Methylisothiazolinone, A Neurotoxic Biocide, Disrupts the Association of Src Family Tyrosine Kinases with Focal Adhesion Kinase in Developing Cortical Neurons

Kai He, Jason Huang, Carl F. Lagenaour, and Elias Aizenman
Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania
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ABSTRACT
Methylisothiazolinone (MIT) is a biocide widely used in industrial and cosmetic products with potential as a neurotoxicant. We previously reported that short acute exposures to relatively high concentrations of MIT (100 μM) lead to widespread and selective neuronal death in vitro. To evaluate the biological properties of chronic exposures to MIT, freshly dissociated rat cortical neurons were continuously exposed to low concentrations (0.1–3 μM) of the biocide in serum-containing media. Although we observed minimal effects on cell viability, MIT induced a dramatic inhibition of neurite outgrowth. Immunoblotting and immunoprecipitation experiments revealed that focal adhesion kinase (FAK) phosphorylation was primarily affected by the MIT treatment. The phosphorylation level at tyrosines 576 and 861 of FAK was significantly decreased and likely contributed to the overall reduction of tyrosine phosphorylation of this protein. MIT inhibited Src family kinases (SFKs) in cell-free assays and led to the physical dissociation of FAK from the signaling complexes that it normally forms with c-Src and Fyn in developing neurons. High-density neuronal cultures were then employed to increase cell-to-cell contact. This approach resulted in an overall enhancement of SFKs and FAK phosphorylation and could overcome the deficits induced by MIT. This study suggests that a disruption of FAK-SFK complexes due to SFK inhibition leads to FAK dysfunction, with detrimental effects to immature neurons. Prolonged exposure to low levels of MIT and related compounds may have damaging consequences to the developing nervous system.

Isothiazolone and its derivatives are a class of common chemical biocides used as preservatives to control microbial growth in a very large number of industrial applications and in household products. These compounds have an active sulfur moiety that can react with and oxidize thiol-containing residues, thereby effectively killing most aerobic and anaerobic bacteria as well as fungi and other microorganisms (Collier et al., 1990). In addition, isothiazolone derivatives can inhibit human immunodeficiency virus replication in vitro (Rice et al., 1995) and can reduce tumor mass in a mouse model of human breast cancer (Wang et al., 2004).

Two widely used isothiazolone biocides are 5-chloro-2-methyl-4-isothiazolin-3-one (CMIT) and 2-methyl-4-isothiazolin-3-one (MIT), which are the active ingredients in a biocide mixture sold commercially as Kathon. They are supplied either as a single chemical solution or in combination at a wide range of concentrations (1.5–50%). As the use of these compounds has risen dramatically in recent years, there is increased concern about their potential risks to humans through inhalation and skin contact. Previous in vitro studies have shown that CMIT and MIT are profoundly cytotoxic to different types of cell lines (Ettorre et al., 2003). In addition, our group has reported that MIT is a highly selective neurotoxin, largely sparing glia, following a short acute exposure in established, mature rat cerebrocortical cultures (Du et al., 2002). MIT, under these conditions, triggered noncaspase-dependent cell death following the liberation of intracellular zinc and sequential activation of 12-lipoxygenase, extracellular signal-regulated kinase (ERK), and NADPH oxidase, leading to reactive oxygen species production, DNA damage, and overactivation of poly(ADP-ribose) polymerase (Du et al., 2002).

In the present study, we sought to investigate the neurotoxic consequences of prolonged applications of MIT at much lower concentrations than those utilized previously. Here, we
present evidence that MIT can inhibit Src family kinases (SFKs) and describe a MIT-induced disruption of the functional interaction between focal adhesion kinase (FAK) and SFKs. This results in a reduced level of tyrosine phosphorylation on FAK that is probably responsible for MIT's neurotoxic actions on developing cortical neurons in vitro.

Materials and Methods

Materials. Antibodies used in this study were from the following sources: mouse anti-FAK monoclonal antibody (clone 4.47) and anti-phosphotyrosine antibody (clone 4G10), Upstate Biotechnology (Lake Placid, NY); mouse anti-FAK monoclonal antibody, MBL International (Woburn, MA); rabbit anti-Src monoclonal antibody (36D10), rabbit anti-ERK1/2, and anti-phospho-ERK1/2 polyclonal antibodies, Cell Signaling (Beverly, MA); mouse anti-PY2K2/cell adhesion kinase β monoclonal antibody, BD Biosciences/Transduction Laboratories (San Diego, CA); rabbit anti-Fyn antibody, mouse anti-Src monoclonal antibody (B-12), and protein A/G plus-agarose, Santa Cruz Biotechnology (Santa Cruz, CA); horseradish peroxidase-conjugated goat secondary antibodies against either mouse or rabbit IgG, Bio-Rad (Hercules, CA); and mouse anti-neuronal class III β-tubulin (TuJ1) monoclonal antibody, Covance (Berkeley, CA). Rabbit anti-Src pY418 and pY529 phosphospecific antibodies and rabbit phosphorylation site-specific anti-FAK antibodies were purchased from Biosource International, Inc. (Camarillo, CA). Enolase and fluorescein isothiocyanate-labeled anti-mouse IgG secondary antibody were from Sigma (St. Louis, MO). Partially purified, active c-Src, and Fyn kinases were from Upstate Biotechnology. Recombinant active FAK kinase was from Biosource. PD98059 and U0126 were from Calbiochem (La Jolla, CA). Alexa Fluor 546 phalloidin for labeling F-actin was from Molecular Probes (Eugene, OR). Mouse monoclonal antibody against tubulin was a generous gift from Dr. Willi Halfter (University of Pittsburgh). Bicinchoninic acid-based protein assay reagents were from Pierce (Rockford, IL). Unless specified, all other chemicals used were of analytical grade quality or better and were obtained from Sigma.

Cell Culture. Cultures were prepared from the cerebral cortex of embryonic day 17 Sprague-Dawley rat fetuses (Hartnett et al., 1997). In brief, dissociated cells were suspended in plating medium composed of 80% Dulbecco’s modified Eagle’s medium (Invitrogen, Gaithersburg, MD), 10% Ham’s F-12 nutrients, and 10% heat-inactivated bovine calf serum with iron supplement (Hyclone, Logan, UT), with 25 mM HEPES, 2 mM 1-glutamine, 24 U/ml penicillin, and 24 μg/ml streptomycin. Cells at various densities were then plated onto poly-l-lysine (PLL)-coated tissue culture plates or culture dishes. Neurons were cultured in a humidified atmosphere at 37°C with 95% air and 5% CO2. All experiments were performed with serum-containing plating medium.

Cell Viability Assay. To evaluate the toxicity of MIT to cortical neurons in culture, fresh cell suspensions (1.5 ml), adjusted to 6.95 × 104 cells/ml, were added to each PLL-coated well of a 24-well plate to produce a low-cell density culture (5.21 × 104 cells/cm2). The cells were cultured for 3 h before beginning a continuous treatment with various concentrations of MIT or inhibitors. After a 14-h incubation, cells were washed with ice-cold PBS buffer three times, and the total activity of lactate dehydrogenase (LDH) in the remaining cells was measured using an in vitro toxicity assay kit (Sigma).

Morphological Analysis. Cells used in morphological studies were cultured and treated under the same conditions as above, except that they were plated onto a 24-well plate containing PLL-coated glass coverslips. After 14-h treatment, cells were fixed in PBS containing 4% paraformaldehyde, 5 mM EDTA, and 0.025% glutaraldehyde for 10 min at room temperature. If necessary, fixed cells were immunostained with appropriate antibodies as indicated in figure legends. Coverslips were mounted onto glass microscope slides, and random microscopic fields in the cultures were photographed. All cell bodies were counted, and all neurites within the microscopic field were measured using IP Labs 3.2 software (Scanco-lytics/BD Biosciences, Rockville, MD). The person performing the morphological analysis was blinded to the experimental conditions.

Cell-Free Tyrosine Kinase Assay. We used the method for tyrosine kinase activity described by O’Hara et al. (2003), with slight modifications. Either partially purified active SFKs or recombinant FAK (2 U for c-Src or Fyn and 0.1 μg for FAK) was included in a kinase reaction buffer (40 μl) containing 20 mM Hepes, 5 mM MgCl2, 2 mM MnCl2, and 0.1 mM ATP. The reaction was then treated with different concentrations of MIT (10 μl) for 10 min at 30°C. Later, the kinase reaction (20 min at 30°C) was started by the addition of 10 μl of kinase buffer, which contained a final concentration of 5.23 μg/ml acid-denatured enolase and 100 μM ATP. The reaction was stopped with the addition of reducing sample buffer. Samples were collected and later subjected to gel electrophoresis.

Preparation of Protein Samples from Cultured Neurons. Freshly dissociated neurons (18.5 ml, 2.51 × 105 cells/ml) were usually plated onto a 100-mm cell culture dish, and this cell density was designated as low-density cell culture. To increase the cell density (see Results), two different approaches were used; one was to keep the same volume (18.5 ml) of the cell suspension on all 100-mm dishes but adjust the number of cells to either 5.01 × 105 (medium cell density) or 1 × 106 (high cell density) cells/ml. Alternatively, the same number of cells (7.74 × 105 cells/ml, 6 ml) was added onto culture dish of different sizes: 100 (low density), 60 (medium density), or 35 (high density) mm. Cell cultures were incubated with MIT (2 μM) at 37°C, 3 h after plating. In the experiments performed with c-Src and Fyn immunoprecipitation, cell suspension was adjusted to 5 × 105 cells/ml before plating, and the same volume of cells was added to a 100-mm culture dish and treated with MIT (4 μM) for 1 h.

To harvest protein, cell-containing dishes were briefly rinsed twice with ice-cold PBS, and cells were then gently scrapped off the dishes on ice after addition of lysis buffer (50 mM HEPES, pH 7.5, 1% Triton X-100, 0.1% SDS, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 2 mM benzamidine). Debris was pelleted by centrifugation for 10 min, and the remaining lysate was used immediately or stored at −20°C. The protein concentrations of the samples were measured with by the bicinchoninic acid method according to the manufacturer’s protocol.

Immunoprecipitation. Equal amounts of total proteins from MIT-treated cell extract or control were mixed with 2 to 3 μg of either mouse anti-FAK or anti-PY2K2 monoclonal antibody and incubated at 4°C overnight with gentle agitation. The immune complex of specific protein and mouse antibody was then mixed in a rotary agitator with 100 μl of 50% (v/v) affinity gel coupled with rabbit anti-mouse IgG antibody at 4°C for an additional 2 h. For SFK protein binding assays, cells were lysed following inactivation of MIT, and samples were precleared with protein A/G plus agarose for 3 h at 4°C before incubation with MIT (10 μl of rabbit antibody against Ssrc or Fyn at 4°C) for 4°C. Protein-A/Protein complexes were precipitated with 40 μl of protein A/G plus-agarose for 3 h at 4°C. All beads were then washed and dissolved in 2× reducing electrophoresis sample buffer and boiled at 100°C for 5 min. With brief centrifugation, the supernatants of protein samples were collected and later subjected to gel electrophoresis.

Electrophoresis and Immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by standard procedures using the Mini Protein 3 System (Bio-Rad). Before electrophoresis, protein samples, with the exception of those samples prepared by immunoprecipitation, were treated with the reducing electrophoresis sample buffer from Pierce and boiled at 100°C for 5 min. Samples with equal amount of protein and a broad-range molecular weight protein marker from Bio-Rad were run in parallel on 10% SDS-PAGE gel. For immunoblotting analysis, separated protein bands were transferred onto a 0.2 μm-nitrocellulose membrane (Bio-Rad).
Protein transfer was confirmed by staining with Ponceau S solution (0.1% Ponceau S in 5% acetic acid). The membranes were then blocked with 1% nonfat milk powder in PBS with 0.05% Tween 20 (PBST) at room temperature for 1 h and probed with appropriated primary antibodies diluted in PBST. After washing (three times) in PBST, blots were incubated with goat secondary antibody conjugated to horseradish peroxidase at room temperature for 1 h. Blots were then visualized using SuperSignal CL-HRP Substrate System (Pierce) and exposed to BioMax films (Eastman Kodak, Rochester, NY). Equal protein loading was evaluated using two approaches. Generally, the blots were stripped [100 mM 2-mercaptoethanol, 2% (w/v) SDS, and 62.5 mM Tris-HCl, pH 6.7] for 30 min at 50°C and then reprobed with primary and secondary antibodies as described above. Alternatively, an identical gel was run in parallel, and the additional blot was used to check protein loading. This method was employed in cases of low levels of protein, in which the stripping method could not provide accurate detection. The developed films were scanned and analyzed using Scion Image software (version 4.02; Scion Corporation, Frederick, MD).

Statistical Analysis. Data are expressed as the mean ± S.E.M. Experimental data were analyzed by one-way analysis of variance (ANOVA) with post hoc comparisons or by pair-wise comparisons (Student’s t test).

Results

Neurotoxic Consequences of MIT Exposure to Developing Cortical Neurons. To assess the toxicity of MIT on developing neurons, cells were plated at a density of 5.21 × 10⁴ cells/cm², allowed to adhere for 3 h (time 0 h in Fig. 3 and subsequent figures), and then treated with varying concentrations of the biocide (0.1, 0.3, 1, and 3 μM) for 14 h. The viability of neurons was determined by measuring total cytoplasmic LDH activity in the remaining cells. Under these conditions, we observed a modest level (~35%) of MIT-induced cell death, but only when the concentration of the biocide reached 3 μM (Fig. 1). At 1 μM MIT or below, no significant cell loss could be detected. Strikingly, however, we observed a profound inhibition of process outgrowth by these low concentrations of MIT (Fig. 2). Therefore, we turned our attention to investigate this phenomenon further. After overnight incubation with 0 to 1 μM MIT, neurons were fixed and stained with a monoclonal antibody against neuronal class III β-tubulin. Comparing the morphological changes between control and treated neurons (Fig. 2, A–C), we found a significant inhibition of process outgrowth by 1 μM MIT (Fig. 2G), although at 0.3 μM, there was an observable trend to shorter neurites as well. In subsequent studies, the medium containing MIT was removed from sister culture plates after the 14-h treatment, and cells were washed, refed with normal growth medium, and maintained for an additional 4 days. Under these conditions, many MIT-pretreated neurons showed shorter and less elaborate processes as they appeared to experience slow growth and decreased cell-to-cell contact, perhaps due to a decrease in neuronal mobility (Fig. 2, compare D with E and F). These results suggest that chronic exposures to low concentrations of MIT had toxic effects on immature cortical neurons in culture, which were manifested primarily by a pronounced inhibition of process outgrowth.

Characterization of FAK as a Signaling Protein Regulated by MIT. We next investigated the biochemical pathways involved in this neurotoxicity by searching for potential MIT-sensitive signaling proteins. We first examined the overall level of protein tyrosine phosphorylation in treated and untreated cultures. Embryonic rat cortical neurons were plated and allowed to adhere to tissue culture plates for 3 h. Cells were then treated with either vehicle or 2 μM MIT for an additional 4 h. Protein samples were then harvested, separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with a phosphotyrosine-specific monoclonal antibody (4G10). Proteins bearing phosphotyrosines exhibited a wide range of molecular weights (Fig. 3A), and there was a basal level of tyrosine-phosphorylated proteins in control cultures (Fig. 3A, 0 h). Interestingly, we found that the level of tyrosine phosphorylation of one single major protein with an approximate molecular mass of ~125 kDa had been substantially affected by MIT treatment (Fig. 3A, arrow). To identify this 125-kDa protein, we first tested if its tyrosine phosphorylation levels were affected upon cell adhesion. We observed that the phosphorylation of this protein was barely detectable in cell extracts generated from cell suspensions (data not shown). In contrast, extracts harvested from cells plated onto PLL-coated dishes had substantial levels of tyrosine phosphorylation, suggestive of an adhesion-related process. This unknown protein thus had a number of characteristic attributes, including a high level of tyrosine phosphorylation upon cell adhesion, seemingly associated with neurite outgrowth, and a molecular mass of approximately 125 kDa. With this information, we hypothesized this protein to be FAK. Indeed, a monoclonal antibody specific for FAK detected a major protein band of 125 kDa in samples treated with or without MIT, which comigrated with the antiphosphotyrosine antibody-reacting band (Fig. 3B). To confirm the identity of this protein as FAK, we performed immunoprecipitation with an anti-FAK antibody. Cell extracts were prepared from MIT or vehicle-treated cultures. Proteins were immunoprecipitated and then immunoblotted
Fig. 2. MIT impairs neurite outgrowth. Freshly dissociated embryonic rat cortical neurons ($5.21 \times 10^5$ cells/cm²) were plated on poly-l-lysine-coated glass coverslips for 3 h and then treated with vehicle (A and D) or 0.3 (B and E) or 1 (C and F) μM MIT. A to C, neurons were cultured overnight in the presence or absence of MIT. D to F, neurons were cultured with or without MIT overnight and then returned to normal growth medium without MIT for an additional 4 days. Neurons were fixed and immunocytochemically stained with mouse antibody specific for neuronal class III β-tubulin. Immunofluorescent labeling of cells was done using a fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody. Representative photographs show morphological changes on neurons as a result of MIT treatment. G, quantification of the concentration-dependent inhibition of neurite outgrowth induced by MIT. Values represent mean ± S.E.M. of five independent experiments (**, p < 0.01, compared with control; ANOVA/Dunnett).

Fig. 3. Deficits in FAK phosphorylation as a result of MIT treatment. Freshly dissociated embryonic (E17) cortical neurons were plated at low-cell density as described in Materials and Methods. A, after 3 h in culture (designated as 0 h, before MIT treatment), cells were treated with MIT (2 μM) or vehicle for 4 h. Protein samples were collected, and equal amounts of proteins were separated by 10% reducing SDS-PAGE and transferred to a nitrocellulose membrane. The resulting blot was probed with a mouse monoclonal antibody specific for phosphotyrosine. Compared with control (0 h), proteins showed an increasing level of tyrosine phosphorylation with time in culture (4 h). The arrow points to a protein band with a substantial reduction of tyrosine phosphorylation in the presence of MIT. Protein loading for all samples was identical as judged by Coomassie Blue-stained SDS-PAGE gels (data not shown). B, samples were prepared after incubation with either vehicle or MIT (2 μM) for 45 min. Two identical protein-bound nitrocellulose membranes were blotted with antibodies against either phosphotyrosine (P-Tyr) or FAK. Arrow, position is the same for the band with decreased phosphotyrosine levels after MIT treatment. C, immunoprecipitation of FAK from cortical neurons. Anti-FAK immunoprecipitated proteins were probed with anti-phosphotyrosine monoclonal antibody (P-Tyr) indicated by arrow (top). After stripping this antibody, blot was reprobed with anti-FAK monoclonal antibody (FAK) to demonstrate equal protein loading (bottom). These data are a representative of results from three independent experiments. D, early and persistent impairment of FAK phosphorylation by MIT. Cells plated at low density as mentioned above were cultured for 3 h (0-h time point) and then treated with MIT (2 μM) or vehicle for 0.5, 2, or 8 h and harvested. Two identical protein-bound nitrocellulose membranes were blotted with antibodies, either anti-phosphotyrosine antibody (P-Tyr) or anti-FAK antibody (FAK), the latter serving as a loading control. Data shown are representative of two independent experiments.
with anti-phosphotyrosine antibody. Figure 3C shows that the level of phosphotyrosine on immunopurified FAK from MIT-treated sample was substantially decreased when compared with control. These data indicate that the MIT-sensitive 125-kDa protein is indeed FAK.

The deficits in tyrosine phosphorylation of FAK could be detected as early as 30 min following MIT treatment, and this inhibitory effect was still present as late as 8 h (Fig. 3D). In contrast, tyrosine phosphorylation of FAK in vehicle-treated cultures continued to increase with time in culture. Hence, the inhibitory actions of MIT on FAK phosphorylation were both rapid and persistent. FAK is a multiple tyrosine-containing enzyme with as many as six tyrosine residues that have been identified as phosphorylation targets. As such, we analyzed the level of phosphorylation at four tyrosine residues of FAK with phosphorylation site-specific antibodies (Fig. 4A). We observed a 60% decrease in the phosphorylation state of Tyr576 within the kinase domain of FAK and approximately a 40% decrease at Tyr861 in MIT-treated neurons (Fig. 4B). In contrast, the phosphorylation levels of tyrosine 407 were not significantly altered when compared with vehicle-treated cultures. We also noted an abundant level of autophosphorylation at tyrosine 397 in samples obtained from MIT-treated cultures.

**Direct Inhibition of MIT on c-Src Kinase Activity in Cell-Free Assays.** Earlier studies have demonstrated that phosphorylation of FAK at Tyr576, Tyr577, and Tyr861 is primarily regulated by the nonreceptor protein tyrosine kinase c-Src and other SFKs (Calalb et al., 1995). We thus tested whether MIT inhibited FAK phosphorylation directly or via SFK inhibition. First, the effects of this biocide on c-Src activity were evaluated using a commercially available, purified recombinant active kinase. Changes of tyrosine kinase activity of c-Src after MIT treatment were measured by detecting the level of autophosphorylation at tyrosine 418, as well as the level of tyrosine phosphorylation of the exogenous substrate enolase using a cell-free assay. As shown in Fig. 5A, using a phosphospecific antibody against Src Tyr418, we found that the autophosphorylation of c-Src was decreased by MIT (50 μM), indicating that the biocide probably directly inhibited c-Src. Consistent with this observation, the tyrosine phosphorylation of enolase was also inhibited at the same concentration of MIT (Fig. 5B). However, no change of the phosphorylation at (Tyr529) on the inhibitory domain of c-Src could be detected (data not shown), suggesting at least a certain degree of specificity to MIT’s inhibitory actions on c-Src kinase activity. We also employed purified recombinant Fyn kinase, another SFKs member, and tested it in a similar fashion. We found that MIT had similar inhibitory effects on this kinase as well (data not shown). The concentrations of MIT required to produce inhibition of c-Src and Fyn were much higher than those needed to observe its deleterious actions in neurons. This is probably due to the need to overcome the high concentration of dithiothreitol present in these commercially available active enzymes. Finally, we proceeded to test any possible direct inhibitory effects of MIT on FAK. However, we found no change in the levels of tyrosine phosphorylation of enolase catalyzed by FAK in the presence of MIT at concentrations as high as 100 μM (data not shown). These results strongly suggest that MIT can inhibit the kinase activity of SFKs and not FAK.
MIT (µM)

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Fig. 5. MIT inhibits autophosphorylation of Src kinase and Src-dependent enolase tyrosine phosphorylation. Protein samples were prepared as described under Materials and Methods using recombinant active Src. In the top panels of A and B, Src autophosphorylation was detected using a rabbit phosphospecific antibody to Src(pY418), whereas the level of phosphorylated enolase was probed using an anti-phosphotyrosine antibody. Background protein loading was checked on respective membranes after stripping, using anti-Src antibody or anti-enolase antibody (A and B, bottom). Data shown are representative of three independent experiments.

Fyn were isolated, and then the presence of FAK in the complexes was analyzed by immunoblotting. As expected, we found that FAK could coimmunoprecipitate with either c-Src or Fyn from normal rat embryonic cortical neurons (Fig. 6). However, we were unable to detect FAK in samples harvested from neurons exposed to MIT. Our results strongly indicated that both c-Src and Fyn no longer bind FAK following MIT treatment, suggesting that the inhibited kinases dissociate from their scaffolding complexes, which were likely formed upon cell plating. This novel finding suggests that MIT may induce a conformational change in the kinases and influences their interaction, providing a plausible explanation for the dephosphorylation of Tyr576 and Tyr861 of FAK following biocide exposure. SFKs must then likely be in a functional complex with FAK for appropriate signaling following cell adhesion.

Enhanced Cellular SFK Activity and FAK Phosphorylation by High-Density Culture in MIT-Exposed Neurons. Cell density can influence the phosphorylation state of c-Src and FAK in cultured cells by facilitating cell-to-cell contact and cell adhesion molecule-dependent signaling (Batt and Roberts, 1998). In preliminary studies, we observed that neurons plated on L1 or neural cell adhesion molecule (NCAM) could partially overcome the deleterious actions of MIT on neurite outgrowth. We thus investigated if neurons grown at various cell densities responded differently to the toxic actions of MIT. Two different methods to increasing cell density were employed and examined the degree of SFKs and FAK tyrosine phosphorylation in the presence or absence of MIT. First, cells were plated in increasing numbers in tissue culture plates of the same size (100 mm). Under these conditions, we observed a higher level of autophosphorylation (Ty418) on endogenous SFKs (the phospho-specific antibody is nonselective for SFKs) in control cells with increasing cell density, although phosphorylation level of Tyr529 remained unchanged (Fig. 7). The inhibitory actions of MIT seen at low-cell density culture could be reversed by the high-density growth conditions (Fig. 7, top). Likewise, the phosphorylation level of FAK increased with higher cell density, with MIT unable to inhibit the phosphorylation of this kinase under these circumstances (Fig. 7B). In the second set of experiments (Fig. 7B), we plated the same number of cells in tissue culture plates of various sizes (35, 60, and 100 mm). This strategy also resulted in different cell densities, but the total number of cells in the culture remained the same. Both the level of FAK tyrosine phosphorylation and the inhibitory actions of MIT essentially reproduced what had been observed using the first strategy. The evidence presented in this series of studies indicates that increased cell-to-cell contact resulting in increased kinase activity of SFKs as well as tyrosine phosphorylation of FAK can overcome the actions of MIT in cultured neurons. Whether higher concentrations of MIT could reverse this effect could not be tested because the previously reported cell death pathway is then rapidly triggered (Du et al., 2002).

Phosphorylation ERK1/2 and PYK2 Are Not Affected by MIT Treatment. FAK can act as an adapter protein, integrating signals to alter cell function via several signaling pathways, including ERK. In our previous study, we reported that the acute toxicity induced by high concentrations of MIT was mediated via ERK activation (Du et al., 2002). We thus sought to determine whether the neurite growth-inhibiting
actions of MIT involved changes in the level of activation of this mitogen-activated protein kinase. Cell lysates harvested from neuronal cultures treated with vehicle or MIT (2 μM) for either 30 min or 2 h were probed with an anti-phosphorylated ERK1/2 polyclonal antibody. In contrast to the results obtained with FAK, we observed no changes in the level of ERK phosphorylation with either plating time or MIT treatment (Fig. 9A). Finally, we observed that U0126 and PD98059 failed to inhibit neurite outgrowth in our cultures (Fig. 9B). These data indicate that the ERK pathway is

Fig. 8. Enhanced cell density prevents MIT-induced impairment of FAK phosphorylation. A, samples from freshly dissociated cells were prepared as same as described in Fig. 7. Two identical immunoblots (top) were performed with anti-phosphotyrosine antibody (P-Tyr) and anti-FAK antibody (FAK), respectively, the latter serving as a loading control. In the bottom, blots were quantified and normalized to control. Values represent the means ± S.E.M. (n = 3). A one-way ANOVA revealed a significant effect of cell density on FAK phosphorylation in MIT-treated cultures (p < 0.001). B, alternative approach to increase cell density. An equal number of cells (7.74 × 10^5 cells/ml; 6 ml) were plated onto culture dishes of different diameters (low, 100 mm; medium, 60 mm; high, 35 mm). Cells were then lysed, and proteins were harvested. Two identical immunoblots (top) were performed with anti-phosphotyrosine antibody (P-Tyr) and anti-FAK antibody (FAK), respectively, the latter serving as a loading control. In the bottom, blots were quantified and normalized to control. Values represent the means ± S.E.M. (n = 3). A one-way ANOVA revealed a significant effect of cell density on FAK phosphorylation in MIT-treated cultures (p < 0.01).

Fig. 9. MIT does not interfere with ERK and Pyk2 signaling. Cells were plated at low density the same as described in Fig. 8 and cultured for 3 h. A, following 3-h plating (0-h time point), cells were treated with either vehicle or MIT (2 μM) for 0.5 and 2 h. At each designated time point, cells were harvested and lysed; protein samples were immunoblotted with anti-phosphorylated Erk1/2 antibody (P-ERK1/2, top) and later reprobed with anti-Erk1/2 antibody (ERK1/2, bottom). No changes in ERK1/2 phosphorylation were noted with MIT treatment. B, cells were treated with either vehicle or the ERK activation inhibitors U0126 (10 μM) and PD98059 (38.7 μM) overnight. Representative phase-contrast photographs show that inhibitors of ERK activation do not interfere with neurite outgrowth in our system. C, following the treatment with either vehicle or MIT (2 μM) for 1 h, cells were lysed, and Pyk2 was immunoprecipitated using an anti-Pyk2 monoclonal antibody. Proteins were separated by 10% reducing SDS-PAGE and transferred onto nitrocellulose membranes. Two identical membranes were immunoblotted with anti-phosphotyrosine antibody (P-Tyr, top) and anti-Pyk2 antibody (Pyk2, bottom).
probably not involved in the neurodevelopmental toxic actions of low concentration of MIT.

In our final set of experiments, we were keen to explore PYK2-related adhesion focal tyrosine kinase/cell adhesion kinase β as a potential downstream target in our developmental MIT toxicity paradigm. PYK2, like FAK, is a non-receptor tyrosine kinase highly expressed in neuronal cells (Girault et al., 1999). It shares a high degree of sequence similarity and biochemical properties with FAK, in addition to possessing multiple sites of tyrosine phosphorylation (Girault et al., 1999). PYK2 was immunoprecipitated from MIT-treated sample and vehicle-treated controls using a mouse anti-PYK2 monoclonal antibody and probed with anti-phosphotyrosine antibody. We observed no difference in PYK2 phosphorylation under both conditions (Fig. 9C), suggesting that this protein was not a target of the toxic actions of MIT.

Discussion

Molecular Mechanism of Neurodevelopmental MIT Toxicity. The regulation and interaction of SFKs with FAK, stimulated by integrins, cell adhesion molecules, and extracellular matrix proteins, is recognized to play an important role in many cellular processes, including neural development. Here, we report a previously unrecognized consequence of SFK inhibition by the thiol-reactive compound MIT, namely a physical dissociation of FAK from the signaling complex, leading to FAK dephosphorylation and probably loss or decrease of function of the signaling cascade. Our results also demonstrate that MIT, at low concentrations, significantly impairs the growth of neurites and can cause neuronal death in freshly dissociated cortical neurons in vitro.

Our studies show that SFKs are sensitive targets for the thiol modification by MIT. In contrast, FAK is not directly inhibited by the biocide, although its phosphorylation level clearly, albeit indirectly, is. The autophosphorylation of Src Tyr418 can be used as an indicator of kinase activity because its phosphorylation level correlates well with enzyme activity (Boerner et al., 1996). MIT decreased the phosphorylation of this tyrosine and impaired its kinase function, whereas the phosphorylation level of another tyrosine in Src, Tyr529, remained unaffected. Our results are highly reminiscent of other studies in which thiol modification on SFKs were reported to affect Tyr418 phosphorylation-associated processes, independently of Tyr529 phosphorylation (Akhand et al., 1999). SFKs have a cluster of cysteine residues in their kinase domains. Of these, cysteine 487 and cysteine 498 are highly conserved and are critical for enzymatic activity (Senga et al., 2000); modification of these sulfhydryl groups can abolish the kinase activities of both Src and Lck (Uehara et al., 1989; Veillette et al., 1993; Senga et al., 2000; Oo et al., 2003). Interestingly, it has been reported that an isothiazole-zolone-derived compound can inhibit Lck activity via precisely this mechanism (Trevillyan et al., 1999). Related to this, we have recently observed that herbimycin A, an SFK inhibitor also possessing thiol reactivity, inhibits neurite outgrowth in cortical neurons. Herbimycin A has been shown to inhibit neuronal migration, differentiation, and FAK tyrosine phosphorylation in rat cortical neurons (Maeda and Noda, 1998).

It is generally believed that after cell adhesion-stimulated autophosphorylation of FAK Tyr397, SFKs are recruited to form a complex via their SH2 binding domain. This results in the activation of SFKs and subsequent phosphorylation of other tyrosines on FAK, including Tyr576 and Tyr577 on the kinase domain, as well as Tyr861 in the C-terminal domain (Calab et al., 1995). Our data suggest that, as a result of MIT exposure, SFKs dissociate from the complex and fail to phosphorylate FAK. The fact that phosphorylation of FAK Tyr397 was unaltered in MIT-treated cultures suggests that the initial cell adhesion process after plating is sufficient for autophosphorylation to occur. SFKs have an additional pair of highly conserved cysteines (Cys238 and Cys245) in the SH2 domain (Oo et al., 2003). Thus, it is also plausible that MIT also interacts with these thiols to promote the dissociation of the signaling complex.

Effects of Plating Density. Increased cell plating densities reversed the effects of MIT on FAK phosphorylation. When cells are plated at a high density, increased cell-to-cell contact may initiate multiple cellular signaling pathways by cell adhesion molecules such as integrins, cadherins, and the Ig superfamily. The effects of these possibly nonoverlapping signaling pathways may then be sufficient to compensate for the detrimental actions of MIT. For instance, NCAM can stimulate neurite outgrowth via a signaling pathway requiring the Src family kinase Fyn (Beggs et al., 1994), and immunoprecipitation studies have demonstrated the association of NCAM with Fyn and FAK, leading to the increased tyrosine phosphorylation of both kinases (Beggs et al., 1997). In contrast, the adhesion molecule L1 stimulates neurite outgrowth via a homophilic binding mechanism involving Src but independently of FAK activation (Igelzi et al., 1994; Schmid et al., 2000). In preliminary studies, when we used L1 and NCAM as coating substrates, we found they could partially reverse the deleterious actions of MIT on neurite outgrowth. Therefore, cross-talk between signaling pathways triggered by cell contact may be sufficient to overcome the actions of MIT, at least at the low concentrations tested. We could not properly evaluate whether higher concentrations of MIT could overcome the high-density plating effect as the biocide then becomes highly lethal via a different pathway (Du et al., 2002).

ERK Activation Is Not Involved in Neurite Outgrowth in Cortical Neurons. In addition to its kinase activity, FAK has been suggested to work as a scaffolding protein to integrate multiple signaling pathways in response to extracellular stimuli (Sieg et al., 2000; Parsons, 2003). One of these downstream signaling pathways from FAK is the mitogen-activated protein kinase ERK (Schaller, 2001). We were not able, however, to detect any changes in the phosphorylation levels of ERK upon MIT exposure, nor did ERK activation inhibitors prevent neurite outgrowth. Since our exposure conditions were performed in the presence of serum, it is likely that trophic factors present in the plating media were sufficient to trigger ERK activation. The fact that ERK activation inhibitors were unable to inhibit neurite outgrowth suggests that, under our plating conditions, the phosphorylation levels of FAK, and not its role in ERK activation via protein scaffolding, are more immediate predictors of its physiological function, vis-à-vis neurite outgrowth. In fact, it has been shown by others that the inhibition of FAK activation has no effect on ERK1/2 phosphorylation (Yang et...
al., 2004). Thus, although neurite outgrowth is associated with the activation of ERK in PC12 cells (Kolkova et al., 2000), chick retinal neurons (Schmid et al., 1999), and rat cerebellar neurons (Schmid et al., 1999), this does not seem to be the case in our system. In addition, the level of tyrosine phosphorylation of the closely related nonreceptor tyrosine kinase Pyk2 remained unaltered by the biocide. Because Pyk2 activation by growth factors is mediated by autophosphorylation in a Src-independent manner (Park et al., 2004), blocking the activities of SFPKs by MIT, as discussed above, probably represents the major effect for the biocide in inhibiting FAK phosphorylation.

**Potential Implications of This Study.** FAK-deficient mice die during the early embryonic period (Ilic et al., 1995). In the nervous system, this kinase is required for the formation of cortical basement membrane (Beggs et al., 2003), axonal branching and synapse formation (Rico et al., 2004), neuronal migration (Xie et al., 2003), and long-term potentiation (Yang et al., 2003). Therefore, drugs or chemicals interfering with FAK signaling are likely to be detrimental to a developing organism. The concentration of Kathon in household products (shampoos, conditioners, hand creams, and lotions) ranges between 15 and 30 ppm (equivalent to 100–200 μM for the combined CMIT and MIT) (Rastogi, 1990). In recent studies, we have found that the neurotoxic potency of CMIT on the inhibition of neurite outgrowth is at least 3-fold higher than that of MIT, attributable to its increased lipophilicity (Jayjock et al., 1996). Unfortunately, CMIT became commercially available only after most of the work described here had been performed. It has also been shown that Kathon is dermally absorbed at rates between 50 and 80% in experimental animals (Jayjock et al., 1996). Thus, the targeted tyrosine dephosphorylation of SFKs and FAK by MIT found in this study may have a direct consequence to in vivo brain development, although this has yet to be experimentally demonstrated. In preliminary studies, CMIT was noted to be lethal at low concentrations (3 μM) to developing Xenopus laevis tadpoles (C.D. Aizenman, personal communication).

Thus, the risk of MIT/CMIT exposure could be particularly high for fetuses of pregnant women exposed to these chemicals in occupational or nonoccupational settings. Indeed, Schettler (2001) has argued that the developing brain may be vulnerable to environmental agents at exposure levels that have no lasting effects in adults. He also states that, with very few exceptions, neurodevelopmental toxicity data are missing for most industrial chemicals in widespread use, even when population-wide exposures are documented. This is a disturbing statement in light of the growing recognition of an apparent increase in the incidence of developmental disabilities in children. In the United States, 17% of children under 18 (12 million) present some form of learning disability, have behavioral problems, or suffer from delays in growth or development, cerebral palsy, speech deficits, epilepsy, deafness, or blindness (Boyle et al., 1994). Astonishingly, learning disabilities afflict 5 to 10% of children in United States public schools (Parrill, 1996). One must question whether some of the large number of environmental agents for which we have no information regarding their effects on the developing nervous system may be partly responsible for these problems. Unfortunately, regulatory agencies generally fail to require neurodevelopmental testing of chemicals (Schettler, 2001). The results presented here suggest that the potential neurodevelopmental consequences of occupational or environmental exposure to isothiazoline derivatives should be closely scrutinized.

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**References**


Rice WG, Supko JG, Malpeis L, Buckheit BW Jr, Clanton D, Bu M, Graham L,


Address correspondence to: Dr. Elias Aizenman, Department of Neurobiology, University of Pittsburgh School of Medicine; E1456 BST, Pittsburgh, PA 15261. E-mail: redox@pitt.edu