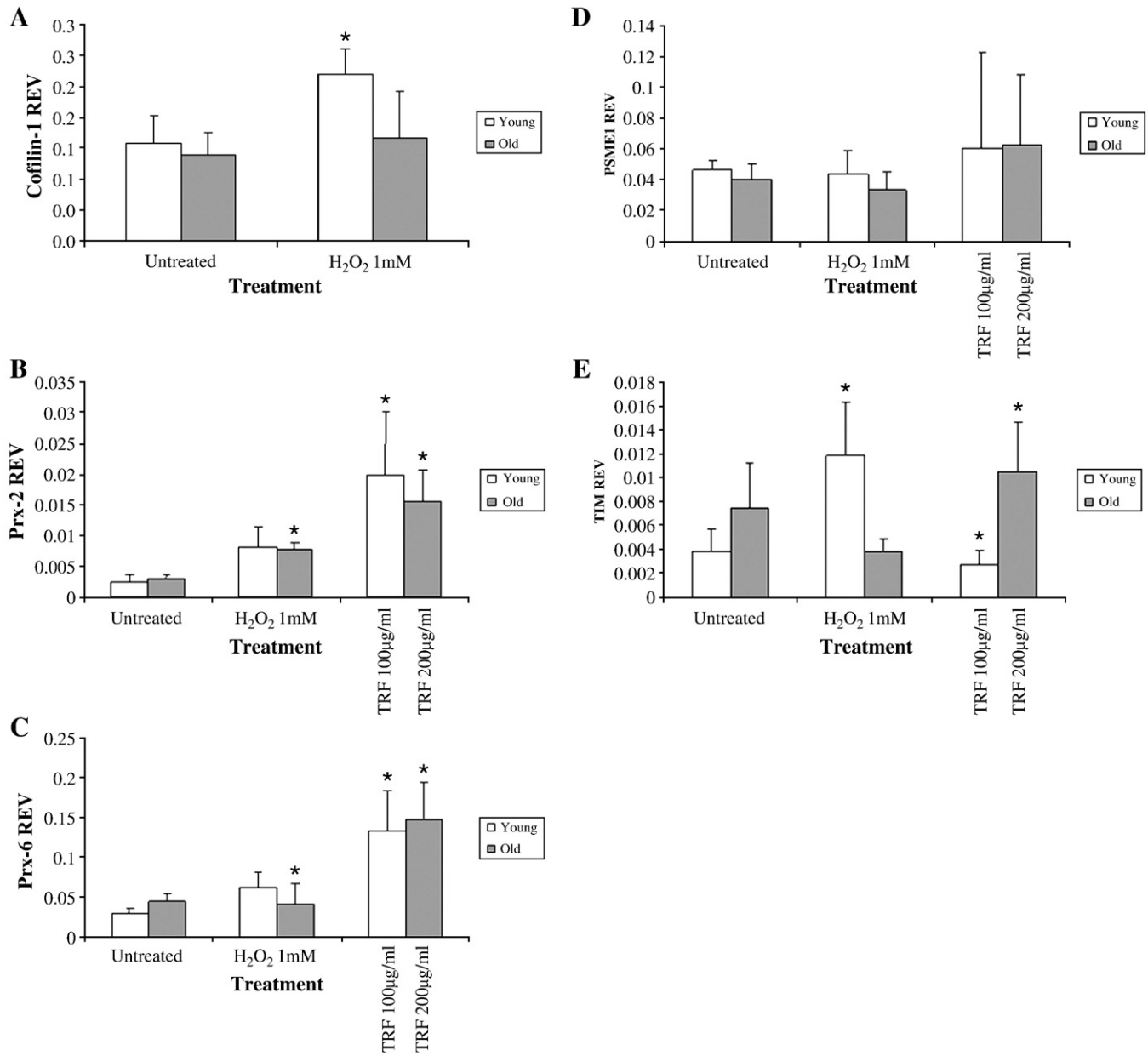

Fig. 4. Results of quantitative RT-PCR showing relative expression of COF-1, PRDX-2, PRDX-6, PA28 alpha and TIM. Total mRNA was extracted from lymphocytes that were untreated (control), H_2O_2-treated and TRF treated from both age groups. Quantitative RT-PCR was then performed using primers described in Table 1. (A) Effect of H_2O_2 exposure on the relative expression of Cofilin-1. (B) Effect of H_2O_2 exposure and TRF treatment on the relative expression of PRDX-2. (C) Effect of H_2O_2 exposure and TRF treatment on the relative expression of PRDX-6. (D) Effect of H_2O_2 exposure and TRF treatment on the relative expression of PA28 alpha. (E) Effect of H_2O_2 exposure and TRF treatment on the relative expression of TIM for both groups. Relative intensities of all genes of interest were determined by using β-actin as an internal standard. Results represent the mean±S.D. for three experiments. *P<.05.

4.1. Proteins that are regulated with H_2O_2 exposure and TRF treatment

4.1.1. Proteins involved in metabolism

Fewer metabolic proteins appear to change in the older group with TRF treatment. These metabolic proteins were all of mitochondrial origin. Mitochondria decay [29] and a decreased rate of protein synthesis in the mitochondria [30] have been shown to occur during the aging process. Thus, the observation that fewer metabolic proteins change in expression in the older group may be attributed to this decreased rate of protein synthesis and increased mitochondrial decay.

TIM is a key enzyme of the glycolytic pathway that catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. It has been shown that posttranslational modification of glycolytic enzymes is an intracellular sensor for oxidative stress [31]. Oxidizing agents such as H_2O_2 have been shown to carbonylate and induce polypeptide degradation in glycolytic enzymes [32]. Such posttranslational modifications may lead to an apparent down-regulation of TIM as the native form is being modified. However, in our analysis, we did not identify an up-regulated protein corresponding to a different/modified isoform of TIM. ECH catalyzes the second step in the breakdown of fatty acids or the second step of β-oxidation in fatty acid metabolism. It generates energy in the form of NADH, which can be converted into ATP that is utilized as a source of energy for the cell during cell damage. The remaining three proteins are all involved in energy production; the change in expression observed suggested that there is a change in metabolic processes and/or energy utilization within the lymphocytes following H_2O_2 exposure and TRF treatment.

4.2. Stress-related proteins

PRDX are a class of thiol-specific antioxidant proteins that utilize conserved cysteine residues to reduce hydrogen peroxide and other organic peroxides. Low levels of the reduced form of PRDX and high levels of ROS may trigger oxidative stress that lead to apoptosis in human lymphocytes. These proteins have received a great deal of attention recently owing to their role in regulating hydrogen peroxide levels and as an intracellular signaling molecule common to many cytokine-induced signal transduction pathways [33]. All types of PRDX share the same catalytic mechanism in which the active site cysteine is oxidized to sulfenic acid with peroxide as a substrate [34]. A second conserved cysteine at the C-terminal end of the other subunit then reacts with the sulfenic acid to form a disulfide bridge. Reduction by thioredoxin (Trx) regenerates PRDX and completes the cycle. Trx is, in turn, regenerated by Trx reductase, with reducing equivalents derived from nicotinamide adenine dinucleotide phosphate (NADPH) [35]. However, the exact molecular mechanism of PRDX regulation remains unclear.

The down-regulation of PRDX observed here may be due to posttranslational modification brought about by overoxidation through its scavenging activity towards H_2O_2. This reaction causes conversion of its active site cysteine into cysteic acid, thereby adding a negative charge to the protein causing a change in its pI [36]. This shift in isoelectric point (pI) would make the reduced form of PRDX appear to be absent or down-regulated (as observed in our 2DE analysis). However, we did not identify an up-regulated protein corresponding to a different isoform of PRDX in any of our analysis.

The apparent up-regulation of PRDX following TRF treatment, on the other hand, may be due to the fact that TRF itself is an antioxidant. Its presence reduces the oxidative burden on PRDX thus, preventing the occurrence of posttranslational modification. This, in turn, would lead to the apparent up-regulation of these proteins when compared to H_2O_2 challenged cells. However, results from RT-PCR showed that the relative messenger RNA (mRNA) levels of PRDX increased with TRF treatment suggesting that there may indeed be an increase in PRDX expression.

The expression of SODC and LGUL was also found to be regulated. SODC eliminates free radicals produced within cells [37], whereas LGUL catalyzes the conversion of hemimercaptal formed from methylglyoxal and glutathione to *S*-lactoylglutathione.

4.3. Structural and structure-related proteins

COF1 controls reversible actin polymerization and is the major component of intracellular and cytoplasmic actin rods. CAP1 directly regulates actin filament dynamics and has been implicated in a variety of complex developmental and morphological processes such as mRNA localization and the establishment of cell polarity. The remaining three regulated proteins — COTL1, GMFG and ARPC5 — are all associated with the regulation of actin polymerization.

Actin is highly conserved and is involved in various types of cell motility and is ubiquitously expressed in all eukaryotic cells. It has been shown to have roles in aging and apoptosis [38]. It has been reported that exposure to ROS caused a remodeling of the actin cytoskeleton [39]. The changes in protein expression observed here would suggest that a remodeling of actin may be taking place.

Actin itself, however, was not shown to be differentially expressed. It is likely that although there is a remodeling of the actin cytoskeleton and a change in actin polymerization, the total actin constituent of cells under oxidative stress and treatment with TRF remained constant.

4.4. Proteins involved in protein degradation

PA28 alpha has been implicated in immunoproteasome assembly and is required for efficient antigen processing. The PA28 activator complex enhances the generation of class I binding peptides by altering the cleavage pattern of the proteasome. The proteasome itself is a major protein-degrading enzyme, which catalyzes degradation of oxidized and aged proteins and signal transduction factor and cleaves peptides for antigen presentation. It exists in the equilibrium of 28s and 20s particles [40]. Ethanol has been shown to cause alterations in proteasome function depending on the oxidative stress levels. Low oxidative stress induces proteasomal activity, while high levels of oxidative stress will reduce it. It has been proposed that the modulation of proteasome activity is related to oxidative modification of proteasomal proteins by primary and secondary products derived from oxidation of the stress inducer [41].

4.5. Other proteins that change in expression

4.5.1. Regulatory proteins and initiation factors

LEG1 has been shown to have functional roles in the regulation of apoptosis, cell proliferation and cell differentiation [42]. MTPN have potential roles in cerebral morphogenesis. SORCN and MLRM are both involved in the regulation of smooth muscle contractility. How these proteins are related to the effects of TRF is, however, unclear.

IF5A1 has multiple roles in the cell such as involvement in the mediation of apoptosis [43] and the regulation of neuronal cell growth [44].

5. Conclusions

Based on our observations here, we draw the following conclusions. In terms of change in protein expression, the younger group is more susceptible to changes due to external stimuli compared to the older group. There was no clear dose–response relationship between proteins that changed in expression and the different concentrations of TRF used. Finally, among the proteins that changed in expression with TRF treatment were the peroxiredoxins (PRDXs). PRDXs are involved in the regulation of oxidative stress. H_2O_2 exposure down-regulated PRDX and treatment with TRF reversed this effect. This was confirmed by quantitative RT-PCR. The fact that both the protein expression and mRNA were up-regulated with TRF treatment suggested that TRF directly influenced the expression dynamics of the PRDX, thus improving the cells ability to resist damage caused by oxidative stress.

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