

Erk1/2 Phosphorylation and Reactive Oxygen Species Formation via Nitric Oxide and Akt-1/Raf-1 Crosstalk in Cultured Rat Cerebellar Granule Cells Exposed to the Organic Solvent 1,2,4-Trimethylcyclohexane

Oddvar Myhre,^{*,†,1} Sigrun Hanne Sterri,^{*} Inger Lise Bogen^{*} and Frode Fonnum^{*}

^{*}Norwegian Defence Research Establishment, Division for Protection and Materiel, P.O. Box 25, N-2027 Kjeller, Norway, and [†]VISTA (The Norwegian Academy of Science and Letters/Statoil), P.O. Box 25, N-2027 Kjeller, Norway

Received March 1, 2004; accepted April 13, 2004

Previously, we have shown that exposure of cultured rat cerebellar granule cells to the hydrocarbon solvent 1,2,4-trimethylcyclohexane leads to formation of reactive oxygen species (ROS). However, the cellular mechanisms responsible for formation of ROS in these cells after exposure to organic solvents are poorly understood. Here, we found that 1,2,4-trimethylcyclohexane induced a time and concentration dependent dephosphorylation of Akt-1 at Ser-473 and Raf-1 at Ser-259. An increased level of phosphorylated extracellular signal-regulated kinases (Erk1/2) at Tyr-204 was observed. By use of the nitric oxide synthase inhibitors N^ω-nitro-L-arginine methylester and diphenyleiiodonium, we found that intracellular formation of nitric oxide was necessary for phosphorylation of Erk1/2 and for the formation of ROS. Furthermore, the ROS formation was inhibited by the Erk1/2 pathway inhibitor U0126. A 1,2,4-trimethylcyclohexane (TMCH)-induced cell death was lowered by U0126 and the free radical scavenger vitamin E. Our results show that Erk1/2 kinases and nitric oxide (NO) may participate in ROS formation induced by 1,2,4-trimethylcyclohexane in cultured rat cerebellar granule cells, and also indicate a crosstalk between Akt and the Raf-Mek-Erk signaling systems.

Key Words: organic solvents; mitogen-activated protein kinases; Akt-1; Raf-1; nitric oxide; reactive oxygen species.

Long-term exposure to organic solvents may lead to cerebellar dysfunction (Lam *et al.*, 2000; Malm and Lying-Tunell, 1980), cerebral atrophy, and changes in white matter (Feldman *et al.*, 1999; Kamran and Bakshi, 1998). The cellular mechanisms that lead to solvent-induced damage are poorly understood; however, recent studies support the hypothesis that formation of reactive oxygen species (ROS) may lead to neuronal cell damage. We have shown that inhibition of ROS formation in cerebellar granule cells (CGC) caused by L-2-chloropropionic

acid (Myhre *et al.*, 2001) and polychlorinated biphenyls (Mariussen *et al.*, 2002) strongly decreased cell death. Intraperitoneal administration of toluene increased formation of ROS in the rat brain (Mattia *et al.*, 1991, 1993a,b), and benzene increased formation of hydroxyl radicals in bone marrow of rats (Khan *et al.*, 1990). Recently, exposure to organic solvents is shown to induce an extensive rise in ROS formation in CGC (Dreiem *et al.*, 2002). However, the complex upstream pathways responsible for formation of ROS in CGC after exposure to organic solvents are poorly understood. In the present work, we have investigated the importance of phosphorylation of extracellular signal-regulated kinases (Erk1/2) for ROS formation and cell death caused by hydrocarbon solvents, since oxidative stress caused by high glutamate (Sato *et al.*, 2000; Stanciu *et al.*, 2000), neuropathological stimulation of the NMDA receptor (Murray *et al.*, 1998), or hydrogen peroxide (Samanta *et al.*, 1998) are associated with increased level of phosphorylation of these kinases in cultured primary neuronal cells.

The classical pathway whereby Erk1/2 types of MAPkinases are activated is through the Ras-Raf-Mek cascade, but some intracellular signalling pathways that transmit signals from the plasma membrane to the nucleus are suggested to be regulated by formation of nitric oxide (NO; Stamler *et al.*, 2001). The present study was in part undertaken to investigate the role of NO in phosphorylation of Erk1/2 in CGCs. Another potential regulator of Erk1/2 activation in our system was considered to be the Akt-1/Raf-1 crosstalk, since it has been shown that Akt-1 may regulate the Raf-Mek-Erk pathway in vascular smooth muscle cells (Reusch *et al.*, 2001), in differentiated myotubes (Rommel *et al.*, 1999) and in a human breast cancer cell line (Zimmermann and Moelling, 1999) through phosphorylation of Raf-1 at the Ser-259 regulatory site. Thus, a possible crosstalk may exist between Akt-1 and the Raf-Mek-Erk cascade in our system.

In the present work, we have studied the cellular mechanisms for ROS formation and cell death in cultured rat CGCs exposed to the hydrocarbon solvent 1,2,4-trimethylcyclohexane. The level of phosphorylated Akt-1 and Raf-1 was investigated to

¹ To whom correspondence should be addressed at Amersham Health AS, Department of Safety, Nycoveien 2, P.O. Box 4220, Nydalen, N-0401 Oslo, Norway. Fax: + 47 23186008. E-mail: oddvar.myhre@amersham.com.

probe the possible roles of an Akt-1/Raf-1 crosstalk in phosphorylation of Erk1/2 and ROS formation. Possible involvement of NO in regulation of Erk1/2 phosphorylation was elucidated by use of specific NO synthase inhibitors.

MATERIALS AND METHODS

Materials. Acetic acid, bromophenol blue, Coomassie Brilliant Blue-G, cytosine- β -D-arabinofuranoside, 2',7'-dichlorofluorescein diacetate (DCFH-DA), diphenyleiiodonium (DPI) chloride, glycerol, L-glutamine, 2-mercaptoethanol, methanol (MeOH), N^o-nitro-L-arginine methylester (L-NAME), N-(1-naphthyl) ethylenediamine dihydrochloride, β -nicotinamide adenine dinucleotide (NADH), S-Nitroso-N-acetyl-D,L-penicillamine (SNAP), phosphoric acid (H₃PO₄), poly-L-lysine, pyruvate, sodium dodecyl sulphate (SDS), sulfanilamide, (\pm) α -tocopherol (vitamin E), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). U0126 was obtained from Promega Corporation (Madison, WI). The solvent 1,2,4-trimethylcyclohexane (TMCH) was purchased from Aldrich Chemical Company (St. Louis, MO). Hanks Balanced Salt Solution (HBSS) [CaCl₂·2H₂O 1.26 mM, KCl 5.37 mM, KH₂PO₄ 0.44 mM, MgCl₂·6H₂O 0.49 mM, MgSO₄·7H₂O 0.41 mM, NaCl 140 mM, NaHCO₃ 4.17 mM, Na₂HPO₄ 0.34 mM, D-glucose 5.55 mM], Eagles Basal Medium, heat inactivated fetal bovine serum, and HEPES buffer were purchased from Gibco BRL (U.K.). Penicillin and streptomycin were from BioWittaker, Europe. Enhanced chemiluminescence (ECL) reagent was from Amersham Biosciences, Norway. The following antibodies were used: mouse phospho-Erk1/2 (Tyr-204) monoclonal antibody (mAb), and rabbit phospho-Akt-1 (Ser-473) polyclonal antibody (pAb) (Santa Cruz Biotech Inc., Santa Cruz, CA), rabbit phospho-Raf (Ser-259) polyclonal antibody (Cell Signalling Technology Inc., MA), horseradish peroxidase-conjugated rabbit anti-mouse IgG- and goat anti-rabbit IgG- antibodies (DAKO A/S, Denmark).

Neuronal cell culture. Primary cultures of rat CGCs were prepared essentially as described previously (Gallo *et al.*, 1982), with a few modifications. Briefly, cerebella were dissected from seven-day-old Wistar rat pups (Møllegaard breeding laboratories, Denmark), meninges were removed, and single cell-suspension was obtained through trypsinizing and mechanical trituration of the tissue. Cells were plated into 50 mm cell culture dishes that had been coated previously for 1 h with poly-L-lysine (10 μ g/ml). The cells were seeded (10⁶/ml) in 4 ml of Eagles Basal Medium with 10% heat inactivated fetal bovine serum, 25 mM KCl, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin, and cultured in an incubator at 37°C with 5% CO₂ and constant humidity. After 16 to 22 h cytosine β -D-arabinofuranoside was added (final concentration 2.5 μ g/ml) to prevent growth of glial cells. Experiments were performed on day 6–7 *in vitro*. The treatment of animals was approved by the national committee for experiments on animals.

Assessment of reactive oxygen species. Assessment of ROS formation in CGCs by use of the fluorescent probe DCFH-DA was performed essentially as described previously (Dreiem *et al.*, 2002), with some modifications. The method is based on the incubation of the CGCs with DCFH-DA, which diffuses passively through the cellular membrane. Intracellular esterase activity results in the formation of DCFH, a nonfluorescent compound, which emits fluorescence when it is oxidized to 2',7'-dichlorofluorescein (DCF).

Prior to use, the CGCs were loaded with 5 μ M DCFH-DA (1:1000 dilution of MeOH stock, stored at –20°C) directly in the cell culture medium for 20 min (37°C with 5% CO₂ and constant humidity). Thereafter, the medium containing the DCFH-DA was replaced with 1.5 ml HEPES-buffered incubation medium (HBM: HBSS supplemented with HEPES 20 mM and glucose 10 mM). Fifteen μ l TMCH (1:100 dilution of MeOH stock) was added, and the cells were harvested with a cell scraper. We have previously reported the dose-response relationship for TMCH (50–800 μ M) in CGCs (Dreiem *et al.*, 2002), and this was therefore omitted in the present work. The cellular mechanisms involved in the formation of ROS were elucidated by

preincubation of the cells (10 min) with an enzymatic inhibitor (the NO synthase inhibitors L-NAME [100–400 μ M] or DPI [3.5–14 μ M]), or the Mek1/2 inhibitor U0126 [1–20 μ M]). Thereafter, TMCH (200 μ M) was added to the CGCs. Three aliquots of 250 μ l were taken from each cell culture dish and placed in the wells of a multiwell plate. Fluorescence was recorded in a Perkin Elmer LS50B luminescence spectrometer (excitation wavelength 485 nm, emission wavelength 530 nm) for 3 h. In each experiment two cell culture dishes were used for each test solution.

Western blot. Prior to use, the culture medium was removed and replaced with 1.5 ml fresh HBM and the cells were left for 5 min to equilibrate. To elucidate the cellular mechanisms involved in activation of Erk1/2, the CGCs were preincubated (10 min) in HBM containing the Mek1/2 inhibitor U0126 (20 μ M), the NO synthase inhibitors L-NAME (300 μ M) or DPI (7 μ M). Thereafter, 15 μ l of TMCH (1:100 dilution of MeOH stock) was added, and the dishes were incubated at 37°C in 5% CO₂ atmosphere for different time-spans (20 min to 90 min, see figure legends). After the indicated incubation period, the CGCs were washed with 0.9% NaCl, and lysed in 150 μ l 20 mM phosphate buffer (pH 7.4) with 0.1% Triton X-100. Before sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), aliquotes of lysates were mixed with loading buffer (final concentration 3% SDS, 5% glycerol, 62.5 mM Tris/HCl pH 6.9, 0.1% bromophenol blue, 6% 2-mercaptoethanol) and boiled for 2 min. Electrophoresis was performed with 3% stacking/12% separating SDS-PAGE gels (5 min at 90 V, thereafter 45 min at 190 V). The separated proteins were then electrophoretically transferred to nitrocellulose membranes (0.45 μ m) overnight (30 V). The nitrocellulose blots were incubated in blocking buffer (Tris-buffered saline containing 0.05% Tween 20 [TBST] and 5% low-fat dry milk) for 1 h and probed with phospho-Erk1/2 mAb (1:200 dilution in blocking buffer), phospho-Akt-1 pAb (1:200 dilution in blocking buffer), or phospho-Raf-1 pAb (1:200 dilution in blocking buffer) for 2 h. The blots were washed in TBST (6 \times 5 min) and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution in blocking buffer) for 2 h at room temperature. After washing in TBST (6 \times 5 min), the blots were exposed to ECL reagent for 1 min. The signals were visualized on X-OmatBlue XB-1 film (Kodak) and scanned in a desktop scanner (Hewlett Packard Scan Jet 3c) at 400 dpi. Densitometric quantification was analyzed in Adobe Photoshop (Version 6.0). The density of the spots was multiplied with the number of pixels to calculate the integrated value.

After electrophoresis, aliquotes of lysates were controlled for similar total protein contents by staining with 0.1% Coomassie Brilliant Blue-G in acetic acid/MeOH (10%/20%) for 2 h. The gel was destained in the same mixture without Coomassie Brilliant Blue-G, and dried in a gel slab dryer at 80°C for 2 h.

Nitrite measurements. Measurements of nitrite in the supernatant of the cultured CGCs, using the Griess reagent (Green *et al.*, 1982), were taken as an indicator of NO generation. In brief, TMCH was added to the plated CGCs in 1.1 ml HBM (37°C in 5% CO₂ atmosphere) for 150 min. The NO donor SNAP (1 mM; Shaffer *et al.*, 1992) was included as a positive control. Thereafter, 1 ml of the cell supernatant was incubated with 100 μ l Griess reagent (equal volumes of 1% N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water and a mixture of 10% sulphanilamide and 50% concentrated H₃PO₄) at room temperature for 30 min. The absorbance of the reaction product was measured at 546 nm. Each experiment was repeated 12 times with cells from different preparations (Table 1).

Survival of cerebellar granule cells. The cellular culture medium was removed from the plated granule cells and replaced by pre-warmed (37°C) HBM containing 25 mM KCl. Thereafter, the granule cells were incubated with TMCH for 18 h prior to determining neuron survival by using the lactate dehydrogenase (LDH) assay (Koh and Choi, 1987). Aliquots of the incubation medium were transferred to the wells of a custom made 48 well microplate with glass bottom. 25 μ l of a 13.6 mM stock solution of pyruvate (final concentration 0.68 mM) was added and the volume was adjusted to 450 μ l with 0.1 M KPO₄ buffer (pH 7.5). The reactions were started by automated

TABLE 1
Nitrite Accumulation (μM Nitrite/h) in the Medium of Rat Cerebellar Granule Cells TMCH and the NO Donor SNAP exposed to (150 min)

Treatment	μM Nitrite/h
Cell control	1.39 ± 0.31
MeOH-control	1.13 ± 0.26
TMCH (200 μM)	$3.17 \pm 0.87^*$
TMCH (800 μM)	$3.92 \pm 1.21^*$
SNAP (1 mM)	$21.2 \pm 2.31^*$

Note. Values are mean \pm SEM, $n = 12$.

* $p \leq 0.05$ versus cell control.

injection of 50 μl of an 846 μM stock solution of NADH (final concentration 84.6 μM). The LDH activity was measured continuously at 28°C by monitoring the decrease in fluorescence emission at 460 nm using a BMG FLUOstar Optima fluorimeter (excitation wavelength 340 nm). The fluorescence decay rate was used to calculate the LDH activity in the samples by referring to 0.1% digitonin as 100% cell death.

For mechanistic studies, cell survival was measured after incubation of the dishes with the potential neuroprotective compounds U0126 (20 μM) and vitamin E (50 μM). The potential neuroprotective compounds were added 30 minutes prior to TMCH (200 μM) exposure. Each experiment was repeated three to six times with cells from different preparations. The control groups used for testing statistical differences were exposed at the same day as the treatment group.

Statistical analysis. The effects of TMCH on ROS formation in the presence or absence of additional drugs and on nitrite accumulation were evaluated using ANOVA, followed by Newman-Keuls post-hoc test. The effects of ROS inhibitors on cell death were evaluated using Student's *t*-test. Differences were considered to be of statistical significance when $p \leq 0.05$.

RESULTS

Preincubation of the CGCs with the Mek1/2 inhibitor U0126 (1, 5, 10, and 20 μM) (Duncia *et al.*, 1998; Favata *et al.*, 1998) resulted in a reduction of the TMCH-stimulated ROS formation by 33, 46, 62, and 85%, respectively. Only the results obtained by use of 20 μM U0126 are presented (Fig. 1A). Neuron survival after exposure to TMCH was elucidated by using the lactate dehydrogenase (LDH) assay (Koh and Choi, 1987). The protective effects of U0126 (20 μM) and vitamin E (50 μM) on TMCH-induced cell death is shown in Figure 1B. These results strongly suggest that Erk1/2 are involved in ROS formation, and that cell death is at least partly dependent of ROS formation.

To investigate the phosphorylation state of Erk1/2, Akt-1, and Raf-1 in the CGCs, phospho-specific antibodies that recognize the phosphorylated Tyr-204 of Erk1/2, phosphorylated Ser-473 of Akt-1, and phosphorylated Ser-259 of Raf-1 were employed. Preliminary dose-response experiments showed that exposure of the cells in the range of 100–800 μM TMCH induced a robust phosphorylation of Erk1 and 2, demonstrated by a large increase in the relative intensities of the two immunodetectable bands (44 kDa and 42 kDa,

respectively) compared to basal levels (Fig. 2A). In a parallel set of immunoblots the time-response aspect of the TMCH-induced Erk1/2 phosphorylation was determined. The CGCs were exposed to TMCH (200 μM) for 20, 40, 60, and 90 min, respectively (Fig. 3A). These data show that the phosphorylation level of Erk1/2 was most pronounced after about 60 min. During subsequent experiments to elucidate the cellular activators upstream of the Erk1/2 kinases, we chose to stimulate the CGCs with 200 μM TMCH for 60 min in presence and absence of different inhibitory agents.

Preincubation of the CGCs with U0126 (20 μM) alone lowered both the DCF-fluorescence (Fig. 1A), and the level of phosphorylated Erk1/2 compared to the cell control (basal) and the MeOH control (Fig. 4). Separate experiments were conducted to rule out the possibility that U0126 affected the DCF-fluorescence by quenching (not shown).

Stimulation of glutamate receptors can activate Erk1/2 signalling (Fukunaga and Miyamoto, 1998), and neuronal NO synthase is coupled to Ca^{2+} influx following NMDA receptor stimulation (Dawson and Dawson, 2000). However, as far as we know, the involvement of NO in activation of Erk1/2 in cultured rat CGCs is not yet elucidated. Thus, we investigated whether ROS formation and the level of Erk1/2 phosphorylation are dependent of NO using the conditions defined below. Pretreatment of the cells with the NO synthase inhibitors L-NAME (300 μM) (Moncada *et al.*, 1991) or DPI (7 μM) (Stuehr *et al.*, 1991) prior to exposure to TMCH lowered the DCF-fluorescence by 67 and 44%, respectively (Fig. 1A). The concentrations of these inhibitors were chosen based on preliminary dose-response experiments. Increasing the concentration of L-NAME to 400 μM or of DPI to 14 μM did not lead to further decrease of the DCF-fluorescence, and lower concentration of L-NAME (100 and 200 μM) or DPI (3.5 μM) gave lower inhibition of the ROS formation (not shown). Preincubation of the cells with U0126 (20 μM), L-NAME (300 μM), and DPI (7 μM) prior to addition of TMCH (200 μM) strongly decreased the phosphorylation level of Erk1/2 (Fig. 4). Intracellular formation of NO was confirmed by use of the Griess method (Green *et al.*, 1982). Exposure of the cells to 200 μM and 800 μM TMCH increased the NO formation by 228 and 282%, respectively, while exposure to the NO donor SNAP (1 mM) (Shaffer *et al.*, 1992) resulted in a 15-fold elevation compared to the cell control (Table 1).

Dose-response experiments showed that exposure of the cells in the range of 200–800 μM TMCH induced decreased phosphorylation state of Akt-1, demonstrated by a marked decrease in the relative intensity of the immunodetectable band (60 kDa) compared to basal levels (Fig. 2B). Furthermore, a time-dependent decrease in the phosphorylation of Akt-1 in the CGCs treated with TMCH (200 μM) was shown, with the most pronounced effect after about 60 min (Fig. 3B). Importantly, as in the case of Akt-1, exposure of CGCs with TMCH (200–800 μM) for 60 min decreased the phosphorylation level of Raf-1 compared to the cell control (basal) and the MeOH control

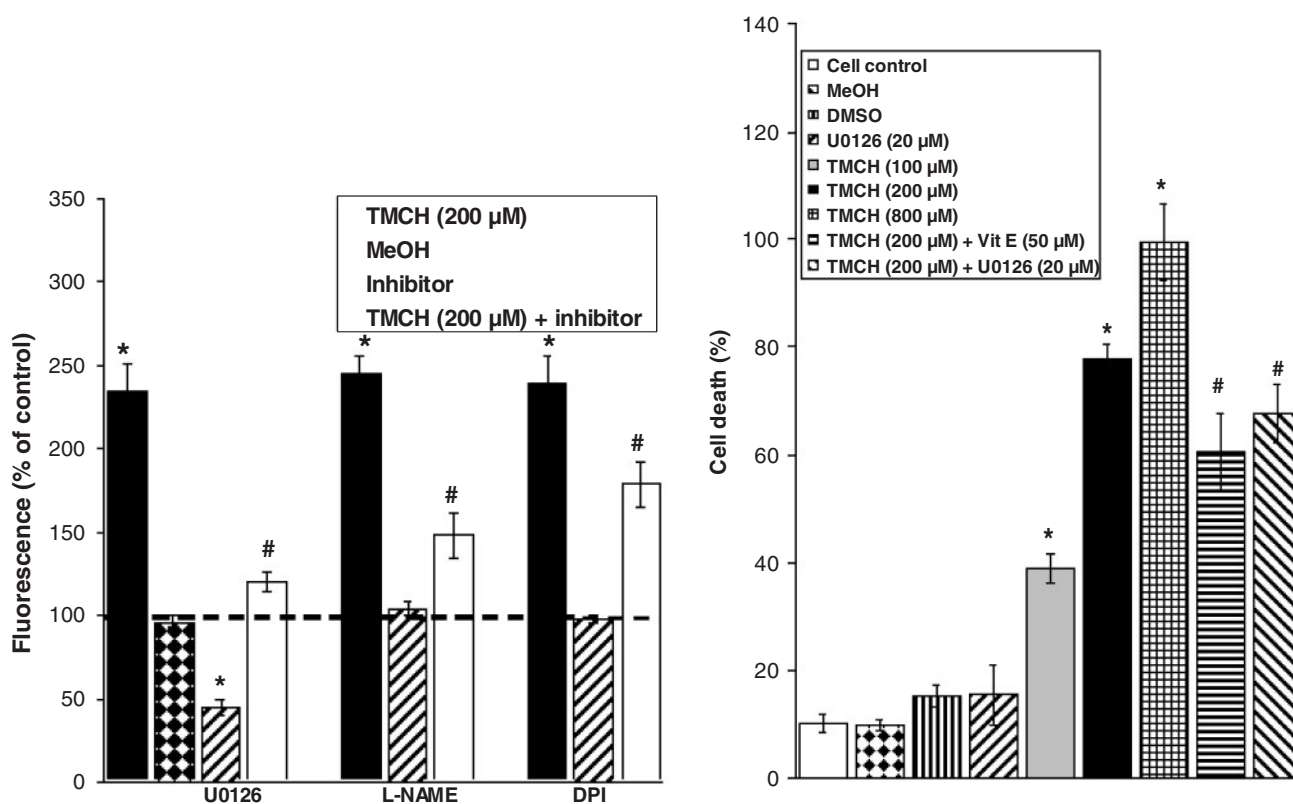


FIG. 1. (A) Pharmacological characterization of the 1,2,4-trimethylcyclohexane (TMCH)-evoked DCF-fluorescence in the CGCs. The CGCs were preincubated (10 min) with the Mek1/2 inhibitor U0126 (20 μ M), and the NO synthase inhibitors L-NAME (300 μ M) or DPI (7 μ M). Thereafter, TMCH (200 μ M) was added to the dishes, and the DCF-fluorescence was recorded in a Perkin Elmer LS50B luminescence spectrometer for 3 h. All values are relative to the cell control (set to 100%). Values are mean \pm SEM, $n = 5$. * $p \leq 0.05$ versus cell control, # $p \leq 0.05$ versus cells treated with TMCH (200 μ M). (B) Effects of U0126 and vitamin E on the TMCH-induced cell death. The CGCs were incubated with TMCH for 18 h prior to determining neuron survival by using the lactate dehydrogenase (LDH) assay. The LDH activity was measured continuously at 28°C by monitoring the decrease in fluorescence emission at 460 nm. The fluorescence decay rate was used to calculate the LDH activity in the samples by referring to 0.1% digitonin as 100% cell death. Values are mean \pm SEM, $n = 3-6$. * $p \leq 0.05$ versus cell control, # $p \leq 0.05$ versus cells treated with TMCH (200 μ M).

(72–76 kDa, Fig. 2C). Akt-1 regulates the activity of Raf-1 through phosphorylation at Ser-259 (Moelling *et al.*, 2002), strongly suggesting a crosstalk between Akt-1 and the Raf-Mek-Erk pathway in our system.

The protein content in all cell lysates used for western blot was similar. Representative Coomassie blue stained gels showing equal loading of proteins are shown in Figure 2.

DISCUSSION

Erk1/2 play critical intermediary roles in mediating signal transduction from the membrane to the nucleus. Therefore, we investigated whether the phosphorylation states of these signalling enzymes were affected by exposure of CGCs to the hydrocarbon solvent TMCH. Possible regulators of Erk1/2 phosphorylation in our system were hypothesized to be NO or an Akt-1/Raf-1 crosstalk, so the involvement of these were elucidated. Furthermore, a possible link between the Erk1/2 phosphorylation level, ROS formation, and cell death was investigated. Our

results show that exposure of cultured rat CGCs to the hydrocarbon solvent TMCH leads to phosphorylation of the Erk1/2 type of MAPKs, resulting in formation of ROS and subsequently cell death. The phosphorylation of the Erk1/2 kinases appears to occur by NO and by an Akt-1/Raf-1 dependent process.

ROS formation in the CGCs was measured as increased DCF-fluorescence (Fig. 1A). We have recently shown that DCFH is sensitive towards oxidation by peroxynitrite, hydrogen peroxide (in combination with cellular peroxidases), peroxidases alone, and hydroxyl radicals, while it is not suitable for measuring NO, hypochlorous acid, or superoxide radicals in biological systems (Myhre *et al.*, 2003). It has been suggested that use of DCF to measure ROS formation in cellular systems may be inappropriate, because it is suggested that the probe itself may generate free radicals. Marchesi *et al.* (1999) have shown that DCF irradiated with visible light in the presence of reduced glutathione or reduced NADH may lead to formation of a semiquinone radical of DCF. Also, DCFH may be oxidized either by horseradish peroxidase (HRP)-compound I or HRP-compound II with the

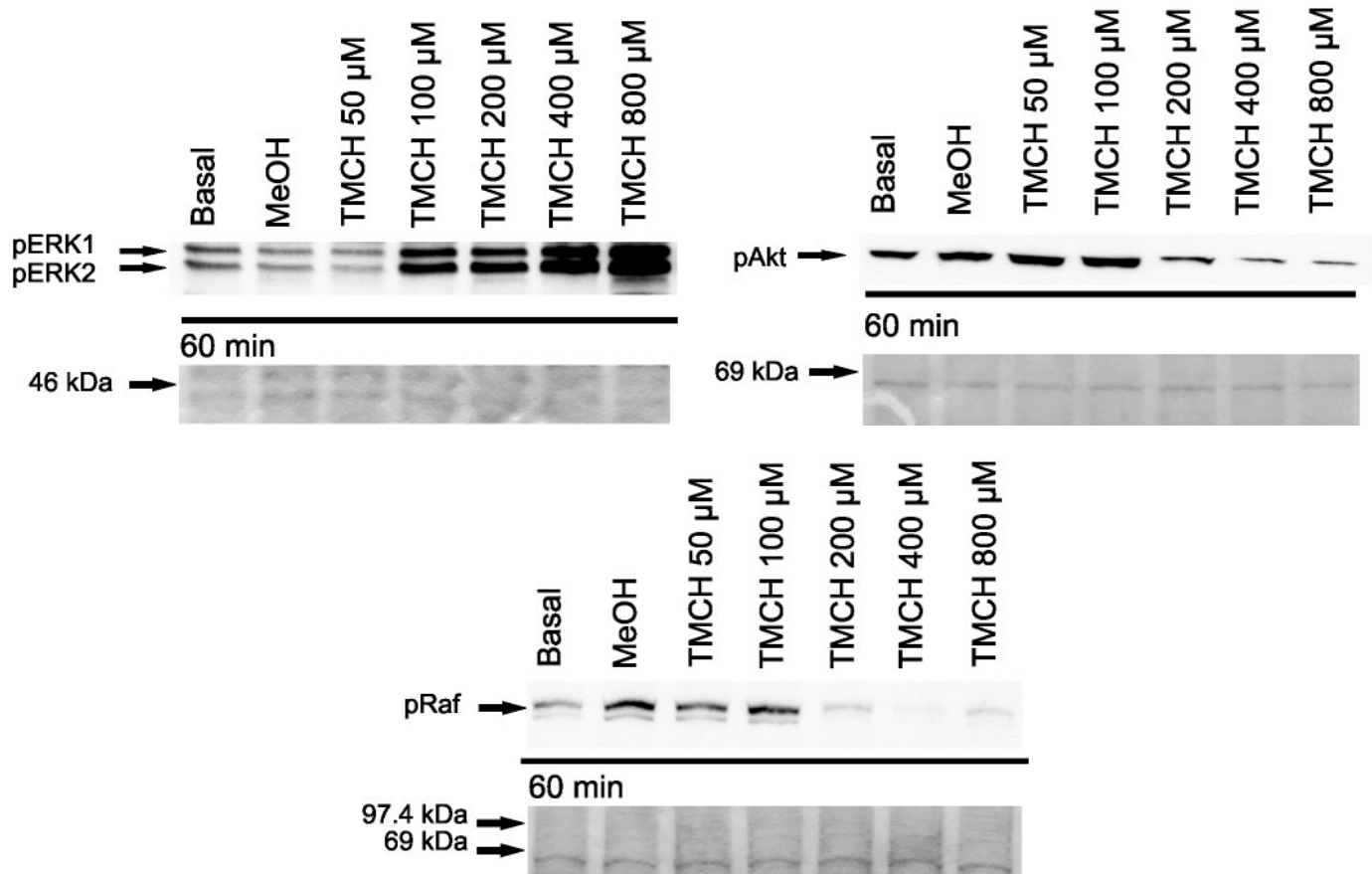


FIG. 2. Dose-dependent phosphorylation of Erk1/2 and dephosphorylation of Akt-1 and Raf-1 kinases in the CGCs exposed to 1,2,4-trimethylcyclohexane (TMCH). (A) The CGCs were exposed for 1 h in HBM (basal), HBM with MeOH (solvent for TMCH), or HBM with the indicated concentrations of TMCH, and immunoblotted with antibodies that recognize the phosphorylated Tyr-204 of Erk1/2. The densitometric quantifications of the bands show that TMCH at 100–800 μ M increased the pErk1/2 levels by 193, 183, 258, and 347% compared to basal, respectively. (B) The CGCs were exposed for 60 min in HBM (basal), HBM with MeOH (solvent for TMCH), or HBM with the indicated concentrations of TMCH, and immunoblotted with antibodies that recognize the phosphorylated Ser-273 of Akt-1. The densitometric quantifications of the bands showed that TMCH at 200–800 μ M decreased the pAkt-1 levels by 37, 68, and 74% of basal, respectively. (C) The CGCs were exposed for 60 min in HBM (basal), HBM with MeOH (solvent for TMCH), or HBM with the indicated concentrations of TMCH, and immunoblotted with antibodies that recognize the phosphorylated Ser-259 of Raf-1. The signals were visualized by an ECL reagent. The densitometric quantifications of the bands showed that TMCH at 200–800 μ M decreased the pRaf-1 levels by 67% or more compared to basal, respectively. The Coomassie blue stained gel shows equal loading of proteins with molecular mass of 42 kDa (Erk 2), 44 kDa (Erk 1), 72–76 kDa (Raf-1), and 60 kDa (Akt-1).

obligate generation of the DCF semiquinone free radical, and hydrogen peroxide may be formed through dealkylation of DCFH-DA by esterases (Rota *et al.*, 1999). When formed in an aerobic system, the semiquinone radical is oxidized by oxygen, which regenerates the dye and forms superoxide. However, since TMCH induces a dose-dependent increase in DCF fluorescence in CGCs (Dreiem *et al.*, 2002), and since the DCF fluorescence is inhibited by specific enzymatic inhibitors, it is unlikely that the above discussed factors make a significant contribution to the DCF fluorescence observed in our test system.

The upstream dual-specificity kinases Mek1/2 are inhibited by U0126 (Duncia *et al.*, 1998; Favata *et al.*, 1998), and pretreatment with this inhibitor resulted in 85% reduction of the TMCH-stimulated DCF-fluorescence (Fig. 1A). The inhibitory effect of U0126 on the phosphorylation level of Erk1/2 may be

due to the fact that it binds to Mek1/2 in a noncompetitive manner (Duncia *et al.*, 1998) and thereby inhibits the catalytic activity of these enzymes. That the ROS formation is dependent on an Erk1/2 stimulated pathway is an interesting finding, since a possible link between Erk1/2 activation and ROS formation has been scarcely investigated. Preincubation with U0126 (20 μ M) and vitamin E (50 μ M) showed a protective effect against cell death in our system (Fig. 1B). These results strongly suggest that Erk1/2 are involved in ROS formation, and that cell death is at least partly dependent of ROS formation. This is in accordance with our previous finding that U0126 protects against ROS formation and cell death of CGCs exposed to L-2-chloropropionic acid (Myhre *et al.*, 2001).

There are at least two important mechanisms whereby phosphorylation and activation of Erk1/2 may lead to ROS formation

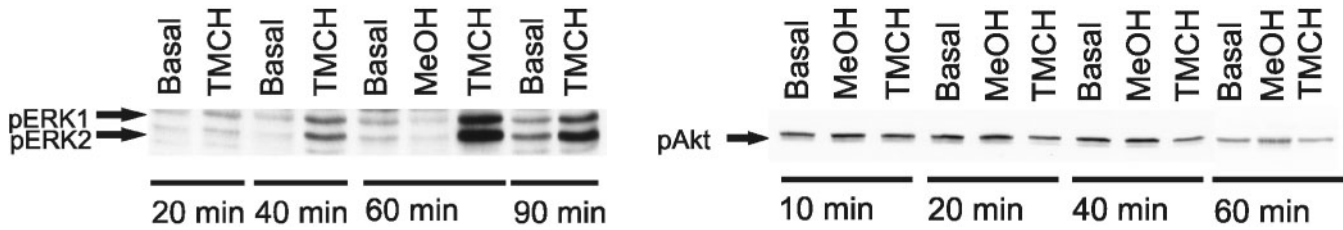


FIG. 3. Time-dependent phosphorylation of Erk1/2 and dephosphorylation of Akt-1 kinase in the CGCs exposed to 1,2,4-trimethylcyclohexane (TMCH). (A) To determine the time-response aspect of the Erk1/2 phosphorylation level, CGCs were exposed to HBM (basal), HBM with MeOH (when included), or HBM with 200 μ M of TMCH for 20, 40, 60, and 90 min, respectively. The CGCs were immunoblotted with antibodies that recognize the phosphorylated Tyr-204 of Erk1/2. The densitometric quantifications of the bands in lanes 2–9 showed 222, 97, 875, 271, 81, 2745, 647, and 1713% of the basal level at 20 min, respectively. (B) To determine the time-response aspect of the Akt-1 phosphorylation level, CGCs were exposed to HBM (basal), HBM with MeOH (when included), and HBM with 200 μ M of TMCH for 10, 20, 40, and 60 min, respectively. The CGCs were immunoblotted with antibodies that recognize the phosphorylated Ser-273 of Akt-1. The signals were visualized by an ECL reagent. The densitometric quantifications showed 108, 66, 53, and 76% of the basal levels after TMCH exposure for 10, 20, 40, or 60 min, respectively.



FIG. 4. Phosphorylation of Erk1/2 kinases in the CGCs exposed to 1,2,4-trimethylcyclohexane (TMCH) is dependent on NO. The CGCs were exposed for 60 min to HBM (basal), HBM with MeOH (solvent for TMCH), or HBM with TMCH (200 μ M) in the presence and absence of the Mek1/2 inhibitor U0126 (20 μ M), or the NO synthase inhibitors L-NAME (300 μ M) and DPI (7 μ M). The CGCs were immunoblotted with antibodies that recognize the phosphorylated Tyr-204 of Erk1/2. The signals were visualized by an ECL reagent. The densitometric quantifications of the bands in lanes 2, 3, 7, and 9 showed 270, 2576, 670, and 236% of the basal level, respectively. The bands in lanes 4, 5, 6, and 8 were not detectable.

in CGCs. First, all five subunits of the superoxide radical producing NADPH oxidase enzyme complex are expressed in neurons, which is a possible mediator of oxidative stress (Tammariello *et al.*, 2000a,b) in our system. This hypothesis is in agreement with Shen *et al.* (2002), who showed that *in vitro* neurotoxicity of methylisothiazolinone occurred through production of ROS downstream from Erk1/2 via activation of the NADPH oxidase. The mechanism for this is probably phosphorylation of p47phox by Erk1/2, which is one of the key steps in assembly and activation of the NADPH oxidase complex (Dewas *et al.*, 2000). Second, the Erk pathway may activate cytosolic phospholipase A₂ (cPLA₂) by phosphorylating Ser-505 (Lin *et al.*, 1993), leading to production of superoxide radicals, and free fatty acids that can stimulate the NADPH oxidase complex directly (Sellmayer *et al.*, 1996). Altogether, the robust Erk phosphorylation observed in our experiments may

lead to ROS formation through phosphorylation of cPLA₂ and the NADPH oxidase enzyme complex.

Since the mechanisms for Erk1/2 activation in cultured rat CGCs are poorly understood, we studied the importance of NO by treatment of the CGCs with the NO synthase inhibitors L-NAME (Moncada *et al.*, 1991) and DPI (Stuehr *et al.*, 1991). Both lowered the DCF-fluorescence (Fig. 2A) and ameliorated the phosphorylation of Erk1/2 (Fig. 4). Furthermore, formation of nitrite in the supernatant of the cultured CGCs exposed to TMCH confirms intracellular production of NO in our system (Table 1). NO is previously reported to play important roles in neuronal signalling (Contestabile, 2000), and may act as a regulator of intracellular pathways that transmit signals from the plasma membrane to the nucleus (Stamler *et al.*, 2001). To our knowledge, we are the first to demonstrate that NO is involved in phosphorylation of Erk1/2 in cultured rat CGCs. Since treatment of the CGCs with U0126 in combination with TMCH almost totally ameliorated the phosphorylation of Erk1/2 (Fig. 4), we suggest that activation by NO takes place at or upstream of Mek1/2.

Another potential regulator of Erk1/2 activation in our system was considered to be the Akt-1/Raf-1 crosstalk, since it has been shown that phosphorylation of Raf-1 at Ser-259 by Akt-1 inactivates the Raf-Mek-Erk pathway in different cell lines (Reusch *et al.*, 2001; Rommel *et al.*, 1999; Zimmermann and Moelling, 1999). The kinase activity of Raf-1 is down-regulated by phosphorylation of a conserved serine residue (Ser-259) in the amino-terminal regulatory domain (Morrison and Cutler, 1997), and the kinase responsible for this phosphorylation of Raf-1 is Akt-1 (Zimmermann and Moelling, 1999; Moelling *et al.*, 2002). Active Raf-1 phosphorylates and activates the Mek-Erk kinase pathway (Kyriakis *et al.*, 1992). We assessed the phosphorylation level of Akt-1 and Raf-1 by the use of antibodies that recognize phosphorylated sites Ser-473 and Ser-259, respectively. Phosphorylation at Ser-473 is required for full activation of Akt-1 (Datta *et al.*, 1999). High basal levels of phosphorylated Akt-1 were detected in the CGCs (Figs. 2B and 3B). This is in

accordance with Chalecka-Franaszek and Chuang (1999), who reported high levels of phosphorylated Akt-1 in CGC grown in the presence of serum. Further, these authors showed that exposure of cells to a ROS-inducing compound such as glutamate induced a rapid loss of Akt-1 phosphorylation. Interestingly, we observed a time-dependent decrease in the phosphorylation level of Akt-1 in the CGCs exposed to TMCH, with the most pronounced effect after 60 min (Fig. 3B), consistent with the timepoint of the highest level of Erk1/2 phosphorylation (Fig. 3A). Further, exposure of CGCs with TMCH (200–800 μ M) for 60 min resulted in a marked reduction of the level of phosphorylated Akt-1 and Raf-1 (Figs. 2B and 2C). However, while Erk1/2 phosphorylation is increased by TMCH compared to basal at 40 min (Fig. 3A), little change is evident for Akt-1 at that same time (Fig. 3B). Furthermore, at 100 μ M TMCH there is a significant increase in Erk phosphorylation (Fig 2A) with little decrease in Akt-1 at the same dose (Fig. 2B). Similarly, Raf-1 phosphorylation is not affected by 100 μ M TMCH (Fig. 2C). These data indicate that an additional mechanism other than Akt-1 and Raf-1 could be responsible for Erk1/2 activation in the CGCs when exposed to low concentrations of TMCH (100 μ M, 40 min).

Since Akt-1 inactivates the activity of Raf-1 through phosphorylation at Ser-259, and because the classical pathway whereby Erk1/2 are phosphorylated is through the Raf-Mek-Erk pathway, our findings strongly suggest a crosstalk between Akt-1 and the Raf-Mek-Erk cascade in the cultured rat CGCs. That Raf-1 is inactivated when phosphorylated at Ser-259 by Akt-1 is in agreement with other findings in vascular smooth muscle cells (Reusch *et al.*, 2001), in differentiated myotubes (Rommel *et al.*, 1999), and in a human breast cancer cell line (Zimmermann and Moelling, 1999), where phosphorylation of Raf-1 at Ser-259 by Akt-1 resulted in reduced Raf-1 kinase activity and a termination of Erk1/2 phosphorylation. We propose that exposure of the cultured rat CGCs to 200 μ M TMCH for 60 min leads to dephosphorylation of Akt-1, and thereby to a decreased level of phosphorylated Raf-1 at Ser-259, resulting in an increased level of Erk1/2 phosphorylation, and increased ROS formation.

In summary, we found that exposure of cultured rat CGCs to the solvent TMCH induced a time and concentration dependent dephosphorylation of Akt-1 at Ser-473 and Raf-1 at Ser-259. Also, an increased level of phosphorylated Erk1/2 was observed. Thus, the cross-regulation between the Akt and Raf-Mek1/2 signalling cascades correlated with the pattern of Erk1/2 phosphorylation. The increased level of phosphorylated Erk1/2 led to an extensive increase in ROS formation. By use of NO synthase inhibitors, we found that intracellular formation of NO was necessary for phosphorylation of Erk1/2 and for the formation of ROS. The increased formation of ROS was at least in part responsible for the TMCH-induced cell death. In addition to the important role of NO in cell signalling, it can also cause cytotoxic effects through formation of reactive nitrogen species. Indeed, it has been postulated that NO is an important agent

mediating the neurotoxicity associated with glutamate receptor stimulation in primary cortical cultures (Dawson *et al.*, 1991). When reactive nitrogen species and oxygen species overwhelm the antioxidant defense of the cell, the resulting oxidative stress may cause damage to biological macromolecules and possibly cell death (Contestabile, 2001; Mariussen *et al.*, 2002; Myhre *et al.*, 2001). Our findings may have toxicological consequences, since the white spirit constituent TMCH is known to accumulate in high concentration in the rat brain after inhalation (Zahlsen *et al.*, 1990). Thus, our results may, at least in part, indicate an underlying mechanism for neuronal damage observed in the cerebellum (Malm and Lying-Tunell, 1980; Lam *et al.*, 2000) after inhalation of organic solvents.

REFERENCES

- Chalecka-Franaszek, E., and Chuang, D. M. (1999). Lithium activates the serine/threonine kinase Akt-1 and suppresses glutamate-induced inhibition of Akt-1 activity in neurons. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 8745–8750.
- Contestabile, A. (2000). Roles of NMDA receptor activity and nitric oxide production in brain development. *Brain Res. Rev.* **32**, 476–509.
- Contestabile, A. (2001). Oxidative stress in neurodegeneration: Mechanisms and therapeutic perspectives. *Curr. Top. Med. Chem.* **1**, 553–568.
- Datta, S. R., Brunet, A., and Greenberg, M. E. (1999). Cellular survival: A play in three acts. *Genes Dev.* **13**, 2905–2927.
- Dawson, V. L., and Dawson, T. M. (2000). Neuronal ischaemic preconditioning. *Trends Pharmacol. Sci.* **21**, 423–424.
- Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S., and Snyder, S. H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6368–6371.
- Dewas, C., Fay, M., Gougerot-Pocidalo, M. A., and El-Benna, J. (2000). The mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway is involved in formyl-methionyl-leucyl-phenylalanine-induced p47phox phosphorylation in human neutrophils. *J. Immunol.* **165**, 5238–5244.
- Dreiem, A., Myhre, O., and Fonnum, F. (2002). Relationship between lipophilicity of C6 to C10 hydrocarbon solvents and their ROS-inducing potency in rat cerebellar granule cells. *Neurotoxicology* **23**, 701–709.
- Duncia, J. V., Santella, J. B., Higley, C. A., Pitts, W. J., Wityak, J., Fietze, W. E., Rankin, F. W., Sun, J. H., Earl, R. A., Tabaka, A. C., Teleha, C. A., Blom, K. F., Favata, M. F., Manos, E. J., Daulerio, A. J., Stradley, D. A., Horiuchi, K., Copeland, R. A., Scherle, P. A., Trzaskos, J. M., Magolda, R. L., Trainor, G. L., Wexler, R. R., Hobbs, F. W., and Olson, R. E. (1998). MEK inhibitors: The chemistry and biological activity of U0126, its analogs, and cyclization products. *Bioorg. Med. Chem. Lett.* **8**, 2839–2844.
- Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., and Trzaskos, J. M. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase. *J. Biol. Chem.* **273**, 18623–18632.
- Feldman, R. G., Ratner, M. H., and Ptak, T. (1999). Chronic toxic encephalopathy in a painter exposed to mixed solvents. *Environ. Health Persp.* **107**, 417–422.
- Fukunaga, K., and Miyamoto, E. (1998). Role of MAP kinase in neurons. *Mol. Neurobiol.* **16**, 79–95.
- Gallo, V., Ciotti, M. T., Coletti, A., Aloisi, F., and Levi, G. (1982). Selective release of glutamate from cerebellar granule cells differentiating in culture. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7919–7923.

- Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.* **126**, 131–138.
- Kamran, S., and Bakshi, R. (1998). MRI in chronic toluene abuse: Low signal in the cerebral cortex on T2-weighted images. *Neuroradiology* **40**, 519–521.
- Khan, S., Krishnamurthy, R., and Pandya, K. P. (1990). Generation of hydroxyl radicals during benzene toxicity. *Biochem. Pharmacol.* **39**, 1393–1395.
- Koh, J. Y., and Choi, D. W. (1987). Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J. Neurosci. Methods* **20**, 83–90.
- Kyriakis, J. M., App, H., Zhang, X. F., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. (1992). Raf-1 activates MAP kinase-kinase. *Nature* **358**, 417–421.
- Lam, H. R., Ladefoged, O., Østergaard, G., and O'Callaghan, J. P. (2000). Inhalation exposure to white spirit causes region-dependent alterations in the levels of glial fibrillary acidic protein. *Neurotoxicol. Teratol.* **22**, 725–731.
- Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993). cPLA₂ is phosphorylated and activated by MAP kinase. *Cell* **72**, 269–278.
- Malm, G., and Lying-Tunell, U. (1980). Cerebellar dysfunction related to toluene sniffing. *Acta Neurol. Scand.* **18**, 67–76.
- Marchesi, E., Rota, C., Fann, Y. C., Chignell, C. F., and Mason, R. P. (1999). Photoreduction of the fluorescent dye 2'-7'-dichlorofluorescein: A spin trapping and direct electron spin resonance study with implications for oxidative stress measurements. *Free Radic. Biol. Med.* **26**, 148–161.
- Mariussen, E., Myhre, O., Reistad, T., and Fonnum, F. (2002). The polychlorinated biphenyl mixture aroclor 1254 induces death of rat cerebellar granule cells: The involvement of the N-methyl-D-aspartate receptor and reactive oxygen species. *Toxicol. Appl. Pharmacol.* **179**, 137–144.
- Mattia, C. J., Adams, J. D. Jr., and Bondy, S. C. (1993a). Free radical induction in the brain and liver by products of toluene catabolism. *Biochem. Pharmacol.* **46**, 103–110.
- Mattia, C. J., Ali, S. F., and Bondy, S. C. (1993b). Toluene-induced oxidative stress in several brain regions and other organs. *Mol. Chem. Neuropathol.* **18**, 313–328.
- Mattia, C. J., LeBel, C. P., and Bondy, S. C. (1991). Effects of toluene and its metabolites on cerebral reactive oxygen species generation. *Biochem. Pharmacol.* **42**, 879–882.
- Moelling, K., Schad, K., Bosse, M., Zimmermann, S., and Schwenecker, M. (2002). Regulation of Raf-Akt crosstalk. *J. Biol. Chem.* **277**, 31099–31106.
- Moncada, S., Palmer, R. M., and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**, 109–142.
- Morrison, D. K., and Cutler, R. E. (1997). The complexity of Raf-1 regulation. *Curr. Opin. Cell Biol.* **9**, 174–179.
- Murray, B., Alessandrini, A., Cole, A. J., Yee, A. G., and Furshpan, E. J. (1998). Inhibition of the p44/42 MAP kinase pathway protects hippocampal neurons in a cell-culture model of seizure activity. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 11975–11980.
- Myhre, O., Andersen, J. M., Aarnes, H., and Fonnum, F. (2003). Evaluation of the probes 2',7'-dichlorofluorescein diacetate, luminol, and lucigenin as indicators of reactive species formation. *Biochem. Pharmacol.* **65**, 1575–1582.
- Myhre, O., Bjugan, B., and Fonnum, F. (2001). The toxic effect of L-2-chloropropionate on cultured rat cerebellar granule cells is ameliorated after inhibition of reactive oxygen species formation. *J. Neurosci. Res.* **66**, 992–997.
- Reusch, H. P., Zimmermann, S., Schaefer, M., Paul, M., and Moelling, K. (2001). Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. *J. Biol. Chem.* **276**, 33630–33637.
- Rommel, C., Clarke, B. A., Zimmermann, S., Nunez, L., Rossman, R., Reid, K., Moelling, K., Yancopoulos, G. D., and Glass, D. J. (1999). Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* **286**, 1738–1741.
- Rota, C., Chignell, C. F., and Mason, R. P. (1999). Evidence for free radical formation during the oxidation of 2'-7'-dichlorofluorescein to the fluorescent dye 2'-7'-dichlorofluorescein by horseradish peroxidase: Possible implications for oxidative stress measurements. *Free Radic. Biol. Med.* **27**, 873–881.
- Samanta, S., Perkinson, M. S., Morgan, M., and Williams, R. J. (1998). Hydrogen peroxide enhances signal-responsive arachidonic acid release from neurons: Role of mitogen-activated protein kinase. *J. Neurochem.* **70**, 2082–2090.
- Satoh, T., Nakatsuka, D., Watanabe, Y., Nagata, I., Kikuchi, H., and Namura, S. (2000). Neuroprotection by MAPK/ERK kinase inhibition with U0126 against oxidative stress in a mouse neuronal cell line and rat primary cultured cortical neurons. *Neurosci. Lett.* **288**, 163–166.
- Sellmayer, A., Obermeier, H., Danesch, U., Aepfelbacher, M., and Weber, P. C. (1996). Arachidonic acid increases activation of NADPH oxidase in monocytic U937 cells by accelerated translocation of p47-phox and co-stimulation of protein kinase C. *Cell. Signal.* **8**, 397–402.
- Shaffer, J. E., Han, B. J., Chern, W. H., and Lee, F. W. (1992). Lack of tolerance to a 24-hour infusion of S-nitroso N-acetylpencillamine (SNAP) in conscious rabbits. *J. Pharmacol. Exp. Ther.* **260**, 286–293.
- Shen, D., McLaughlin, B. A., Pal, S., and Aizenman, E. (2002). *In vitro* neurotoxicity of methylisothiazolinone, a commonly used industrial and household biocide, proceeds via a zinc and extracellular signal-regulated kinase mitogen-activated protein kinase-dependent pathway. *J. Neurosci.* **22**, 7408–7416.
- Stamler, J. S., Lamas, S., and Fang, F. C. (2001). Nitrosylation: The prototypic redox-based signalling mechanism. *Cell* **106**, 675–683.
- Stanciu, M., Wang, Y., Kentor, R., Burke, N., Watkins, S., Kress, G., Reynolds, I., Klann, E., Angiolieri, M. R., Johnson, J. W., and DeFranco, D. B. (2000). Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *J. Biol. Chem.* **275**, 12200–12206.
- Stuehr, D. J., Fasehun, O. A., Kwon, N. S., Gross, S. S., Gonzales, J. A., Levi, R., and Nathan, C. F. (1991). Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs. *FASEB J.* **5**, 98–103.
- Tammariello, S. P., Snyder, K. A., Bu, J., Doonan, R., Quinn, M. T., and Estus, S. (2000a). NADPH oxidase and neuronal apoptosis. *Soc. Neurosci. Abstracts* **26**(1–2), Abstract No. 700.19.
- Tammariello, S. P., Quinn, M. T., and Estus, S. (2000b). NADPH oxidase contributes directly to oxidative stress and apoptosis in nerve growth factor-deprived sympathetic neurons. *J. Neurosci.* **20**, RC53.
- Zahlsen, K., Nilsen, A. M., Eide, I., and Nilsen, O. G. (1990). Accumulation and distribution of aliphatic (n-nonane), aromatic (1,2,4-tritoluene) and naphthenic (1,2,4-trimethylcyclohexane) hydrocarbons in the rat after repeated inhalation. *Pharmacol. Toxicol.* **67**, 436–440.
- Zimmermann, S., and Moelling, K. (1999). Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* **286**, 1741–1744.