Molecular mechanisms of oxidative stress resistance induced by resveratrol: Specific and progressive induction of MnSOD

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Abstract

trans-Resveratrol (3,4′,5-trihydroxystilbene; RES), a polyphenol found in particularly high concentrations in red wine, has recently attracted intense interest for its potentially beneficial effects on human health. Here, we report the effects of long-term exposure to micromolar concentrations of RES on antioxidant and DNA repair enzyme activities in a human cell line (MRC-5). RES had either no effect on, or reduced the activities of glutathione peroxidase, catalase and CuZn superoxide dismutase (SOD), in treatments lasting up to 2 weeks. RES failed to induce activities of the DNA base excision repair enzymes apurinic/apyrimidinic endonuclease and DNA polymerase β. However, it dramatically and progressively induced mitochondrial MnSOD expression and activity. Two weeks exposure to RES increased MnSOD protein level 6-fold and activity 14-fold. Thus, long-term exposure of human cells to RES results in a highly specific upregulation of MnSOD, and this may be an important mechanism by which it elicits its effects in human cells.
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trans-Resveratrol (3,4′,5-trihydroxystilbene; RES), a polyphenol found in wine, has recently attracted intense interest as a caloric restriction mimetic [1]. In addition to its ability to extend lifespan and to inhibit the growth of cancerous cells RES is protective against cardiovascular disease, ischaemic injury, and diabetes (reviewed in [2]). Some of the beneficial effects of RES have been attributed to its antioxidant effects. It is, however, a rather weak chemical antioxidant [3]. It therefore appears that reported antioxidant effects of RES may be due to its direct interactions with biomolecules that confer cellular stress resistance.

One mechanism by which RES exerts its beneficial effects may be its ability to attenuate oxidative DNA damage, as has been shown in a wide variety of cells [4–6]. However, whether RES acts by enhancing DNA repair or simply by preventing oxidative DNA damage from occurring is currently unknown. Oxidative DNA damage, such as base modifications and abasic sites, is efficiently removed by the base excision repair (BER) pathway [7], and it is therefore of interest to determine whether RES is capable of enhancing the activity of this pathway. Many simple DNA lesions are effectively removed by short patch BER, the first step of which involves removal of the damaged base, leaving an abasic site. This can subsequently be processed by the enzyme apurinic/apyrimidinic endonuclease (APE) to yield a DNA strand break 5′ to the baseless sugar. Polymerase β (polβ) is then able to fill the remaining nucleotide gap. This latter enzyme appears to be rate-limiting to overall BER pathway activity [8].

Here we have used MRC-5 cells (human lung fibroblasts) as a representative normal, non-cardiovascular cell line in which to study effects of RES on both cellular antioxidant capacity and DNA BER. The majority of studies of the effects of RES on human cell biology have focused on relatively short-term exposures, though this

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nutraceutical is being marketed for long-term consumption. We therefore investigated both short- and long-term effects of RES on MRC-5 cells. We observed that long-term exposure to RES had no effect on APE and actually decreased DNA polβ activity. Similarly, RES treatment had little effect on the activities of glutathione peroxidase and CuZn superoxide dismutase (CuZnSOD), while decreasing CAT activity. In contrast, RES treatment elicited a profound and progressive induction of mitochondrial superoxide dismutase (MnSOD). This observation is significant because MnSOD overexpression alone is capable of reducing intracellular oxidative stress and conferring stress resistance [9]. Thus, it can elicit many of the same beneficial effects as RES treatment. We therefore suggest that the specific induction of MnSOD is an important mechanism by which RES exerts its biological effects.

Materials and methods

Reagents. Resveratrol (>98% purity) was purchased from A.G. Scientific, (San Diego, USA). Antibodies to human CuZnSOD and human MnSOD were purchased from StressGen Biotechnologies (Ann Arbor, USA). IR dye-conjugated secondary antibodies to rabbit and mouse were purchased from Rockland Immunochemicals, (Glibertsville, USA). Pure human polβ and APE were purchased from Trevigen (Gaithersburg, USA).

Cell line and growth conditions. Normal human lung fibroblasts (MRC5) were obtained from the Coriell Cell Repository and grown in Modified Eagle's Media containing L-glutamine, supplemented with penicillin/streptomycin, non-essential amino acids and 15% fetal bovine serum (FBS). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. RES was administered in a sterile DMSO solution (0.07%; <1 μM). Cell cultures were incubated with 0, 50 μM or 100 μM RES in DMSO. The culture medium was refreshed every 2d and newly prepared RES was added each time. Cohorts of cells were harvested after 24 h, 72 h and 2 weeks. Whole cell extracts were prepared essentially as in [22]. Protein concentrations were determined by the Bio-Rad™ assay.

Western blots. Equal amounts of protein extracts were separated by SDS–PAGE and electro-transferred to a PVDF membrane. Membranes were stained with Memcode reversible protein stain to ensure even transfer. MnSOD and CuZnSOD were visualized using an Odyssey infrared imaging system (LI-COR Biosciences) and quantified using Odyssey imaging system software.

DNA BER assays. The activity of APE was measured essentially as in [10], by incubating 100 ng of cellular protein with 1 pmol of ^{32}P-end labeled tetrahydrofuran (THF) containing double stranded 30 mer oligonucleotide or negative control oligonucleotide (dsC; see Table 1) for 5, 10, and 15 min time intervals at 37 °C. Polβ repair activity was assayed essentially as in [10], using a double-stranded gapped 34-mer oligonucleotide (GAP) or full-length control oligonucleotide (dsG; see Table 1). The reactions were initiated by addition of cell lysate (0.5 μg). After incubation for 30, 60, and 90 min at 37 °C the reactions were terminated by the addition of 5 μg proteinase K and 1 μl of 10% SDS and incubation at 55 °C for 30 min. DNA was precipitated overnight at −20 °C. Samples were electrophoresed at 20 W for 2 h 30 min, then visualized with a Fuji posphorImager and analyzed with Image Gauge™ (Fuji Film). Polβ activity was quantified as the incorporation of ^{32}P-labeled dCTP.

In-gel superoxide dismutase activity assay. An in-gel assay was used to separate and quantify MnSOD and CuZnSOD activities as described by Fridovich and Beauchamp [11]. To distinguish between CuZnSOD and MnSOD bands, 5 mM KCN was added to the staining solution in some gels. An in-gel standard curve was constructed using a dilution series of pure bovine liver SOD (Sigma). Gels were scanned and SOD activities were quantified using Bio-Rad's Quantity One® software.

Table 1
Oligonucleotides used in BER assays

Name	Sequence
THF	5′-ATA TAC CGC GG(AP) CGG CCG ATC AAG CTT ATT-3′ 3′-TAT ATG GCG CCG GCC GGC TAG TTC GAA TAA-5′
dsC	5′-ATA TAC CGC GGC CGG CCG ATC AAG CTT ATT-3′ 3′-TAT ATG GCG CCG GCC GGC TAG TTC GAA TAA-5′
GAP	5′-CTG CAG CTG ATG CGC-OH P-GT ACG GAT CCC CGG CTA C-3′ 3′-GAC GTC GAC TAC GCG GCA TGC CTA GGG GCC CAT G-5′
dsG	5′-CTG CAG CTG ATG CGC CGT ACG GAT CCC CGG GTA C-3′ 3′-GAC GTC GAC TAC GCG GCA TGC CTA GGG GCC CAT G-5′

Underlined regions represent area of interest. dsC, double stranded 30-mer control oligonucleotide with no damage; THF, double-stranded THF oligo containing a tetrahydrofuran apurinic/apyrimidinic (AP) site analogue; GAP, double-stranded gapped-oligo; dsG, double-stranded 34-mer control with no damage.

Other enzyme assays. All enzyme assays were performed at 30 °C, using a Varian Cary 100 Bio UV–Visible Spectrophotometer. The citrate synthase (CS) and lactate dehydrogenase (LDH) assays were as in [12]. Glutathione peroxidase and catalase assays were as in [10].

Cellular oxygen consumption. Cells were dissociated using trypsin, centrifuged at 500g and washed twice with PBS. Viable and non viable cell counts were determined in an aliquot of the cell suspension by trypan blue exclusion prior to measurement. The rate of oxygen consumption in whole cells was measured in fresh MEM (without FBS) at 37 °C using a Rank Brothers Limited Dual digital Model 20 oxygen sensing electrode.

Statistical analysis. Enzyme activity data were analyzed by means of a 2-way ANOVA test with replication using Microsoft Excel 2004 ($α = 0.05$). Bonferroni's post hoc test was used to determine where statistical differences existed. Cellular respiration data were analyzed using Student's *t*-test.

Results

The effects of RES on DNA repair were assessed as the activity of two essential enzymes in short patch BER, polβ and APE. Continuous treatment with any concentration of RES for up to 2 weeks had no effect on APE activity (Fig. 1A). Similarly, short-term treatment with RES did not alter polβ activity in MRC-5 cells. However, a significant decrease in polβ activity was observed following 2 weeks incubation with either concentration of RES (Fig. 1B).

The effects of RES on cellular antioxidant enzymes were also determined. RES treatment at any of the concentrations or incubation times tested reduced the total catalase activity of cell lysates (Table 2). A subtle stimulation of glutathione peroxidase activity was observed in cells exposed to higher concentrations of RES for 2 weeks (Table 2). CuZnSOD protein levels were quantified by Western blot; however no significant change was observed for any RES concentration or incubation time (Fig. 2B).

In contrast, MnSOD was progressively and profoundly induced by either concentration of RES (Fig. 2D). MnSOD
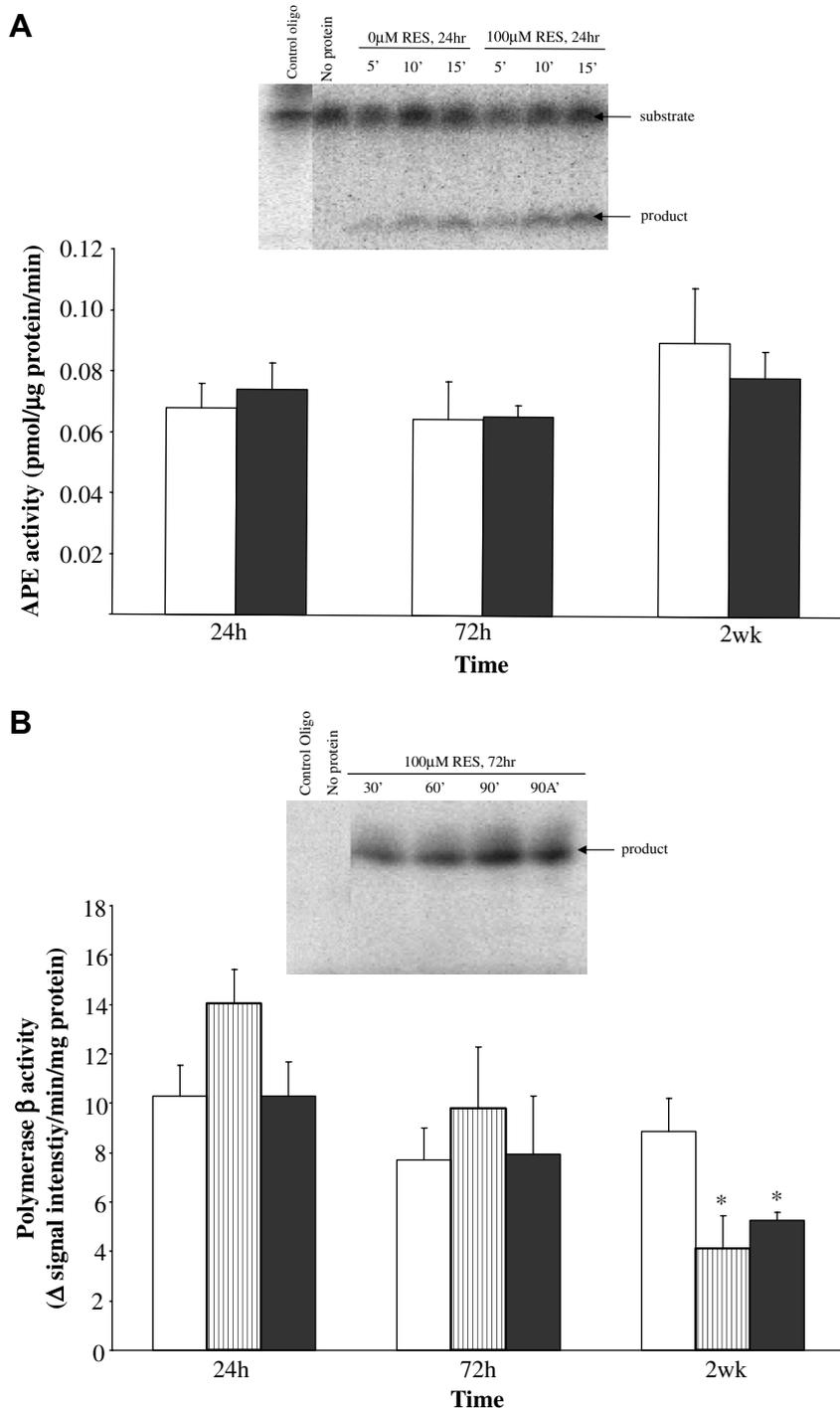
Fig. 1. DNA repair enzyme activities in control and RES treated MRC-5 cells. (A) APE activity and (B) polβ activity in lysates prepared from MRC cells treated with 0 μM, 50 μM, and100 μM RES for 24 h, 72 h or 2 weeks. Open bars = 0 μM RES; vertically hatched bars = 50 μM RES; solid bars = 100 μM RES. Values shown are means ± SEM of duplicate measurements on each of three lysates. *Significantly different from 0 μM RES control ($P < 0.05$). Insets show representative gels.

protein levels were elevated almost 3-fold by 72 h of incubation with any concentration of RES. By two weeks, an approximately 6-fold induction of MnSOD protein was observed. A parallel upregulation of MnSOD activity was observed using native gel electrophoresis to separate this protein from CuZnSOD. MnSOD activity was 14-fold greater than a 0 μM RES control by two weeks

(Table 2). In the same analysis, however, no statistically significant effect of RES was observed for CuZnSOD activity at any concentration or time point (Table 2). Thus, long-term RES exposure elicited a highly specific upregulation of MnSOD expression and activity.

MnSOD localizes exclusively to the mitochondrial matrix. To determine whether the increases in MnSOD

Table 2
Antioxidant enzyme activities in lysates prepared from control and RES treated MRC-5 cells

Enzyme	[RES] (µM)	Activity		
		24 h	72 h	2 weeks
Catalase (nmol/mg/min)	0	37.8 ± 1.2	36.6 ± 1.6	41.5 ± 4.1
	50	26.7 ± 1.4[*]	24.1 ± 3.1[*]	21.9 ± 1.3[*]
	100	21.3 ± 1.8[*]	24.8 ± 2.2[*]	23.3 ± 3.9[*]
Glutathione peroxidase (nmol/mg/min)	0	887 ± 30	987 ± 86	836 ± 39
	50	915 ± 27	964 ± 42	892 ± 72
	100	895 ± 37	852 ± 28	1250 ± 38[*]
Mn superoxide dismutase (U/mg cellular protein)	0	2.2 ± 0.3	1.8 ± 1.0	1.0 ± 0.3
	50	2.7 ± 0.2	5.4 ± 1.8	13.7 ± 4.0[*]
Cu/Zn superoxide dismutase (U/mg cellular protein)	0	1.9 ± 0.4	1.7 ± 0.4	0.8 ± 0.5
	50	2.4 ± 0.3	1.7 ± 0.4	1.4 ± 1.7

Values shown are means ± SEM of duplicate measurements on each of three lysates.

[*] Significantly different from 0 µM RES control ($P < 0.05$).

were simply due to increased mitochondrial density we measured the activity of CS, a marker of cellular mitochondrial content [13]. An approximately 20% increase in CS activity occurred in cells treated with any concentration of RES for two weeks (Fig. 2E), indicating a slight increase in mitochondrial density. This increase, however, was insufficient to account for the observed change in MnSOD. Therefore, the apparent level of MnSOD per unit mitochondrion was significantly increased.

To address the possibility that the concentrations of RES used in this study could have been toxic, we assessed the function and viability of cells used to measure enzyme levels and activities in several different ways. Firstly, the ability to exclude trypan blue indicated that >99% of cells were viable, and this was not different between control and RES treated cells (data not shown). Secondly, although population doubling time was increased, cells continued to replicate throughout the study. Thirdly, as RES is known to inhibit several of the mitochondrial respiratory complexes [14], we measured mitochondrial oxygen consumption in cells treated with 75 µM RES for 24 h, 72 h, or 2 weeks. Cellular respiration was reduced by approximately 20% at 24 h in RES-treated cells. However, this effect was transient (Fig. 3A). LDH was measured as an indicator of reliance on glycolysis, which would be expected to increase if RES significantly inhibited oxidative phosphorylation. LDH activity was almost double control values at 24 h in cells treated with 100 µM RES (Fig. 3B). However, at 72 h and 2 weeks, no differences were observed between control and RES-treated cells.

Discussion

The most striking, and important result we report here is a highly specific, progressive and dramatic induction of MnSOD expression and activity elicited by treatment of an untransformed human cell line with micromolar concentrations of RES. The broad significance of MnSOD has

been well established. As the sole SOD of the mitochondrial matrix, MnSOD reduces intracellular oxidative stress and confers resistance to oxidative stress-induced mitochondrial dysfunction, permeability transition and apoptotic death in various disease contexts (reviewed in [15]). As such, MnSOD overexpression has been associated with extension of lifespan [16,17], resistance to ischemia–reperfusion injury and resistance to irradiation-induced damage [18]. In addition, overexpression of MnSOD has been reported to inhibit cancer cell growth. Interestingly, most of these same observations have been made for RES (Table 3). We therefore propose that the RES-mediated induction of MnSOD may be an important mechanism by which this polyphenol confers its beneficial effects.

While we do not know the mechanism by which RES stimulated MnSOD expression and activity in MRC-5 cells, various authors have reported that RES stimulates migration of FOXO transcription factors to the nucleus [19]. RES has been shown to enhance the sirtuin-catalyzed deacetylation of FOXO3a [20], which stimulates its transcriptional activity [21]. MnSOD is known to be a target of FOXO3a, and MnSOD expression is strongly induced in cells overexpressing FOXO3a [21]. It is therefore possible that chronic treatment with RES stimulates a sustained induction of FOXO3a activity, and thus MnSOD transcription, in MRC-5 cells. This should be the focus of future investigations.

There was no evidence that long-term exposure to the concentrations of RES used here was toxic to MRC-5 cells. Although RES-treated cells had longer population doubling times (data not shown), they continued to replicate throughout the experiment. Also, despite an initial depression of metabolic rate and induction of LDH activity in 24 h RES-treated cells, suggesting a general reduction in oxidative activity, both values returned to normal during continuous treatment with any concentration of RES. We interpret these results as indicating that RES causes a short-term, transient suppression of cellular metabolic rate. Interactions between RES and mitochondrial respiratory complexes have been reported [14], as has an increase in mitochondrial abundance [13], which we observed here using CS activity as proxy. Therefore, while resveratrol clearly elicits a number of alterations in cellular metabolism, there is no evidence that it is toxic to MRC-5 cells at the concentrations used here.

One of the purposes of the present study was to determine whether RES treatment of human cells stimulated DNA BER activities, given the widely reported reductions in levels of oxidative DNA damage in RES-treated cells [4–6]. However, we found no evidence of a RES-induced increase in BER enzyme activities. In fact polβ, which catalyzes the rate-limiting step in BER, was significantly reduced by long-term RES treatment. Polβ has been shown to be regulated by intracellular oxidative stress [10,22]. Thus, we suggest that lower polβ activities are related to generally lower levels of intracellular oxidative stress secondary to the upregulation of MnSOD, and that lower
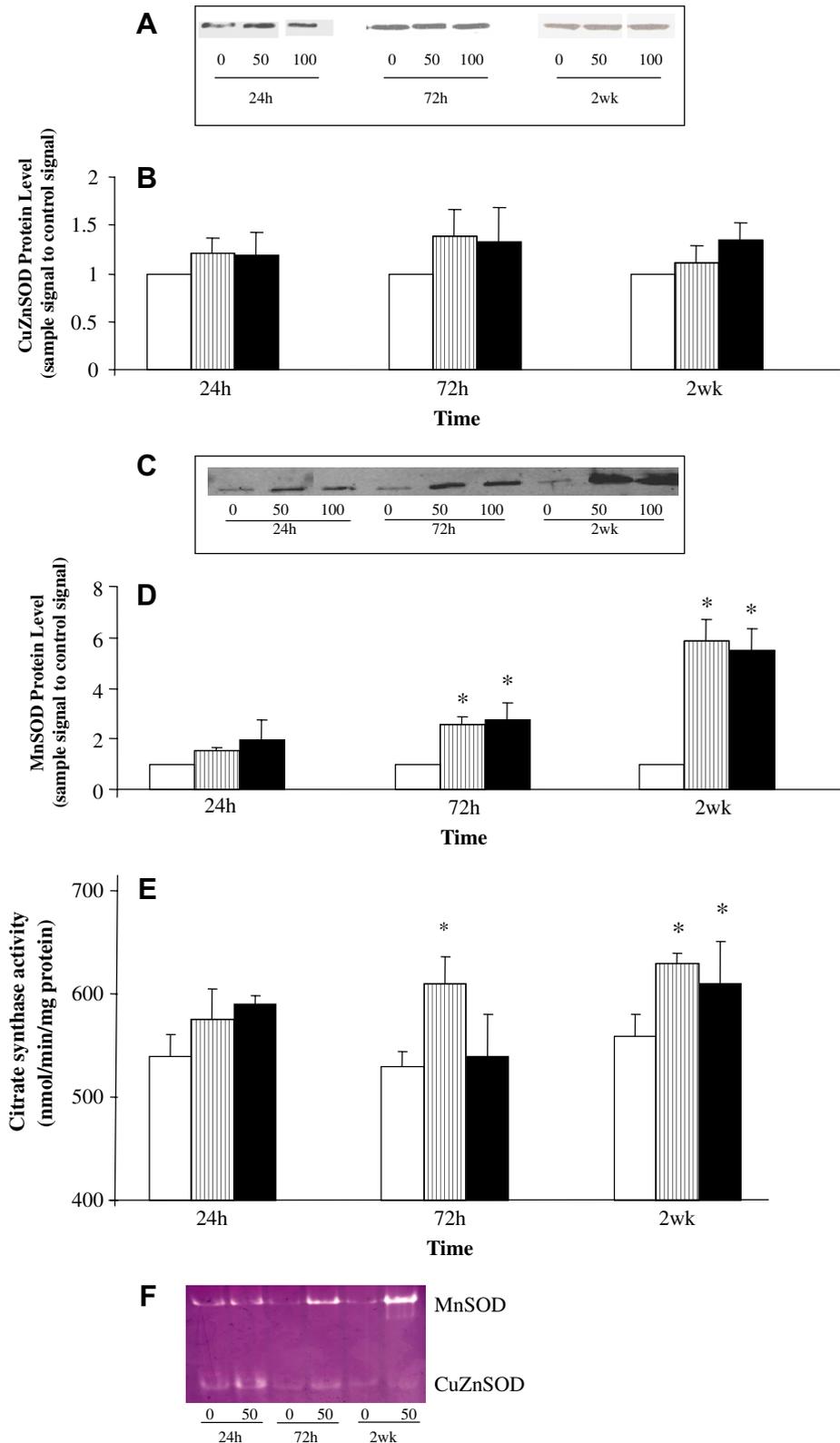
Fig. 2. CuZnSOD and MnSOD protein levels in control and RES treated MRC-5 cells. (A) Representative Western blot showing CuZnSOD protein band. (B) Relative changes in the levels of CuZnSOD in lysates prepared from MRC-5 cells. (C) Representative Western blot showing MnSOD protein band. (D) Relative changes in the levels of MnSOD in lysates prepared from MRC-5 cells. Relative change was measured using control (0 μM RES) as a reference and values were interpolated from a standard curve. Identities of bars are as in Fig. 1. Values shown are means ± SEM of one Western blot per lysate. *Significantly different from control (0 μM RES at the corresponding time). (E) Citrate synthase activity in lysates prepared from MRC-5 cells treated with 0 μM, 50 μM and 100 μM RES for 24 h, 72 h or 2 weeks. Identity of bars is as in Fig. 1. Values shown are means of duplicate measurements on each of three lysates. *Significantly different than 0 μM RES control at the corresponding time ($P < 0.05$). (F) Representative SOD activity gel showing MnSOD and CuZnSOD activities.
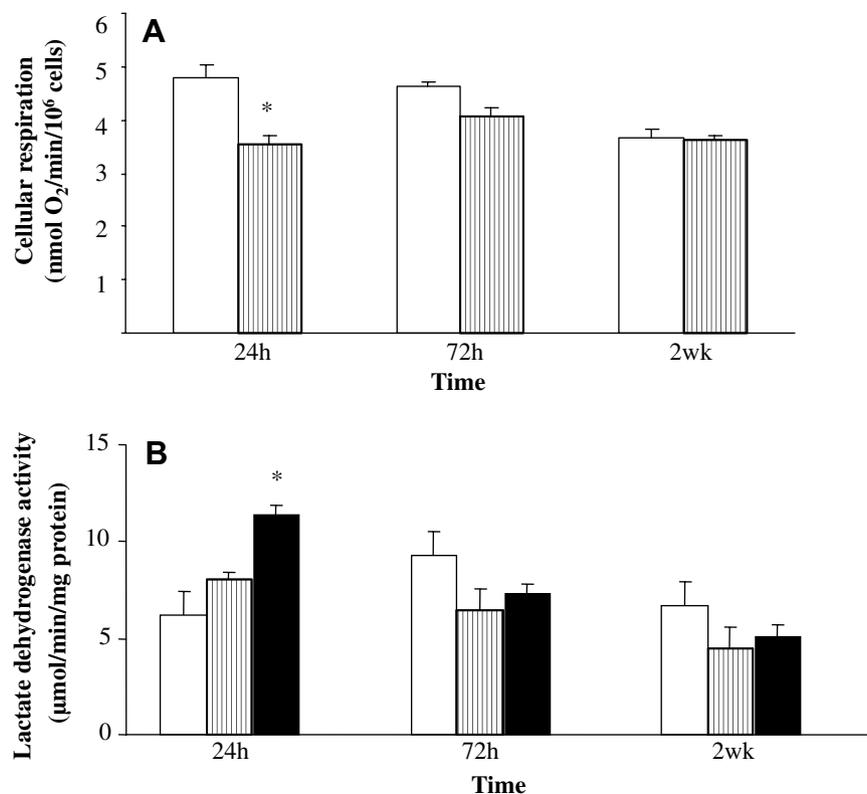
Fig. 3. Metabolic characterization of MRC-5 cells treated with 0–100 μM RES. (A) Rates of oxygen consumption in cells treated with 75 μM RES for 24 h, 72 h, and 336 h. Open bars = 0 μM RES; vertically hatched bars = 75 μM RES Values shown are means ± SEM of six independent measurements. *Significantly different than 0 μM RES control at the corresponding time ($P < 0.05$); (B) Lactate dehydrogenase activity in lysates prepared from MRC-5 cells incubated with 0 μM, 50 μM, and 100 μM RES for 24 h, 72 h or 2 weeks. Identity of bars is as in Fig. 2. Values shown are means ± SEM of duplicate measurements of three individually prepared lysates. *Significantly different from control value at 24 h ($P < 0.05$).

Table 3
Comparison of the effects of resveratrol administration and MnSOD overexpression in cell culture and animal models of longevity and stress resistance

Observation	Resveratrol administration	MnSOD overexpression
Lifespan extension	*Caenorhabditis elegans* [2]	*Drosophila melanogaster* [16]
	Drosophila melanogaster [2]	Mice [17]
	Nothobranchius furzeri [2]	
Inhibition of cell proliferation	Pancreatic cancer cells [25]	Human pancreatic carcinoma cells [26]
Protection against ischemia/reperfusion injury	Isolated rat hearts [27]	Myocardial injury *in vivo* [28]
Neurodegeneration models	Attenuation of MPTP toxicity in PC12 cells [29]	Attenuation of MPTP toxicity in mice [30]

levels of DNA oxidative damage reported in RES treated cells result from reduced mitochondrial ROS production.

An important result is that most of the effects observed with RES treatment, including the induction of MnSOD, had reached a maximal level at 50 μM RES, and were not increased further at higher concentrations. This suggests that lower concentrations of RES would also have elicited these effects. This is an important consideration for dietary introduction of RES, which typically results in low micromolar concentrations in tissues [23]. However, it may be possible to enhance these levels using other means of delivery, particularly those that target specific tissues. We are currently investigating various avenues of RES administration that stimulate MnSOD activity in mice, and find that it is also able to upregulate MnSOD in some mouse tissues (unpublished results).

In conclusion, we have identified MnSOD as a specific target of RES in human cells chronically exposed to this polyphenol. Given the notable overlap between the biological effects of MnSOD overexpression and those reported for RES treated cells and animals, we propose that modulation of MnSOD activity may be an important mechanism underlying the actions of RES and this possibility should be investigated in detail.

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