Cytoprotective effect of γ-tocopherol against tumor necrosis factor α induced cell dysfunction in L929 cells

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Abstract. The antioxidant vitamin γ-tocopherol exerts protective and anti-inflammatory effects in various models of critical illness. The combination of actinomycin D and tumor necrosis factor α (TNFα) in the immortalized fibroblast cell line L929 is a well-established method to model pro-inflammatory cytotoxicity in cultured cells *in vitro*. The present study had two aims. First, we wished to characterize the contribution of reactive oxygen species (ROS) to the cell dysfunction and this commonly used model system of cell death. Second, we wished to investigate the effects of γ-tocopherol on this response. Cells were exposed to actinomycin D (0.5 μg/ml) + TNFα (100 pg/ml) in the absence or presence of 1 h of γ-tocopherol pre-treatment. The earliest change that was detected in our system in response to TNFα was an increase in mitochondrial oxidant production, already apparent at 45 min. Changes in glycolysis and oxidative phosphorylation parameters were already apparent at 2 h, as detected by the Seahorse Biosciences XF24 Flux Analyzer. By 6 h, a slight decrease in Cell Index was detected by impedance-based analysis, employing an electronic sensor array system (XCelligence). At the same time, a slight decrease in cell viability was detected by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method, along with a significant increase in lactate dehydrogenase (LDH) release into the culture medium, and a detectable degree of mitochondrial membrane depolarization. Between 12 and 24 h, the cell viability (already at a low level) further declined, which coincided with a secondary, marked decline in the mitochondrial membrane potential. Pre-treatment of the cells with γ-tocopherol (10-300 μM) provided a significant protection against all of the functional alterations induced by actinomycin D and TNFα. The current study provides direct evidence that reactive oxidant formation plays an important role in the current experimental model of cell dysfunction, and demonstrates the protective effects of the potent endogenous antioxidant vitamin, γ-tocopherol. The mechanisms described in the current study may, in part, contribute to the protective effects of γ-tocopherol in various models of critical illness.

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Abbreviations: AUC, area under the curve; DTT, dithiothreitol; ECAR, extracellular acidification rate; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolo-carbocyanine iodide; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; OCR, oxygen consumption rate; PAR, poly(ADP-ribose) polymer; PARP, poly(ADP-ribose) polymerase; TNFα, tumor necrosis factor α

Key words: antioxidants, cell death, necrosis, apoptosis, PARP, reactive oxygen species, vitamin E

Introduction

Multiple studies have demonstrated the potent anti-oxidant effects of γ-tocopherol, the minor component of the anti-oxidant vitamin E. As opposed to γ-tocopherol (which is the principal component of vitamin E), γ-tocopherol exerts protective effects against both reactive oxygen (ROS) and reactive nitrogen species in various cell types, resulting in potent cytoprotective effects in various experimental systems (1-3).

The biochemical reactions and the potential therapeutic utility of γ-tocopherol has become a subject of intensive investigations in recent years (4). Several studies have demonstrated the therapeutic potential of γ-tocopherol in various models of critical illness as well as in various chronic inflammatory and metabolic diseases (4-6).

The translational relevance of γ-tocopherol is underlined by recent studies demonstrating the depletion of this antioxidant in severe burn injury and other disease conditions (7,8); the association of high levels of this antioxidant with disease prevention (8,9); and the therapeutic effects of tocopherol supplementation in pilot clinical studies (10,11).

Incubation of cultured cells with the pro-inflammatory cytokine tumor-necrosis factor α (TNFα) has been used in multiple studies to characterize the mechanisms of inflammatory cell injury. In L929 cells TNFα is often combined with the RNA synthesis inhibitor actinomycin D, which potentiates the degree of cell death/cell injury (12-15). The current investigation had two main goals. The first goal was to explore the contribution of ROS to the development of cell dysfunction in this commonly used model of cell injury. The second goal of the study was to test the potential cytoprotective effect of the antioxidant γ-tocopherol in this model. The results demonstrate

a strong pro-oxidative component of the pro-inflammatory cell death in this model system, and the marked cytoprotective effect of γ-tocopherol.

Materials and methods

Materials. All reagents (unless specified otherwise) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell culture. L929 mouse fibroblasts cells were maintained in 25 mM glucose containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37˚C in a 5% CO_2 atmosphere. For the metabolic studies, L929 cells were seeded into special 24-well Seahorse Bioscience V7 tissue culture plates at the indicated density and allowed to adhere and grow for 24 h. Cells were treated with TNFα (recombinant human TNFα at a final concentration of 100 pg/ml) and actinomycin D (at a final concentration of 0.5 μg/ml) in the absence or presence of 1 h of γ-tocopherol (in most experiments, at a final concentration of 100 μM) on 96-well plates. In a series of concentration-response studies, the effect of γ-tocopherol (alone or as a pre-treatment before actinomycin D and TNFα) was also evaluated at concentrations of 1 μM-1 mM. In a separate set of experiments, the effect of γ-tocopherol (1 μM-1 mM) was assessed on the cytotoxicity elicited by TNFα alone, when applied alone, at a higher concentration of 1000 pg/ml. Analysis was performed at various times thereafter (45 min - 24 h), depending on the experimental protocol.

Measurement of cell viability using the MTT method. To estimate the number of viable cells 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the cells at a final concentration of 0.5 mg/ml and cultured at 37˚C for 1 h. Cells were washed with PBS and the formazan dye was dissolved in isopropanol. The amount of converted formazan dye was measured at 570 nm with a background measurement at 690 nm on a Spectramax microplate reader (Molecular Devices, Mountain View, CA) (16). Viable cell count was calculated as a percent of vehicle-treated control cells.

Measurement of cell death using the lactate dehydrogenase (LDH) method. Cell culture supernatant (50 μl) was mixed with 100 μl freshly prepared LDH assay reagent to reach final concentrations of 85 mM lactic acid, 1040 mM nicotinamide adenine dinucleotide (NAD^+), 224 mM N-methylphenazonium methyl sulfate, 528 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride and 200 mM Tris (pH 8.2). The plates were incubated for 15 min and read at 492 nm with a background measurement at 690 nm (endpoint assay) on a microplate reader as described (16). The LDH activity was expressed as percent of LDH activity measured in the supernatant of vehicle-treated control cells.

Measurement of the mitochondrial membrane potential using the JC-1 method. To monitor the mitochondrial membrane potential, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was used as described (17). The green fluorescent JC-1 probe exists as a monomer at low membrane potential. However, at higher potentials, JC-1 forms red-fluorescent aggregates. Thus, the emission of this cyanine dye can be used as a sensitive measure of membrane potential. In healthy cells, JC-1 exists as a monomer in the cytosol (green fluorescent signal) and also accumulates as aggregates in the mitochondria (red fluorescence signal). In apoptotic and necrotic cells, more JC-1 exists in monomeric form and stains the cytosol green. The ratio of green-red fluorescence is a parameter that is dependent on the membrane potential. After treatment, cells were first loaded with JC-1 for half an hour at 37˚C in DMEM then washed with PBS. Reading was carried out in PBS. Fluorescence intensity measurements were made with a BioTek plate reader with the following settings: Ex: 485/530 nm, Em: 528/590 nm.

Measurement of mitochondrial ROS production using MitoSOX Red. MitoSOX™ Red mitochondrial superoxide indicator is a fluorogenic dye used for the highly selective detection of superoxide in the mitochondria of live cells. MitoSOX™ Red reagent is live-cell permeant and is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX Red reagent is oxidized by superoxide and exhibits red fluorescence. MitoSOX Red reagent is readily oxidized by superoxide but not by other ROS or reactive nitrogen species generating systems, and oxidation of the probe is prevented by superoxide dismutase (18). After treatment, cells were washed and loaded with 5 μM of staining solution (in DMEM) for 30 min at 37˚C. Cells were washed and 100 μl PBS were added. Fluorescence reading was performed on a microplate reader (BioTek), Ex/Em: 510/580 nm.

Cell impedance measurements using the XCelligence® method. To monitor cellular events in real time without the incorporation of labels we used the XCelligence® system to measure electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture E-Plates. The impedance measurement provides quantitative information about the biological status of the cells, including cell number, viability, morphology and adherence. Cells (20,000 cells/well) were seeded into 180 μl of media in a 96X E-Plate. The attachment, spreading and proliferation of the cells were monitored every 15 min using the XCelligence system as described (16). Approximately 24 h after seeding, cells were treated with addition of 20 μl test compounds dissolved in cell culture media.

Bioenergetic measurements using the Seahorse method. An XF24 Analyzer (Seahorse Biosciences) was used to measure the bioenergetic function in intact L929 cells. The XF24 creates a transient 7 μl chamber in specialized microplates that allows for oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) to be monitored in real time (19). For all bioenergetic measurements, the culture medium was changed 1 h prior to the assay run with unbuffered 25 mM glucose containing DMEM (pH 7.4) supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate. First, the optimal number of L929 cells/well was determined to allow the appropriate detection of changes in OCR and ECAR for the subsequent experiments as 50,000 cells/well. Next, an assay protocol was developed to measure indices of mitochondrial function. TNFα plus actinomycin D, oligomycin, FCCP and antimycin A were injected sequentially through ports of the

Seahorse Flux Pak cartridges to reach final concentrations of 100 pg/ml plus 0.5 μg/ml, 1 μg/ml, 0.3 μM and 2 μg/ml, respectively. A final concentration of 100 μM of γ-tocopherol was applied as a pre-treatment 1 h prior to the administration of TNFα and actinomycin D. Oligomycin, FCCP and antimycin A, when used in the appropriate manner, as previously described (20) allow the determination of the basal level of oxygen consumption, the amount of oxygen consumption linked to ATP production, the level of non-ATP-linked oxygen consumption (proton leak) as well as the maximal respiration capacity and the non-mitochondrial oxygen consumption, respectively. The values of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) reflect the metabolic activities of the cells with respect to oxidative phosphorylation and glycolysis, respectively.

Immunoblot analysis. Cells were treated with TNFα and actinomycin D in the presence or absence of γ-tocopherol (as above) and processed for Western blotting of poly(ADP-ribose), the product of the nuclear enzyme poly(ADP-ribose) polymer (PAR) as previously described (21). Cells were washed once in PBS and collected by scraping into 100 μl ice-cold RIPA buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT), 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, and a cocktail of protease inhibitors [4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin and aprotinin]. Supernatants were collected after centrifugation. Protein was loaded onto 4-12% polyacrylamide gels. Proteins were separated by electrophoresis and then transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 10% non-fat dried milk in Tris-buffered saline-Tween-20 (TBST) for 90 min. Primary antibodies against PAR (Trevigen, anti-rabbit), and mouse monoclonal α-actin (for loading control) were applied at 1 μg/ml concentrations overnight at 4˚C. After washing 3 times in TBS containing 0.05% Tween-20 (TBST), secondary antibodies (peroxidase-conjugated goat anti-mouse and goat anti-rabbit) were applied at 1:2,000 dilution for 1 h. Blots were washed 3 times in TBST, once in TBS, and incubated in enhanced chemiluminescence reagents (Pierce ECL Western Blotting Substrate) and signals were detected and analyzed using the Syngene Detection system (GBox) with a corresponding software package (GeneSnap).

Statistical analysis. Summary statistics of data are expressed as mean ± SEM. Comparisons were made using the two-way analysis of variance with a Tukey-Kramer post hoc procedure. P-values of ≤0.05 and ≤0.01 were considered statistically significant.

Results

Time-course of the TNFα + actinomycin D-induced cellular responses. Changes in the viability in response to actinomycin D + TNFα treatment, using two standard methods, the MTT reduction method and the LDH release method are depicted in Fig. 1. Actinomycin D + TNFα induced a deterioration of all three parameters, indicative of cell dysfunction. However, the two methods showed a different time-course of the response. None of the parameters measured were significantly altered during the first 3 h. At 6 h, there was a slight decrease in MTT, concomitant with a slight, but statistically significant increase in the LDH content into the supernatant of the cells. By 12 h, the MTT method showed a marked suppression of mitochondrial respiration (over 80% decrease from control); the LDH content of the supernatant continued to rise (an approximately doubling of the value from the value detected at 6 h). Between 12 and 24 h, the already low MTT values showed a slight further decline. The LDH levels in the supernatant did not increase any further over the same time period.

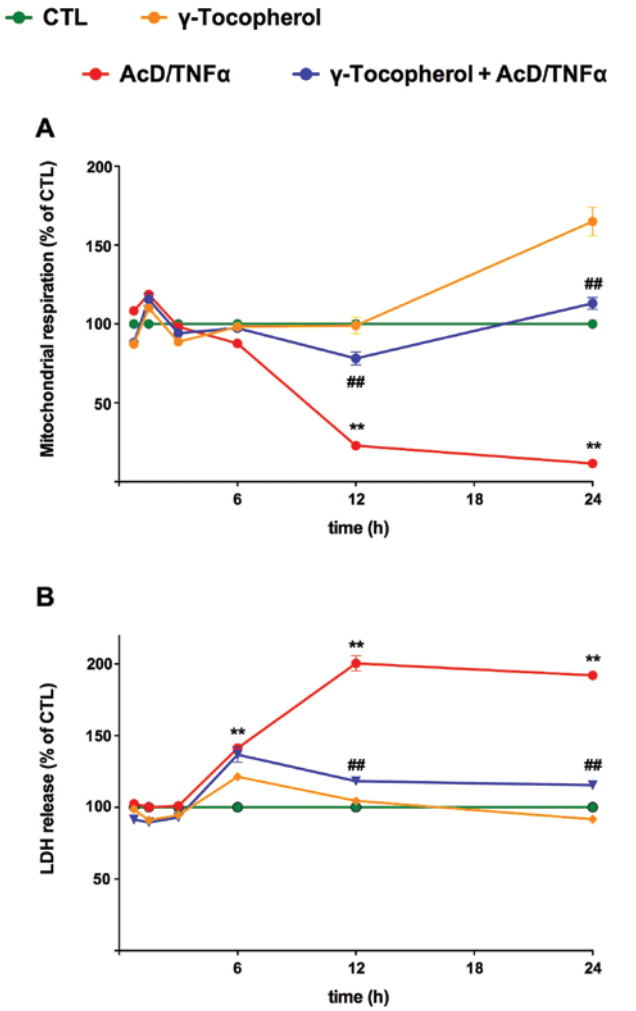

Figure 1. γ-tocopherol reduces L929 cell injury in response to actinomycin D and TNFα in L929 cells. Confluent L929 cultures were pre-treated with γ-tocopherol (100 μM) or vehicle and subjected to combined treatment with actinomycin D (0.5 μg/ml) and TNFα (100 pg/ml) (AcD/TNFα) for the indicated time periods. Viability was determined by (A) the MTT assay and (B) the LDH assay. There was a progressive deterioration of both viability parameters after AcD/TNFα treatment, compared to the untreated control (CTL) cells (**P<0.01) and pre-treatment with γ-tocopherol significantly protected against these alterations ($^{\#\#}$P<0.01). Data are shown as the mean ± SEM values of n=9 wells collected from n=3 experiments performed on 3 different experimental days.

Time-course of the mitochondrial membrane depolarization and mitochondrial superoxide production after TNFα + actinomycin D treatment. Mitochondrial membrane depolarization (as measured by the JC-1 method) did not commence until well after the mitochondrial ROS production had begun: there was no change in JC-1 staining at 3 h

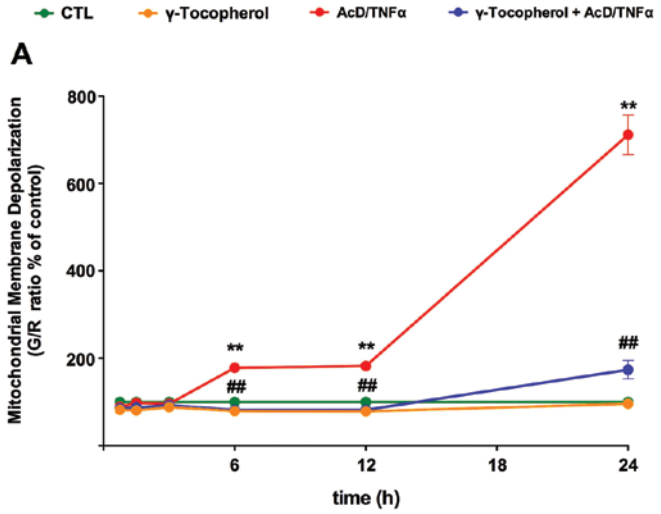

Figure 2. γ-tocopherol preserves the mitochondrial membrane potential (ΔΨm) in response to actinomycin D and TNFα treatment of L929 cells. Confluent L929 cultures were pre-treated with γ-tocopherol (100 μM) or vehicle and subjected to combined actinomycin D (0.5 μg/ml) and TNFα (100 pg/ml) (AcD/TNFα) treatment for the indicated time periods. Mitochondrial membrane potential was evaluated by measuring the green/red fluorescent ratio of JC-1. There was a progressive deterioration of mitochondrial membrane potential after AcD/TNFα, compared to the untreated control (CTL) cells (**P<0.01) and pre-treatment with γ-tocopherol significantly protected against these alterations ($^{\#\#}$P<0.01). Data are shown as the mean ± SEM values of n=9 wells collected from n=3 experiments performed on 3 different experimental days.

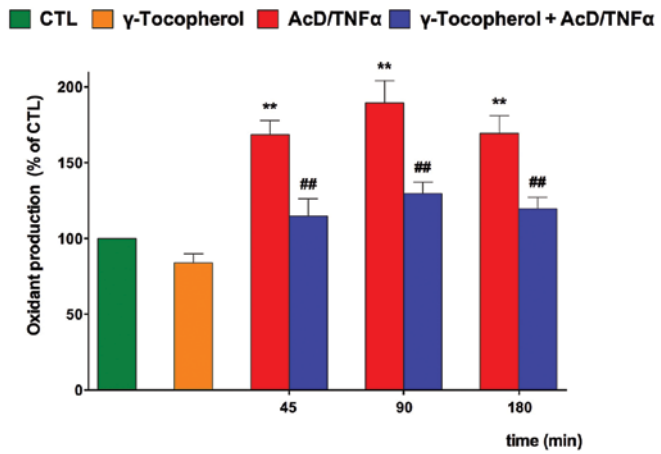

Figure 3. γ-tocopherol reduces mitochondrial superoxide production in response to actinomycin D and TNFα in L929 cells. Confluent L929 cultures were pre-treated with γ-tocopherol (100 μM) or vehicle and subjected to combined actinomycin D (0.5 μg/ml) and TNFα (100 pg/ml) (AcD/TNFα) treatment for the indicated time periods. Mitochondrial superoxide production was measured by the MitoSOX Red method. AcD/TNFα induced a rapid increase in mitochondrial superoxide production, compared to untreated control (CTL) cells (**P<0.01) and pre-treatment with γ-tocopherol significantly reduced this response ($^{\#\#}$P<0.01). Data are shown as the mean ± SEM values of n=9 wells collected from n=3 experiments performed on 3 different experimental days.

after stimulation, while there was a slight increase at 6-12 h (Fig. 2). However, there was a significant and marked further increase in mitochondrial membrane depolarization between 12 and 24 h (Fig. 2). Mitochondrial superoxide production, as measured by the MitoSOX Red method, showed an early

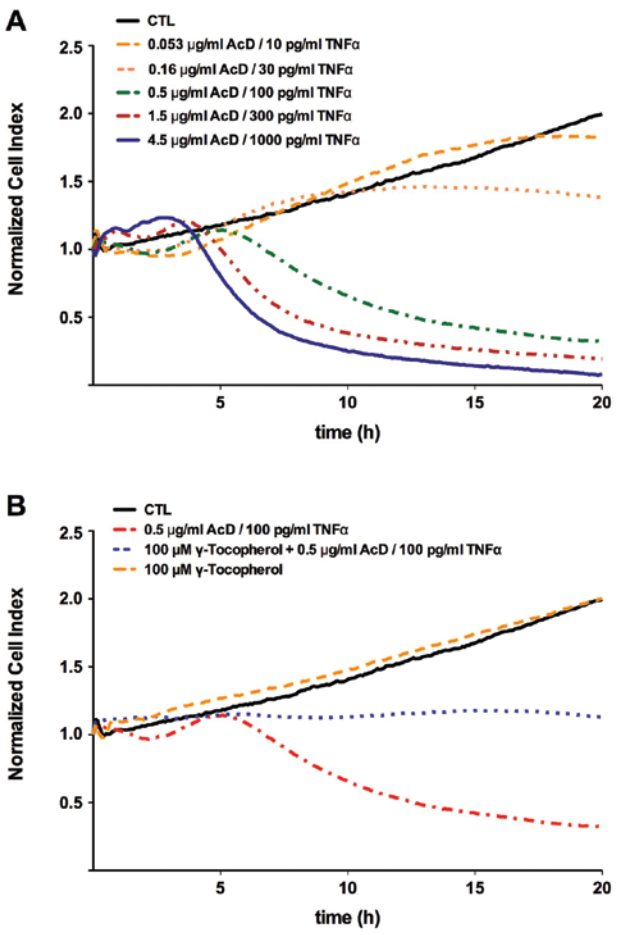

Figure 4. γ-tocopherol reduces the changes in Cell Index in response to actinomycin D and TNFα treatment of L929 cells. Confluent L929 cultures were pre-treated with γ-tocopherol (100 μM) or vehicle and subjected to combined actinomycin D (0.5 μg/ml) and TNFα (100 pg/ml) (AcD/TNFα) treatment for the indicated time periods. Cell Index, as a biophysical indicator of cell impedance, was measured by the XCelligence method. AcD/TNFα induced a decline in Cell Index, compared to the untreated control (CTL) cells and pre-treatment with γ-tocopherol prevented this response. A representative experiment representing mean values of n=3 wells per condition is shown for each experimental group. Similar results were found in three independent experiments performed on different experimental days.

increase (measured at 45 min after stimulation). Among all of the parameters studied in the current series of experiments, this change was the earliest detected alteration. The degree of superoxide production increased by 90 min, plateaued by 3 h (Fig. 3), and failed to further increase throughout the rest of the experimental period (data not shown).

Time-course of the changes in Cell Index, as measured by the XCelligence method after TNFα + actinomycin D treatment. There were no changes in Cell Index until approximately 6 h, at which time point this parameter began to decline (Fig. 4), indicative of an increased cellular conductance, most likely due to a loss of paracellular connections due to an early cell dysfunction. Cell Index substantially declined by 12 h, and showed a slight additional decline in the time period thereafter. The time course of these changes most closely paralleled the changes in MTT after actinomycin D + TNFα treatment in the current experimental system (Fig. 1A vs. Fig. 4). In this experimental system we also assessed the dose-response to actinomycin D

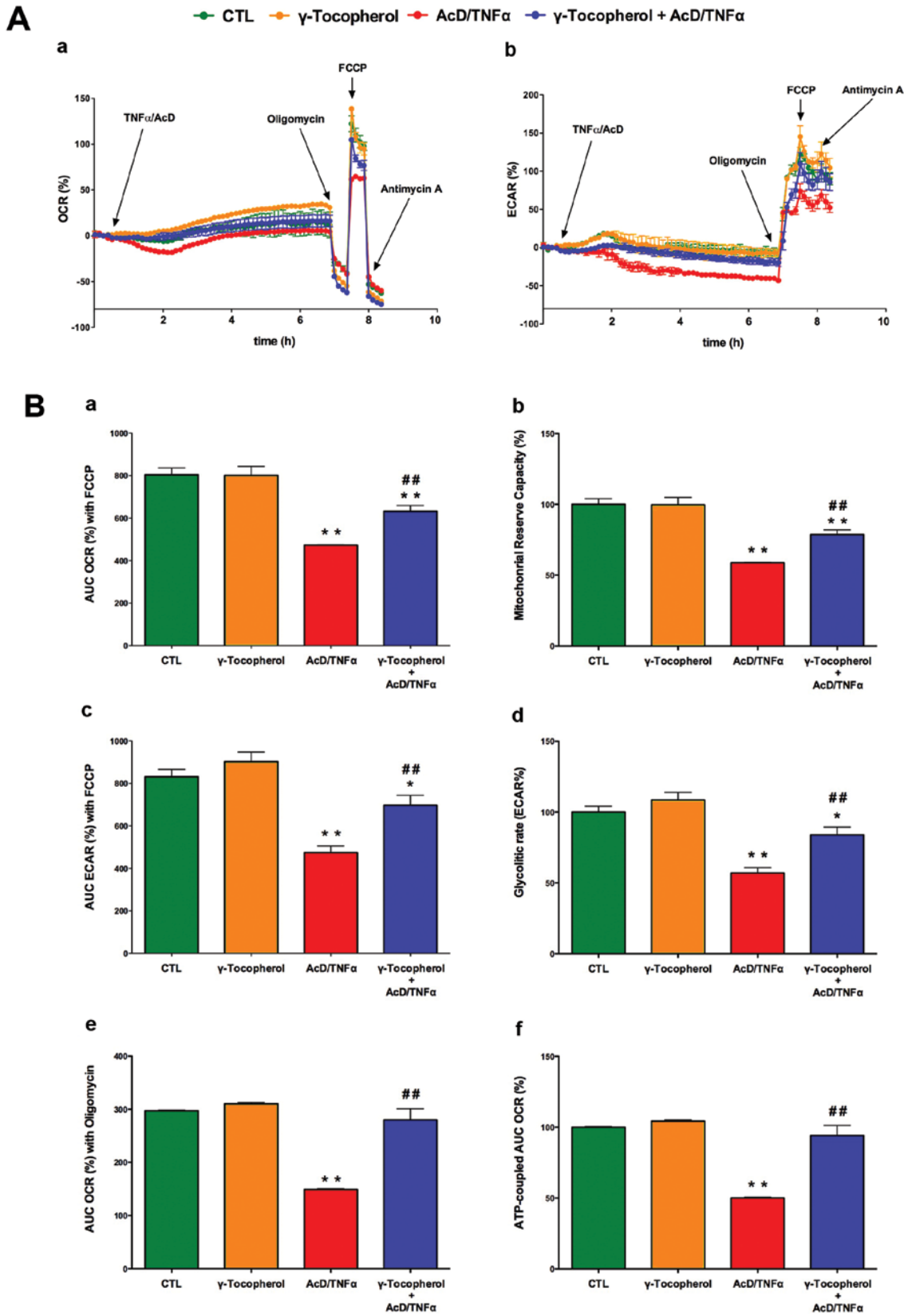

Figure 5. Measurement of mitochondrial function in L929 cell cultures using the Seahorse XF24 Analyzer; effect of γ-tocopherol on the bioenergetic changes induced by actinomycin D and TNFα. Time course of the OCR and ECAR measurements for 50,000 cells under the basal condition followed by the sequential addition of actinomycin D + TNFα (AcD/TNFα), oligomycin (1 μg/ml), FCCP (0.3 μM) and antimycin A (2 μg/ml). Part (A) of (a) represents OCR values and (b) represents ECAR values, both expressed as percentage of baseline. In (B) AUC analyses are shown. (a) The overall amount of oxygen consumption (AUC OCR %) during the treatment of FCCP. (b) The calculated mitochondrial reserve capacity compared to the CTL group based on the AUC OCR % data. (c) The AUC analyses were also applied to determine the overall amount of acidification rate (AUC ECAR %) or proton production after the administration of FCCP. (d) Based on the AUC ECAR % data the calculated glycolytic rate is shown. (e and f) The ATP production coupled oxygen consumption was also determined with the administration of the ATP synthase inhibitor oligomycin. AcD/TNFα induced a decrease in both OCR and ECAR parameters (*P<0.05, **P<0.01) and pre-treatment with γ-tocopherol attenuated these alterations ($^{\#}$P<0.05, $^{\#\#}$P< 0.01). Data are shown as the mean ± SEM values of n=15 wells collected from n=5 experiments performed on 3 different experimental days.

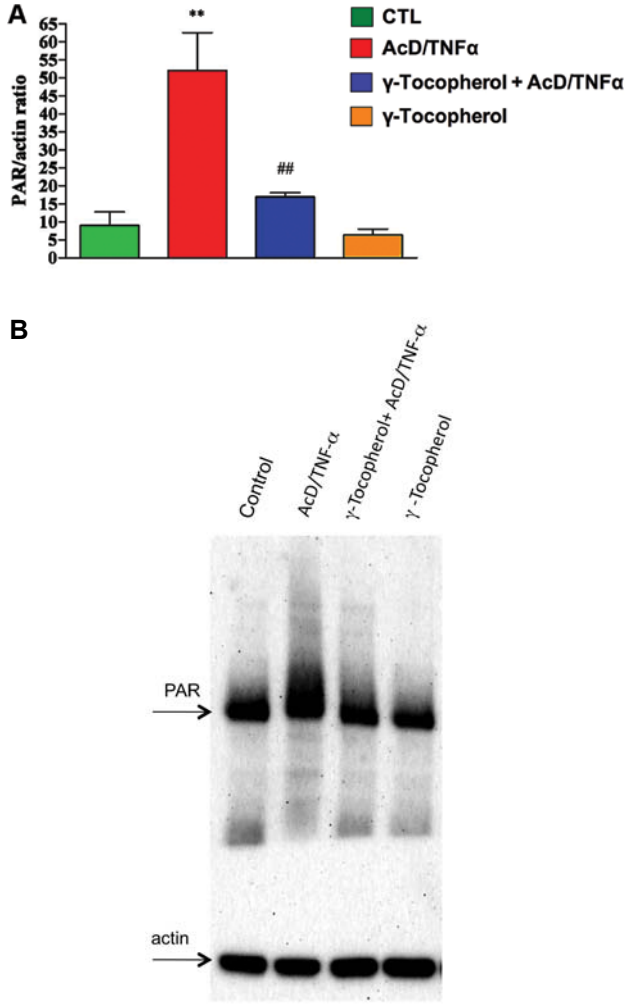

Figure 6. γ-tocopherol reduces PARP activation in response to actinomycin D and TNFα in L929 cells. Confluent L929 cultures were pre-treated with γ-tocopherol (100 μM) or vehicle and subjected to combined actinomycin D (0.5 μg/ml) and TNFα (100 pg/ml) (AcD/TNFα) for 24 h. PARP activation was detected by Western blotting for poly(ADP-ribose) (PAR), the product of the PARP enzyme. PARP activity is expressed as PAR/actin ratio. Data in (A) are shown as mean ± SEM values of n=9 wells collected from n=3 experiments performed on 3 different experimental days. (B) An individual representative Western blot.

and TNFα, and found that substantial (up to 10-fold) increases in TNFα concentration only result in a slight enhancement of the response. However, even in response to these elevated concentrations of TNFα + actinomycin D, the cells did not show any noticeable change in Cell Index for until approximately 5-6 h after stimulation. A decrease in TNFα and actinomycin D concentration to 30 or 10 pg/ml, on the other hand, resulted in a markedly diminished cytotoxic response (Fig. 4).

Bioenergetic changes after actinomycin D + TNFα administration. After obtaining the basal respiration rate in each group, cells were treated with actinomycin D and TNFα and mitochondrial and glycolytic activities were monitored for 10 h. Actinomycin D and TNFα induced an immediate decline in the oxygen consumption rate (OCR) and the cellular glycolytic rate (ECAR) that stabilized at 2 h after the administration of actinomycin D + TNFα (Fig. 5A). While these drops in the OCR and ECAR were relatively modest, actinomycin D + TNFα significantly diminished the mitochondrial respiratory reserve capacity and glycolytic rate of the cells as assessed by the mitochondrial uncoupling agent FCCP at 6 h (Fig. 5Ba-d). It is important to point out that all of these energetic alterations occurred in a time period at which the more conventional viability assays (MTT, LDH) or the XCelligence-based detection of cell conductance had not demonstrated any overt alterations, and at which time point no marked mitochondrial membrane depolarization (as detected by JC-1) had yet occurred. These observations are consistent with the hypothesis that an early oxidative stress response, induced by actinomycin D + TNFα, leads to a substantial, simultaneous defect of both oxidative phosphorylation and glycolysis in L929 cells, which already begins at an early time at which no overt cell toxicity or cell dysfunction is apparent.

The ATP production coupled oxygen consumption was also determined with administration of the ATP synthase inhibitor oligomycin using the Seahorse XF24 Analyzer. The oxygen consumption rate coupled to the ATP production was severely diminished in response to actinomycin D + TNFα. (Fig. 5Be-f). Actinomycin D + TNFα reduced the OCR linked to ATP production by 50% compared to the controls. Actinomycin D and TNFα reduced both the OCR and the ECAR by 40%, while γ-tocopherol exerted a significant protection against these alterations. Tocopherol restored the mitochondrial reserve capacity to approximately 90% of control values, whereas proved less efficient to revert the ECAR values representing the glycolytic changes in the cells. Taken together, the above observations are consistent with the hypothesis that an early oxidative stress response, induced by actinomycin D + TNFα, leads to a substantial, simultaneous defect of both oxidative phosphorylation and glycolysis in L929 cells, which already begins at an early time at which no overt cell toxicity or cell dysfunction is yet apparent.

Poly(ADP-ribose) polymerase (PARP) activation after actinomycin D + TNFα administration. Activation of PARP, a nuclear enzyme, has been implicated in many forms of oxidative cell injury (34), as well as in the current model of actinomycin D + TNFα induced cell dysfunction/cell death in L929 cells (13). Consistent with these observations, we have detected a substantial increase in PARylation of the cells. The most pronounced increase was seen at 24 h, which was a doubling of the PARylation response (Fig. 6); at 12 h the degree of the increase in PARylation was approximately 50% of the value seen at 24 h, whereas at 1, 3 and 6 h no significant increase in PARylation was detected (data not shown).

Effect of γ-tocopherol on the cellular responses induced by actinomycin D + TNFα. The presence of γ-tocopherol (100 μM), on its own, failed to influence any of the baseline parameters studied in the current series of experiments. This finding is consistent with the view that the antioxidant, at this concentration (100 μM), is devoid of any intrinsic cytotoxic effects. Pre-treatment with γ-tocopherol exerted a significant, and in most cases, complete or near-complete protection against the cellular responses induced by actinomycin D + TNFα: it completely prevented the suppression of MTT (Fig. 1A), the release of LDH (Fig. 1B), the increase mitochondrial superoxide formation (Fig. 2), the development of mitochondrial

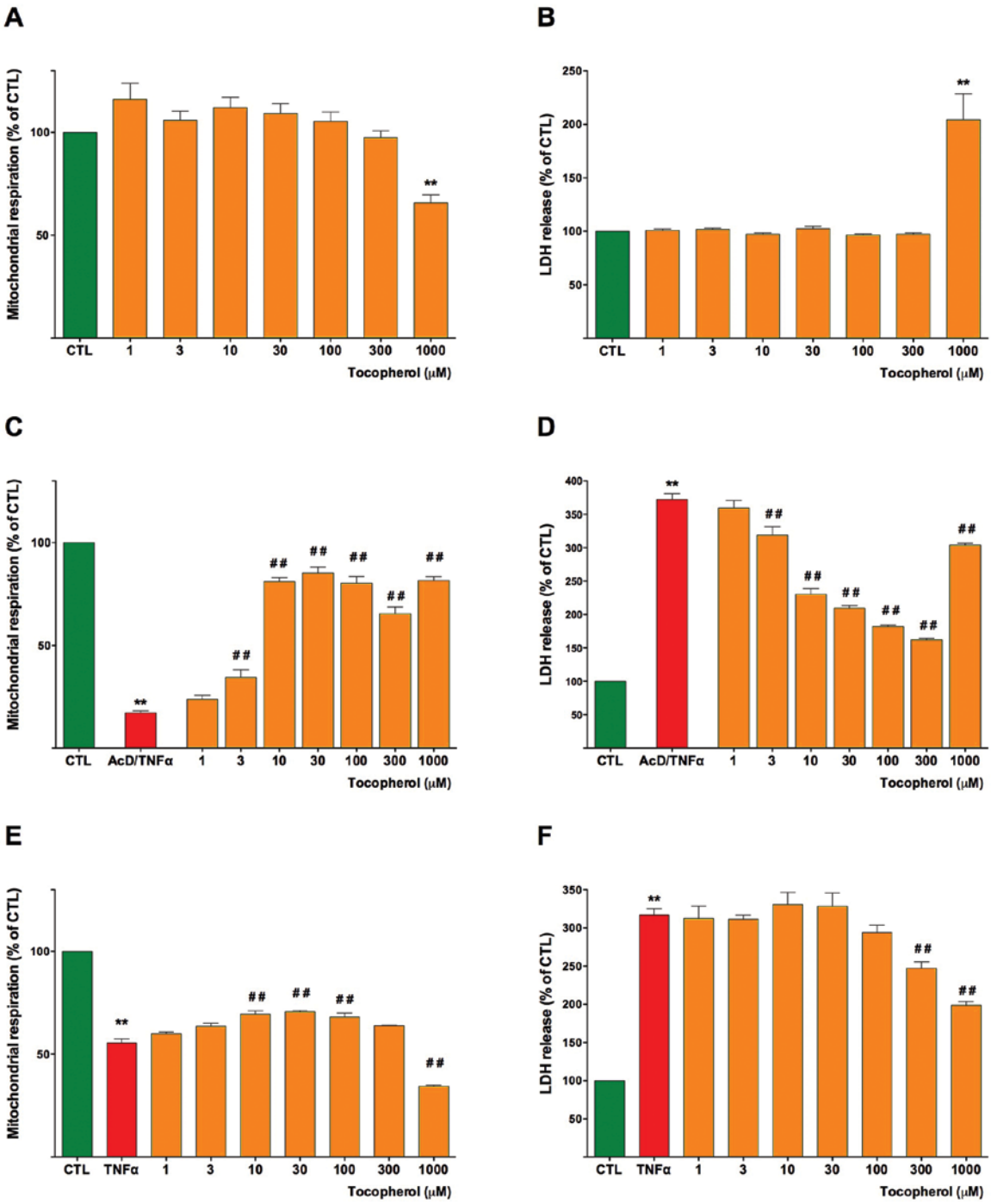

Figure 7. γ-tocopherol reduces L929 cell injury in response to actinomycin D and TNFα in L929 cells: a concentration-response study. Confluent L929 cultures were pre-treated with γ-tocopherol (1 μM - 1 mM) or vehicle and subjected to either vehicle treatment (A and B), or combined actinomycin D (0.5 μg/ml) and TNFα (100 pg/ml) (AcD/TNFα) treatment (C and D) or TNFα alone (1000 pg/ml) (E and F) for 24 h. Viability was determined by the MTT assay (left panels) and the LDH assay (right panel). There was a deterioration of both viability parameters after AcD/TNFα or TNFα compared to untreated control (CTL) cells (**P<0.01) and pre-treatment with γ-tocopherol significantly protected against these alterations ($^{\#\#}$P<0.01). γ-tocopherol (at 1 mM, but not at the concentrations tested below) exerted intrinsic cytotoxic effects. Data are shown as the mean ± SEM values of n=9 wells collected from n=3 experiments performed on 3 different experimental days.

depolarization (Fig. 3), the deterioration of Cell Index (Fig. 4), as well as the PARP activation (Fig. 6). As far as the changes in cellular energetics are concerned, γ-tocopherol markedly improved all parameters of oxidative phosphorylation and glycolysis; γ-tocopherol provided an approximately 75% preservation of the actinomycin D + TNFα-induced alterations in mitochondrial respiratory and glycolytic capacity (Fig. 5). The effect of γ-tocopherol was also examined in a concentration-response experiment on mitochondrial respiration and LDH release (Fig. 7). The optimal cytoprotective concentrations were 100 and 300 μM; at 30 and 10 μM the protective effect diminished, while at the top concentration tested (1 mM), the compound exerted a slight intrinsic suppressive effect on cell viability. In viability assays when cell injury was induced

by TNFα alone (at a higher concentration of 1000 pg/ml), γ-tocopherol continued to exert statistically protective effects starting at the concentration of 10 μM, although, in this series of studies, the extent of the protection remained partial in the entire concentration range of 10-1000 μM tested (Fig. 7).

Discussion

The model of actinomycin D and TNFα exposure in L929 cells has been often used to study cell death/cell injury in response to pro-inflammatory stimulation (12-15). The time-course of the response and many of the cellular effectors have been characterized by multiple experiments. Although the literature is somewhat controversial, according to the more recent body of published studies, the mode of cell death shows many features of necrotic phenotype (22), while caspase activation and apoptotic processes appear to play a minor role (23). The current findings (such as the LDH release into the medium after AcD/TNFα exposure) are consistent with cell membrane disruption and cell death via the necrotic route.

Mitochondrial generation of reactive oxygen species (24,25) as well as mitochondrial permeability transition (23) have been previously implicated in the pathogenesis of cell injury in the current model. The current findings (early reactive oxygen production, mitochondrial permeability transition) are consistent with these findings. However, it is important to point out that some of the earliest alterations we have observed in response to AcD/TNFα have occurred at the level of cellular metabolism: both oxidative phosphorylation and glycolysis were found to be impaired in a simultaneous manner, and these alterations appeared to be dependent on the formation of reactive oxidant species (as evidenced by the protection by γ-tocopherol against these alterations). The cellular energetic alterations induced by actinomycin D and TNFα observed in the current study are consistent with several studies in the literature demonstrating that the oxidant radicals can cause impaired mitochondrial function eventuating in a decrease in respiratory reserve capacity (20,26). Furthermore, many research groups have demonstrated that deleterious oxygen species have a crucial role in diminishing mitochondrial function via oxidative impairment of mitochondria, especially complex I (27,28) and complex III proteins (29). It is generally accepted that during oxidative and nitrosative stress, mitochondria are capable of using 'reserve capacity' which is available to serve the increased energy demands for maintenance of organ function, cellular repair or detoxification of reactive species. Consequently, impairment or depletion of this putative reserve capacity ultimately leads to excessive protein damage and cell death.

According to our results, γ-tocopherol pre-treatment significantly ameliorated the changes in the respiratory reserve capacity in L929 cultures subjected to actinomycin D and TNFα treatment, which suggests that γ-tocopherol acts on an upstream process, possibly, at least in part within the mitochondria. The fact that γ-tocopherol is a potent reactive oxygen species scavenger (antioxidant agent), which has the potential to reduce oxidative damage in various fields of cellular injury and recent studies demonstrating that γ-tocopherol improves mitochondrial function via reduction of reactive oxygen species produced by mitochondria (30) are consistent with this hypothesis. Indeed, mitochondrial oxidant production was diminished in γ-tocopherol-treated cells exposed to actinomycin D and TNFα, which may reflect a very effective intramitochondrial scavenger function, a reduced intramitochondrial ROS production or both. In this context it is important to emphasize that we have also demonstrated here that the mitochondrial oxygen consumption is more efficiently coupled to ATP production in the presence of γ-tocopherol that may represent a key step in the mitochondrial reserve capacity preservation and cell survival. We do not have any evidence for, and we do not propose that γ-tocopherol selectively concentrates in the mitochondria. Subcellular distribution studies demonstrate that the cellular distribution of tocopherols directly corresponds to the lipid distribution (31). Therefore, it is conceivable that some of the γ-tocopherol that we applied to the cells will be present in the mitochondrial membrane, and may be localized in the vicinity of the sites where ROS are produced (e.g. the mitochondrial respiratory chain).

In addition to resulting in a decreased level in mitochondrial respiratory capacity, the results obtained with the Seahorse XF24 analyzer also demonstrated that actinomycin D and TNFα lowers the glycolytic function of the cells. There are several series of data in the literature implicating that reactive oxygen species can diminish GAPDH enzyme function by activating PARP causing reduced GAPDH enzyme activity and subsequently attenuated glycolytic function (32). GAPDH may also be inactivated by S-thiolation induced by a respiratory burst or exposure to oxidants (33). According to our data γ-tocopherol exerted a statistically significant protective effect against the actinomycin D and TNFα-induced glycolytic impairment, which is potentially the result of a direct antioxidant effect of the molecule.

Activation of PARP has been shown to contribute to many forms of oxidant-induced cell injury, at least in part via modulation of cellular metabolism, through the activation of suicidal ATP-consuming energetic cycles triggered by oxidative and nitrosative DNA injury (34). In the current model, Agarwal *et al* (13) have demonstrated an increase in PARP activation and increased poly(ADP-ribosyl)ation. However, the time-course of PARP activation (which is not apparent in the first 6 h) is such, that it is unlikely that it plays a significant role in the early metabolic alterations and in the early phase of cell injury. Based on the results of the previous studies (demonstrating that cellular NAD^+ depletion and ATP depletion only starts around 6 h post actinomycin D and TNFα exposure, we believe that PARP activation is likely to play a more significant role in the second phase of the cell injury (at 12-24 h). The inhibitory effect of γ-tocopherol on PARP activation is consistent with the hypothesis that the antioxidant vitamin inhibits oxidant-induced DNA injury, which is an obligatory trigger for an increase in the catalytic activity of the constitutively expressed PARP-1 enzyme (the major PARP isoform). These findings are also consistent with *in vivo* studies in an acute lung injury model showing that treatment of the animals with γ-tocopherol inhibits tissue PARP activation (6). PARP is certainly not the only oxidant-induced downstream pathway of inflammation/cell injury that is triggered by actinomycin D and TNFα exposure in the current model; several studies have demonstrated protein kinase C activation and p38 activation (35). In this context it is noteworthy that in the current model,

γ-tocopherol treatment (100 μM) also blocked the phosphorylation of p38, as measured at 24 h (unpublished data).

Several limitations to the current study need to be acknowledged. First of all, the current study is an entirely *in vitro* investigation, and, as such, can only mimic to a limited extent the various pathophysiological conditions where γ-tocopherol has been shown to be effective. Therefore, the conclusions made by the current set of studies may not be fully applicable to the *in vivo* conditions. Second, the current experimental model (combination of a pro-inflammatory cytokine and a transcription inhibitor), even though a frequently used and studied experimental model, has certain limitations, and does not fully mimic pathophysiological states (where transcriptional inhibition does not normally occur).

In order to overcome this limitation, we have also assessed the effect of γ-tocopherol in L929 cells exposed to TNFα alone (in the absence of a transcriptional inhibitor). Although the protection by γ-tocopherol against cell injury in this model was still statistically significant, it was less pronounced than when the protocol utilized the combined application of actinomycin D and TNFα (Fig. 7). There are substantial differences in the molecular mechanisms of the cell death/cell injury, depending on the absence or presence of protein synthesis inhibitors (15), and we hypothesize that under the conditions of TNFα alone (where the cytokine needed to be applied at a substantially higher concentration, and even at this concentration, the degree of cell death was less pronounced than when actinomycin D and TNFα were used together), multiple additional pathways of cell injury and cell death become activated. Some of these pathways (caspases, PARP and other effectors) may not be entirely dependent on oxyradicals, and this may explain the reduced effectiveness of γ-tocopherol. Nevertheless, we must point out that even in this native system, γ-tocopherol, at a physiologically and therapeutically relevant concentration (as low as 10 μM) afforded statistically significant cytoprotection, supporting the overall conclusions of the current study.

In summary, the current studies have demonstrated that γ-tocopherol exerts protective effects in cells exposed to various types of oxidative stress, as well as in various *in vivo* models of acute and chronic diseases which are, at least in part, developing on the basis of an increased oxidative or nitrosative stress. The current results further underline the antioxidant potency of γ-tocopherol, and may explain some of the protective mode of action for this compound.

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