Nitroxides Tempol and Tempo Induce Divergent Signal Transduction Pathways in MDA-MB 231 Breast Cancer Cells*

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Simeng Suy‡, James B. Mitchell§, Desiree Ehleiter¶, Adriana Haimovitz-Friedman¶, and Usha Kasid∥

From the ‡Departments of Radiation Medicine and Biochemistry and Molecular Biology, Lombardi Cancer Center, Georgetown University Medical Center, Washington D.C. 20007, the ¶Radiation Biology Division, NCI, National Institutes of Health, Bethesda, Maryland 20892, and the ∥Department of Radiation Oncology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Tempol and tempo are stable free radical nitroxides that possess antioxidant properties. In this study, we examined the effects of these compounds on components of the mitogen-activated protein kinase signal transduction cascade. Tempo treatment (15 min) of MDA-MB 231 human breast cancer cells resulted in significant levels of tyrosine phosphorylation of several as yet unidentified proteins compared with equimolar concentration of tempol (10 μM). Both compounds caused tyrosine phosphorylation and activation of Raf-1 protein kinase (30 min, 2-3-fold). Interestingly, however, only tempol caused increased extracellular signal-regulated kinase 1 activity (2 h, >3-fold). On the other hand, tempo, but not tempol, potently activated stress-activated protein kinase (2 h, >3-fold). Consistent with these data, tempol was found to be noncytotoxic, whereas tempo induced apoptotic cell death (2 h, >50%). Tempo treatment also resulted in significant elevation of ceramide levels at 30 min (54% over control) and 1 h (71% over control) posttreatment, preceding stress-activated protein kinase activation and apoptosis. These data suggest that in the absence of an environmental oxidative stress, tempol and tempo elicit distinct cellular signaling pathways. The recognition of the molecular mechanisms of nitroxide action may have important implications for biological effectiveness of these compounds.

A cellular antioxidant defense system composed of enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione protects cells against toxic oxygen metabolites. Exogenously added free radical scavengers have also been shown to alleviate the deleterious effects of oxygen free radicals (1–4). Nitroxide compounds, including tempol and tempo (Fig. 1), are low molecular weight, membrane permeable, stable free radicals that are electron paramagnetic resonance detectable (5) and have been used classically as probes for biophysical and biochemical processes; they have been used as paramagnetic contrast agents in NMR imaging (6, 7), as probes for membrane structure (8), and as sensors of oxygen in biological systems (9). However, over the past few years, novel applications of nitroxides have been demonstrated. Nitroxides have been shown to possess antioxidant activity and protect cells against a variety of agents that impose oxidative stress, including superoxide, hydrogen peroxide, and ionizing radiation (10–20). A variety of chemical mechanisms have been proposed to account for nitroxide antioxidant activity, including superoxide dismutase mimic activity (21), oxidation of reduced metals that would otherwise catalyze the formation of hydroxyl radicals (10) from hydrogen peroxide (10), catalase mimic activity (22), radical-radical interactions (13), and detoxification of secondary organic radicals (13). Although significant research has been conducted at the whole cell level and in animals with nitroxides, little is known at the molecular level of how this novel class of antioxidants affects signal transduction pathways.

Members of the mitogen-activated protein kinase (MAPK) family, including ERKs (p42/44 MAPKs), the stress-activated protein kinases (SAPKs) (also called c-Jun NH₂-terminal kinases (p46/54 JNKs/SAPK1)), and p38 MAPK (also termed reactivating kinase (p38RK)), are activated in response to a variety of cellular stresses, such as changes in osmolality and metabolism, DNA damage, heat shock, ischemia, UV radiation, ionizing radiation, or inflammatory cytokines (23–44). In many of these instances, free radicals and derived species play an important role in initiating a cellular signal transduction response (45). Unlike the ERK signaling pathway, which primarily promotes growth and proliferation/survival, the SAPK and p38 MAPK pathways result in growth arrest and apoptotic or necrotic cell death. Because nitroxides protect against diverse oxidative insults and may have utility in clinical biomedical research, we have investigated the effects of tempol and tempo on MAPK signal transduction pathways in an attempt to better understand their mechanism of action. Evidence presented here demonstrates that tempol and tempo stimulate distinct pathways of the MAPK signaling cascade. Tempol stimulated the ERK activity and was noncytotoxic, whereas tempo induced ceramide generation, SAPK/JNK activation, and apoptotic death of MDA-MB 231 human breast cancer cells. The cytotoxic effect of tempo was also observed in two other cancer cell types, PCL-04A laryngeal squamous carcinoma cells and PC-3 prostate cancer cells. These findings provide new insight into the

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∥To whom correspondence should be addressed: E208, Research Bldg., Georgetown University, 3970 Reservoir Rd., N.W., Washington D.C. 20007. Tel.: 202-687-2226; E-mail: Kasidu@gunet.georgetown.edu.

1 The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; FITC, fluorescein isothiocyanate; PI, propidium iodide; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; WCL, whole cell lysate; KBB, kinase binding buffer; 20 μM 4-morpholinopropanesulfonic acid, pH 7.2, 25 μM β-glycerol phosphate, 5 μM EGTA, 1 mM Na₂VO₄, 1 mM dithiothreitol; MBP, myelin basic protein; DAG, diacylglycerol.
mechanism of action of nitroxide antioxidants, which will be valuable in understanding their biological effectiveness.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The following antibodies were used in this study: anti-SAPK polyclonal antibody (α-NT), anti-phosphotyrosine monoclonal antibody (mAb) (α-PY, 4G10), and agarose-conjugated α-PY (Upstate Biotechnology, Lake Placid, NY); agarose-conjugated anti-ERK1 (C-16, sc-93a), anti-JNK1 (C-17 sc-474a), and anti-Raf-1 (C-12) polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-Raf-1 mAb (α-Raf1) and anti-ERK1 mAb (MK12) (Transduction Laboratories, Lexington, Kentucky). Protein A-agarose and Syntide-2 were obtained from Santa Cruz Biotechnology, Inc. The nitroxide compounds tempol (2,2,6,6-tetramethylpiperidine-N-oxyl) and tempo (4-hydroxy-tempol) were obtained from Aldrich. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and 5% TGS electrophoretic buffer (49 mM Tris, 384 mM glycine, 0.1% SDS) were purchased from Bio-Rad Laboratories, Inc. and premixed 10% Tris-glycine transfer buffer was obtained Bio-Rad. All other reagents were obtained from Sigma unless otherwise indicated.

Cell Culture, Treatments with Tempol and Tempo, and Preparation of Cell Lysates—MIA-MB 231 human breast cancer cells were grown to near confluence in 75-cm² tissue culture flasks in improved minimum essential medium (Cellgro) containing 10% fetal bovine serum and 2 mM l-glutamine in a humidified atmosphere of 5% CO₂:95% air at 37 °C. Cells were trypsinized and plated onto a 150-mm tissue culture dish (two dishes per flask) overnight in medium containing 10% fetal bovine serum followed by two washes with phosphate-buffered saline (PBS). Cultures were maintained in serum-free medium overnight prior to tempol (10 μM) or tempo (10 μM) treatment. Both nitroxide radicals were dissolved in ethanol before use. For the extraction of whole cell lysates (WCLs), cells with or without nitroxide treatment were washed three times with ice-cold PBS containing 0.5 mM MgCl₂ and 0.5 mM Na₃VO₄ and lysed in lysis buffer (50 mM HEPES, pH 7.5; 1% Nonidet P-40; 10% glycerol; 4 mM Na₃VO₄) and lysed in lysis buffer (50 mM HEPES, pH 7.5; 1% Nonidet P-40; 10% glycerol; 4 mM Na₃VO₄). The cell pellet was washed once with PBS and twice with phosphate buffered saline and refed in serum-free medium. Cells were maintained overnight in serum-free medium. At the end of the incubation, reaction mixture was centrifuged briefly in a benchtop microcentrifuge, and 5 μl of the supernatant was spotted in triplicate onto P81 filter paper, air dried, washed, and counted as described above.

ERK and SAPK/JNK Activities—Whole cell lysates prepared as described above were immunoprecipitated (1 μg) with an agarose-conjugated anti-ERK1 antibody or an agarose-conjugated anti-JNK1 antibody for 2 h at 4 °C with constant agitation. The immune-complexes were washed three times in lysis buffer and once in PBS as mentioned earlier. ERK or JNK activity assay was carried out according to manufacturer’s procedures (Upstate Biotechnology). Briefly, the ERK1 immunoprecipitates were incubated for 10 min at 30 °C in a kinase reaction containing 10 μl of MBP as substrate (2 mg/ml stock), 10 μl of inhibitor mixture (20 μM protein kinase C inhibitor peptide, 2 μM protein A inhibitor peptide, and 20 μM compound R24571), and 10 μl of magnesium-ATP mixture (1 μM of [γ-32P]ATP generated by 1.10 dilution of stock (3000 Ci/mmol) in 75 mM magnesium chloride and 500 μM cold ATP). The immune-complexes were centrifuged briefly in a benchtop centrifuge, and 5 μl aliquots of the supernatant were spotted in triplicate onto P81 filter papers. The radioactive filters were washed and counted as described above. Alternatively, to visualize the incorporation of radiolabel into MBP, the kinase reaction was quenched by addition of 20 μl of electrophoresis sample buffer and boiled for 5 min, and proteins were resolved by 15% SDS-PAGE, followed by autoradiography. For JNK activity assay, JNK1 immunoprecipitates were incubated for 30 min at 30 °C in 40 μl of kinase reaction mixture containing 10 μl of KBB, 20 μl of the GST-c-Jun fusion protein (0.2 μg/ml stock), and 10 μl of the diluted [γ-32P]ATP as described above. The kinase reaction was quenched by addition of 20 μl of electrophoresis sample buffer and boiled for 5 min, and proteins were resolved by 15% SDS-PAGE, followed by autoradiography. The labeled GST-c-Jun fusion protein was detected by autoradiography.

Cell Viability Assay—Effects of nitroxide compounds on cell viability and proliferation were determined using a cell viability detection kit (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1-benzene disulfonate, WST-1) according to the manufacturer’s instructions (Boehringer Mannheim). Briefly, MDA-MB 231 cells were seeded onto 96-well plates at density of 10,000 cells/well and maintained overnight in 10% fetal bovine serum-containing medium. The cells were then washed twice with phosphate buffered saline and refed in serum-free medium. The following day, cells were treated for various times with tempol (10 μM) or tempo (10 μM), using six wells per treatment condition. At the end of treatment, medium containing the nitroxide compound was removed and replaced with fresh serum-free medium (100 μl), followed by addition of WST-1 (10 μl). Plates were incubated for 2 h at 37 °C and analyzed at A = 450/600 using a MR 700 microplate reader.

ApoAlert Annexin V apoptosis detection system (CLONTECH, Palo Alto, CA) was used to measure the relative distribution of apoptotic and necrotic cells. Briefly, cells were seeded at a density of 10⁵ cells/cm² in 100-mm tissue culture dishes and maintained in serum-free medium containing 10% fetal bovine serum overnight, followed by washing twice in serum-free medium. Cells were maintained overnight in serum-free medium and exposed to tempol (10 μM) or tempo (10 μM) for various times. This was followed by rinsing twice with serum-free medium prior to trypsinization, dilution in two volumes of serum-free medium, and centrifugation at 10,000 × g. The cell pellet was washed once with PBS.
Tempo and Tempo Stimulate Tyrosine Phosphorylation and Activity of Raf-1 in Vivo—Previously, we demonstrated that ionizing radiation, a well known stress-inducing agent, causes tyrosine phosphorylation of Raf-1 in MDA-MB 231 breast cancer cells (43). Here, we examined the possibility of tyrosine phosphorylation and activation of Raf-1 protein kinase in response to tempol and tempo. Interestingly, both tempol and tempo treatments led to an increase in the level of tyrosine phosphorylated Raf-1 (RafP) (Fig. 3A, top panel). The level of total Raf-1 protein remained unchanged (Fig. 3A, bottom panel). The immunoreactive RafP bands were quantified. Densitometric analysis indicated that increase in the level of RafP detected at 15 min was ~5–8-fold, and RafP content was comparable to the basal level by ~60–120 min (data not shown). The activity of Raf-1 protein kinase was determined by a kinase cascade assay or by the Syntide-2 phosphorylation assay (Fig. 3B). In agreement with the enhanced tyrosine phosphorylation of Raf-1, tempol or tempo treatment resulted in ~2–3-fold increase in the Raf-1 protein kinase activity.

Tempol Stimulates ERK Activity—Because Raf-1 activation generally leads to ERK (p42/44 MAPK) activation, we examined the effects of tempol and tempo on ERK1 enzymatic activity. Representative experiments are shown in Fig. 4. An approximately 3-fold increase in the enzymatic activity of ERK1 was detected by 2 h in cells treated with tempol (Fig. 4A). Interestingly, however, no change in ERK1 activity was noted following tempo treatment compared with control cells (Fig. 4A). In addition, ERK1 phosphorylation was seen as a shift to a more slowly migrating phosphorylated form (ERK1P) on immunoblots using ERK1 immunoprecipitates at 2 h after tempol exposure (10 min), but we were unable to identify a shift in the mobility of ERK1 in tempo-treated cells (data not shown).

Tempo Treatment Results in Enhanced Phosphorylation and Activation of SAPK in Vivo—We next measured the effects of tempol and tempo on SAPK/GNK, a well-known component of the stress-induced signal transduction pathway. The time course experiments indicated that tempol treatment resulted in a significant increase in the level of phosphorylated SAPAK (~54 kDa, SAPK*) compared with tempol treatment or untreated controls (Fig. 5A and B). Consistent with these data, SAPK enzymatic activity was significantly induced in tempol-treated cells as shown by the level of phosphorylated GST-c-Jun (Fig. 5C). Densitometric analysis of three independently performed experiments indicated a 3–7-fold increase in the phosphorylated GST-c-Jun fusion protein detectable after tempol exposure (10 min, 2 h) compared with tempol (10 min, 2 h) or control (1% ethanol, 2 h) treatment.

Tempo Induces Apoptotic Cell Death—Several studies have reported that activation of the SAPK signaling cascade is associated with induction of apoptotic cell death (32). To examine the possible cytotoxic effects of tempo, we first used a colorimetric assay to determine the cell viability and proliferation. Treatment of cells with 10 mM tempo resulted in >50% decrease in the number of viable cells within 2 h. In parallel experiments, the number of viable cells in cultures treated with 10 mM tempol was comparable to control cells treated with 1% ethanol (Fig. 6). These observations prompted us to evaluate whether decrease in the number of viable cells following tempo treatment was due to apoptotic and/or necrotic cell death.

Apoptosis is a process of cell death characterized by cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation (48). Several reports suggest that an early event leading to apoptosis is accompanied by a loss of cell membrane phospholipid asymmetry as a result of translocation of phosphatidylinerine from the intracellular membrane to the extracellular membrane while leaving the cell membrane intact (49). A phosphorus...
phosphatidylserine-binding protein, annexin V, has been used as a specific probe to detect externalization of this phospholipid in a variety of murine and human cell types undergoing apoptosis (50, 51). Cell necrosis, on the other hand, is associated with both the translocation of phosphatidylserine to the external cell surface and the loss of membrane integrity (52). The cell membrane integrity of apoptotic cells can be established with a dye exclusion test using PI. In the following experiments, we used FITC-conjugated annexin V and PI as markers for the evaluation of apoptosis and necrosis. MDA-MB 231 cells treated with tempol or tempo were double-labeled with FITC-conjugated annexin V and PI and then subjected to flow cytometric analysis. Representative cytogram analysis of MDA-MB 231 cells with or without nitroxide compound is shown in Fig. 7A. The lower left quadrant represents viable cells (V), which were negative for annexin V and PI. The lower right quadrant represents apoptotic cells (A), which were positive for annexin V staining. The upper right quadrant represents necrotic cells (N), which were positive for both annexin V and PI stains. Tempo treatment (10 mM for 2 h) resulted in a significant increase in the number of apoptotic cells for up to 2 h, followed by a considerable increase in the number of necrotic cells by 3 h (Fig. 7B). Tempo treatment did not induce apoptosis or necrosis for the duration of the study (3 h) (Fig. 7B). These data suggest that time-stimulated SAPK phosphorylation and activation may be associated with apoptotic cell death in MDA-MB 231 cells.

To determine the generality of the cytotoxic effect of tempo in cancer cells, we have examined two other cancer cell lines: PCI-04A, a human laryngeal squamous carcinoma-derived cell line (53), and PC-3, a human prostate cancer cell line. The data shown in Fig. 7, C and D, demonstrate a significant level of apoptosis and necrosis at 2 h post-tempo treatment (10 mM) in PCI-04A cells. In PC-3 cells, 10 mM tempo treatment resulted in ~84% necrotic cells by 2 h, implying that this treatment condition was highly toxic (Fig. 7E and F). Tempo also induced apoptotic cell death in bovine aortic endothelial cells, as measured by the bisbenzamide trihydrochloride/Hoechst-33258 staining method, as previously described (46) (control: 4 h, 1.55 ± 0.02%; 8 h, 1.99 ± 0.43%; tempo (5 mM): 4 h, 3.97 ± 0.33%; 8 h, 38.85 ± 1.69%). These results clearly demonstrate that tempo but not tempol induces cell death in different types of cells.
and the experiment was repeated three times. Determinations per treatment condition in a representative experiment, cells were grown in serum-free medium overnight in 96-well plates and treated with tempol (10 mM) or tempo (10 mM) for 2 h. WCLs (1 mg) were immunoprecipitated with anti-JNK1 antibody, and the JNK1 activity in immunoprecipitates was measured using GST-c-Jun (~41 kDa) as a substrate. UT, untreated cells grown overnight in serum-free medium; CONT/Control, cells grown in serum-free medium overnight and treated with 1% ethanol for 2 h.

Ceramide Generation in Tempo-treated MDA-MB 231 Cells—Ceramide, a second messenger molecule generated as a result of hydrolysis of the plasma membrane phospholipid sphingomyelin or via de novo synthesis, has been implicated in a variety of biological responses to environmental cues (54). Increase in ceramide has been correlated with increased SAPK/JNK activity, and ceramide and SAPK/JNK have been shown to participate in a signal transduction pathway leading to cell death (26, 44, 55, 56). To assess the possibility of a role of ceramide in tempo-induced SAPK and apoptosis, we used a DAG kinase assay to quantify the ceramide levels in MDA-MB 231 cells treated with or without the nitroxide compound. A 54% increase over control (normalized to 100%) in ceramide level was observed at 30 min, and ceramide level reached 71% over control at 1 h post-tempo treatment (Fig. 8). The level of ceramide generated in tempol-treated cells was not significantly higher compared with control cells at all time points. A significant increase in ceramide level was also noted in tempol-treated (5 mM) bovine aortic endothelial cells, but with slightly different kinetics (data not shown). Ceramide production preceded maximal stimulation of SAPK/JNK and apoptosis, implying its involvement in tempo-induced signaling in MDA-MB 231 cells.

**DISCUSSION**

This study reports, for the first time to our knowledge, signal transduction mechanisms of cellular response to two nitroxides, tempol and tempo, that are well known for their antioxidant properties. Initially, we hypothesized that because ERK pathway is used by a wide variety of cell types for transducing survival or proliferative signals, the antioxidant effects of tempol and tempo may be complemented by stimulation of the ERK signaling pathway. Previous *in vitro* studies suggested that at least 5–10 mM tempol is required to provide radioprotection, and a protection factor as high as 2.2 was achieved with 100 mM tempol (15). Our data showing activation of ERK1 by tempol (10 mM) are consistent with these and other reports of a protective role of tempol against radiation-induced mutagenicity and double strand breaks and hydrogen peroxide-induced mutagenicity (15, 57–59). Surprisingly, however, tempo (10 mM) had no detectable effect on ERK1 activity, suggesting that a dissociation may also exist between ERK signaling and antioxidant activity of certain nitroxides.

Enhanced protein tyrosine phosphorylation, generation of ceramide, activation of SAPK, and induction of apoptosis by tempo are unexpected and novel observations. One possibility for further evaluation is that there may be differential intracellular reduction rate of tempol versus tempo. In this situation, tempo-treated cells may have higher tempo free radical concentration. Free radicals, as second messengers, would then find appropriate cellular targets and turn on a signaling pathway. In this context, it is noteworthy that addition of platelet-derived growth factor to vascular smooth muscle cells results in increased intracellular levels of hydrogen peroxide and reactive oxygen species, and these events have been correlated with platelet-derived growth factor-induced tyrosine phosphorylation, MAPK stimulation, and DNA synthesis (60). In other reports, induction of protein tyrosine phosphorylation in neutrophils is dependent on NADPH oxidase activation (61), and stimulation of as yet unidentified protein tyrosine kinases has been linked to apoptotic death of B-lymphocytes (62). The short time required to observe the apoptosis (2 h) (Fig. 7) suggests that cell cycle, DNA synthesis, or significant transcription/translation may not be a prerequisite for tempol-initiated cell death. It seems possible that posttranslational modification of existing proteins required for the induction of apoptosis is regulated by a free radical-mediated protein kinase pathway(s) involving SAPK.

Endogenous sphingolipid metabolites, such as ceramides and sphingosines, have been recognized as lipid mediators of cell growth, differentiation, and apoptosis (46, 63–67). Apoptosis
Tempo, but Not Tempol, Induces SAPK and Apoptosis

Fig. 7. Tempo induces apoptotic cell death. Cells were grown in serum-free medium overnight in 60-mm dishes and treated with tempol (10 mM) and tempo (10 mM) for various times, trypsinized, and then resuspended in 200 μL of 1× binding buffer as described under “Experimental Procedures.” The cell suspension was double-stained with annexin V-FITC staining) or necrosis (propidium iodide staining). Values shown are the mean ± S.D. of quadruplicate determinations; the value at each time point is normalized to control (100%).

Fig. 8. Ceramide production in tempo-treated MDA-MB 231 cells. Logarithmically growing cells were cultured in serum-free medium in 60-mm dishes and treated with tempol (10 mM) and tempo (10 mM) for indicated times, followed by lipid extraction and quantitation of ceramide by DAG kinase assay as described under “Experimental Procedures.” The organic phase extract containing the γ-32P-labeled ceramide was quantitated. Control cells were grown overnight in serum-free medium, followed by treatment with 1% ethanol for various times ranging from 0.5 to 2 h. Tempo/tempo treatment values shown are the mean ± S.D. of triplicate determinations; the value at each time point is normalized to control (100%).
initiate a signal transduction cascade distinct from tempol, which is more water soluble and more evenly distributed throughout the cell. Although both tempol and tempol stimulated Raf-1, ERK1 activity was increased only in tempol-treated cells. Raf activation was short-lived compared with ERK1.

Raf-1 activity peaked at 30 min, whereas ERK activity began to rise at 15 min and continued to rise for at least 120 min. This lack of correlation between the kinetics of Raf-1 activation and ERK activation has been observed earlier (29, 43) and may be due to multiple effectors, including Raf-1, upstream of ERK. At present, the significance of Raf-1 activity in a nitroxide-induced response is unclear. Mitogen-activated ERK kinase, a known physiological substrate of Raf-1 protein kinase, (45) tempol and tempol stimulate tyrosine phosphorylation and activity of Raf-1 protein kinase, (45) tempol and tempol results to explore this possibility.

Clinical utility. Studies are presently under way in our laboratories to explore this possibility.

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