Available online at: http://brsj.cepsbasra.edu.iq

ISSN –1817 –2695

Received 14-1-2019, Accepted 18-2-2019

GARLIC AND TEA TREE OIL COMPOUNDS PROMOTE OXIDATION OF THE TYPICAL 2-CYS PEROXIREDOXIN PROTEINS IN JURKAT T-LYMPHOCYTES

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ABSTRACT

The mammalian typical 2-Cys peroxiredoxin (Prx) proteins have emerged as important sensors and transducers in the cellular response to oxidative stress. Plant natural products have been shown to have both pro- and anti-oxidant effects but their exact mechanisms of action are not fully understood. Here we show that selected compounds from garlic and tea tree oils promote either oxidised dimer formation or hyperoxidised monomer formation in the typical 2-Cys Prx proteins. Diallyl disulphide (DADS) and diallyl trisulphide (DATS) were the main constituents of garlic oil and showed similar effects to their parent oil but DATS was more effective than DADS that indicating to the importance of the number of sulphur atoms. Tea tree oil was a less potent promotor of the oxidation of the Prx proteins than garlic oil. In contrast, terpinen-4-ol, the major constituent of tea tree oil, was a very effective promotor of the oxidation of the Prx proteins. We show that the results could partly, but not entirely, be explained by differences in the effects of the tested compounds on the activity of thioredoxin reductase (TrxR), the enzyme required for the regeneration of the reduced monomer form of the Prx proteins from their oxidised dimer form. The results are discussed in terms of the involvement of the typical 2-Cys Prx proteins in the cellular response oxidative stress.

Keywords: *Typical 2-Cys peroxiredoxin, Garlic oil, Diallyl disulphide, Diallyl trisulphide, Tea tree oil, Terpinen-4-ol*

1. INTRODUCTION

The typical 2-Cys peroxiredoxin (Prx) proteins are enzymes that catalyse the reductive decomposition of hydrogen peroxide (H_2O_2) and various organic peroxides to less reactive products [1, 2]. They were first described as antioxidant enzymes that protect cells against oxidative stress but they are now thought to be involved in H_2O_2-mediated cell signalling as well [3]. In mammals, there are four

different isoforms, Prx1 located in the cytosol or nucleus, Prx2 located in the cytosol or associated with the erythrocyte membrane, Prx3 located in the mitochondria and Prx4 located in the cytosol or the endoplasmic reticulum [1, 4]. All four isoforms are characterised by interacting active site Cys residues located on separate subunits known as the peroxidatic Cys (C_P) and the resolving Cys (C_R) [1]. During catalysis, C_P in its reduced/thiol state (C_P-SH) attacks the peroxide substrate and becomes oxidised to a Cys-sulphenic acid residue (C_P-SOH). C_P-SOH then forms a disulphide bond with C_R on a separate subunit. Subsequently, the disulphide bonded is broken with the help of thioredoxin (Trx) and Trx reductase (TrxR) and C_P-SH is regenerated ready to begin another cycle of catalysis. The cycle described above occurs in the presence of low concentrations of H_2O_2 but in the presence of high H_2O_2 concentrations, C_P-(SOH) can react with a second molecule of H_2O_2 and become hyperoxidised to a Cys sulphinic acid residue (C_P-SO_2H) [1, 5]. C_P-SO_2H cannot react with C_R and as a result the hyperoxidised form of the enzyme has lost its peroxidase activity. The peroxidase activity is only very slowly restored with the help of a family of enzymes known as the sulfiredoxins [6, 7]. This unusual behaviour has been proposed to allow H_2O_2 to accumulate to act as a cell signalling molecule [8].

When C_P is in its reduced/thiol state (C_P-SH) or its hyperoxidised/sulphinic acid state (C_P-SO_2H), Prx1, 2 and 3 run as monomers (at approximately 22 kDa) on non-reducing polyacrylamide (NR-PAGE) gels whereas when C_P-SH becomes oxidised to C_P-SOH and forms a disulphide bond with C_R, these proteins run as dimers (at approximately 44 kDa) [9]. The reduced and hyperoxidised monomer forms of these enzymes can be readily distinguished using alkylating agents that block the oxidation of the sulfhydryl group of C_P-SH during cell extraction and antibodies that recognise C_P in its hyperoxidised state (C_P-SO_2H). Thus, employing NR-PAGE and appropriate cell extraction conditions and antibodies, the redox/oligomerization state of the typical 2-Cys Prx proteins can be used as a sensitive endogenous indicator of oxidative stress [10].

Previous studies have shown that treatment of Jurkat-T lymphocytes, a model for human cancer cells, with low concentrations of H_2O_2 (< 20 µM) promotes conversion of the reduced monomer form of Prx1, 2 and 3 to the oxidised dimer form while treatment with higher concentrations (≥ 20 µM) promotes conversion of the oxidised dimer form to the hyperoxidised monomer form [9]. Studies have also shown that plant compounds such as phenethyl isothiocyanate, found in cruciferous vegetables (e.g. broccoli), can promote oxidised dimer formation in the Prx3 protein in Jurkat T-lymphocytes and promyelocytic leukemia cells (the human HL-60 cell line) (Brown et al., 2010; Brown et al., 2008). Allyl sulphides found in garlic (*Allium sativum*) oil, such as diallyl disulfide (DADS) and diallyl trisulfide (DATS), and terpenoids found in tea tree (*Melaleuca alternifolia*) oil, such as terpinen-4-ol, have both pro- and anti-oxidant effects but their exact mechanisms of action are not fully understood [11-13]. Thus, the aims of the present study were to investigate the effects of garlic oil, DADS, DATS, tea tree oil and terpinen-4-ol on the *in vivo* redox/oligomerization state of the Prx1, 2 and 3 proteins in Jurkat-T lymphocytes and also to investigate whether the oils/compounds exerted their effects on the typical 2-Cys Prx proteins by inhibiting the activity of TrxR, the enzyme required to regenerate the reduced/thiol state of C_P from its disulphide-bonded state.

2. MATERIALS AND METHODS

2.1 Jurkat T-lymphocyte culture

All cell culture reagents were purchased from Sigma-Aldrich. The cells used in this study were from the Jurkat T-lymphoma cell line. Unless otherwise stated, they were cultured at a temperature of 37^{o}C in an atmosphere of 5% (v/v) CO_2 in air in a medium consisting of RPMI 1640 culture medium supplemented with 10% (v/v) foetal bovine serum (FBS, Life Technologies), 100 units ml^{-1} penicillin/streptomycin and 4 mM L-glutamine. This medium is hereafter referred to as the RPMI culture medium. The number of viable cells was determined by staining the cells with Trypan blue dye and then counting them using a haemocytometer [14].

2.2 Investigating the effects of various pro-oxidant treatments on the in vivo redox/oligomerization state of the typical 2-Cys Prx proteins in Jurkat T-lymphocytes

The effects of hydrogen peroxide (37%, v/v; Thermo Fisher Scientific Pty Ltd), garlic (*Allium sativum*) oil (100%, v/v; Mystic Moments, Fordingbridge, Hampshire, United Kingdom), diallyl disulfide (DADS, 80%, v/v; Sigma-Aldrich), diallyl trisulfide (DATS, 95%, v/v; Cayman Chemical), tea tree (*Melaleuca alternifolia*) oil (100%, v/v; Integria Healthcare Australia Pty Ltd) and terpinen-4-ol (97%, v/v; VWR International Pty Ltd, Australia) on the redox/oligomerization state of the typical 2-Cys proteins in Jurkat T-lymphocytes were investigated. Stock solutions of the test substances, except H_2O_2, were prepared in dimethyl sulfoxide (DMSO). Prior to each experiment, the stock solutions and H_2O_2 were diluted in Jurkat cell culture medium and added to the appropriate wells of a 6-well plate. This was followed by the addition of 4 x 10^6 Jurkat T-lymphocytes per well. The cells maintained as described above had been pelleted by centrifugation at 1,500 *g* for 1 min and then resuspended in Jurkat cell culture medium and counted prior to being added to the wells. The total volume of liquid in each well was 4 ml and the maximum concentration of DMSO was 0.14% (v/v), which we had previously shown not to affect the proliferation of the cells (data not shown). The cells were incubated for 10 min at 37^{o}C in 5% (v/v) CO_2 in air and then harvested by centrifugation at 1,500 *g* for 1 min. Following this incubation, crude cell extracts were prepared as described below. The experiments were repeated four times for H_2O_2 and garlic oil, three times for DADS, twice for DATS and three times for tea tree oil and terpinen-4-ol. The results of representative experiments are shown here.

***2*.3 Preparation of Jurkat T-lymphocyte extracts for the determination of the relative amounts of the reduced, oxidised and hyperoxidized forms of the typical 2-Cys Prx proteins**

The cell extracts were prepared essentially as described by Cox *et al*. [15]. In order to prevent artifactual oxidation of the sulfhydryl (–SH) group of C_P-SH during cell extraction, it is important to 'block' this group using an alkylating agent such as N-ethylmaleimide (NEM). Oxidation of the –SH group of C_P-SH in the typical 2-Cys Prx proteins occurs very rapidly even in the presence of only trace amounts of H_2O_2 [15]. Thus, the harvested cells were incubated for 15 min in a buffer containing 40 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (pH 7.4), 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 2 mM phenylmethanesulfonyl fluoride (PMSF) and 100 mM NEM. Following this, the cells were disrupted by the addition of 1% (w/v) 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate (CHAPS) to the incubation mixture. The cell extract was then clarified by centrifugation at 15,000 *g* for 5 min at 4^{o}C and the supernatant, which contained the Prx proteins, was stored at -80^{o}C until it could be analysed. The protein concentration in the cell extracts was determined using the BCA protein assay (Thermo Fisher Scientific).

2.4 Separation of the reduced, oxidised and hyperoxidised forms of the typical 2-Cys Prx proteins using non-reducing polyacrylamide gel electrophoresis

The reduced, oxidised and hyperoxidised forms of the typical 2-Cys Prx proteins were separated utilizing non-reducing polyacrylamide gel electrophoresis (NR-PAGE) according to the protocol described by Cox, Winterbourn [15]. When subjected to NR-PAGE, the reduced and hyperoxidised forms of Prx1, 2 and 3 run as monomers with a molecular weight of approximately 22 kDa whereas the oxidised form runs as a dimer with a molecular weight of approximately 44 kDa [9]. These different sizes can be resolved using NR-PAGE. Thus, the extracts prepared as described above were first of all mixed with an equal volume of loading buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol and 0.025% (w/v) bromophenol blue. These

mixtures were then loaded into the wells of a NR-PAGE gel (45 µg protein per well). The NR-PAGE gels consisted of a stacking gel containing 0.625 M Tris-base (pH 6.8), 4% (w/v) acrylamide:N,N′-methylenebisacrylamide (37.5:1), 0.125% (v/v) N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and 0.05% (w/v) ammonium persulphate and a resolving gel containing 0.375 M Tris-base (pH 8.8), 15% (w/v) acrylamide:N,N′-methylenebisacrylamide (37.5:1), 0.1% (v/v) TEMED and 0.05% (w/v) ammonium persulphate. The gels were run in a buffer containing 25 mM Tris-base (pH 8.3), 192 mM glycine and 0.1% (w/v) SDS in a Bio-Rad Mini-PROTEAN®II electrophoresis apparatus set to deliver 200 V. At the end of the run, the gels were either stained for protein using Coomassie Blue or used for immunoblotting (see below). The Coomassie Blue protein staining solution contained 0.1% (w/v) Coomassie Brilliant Blue R-250 stain, 50% (v/v) methanol and 10% (v/v) glacial acetic acid. The destaining solution contained 50% (v/v) methanol and 10% (v/v) glacial acetic acid. Imaging of the gels was performed using a Bio-Rad Gel Doc™ EZ Imager.

2.5 Detection of the reduced, oxidised and hyperoxidised forms of the typical 2-Cys Prx proteins using immunoblotting

The reduced, oxidised and hyperoxidised forms of the typical 2-Cys Prx proteins separated as described above were transferred to a nitrocellulose membrane (Bio-Rad) using a Bio-Rad Mini Trans-Blot® apparatus set to deliver a constant current of 200 mA for 2 hours. The transfer buffer contained 50 mM Tris-base, 380 mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol. Following the transfer, the membrane was blocked for one hour at room temperature in a blocking buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2% (v/v) Tween-20 and 5% (w/v) skim milk powder. Subsequently, the blocked membrane was incubated overnight at 4°C in the presence of one of three different kinds of primary antibodies diluted either 1:1000 or 1:2000 in blocking buffer. The primary antibodies, which were rabbit polyclonal antibodies, recognised different isoforms of the Prx proteins or the hyperoxidised state of C_P. The first set of antibodies, which we refer to as the anti-(typical 2-Cys Prx) antibodies, had been produced by immunizing a rabbit with a full-length, recombinantly-expressed and highly purified typical 2-Cys Prx protein which showed 81%, 78%, 64% and 72% identity at the amino acid level to the human Prx1, 2, 3 and 4 proteins, respectively [16]. We expected this set of antibodies to recognise all four isoforms of the typical 2-Cys Prx proteins. The second set of antibodies, which we refer to as the anti-(Prx1) antibodies, had been produced by immunising a rabbit with the peptide PLVSDPKRTIAQDY which is unique to the human Prx1 protein (Antibody Technology Australia Pty Ltd). We expected this set of antibodies to recognise only Prx1. The third set of antibodies which we refer to as the anti-(peroxiredoxin-SO_3) antibodies had been produced by immunising a rabbit with a synthetic sulfonylated peptide with an amino acid sequence corresponding to that of the active site of the human typical 2-Cys Prx proteins in its hyperoxidised state (Abcam®, catalogue number ab16830). We expected this set of antibodies to recognise only the hyperoxidised form of the typical 2-Cys Prx proteins. Following the overnight incubation with the primary antibody preparation, the membrane was washed 5 x 5 minutes with washing buffer (blocking buffer minus the milk powder) and then incubated for 1-2 hours at room temperature with a secondary antibody preparation diluted 1:1000 in blocking buffer. The secondary antibody preparation contained goat anti-(rabbit IgG) conjugated to horseradish peroxidase (Rockland Immunochemicals for Research). Any cross-reacting proteins were detected using the SuperSignal® West Pico Chemiluminescent substrate kit (Thermo SCIENTIFIC) and images of the blots were made using a Bio-Rad ChemiDoc™ MP imaging system.

2.6 Thioredoxin reductase assay

Thioredoxin reductase (TrxR) is required to regenerate the reduced monomer form of the typical 2-Cys Prx proteins from their oxidised dimer form [3]. For the TrxR assays, Jurkat T-lymphocytes were seeded into 75 cm^2 cell culture flasks (Corning) at a density of 5 x 10^5 cells per

flask in 30 ml Jurkat cell culture medium. Following this, the cells were cultured for a period of approximately 60 hours at 37^{o}C in 5% (v/v) CO_2 in air. At the end of the culture period, the cells were pelleted by centrifugation at 1,500 *g* for 1 min and then washed twice in phosphate buffered saline (PBS, pH 7) before being disrupted in a lysis buffer containing 40 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (w/v) CHAPS and 2 mM PMSF. Insoluble material was removed by centrifugation at 15,000 *g* for 5 min at 4^{o}C and the supernatant was retained for analysis. Cell extracts prepared in this way were stored at -80^{o}C until they could be analysed for TrxR activity. TrxR activity was assayed, in the presence and absence of various concentrations of DADS, DATS or terpinen-4-ol using a commercially available assay kit (Sigma-Aldrich®, catalogue number CS0170). The kit coupled the oxidation of NADPH to the reduction of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) to 5-thio-2-nitrobenzoic acid (TNB). The assays were run in triplicate at 25^{o}C in 96-well plates with a total assay volume of 200 µl per well and 120 µg of Jurkat T-lymphocyte protein per assay. The increase in absorbance due to the production of TNB was monitored at 412 nm using a FLUOstar® plate reader (BMG Labtech Pty Ltd). TrxR enzyme activity was calculated by determining the difference in TNB production rate in the presence and absence of a specific inhibitor of TrxR activity supplied with the kit. The protein concentration in the cell extracts was determined using the BCA protein assay (Thermo Fisher Scientific). The concentration of each test substance required to inhibit TrxR activity by 50% (IC_{50}) was calculated as previously described [17]. In brief, a linear trend line was fitted to a plot of % inhibition of the TrxR activity (relative to the control) against the concentration of the test substance and the equation of the line thus generated was used to determine the test substance concentration that gave 50% inhibition of the TrxR activity. All experiments were repeated twice, i.e., with different batches of cells.

2.7 Statistical analyses

Statistical analyses were conducted using the IBM SPSS Statistics 19 software package. The data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* multiple comparisons test. Differences were considered to be significant when $p < 0.05$.

3. RESULTS

3.1 Effects of pro-oxidant treatments on the total soluble proteins extracted from the Jurkat T-lymphocytes and on the in vivo redox/oligomerization state of the typical 2-Cys Prx proteins in these cells

Jurkat T-lymphocytes were treated with various concentrations of H_2O_2, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol and the effects on the total soluble proteins extracted from the cells (Figure 1) and on the *in vivo* redox/oligomerization state of the typical 2-Cys Prx proteins (Figure 2) were determined. Treatment of the cells with H_2O_2, garlic oil, DADS or DATS, regardless of the concentration, had no effect on the total soluble proteins extracted from the cells (Figures 1A-D). However, at the highest concentrations tested, treatment with tea tree oil or terpinen-4-ol caused apparent protein denaturation. This was evidenced by the loss of most of the smaller molecular weight proteins (less than approximately 40 kDa) from the gels stained for total protein when the cells had been treated with either 0.24% (v/v) tea tree oil or 9.0 mM terpinen-4-ol (Figures1E-F).

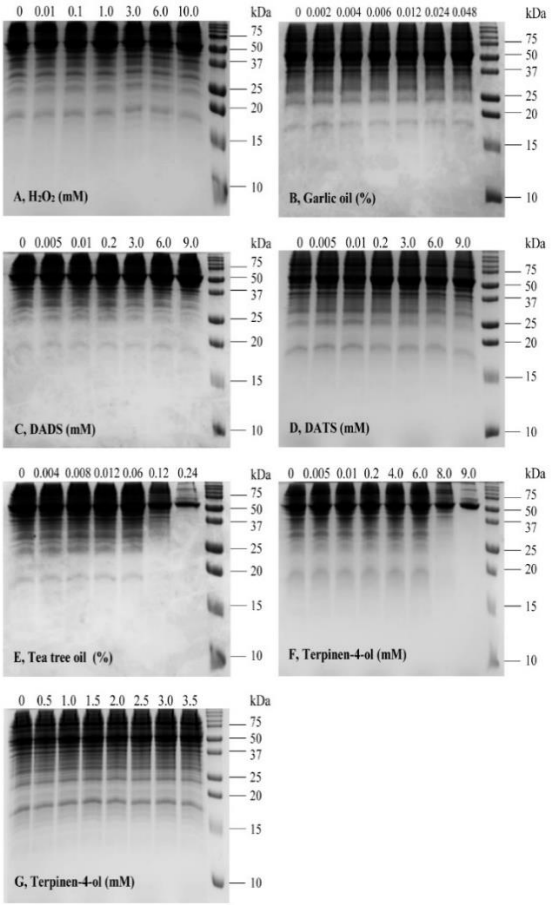

Figure 1. The effects of treating Jurkat T-lymphocytes with H_2O_2, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol on the total soluble proteins extracted from the cells. The proteins (30 µg protein per well) were separated using NR-PAGE and then stained using Coomassie Blue.

Figure 2 shows the effects of the various oils/chemicals on the redox/oligomerization state of the typical 2-Cys Prx proteins. In all cases, the control cells contained approximately equal amounts of the reduced monomer and oxidised dimer forms of these proteins. When the cells were treated with increasing concentrations of H_2O_2, there was increasing conversion of the oxidised dimer form of the proteins to the hyperoxidised monomer form (Figure 2A). In contrast, when the cells were treated with increasing concentrations of garlic oil, DADS or DATS, there was increasing conversion of the reduced monomer form to the oxidised dimer form (Figures 2B-D). DATS was more effective than DADS in promoting oxidised dimer formation. This reinforces the conclusion that the greater the number of sulphur atoms in the allyl sulphides, the greater their effectiveness.

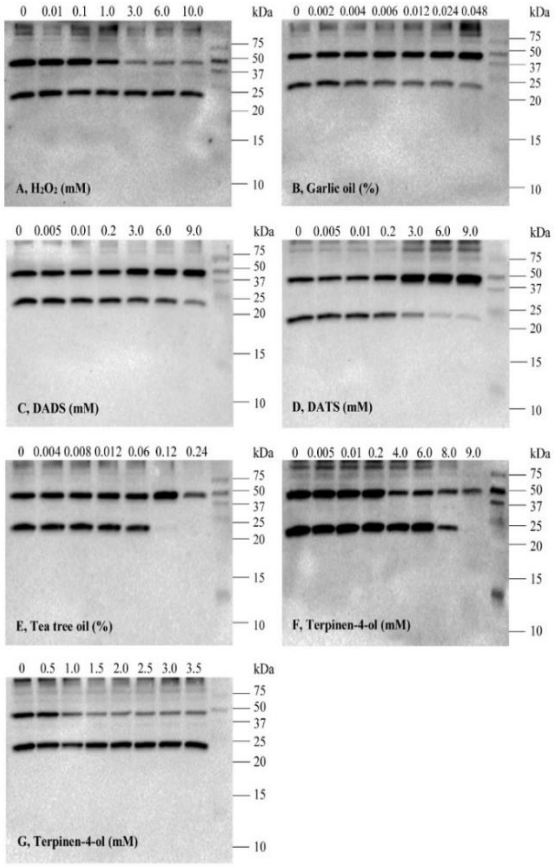

Figure 2. The effects of treating Jurkat T-lymphocytes with H_2O_2, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol on the redox/oligomerization state of the typical 2-Cys Prx proteins. Gels identical to those shown in Figure 1 were run and the proteins thus separated were subjected to immunoblot analysis using the anti-(typical 2-Cys Prx) antibodies (see antibody details).

Unlike garlic oil, tea tree oil had no discernible effect on the redox/oligomerization state of the typical 2-Cys Prx proteins (Figure 2E). At all of the tea tree oil concentrations, except for the highest, there were approximately equal amounts of the monomer and dimer forms of the Prx proteins present. The loss of the monomer form in the cells treated with the highest tea tree oil concentrations (0.12 and 0.24% (v/v)) was probably due to the general loss of smaller molecular weight proteins that occurred in the presence of these high concentrations (Figure 1E). As stated above, we assume that this was due to protein aggregation or denaturation at high tea tree

oil concentrations.

In contrast to its parent oil, terpinen-4-ol had a very strong oxidising effect on the typical 2-Cys Prx proteins (Figures 2F-G). With increasing concentrations of terpinen-4-ol, there was increasing conversion of the oxidised dimer form of the typical 2-Cys Prx proteins to a monomer form. Initially, we were unsure whether this was the reduced monomer or the hyperoxidised monomer. Thus, we tested this using antibodies that specifically recognise the hyperoxidised monomer (Figure 3). As a control, we also tested the effects of exposing the cells to high concentrations of H_2O_2. This experiment showed that terpinen-4-ol, like H_2O_2, promoted conversion of the typical 2-Cys Prx proteins to their hyperoxidised monomer form.Thus, terpinen-4-ol had a stronger oxidising effect than either DADS or DATS and was similar in its effects to high concentrations of H_2O_2. In summary, whereas DADS and DATS promoted oxidised dimer formation in the typical 2-Cys Prx proteins, terpinen-4-ol promoted hyperoxidised monomer formation.

3.2 Effects of pro-oxidant treatments on the redox/oligomerization state of Prx1 in Jurkat T-lymphocytes

To confirm our results, we repeated a subset of the experiments described above but this

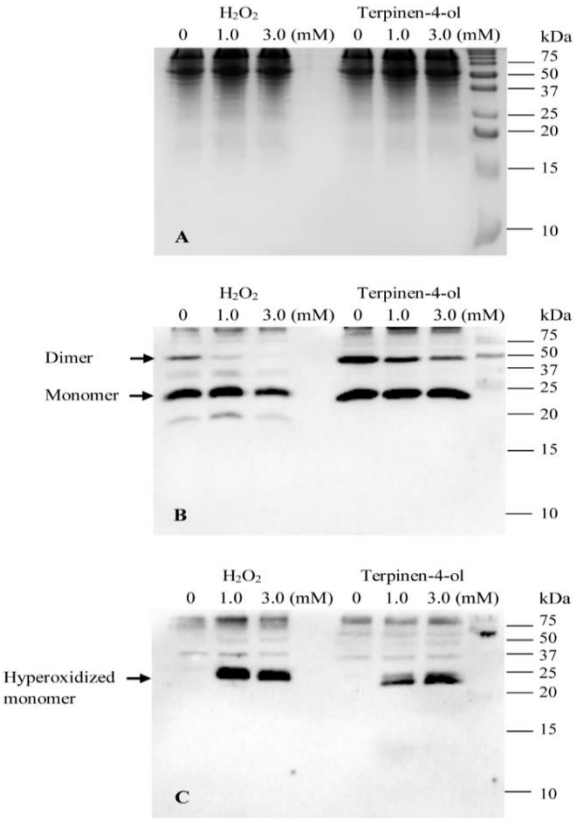

Figure 3. The effects of treating Jurkat T-lymphocytes with H_2O_2 or terpinen-4-ol on the redox/oligomerization state of the typical 2-Cys Prx proteins. The total soluble proteins were extracted from the cells and separated using NR-PAGE. In panel A, the proteins were stained using Coomassie Blue. In panel B, the proteins were subjected to immunoblot analysis using the same antibodies as described in the caption to Figure 2. In panel C, the hyperoxidised form of the typical 2-Cys Prx proteins was detected using commercially available anti-(Prx-SO_3) antibodies (see antibody details).

time using antibodies that were specific for Prx1 (Figure 4). For H_2O_2, garlic oil, DADS and DATS, the results were the same regardless of which type of antibodies were used. In contrast, for tea tree oil and terpinen-4-ol, the results were somewhat different. When using the antibodies expected to recognise all of the four different isoforms of the typical 2-Cys Prx proteins, tea tree oil appeared have no effect on the redox/oligomerization state whereas terpinen-4-ol promoted hyperoxidised monomer formation. In contrast, when using the antibodies that recognised only Prx1, both tea tree oil and

terpinen-4-ol promoted hyperoxidised monomer formation. This is consistent with the data shown in Figure 3 where terpinen-4-ol promoted hyperoxidised monomer formation. Thus, we conclude that tea tree oil and terpinen-4-ol promote hyperoxidized monomer formation in Prx1 whereas garlic oil, DADS and DATS promote oxidised dimer formation.

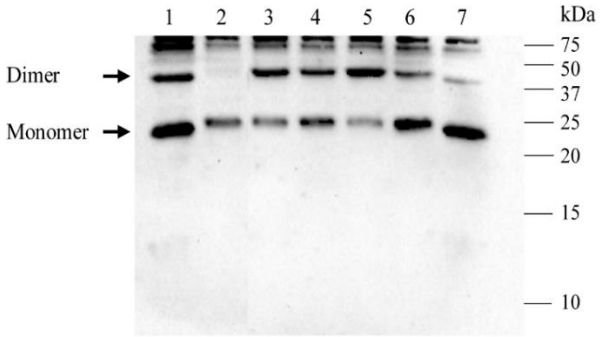

Figure 4. The effects of treating Jurkat T-lymphocytes with H_2O_2, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol on the redox/oligomerization state of Prx1. The total soluble proteins were extracted from the cells and then separated using NR-PAGE. Prx1 was detected using antibodies raised against the peptide PLVSDPKRTIAQDY which is unique to human Prx1. Lane 1 contains the extract from the control cells. Lanes 2 through 7 contain extracts from cells treated with 3 mM H_2O_2 (Lane 2), 0.048% (v/v) garlic oil (Lane 3), 3 mM DADS (Lane 4), 3 mM DATS (Lane 5), 0.06% (v/v) tea tree oil (Lane 6) and 3 mM terpinen-4-ol (Lane 7).

3.3 Effects of selected pro-oxidants on TrxR enzyme activity in Jurkat T-lymphocytes

The regeneration of the reduced monomer form of the typical 2-Cys Prx proteins from their oxidised dimer form requires TrxR activity. Thus, the differing effects of the various oils/compounds on the redox/oligomerization state of the typical 2-Cys Prx proteins could be due to differing effects on TrxR activity. Greater inhibition of TrxR activity should lead to a greater relative abundance of the oxidised dimer or hyperoxidised monomer form of the typical 2-Cys Prx proteins. Thus, we tested the effects of DADS, DATS and terpinen-4-ol on TrxR enzyme activity in extracts of Jurkat-T lymphocytes to determine whether there was a correlation between inhibition

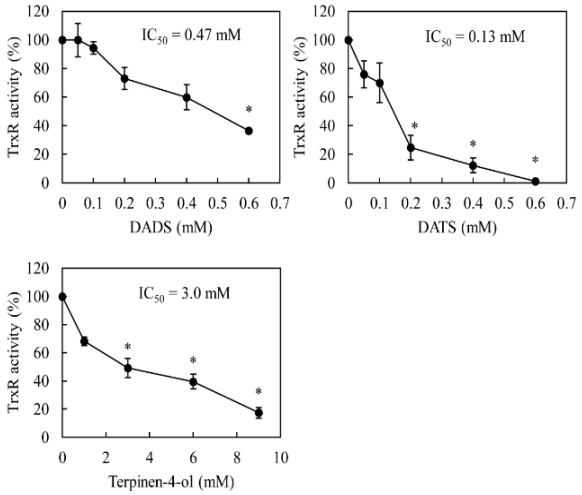

Figure 5. The effects of DADS, DATS and terpinen-4-ol on TrxR enzyme activity in crude extracts from Jurkat T-lymphocytes. The data are expressed as % of the untreated control and each data point is the mean value for 3 replicate wells. The vertical bars represent ± standard error of the mean (n = 3). A star indicates a significant difference from the untreated control ($p < 0.05$).

of TrxR activity and the promotion of the oxidation/hyperoxidation of the typical 2-Cys Prx proteins (Figure 5). The most effective inhibitor of TrxR activity was DATS (IC_{50} = 0.13 mM) followed by DADS (IC_{50} = 0.47 mM) and then terpinen-4-ol (IC_{50} = 3.0 mM). This would suggest that DATS should be more effective than DADS in promoting oxidation of the Prx proteins and indeed it is. In contrast, it would suggest that terpinen-4-ol should be less effective at promoting oxidation of the Prx proteins than either DADS or DATS. However, this was not the case. Instead, terpinen-4-ol was considerably more effective at promoting oxidation of the Prx proteins than either DADS or DATS. Thus, it appears that differential inhibition of TrxR activity is not the only explanation for the different effects of these various oil/chemicals on the redox/oligomerization state of the Prx proteins.

4. DISCUSSION

The redox/oligomerization state of the typical 2-Cys Prx proteins can be used as a sensitive endogenous indicator of oxidative stress [10]. It is

known that treatment of Jurkat-T lymphocytes with H_2O_2, one of the first products of oxidative stress, results in either oxidised dimer formation or hyperoxidized monomer formation in the Prx proteins in these cells, depending upon the concentration of H_2O_2 [3]. At low concentrations (< 20 µM) H_2O_2 treatment results in oxidised dimer formation in Prx1, 2 and 3 whereas at high concentrations (≥ 20 µM) it results in hyperoxidised monomer formation [9]. Here we have obtained similar results but the concentrations of H_2O_2 required were somewhat higher than those previously reported. This may be due to slight differences in experimental methods. Broadly speaking though, we have confirmed that low concentrations of H_2O_2 promote oxidised dimer formation in the typical 2-Cys Prx proteins whereas high concentrations promote hyperoxidised monomer formation.

Phytochemicals, such as the allyl sulfides found in garlic oil and the terpenoids found in tea tree oil are known to have both pro- and anti-oxidant effects but it is unknown whether their mechanisms of action involve the typical 2-Cys Prx proteins [12, 13, 18]. Here we have shown that garlic oil and its major constituents DADS and DATS, as well as tea tree oil and one of its major constituents terpinen-4-ol, can promote oxidation/hyperoxidation of the typical 2-Cys Prx proteins in Jurkat T-lymphocytes. Garlic oil was more effective than tea tree oil and DATS was more effective than DADS in these respects. This suggested that the number of sulphur atoms in the molecule is important. It is known that both DADS and DATS can promote H_2O_2 formation via redox cycling and that DATS is more effective than DADS in this respect [13, 19]. This is consistent with our observation that DATS was more effective than DADS in promoting oxidation/dimerization of the Prx proteins in Jurkat T-lymphocytes and it supports the conclusion that the effects of DADS and DATS on these proteins are due to their ability to promote the production of H_2O_2 which reacts readily with C_P in the Prx proteins.

The effects of tea tree oil and terpinen-4-ol on the redox/oligomerization state of the typical 2-Cys Prx proteins are more difficult to explain. Terpinen-4-ol was a very effective promoter of the oxidation of the typical 2-Cys Prx proteins. Whereas DADS and DATS promoted oxidised dimer formation in the typical 2-Cys Prx proteins, terpinen-4-ol promoted hyperoxidised monomer formation. Thus, terpinen-4-ol was more effective at promoting oxidation of the typical 2-Cys Prx proteins than either DADS or DATS. This suggests that terpinen-4-ol is more effective at promoting H_2O_2 formation in the cells or alternatively that it is more effective at inhibiting TrxR. We tested this second hypothesis by examining the effects of DADS, DATS and terpinen-4-ol on TrxR activity in crude extracts of Jurkat T-lymphocytes. Contrary to our hypothesis, terpinen-4-ol was a relatively poor inhibitor of TrxR activity compared with either DADS or DATS. Thus, greater effectiveness in inhibiting TrxR activity does not seem to explain the greater effectiveness of terpinen-4-ol in promoting oxidation of the typical 2-Cys Prx proteins. On the other hand, DATS was more effective than DADS in inhibiting TrxR activity and it was also more effective in promoting oxidation of the typical 2-Cys Prx proteins. It was known that DADS and DATS inhibit TrxR activity but it was not known how these phytochemicals affect the redox/oligomerization state of the typical 2-Cys Prx proteins [20]. Thus, ours is the first report that shows that garlic oil, DADS and DATS promote the oxidation of the typical 2-Cys Prx proteins. Our observation that DATS is more effective than DADS in this respect and also that DATS is more effective at inhibiting TrxR activity suggests that in the case of these allyl sulphides, the capacity to inhibit TrxR activity is indeed linked to the capacity to promote the oxidation of the typical 2-Cys Prx proteins.

Previous studies have shown that phenethyl isothiocyanate (PEITC) and sulforaphane (SFN), cancer chemopreventive compounds found in cruciferous vegetables such as broccoli, are both very effective inhibitors of TrxR activity but yet PEITC is substantially more effective than SFN in promoting the oxidation of Prx3, a mitochondrial typical 2-Cys Prx protein [21]. This led the authors to conclude that differential inhibition of TrxR activity does not completely explain the differences in the effects of these phytochemicals on the redox/oligomerization state of the typical 2-Cys Prx proteins. The results of our experiments

with terpinen-4-ol support this conclusion. Subsequently, the same authors showed that a functional mitochondrial electron transfer chain is required for the oxidation of Prx3 in response to the treatment of cells with either PEITC or auranofin, the latter being a well established inhibitor of TrxR activity [22]. The mitochondrial electron transfer chain is an important source of ROS, including H_2O_2. Thus, the results of these two studies support the conclusion that the oxidation of Prx3 in the presence of either PEITC or auranofin is due to perturbation of the mitochondrial electron transfer chain leading to increased H_2O_2 production which in turn leads to increased Prx3 oxidation. The results of our study suggest that this conclusion can be extended to apply to other typical 2-Cys Prx proteins and also other phytochemicals as well.

Whereas DADS and DATS promoted oxidised dimer formation in the typical 2-Cys Prx proteins, terpinen-4-ol promoted hyperoxidised monomer formation. This suggests that terpinen-4-ol is more effective at increasing the cellular H_2O_2 concentration than either DADS or DATS. Terpinen-4-ol is a monoterpene and therefore very different in its chemical structure to the sulfur-containing DADS and DATS [12, 23]. It is known that monoterpenes inhibit farnesyl transferases and that these enzymes catalyse the transfer of a 15 carbon isoprenyl lipid moiety onto a conserved Cys residue near to the C-terminus of various proteins including protein-tyrosine phosphatases [24]. Protein farnesylation promotes membrane association and contributes to protein-protein interactions. Recently it was shown that the peroxidase activity of Prx1 is lost when it is phosphorylated by certain protein-tyrosine kinases and that this phosphorylation/inactivation occurs only when Prx1 is associated with lipid rafts [25]. The phosphorylation/inactivation of Prx1 can be reversed by protein-tyrosine phosphatases and it is proposed that inactivation of the lipid raft-associated Prx1 allows localised accumulation of H_2O_2 which in turn allows inactivation of the protein-tyrosine phosphatases. Protein tyrosine phosphatases are known to be inactivated as a result of oxidation of their catalytic Cys residue by H_2O_2 and this allows their counterparts, the protein tyrosine kinases, to phosphorylate their targets and thereby propagate various intracellular signals. It could be that inhibition of protein-tyrosine phosphatase association with lipid rafts due to inhibition of their farnesylation by terpinen-4-ol disrupts the interaction between the protein-tyrosine phosphatases and Prx1 such that Prx1 is inactivated by protein-tyrosine kinases and cannot be reactivated. If this occurs, there would be increasing accumulation of H_2O_2 resulting in hyperoxidation of Prx 1, and possibly other typical 2-Cys Prx proteins. This remains to be investigated.

In conclusion, we have shown that garlic oil and its major constituents DADS, and especially DATS, are potent promotors of the oxidation of the typical 2-Cys Prx proteins. We propose that the greater effectiveness of DATS compared with DADS is due to its greater number of sulphur atoms and consequent greater capacity to promote redox cycling and associated H_2O_2 production. Alternatively, or additionally, the greater effectiveness of DATS compared with DADS may be due to its stronger inhibition of TrxR activity. In contrast to DADS and DATS, terpinen-4-ol was a relatively weak inhibitor of TrxR activity and yet was the strongest of all of the tested chemicals in promoting the oxidation of the typical 2-Cys Prx proteins. Thus, an alternative mechanism needs to be invoked to explain the effects of terpinen-4-ol. This requires further investigation.

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مركبات زيت الثوم وزيت الشاي تحفز اكسدت بروتينات البيرووكسي ريدوكس ذات السستينين المتماثلين في خلايا جركت التائية اللمفاوية

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الخلاصة

برزت بروتينات البيرووكسي ريدوكسن ذات السستينين المتماثلين في اللبائن كمتحسسات ومحولات في الاستجابة الخلوية للاجهاد التأكسدي. تمتلك الزيوت النباتية ومركباتها قابلية للعمل كمولدات للجذور الحرة وكمضادات للاكسدة ولكن الالية الحقيقية لهذه الزيوت غير معروفة بشكل دقيق. لهذا فحصت الدراسة الحالية تاثير زيت الثوم والشاي بالاضافة الى بعض المركبات المختارة من تلك الزيون على انزيمات البيرووكسي ريدوكسن ذات السستينين المتماثلين. اذ اظهرت نتائج الدراسة الحالية بان كلا الزيتين ومركباتهما حفزا اما تشكيل دايمر متاكسد او تشكيل مونمر فاقد لقابلية الاكسدة في بروتينات البيرووكسي ريدوكسن ذات السستينين المتماثلين. كما اظهرا المركبين diallyl disulphide (DADS) و diallyl trisulphide (DATS) التشكيلة الرئيسية لزيت الثوم تاثيرا مشابه لزيتهما الابوي ولكن DATS كان اكثر تاثيرا من DADS مما يدل على اهمية ذرات الكبريت في تركيبة مركبات زيت الثوم. كما بينت الدراسة الحالية ان زيت الثوم اكثر فعالية من زيت الشاي في تحفيز اكسدت بروتينات البيرووكسي ريدوكسن ذات السستينين المتماثلين. على العكس من ذلك اظهر terpinen-4-ol المركب الرئيسي في تشكيلة زيت الشاي فعالية اتجاة بروتينات البيرووكسي ريدوكسن ذات السستينين المتماثلين تفوق الزيت الابوي. اكدت نتائج الدراسة الحالية بشكل جزئي اختلاف المركبات في التاثير على بروتينات البيرووكسي ريدوكسن ذات السستينين المتماثلين فمن الممكن ان يعود الى الاختلاف في تاثيرهما على نشاط الثايروكسين ريدكتيز الانزيم المسؤول عن اعادة فعالية بروتينات البيرووكسي ريدوكسن ذات السستينين المتماثلين الى المونمر الاختزالي. نوقشت نتائج الدراسة الحالية في ضوء استلزام بروتينات البيرووكسي ريدوكسن ذات السستينين المتماثلين في الاستجابة الخلوية للاجهاد التاكسدي.