Biochemical and Biophysical Research Communications **271,** 181–185 (2000)
doi:10.1006/bbrc.2000.2524, available online at http://www.idealibrary.com on IDEAL®

Monoamine Oxidase B Induces ERK-Dependent Cell Mitogenesis by Hydrogen Peroxide Generation

Cécile Vindis, Marie-Hélène Séguélas, Pascale Bianchi, Angelo Parini, and Claudie Cambon[1]
INSERM U388, Institut Louis Bugnard, CHU Rangueil, Bâtiment L3, 1, Avenue J. Poulhès 31403 Toulouse Cedex 4, France

Received March 13, 2000

The mitochondrial enzyme monoamine oxidase (MAO) A and B catalyze the oxidative deamination of various endogenous and exogenous biogenic amines. In the present study, we used human embryonic kidney 293 (HEK 293) cells stably transfected with human MAO-B cDNA to investigate the potential role of hydrogen peroxide (H_2O_2) produced by MAO-B isoform as an intracellular messenger involved in regulation of cell signaling and function. The MAO substrate tyramine induced tyrosine phosphorylation of Shc, ERK activation, and an increase in DNA synthesis in HEK 293 expressing MAO-B, but not in wild type HEK 293 cells, which do not express MAO. Tyramine effects were fully prevented by cell pretreatment with the MAO inhibitor pargyline or the antioxidant N-acetylcysteine. These results show that MAO-B induces MAPK/ERK activation and cell mitogenesis through H_2O_2 production.

Key Words: **monoamine oxidase; hydrogen peroxide; human embryonic kidney 293 cells; extracellular signal-regulated protein kinase; mitogenesis.**

The mitochondrial enzyme monoamine oxidases (MAO-A and MAO-B) are flavoprotein catalysing the oxidative deamination of some neurotransmitters (i.e., noradrenaline, dopamine and serotonin) and different exogenous and endogenous amines, such as tyramine, kynuramine, phenylethylamine and benzylamine. MAO-A and MAO-B are encoded by two distinct genes. Both isoforms display similar activity towards dopamine and tyramine whilst MAO-A preferentially metabolises serotonin and kynuramine and MAO-B has a greater affinity for phenylethylamine and benzylamine. The two MAO isoforms can be also differentiated according to their inhibition by synthetic compounds (clorgyline and Ro 41-1049 for MAO-A; selegiline and Ro 19-6327 for MAO-B) (1–3).

Monoamine oxidases are widely distributed in the central nervous system and in the periphery (4, 5) and play a central role in the control of substrate availability and activity. These enzymes are a potential source of hydrogen peroxide (H_2O_2) during substrate degradation. Reactive oxygen species (ROS) such as H_2O_2 were first described as causative factors of DNA strand breaks, lipid peroxidation and oxidative damage of proteins involved in different pathological processes including neurodegenerative and inflammatory diseases, cancer and atherosclerosis (6–10). Recent works demonstrated that ROS, at non-toxic levels, caused growth in several cell types (11–13). In addition, H_2O_2 can act as a second messenger molecule in mitogen-induced cellular events (14, 15) through the activation of protein tyrosine kinases, serine/threonine kinases and transcription factors (16–21).

At present, the generation of H_2O_2 by MAOs has been mostly considered as a cytotoxic factor involved in oxidative stress and nigral cell degeneration in Parkinson's disease (22). However, we have recently obtained evidences suggesting that H_2O_2 generated by MAOs may be a potential intracellular messenger. Indeed, we showed that, in various cell types, the H_2O_2 produced by MAOs during tyramine degradation is not fully scavenged by intracellular antioxidant systems (23).

In this study, we used human embryonic kidney 293 (HEK 293) cells stably transfected with human MAO-B cDNA (HEK 293-MAO-B) to investigate the consequences of H_2O_2 produced by MAO-B on cell signaling and function. Our results show that amine degradation by MAO-B induces H_2O_2-dependent tyrosine phosphorylation of Shc, ERK activation and subsequent mitogenesis of HEK 293-MAO-B cells.

Abbreviations used: ROS, reactive oxygen species; MAO, monoamine oxidase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; MEK, MAPK kinase; CL, chemiluminescence.

[1] To whom correspondence should be addressed. Fax: 33-5-62-17-25-54. E-mail: cambon@rangueil.inserm.fr.

0006-291X/00 $35.00

MATERIALS AND METHODS

Materials

BSA (bovine serum albumin), pargyline hydrochloride, tyramine hydrochloride, N-acetyl-cysteine, hygromycine B were purchased from Sigma Chemical Co. (St. Louis, MO), Dulbecco's modified Eagle's and Fetal calf serum (FCS), were from Gibco-BRL (Eragny, France), [Me^{3}H] Thymidine, 74 Ci/mmol, 37 Mbq, 2.7 Tbq/mmol was from ICN Pharmaceuticals Inc (Costa Mesa, CA), PD 98059 was from Calbiochem (La Jolla, CA), PVDF transfer membrane was from NEN Life Science Products was from (Boston, MA), anti-active MAPK polyclonal antibody was from Promega (Madison, WI), Proteine A was from Transduction Laboratories (Lexington, KY), Kit ECL detector reagents and ECL phosphorylation module anti-phosphotyrosine HRP conjugate were from Amersham (Buckinghamshire, UK), anti-rabbit IgG-HRP conjugated and anti-rabbit polyclonal IgG ERK2 were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-human Shc transforming protein rabbit polyclonal IgG was from Upstate Biotechnology (Lake Placid, NY), Bio-Rad DC protein assay reagents was from Bio-Rad Laboratories (Ivry-sur-Seine, France).

HEK 293 Cell Transfection and Culture

HEK cells were obtained from American Type Tissue Culture and grown in Dulbecco's modified Eagle's medium at 37°C (5% CO_2) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). The human cDNA for MAO-B was obtained from Dr. Jean Shih in the pECE vector. pECE MAO-B was restricted with Kpn I and Xba I (sites in the pECE polylinker) and the insert purified and ligated into the Kpn I and Xba I sites of pCDNAIII. Cells were stably transfected with DNA by calcium phosphate coprecipitation as previously described (24).

Assay of H_2O_2 Production

H_2O_2 production was measured by chemiluminescence (CL) in the presence of luminol (30 μM) and Horse Radish Peroxidase (HRP) (0.1 U/ml) (25) using a thermostatically (37°C) controlled luminometer (Bio-Orbit 1251) as described previously (23). The generation of CL in cells triggered with 10 μM of tyramine, was continuously monitored for 60 min, and the area under the curve (total CL emission) was analyzed by the Bio-Orbit MultiUse program.

Cell Proliferation Studies

[^{3}H]thymidine incorporation assay. HEK 293 cells were plated at a density of 25×10^3 cells/well in 24 well plates (Nunc). After a 48-h period to allow attachment, cells were made quiescent by incubation for an additional 24-h period in serum free medium Dulbecco's modified Eagle's medium. Cells were pretreated with or without appropriate compounds [the monoamine oxidase inhibitor pargyline, the antioxydant N-acetylcysteine (NAC) (19), the MEKK inhibitor PD 98059 (26)] for 30 min and treated with tyramine in the presence of 1 μCi/ml of [^{3}H]thymidine for 24 h. Cells were then washed twice with 1 ml HBSS (Hanks' balanced salt solution) to remove unincorporated [^{3}H]thymidine and subjected to ice-cold 5% trichloracetic acid (wt/vol) for 15 min. The precipitate was washed with 95% ethanol and dissolved in 100 μl of 0.1 N NaOH/0.1% SDS at 37°C for 30 min. Radioactivity was measured by liquid scintillation counting (Packard Tricarb counter).

Cell number counting. For cell counting, quiescent HEK 293 cells (plated at a density of 80×10^3 cells/well in 6 well plates (Nunc)) were pretreated in the same conditions used for [^{3}H]thymidine incorporation assay, with or without pargyline, NAC or PD 98059 before tyramine addition for 24 h. The cells were then trypsined, resuspended in Isoton and counted in a cell counter (Coulter Counter ZM) as described previously (27). Cell viability was probed by the trypan blue exclusion test.

Western Blot Analysis

HEK 293 cells grown in 100-mm plates were appropriately conditioned and pretreated with or without various inhibitors (pargyline, NAC). At specified times after tyramine treatment, cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2 mM Na_3VO_4, 10 μg/ml phenylmethylsulfonyl fluoride, 3 μg/ml aprotinin and 3 μg/ml leupeptin. Lysate samples (30 μg of protein/lane) were run in 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 1% BSA in Tris-buffered saline-Tween 20 (0.1%) (TBST) overnight at 4°C. Immunoblots were probed using different antibodies. Membranes were washed again in TBST and bands were detected using the ECL reaction. After extensive washing, the blots were reblocked prior to reprobing.

Shc Immunoprecipitation

After tyramine treatment, cells were lysed in RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM Na_3VO_4, 0.1% SDS, 1% Nonidet P40, 1% Na deoxycholate, 10 μg/ml phenylmethylsulfonyl fluoride). Shc proteins were immunoprecipitated from cells lysates (500 μg of protein) overnight with 5 μg of rabbit polyclonal anti-Shc antibody and 50 μl of protein A agarose. Bound proteins were eluted with 40 μl of Laemmli sample buffer. For Western blot analysis, samples were electrophoresed on 10% SDS-polyacrylamide minigels and transferred to polyvinylidene difluoride membranes. Phosphorylated Shc proteins were detected using anti-phosphotyrosine-HRP conjugate antibody. After stripping, blots were reblocked prior to reprobing with a rabbit polyclonal anti-Shc antibody.

Quantitation of Proteins

Concentrations of protein were determined with the Bio-Rad DC protein assay reagents (Bio-Rad Laboratories), with γ-globulins as the standard.

Statistical Analysis

All values are presented as means ± SEM. ANOVA *t* test was used for statistical analysis, and differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Experiments have been performed using the MAO substrate tyramine. Tyramine, which is a dietary component and also derives form tyrosine metabolism (28), is a potent catecholamine releasing factor. This amine appears to be an ideal substrate to specifically define the cell events related to H_2O_2 production by MAOs for two reasons: first, in contrast to catecholamines and serotonin which could activate signaling cascades through the interaction with membrane receptors, tyramine does not interact with specific receptors in mammalian cells (29); second, because of the lack of the catechol grouping (30), tyramine is unable, unlike dopamine, to generate ROS by autoxidation.

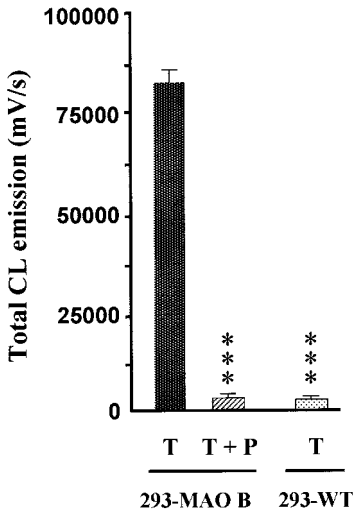

FIG. 1. MAO-dependent H_2O_2 production in intact HEK 293 cell lines. The total chemiluminescence (CL) emission (area under the curve) was measured for 60 min in HEK 293 cells after tyramine (10 μM) (T) addition as described under Materials and Methods. In parallel experiments, HEK 293-MAO B cells were preincubated with 1 μM of pargyline (P) for 30 min prior to tyramine addition. Data are means ± SEM of three separate experiments. ***$p < 0.001$.

The activity of MAO B in wild type and transfected HEK cells was assessed by the measure of H_2O_2 production using a continuous luminol-amplified CL assay. We have previously shown that, using tyramine as a common substrate for MAO-A and MAO-B, this technique allows the measure of the relative expression of each MAO isoform in intact cells (23). As shown in Fig. 1, tyramine (10 μM) did not induce H_2O_2 production in wild type HEK 293 cells suggesting that this cell line does contain MAO activity. This conclusion was also supported by enzyme assay and Western blot analysis (data not shown). In contrast, tyramine induced an immediate H_2O_2 production in HEK 293-MAO B cells. This effect was completely prevented by cell pretreatment with pargyline indicating the specific involvement of MAO in H_2O_2 generation. These results show that HEK 293 wild type and HEK 293-MAO B cells represent an appropriate model to investigate the cell events following H_2O_2 production by MAO.

As mentioned above, there is now a growing body of evidence suggesting that low levels of ROS act as intra- and intercellular "messengers" able to promoting growth responses in a variety of mammalian cell types (11–13). As showed in Fig. 2, cell incubation with tyramine during 24 h resulted in a dose dependent increase in cell proliferation, investigated by [^{3}H]thymidine incorporation (Fig. 2A) and cell counting (Fig. 2B), in HEK 293 MAO B cells but not in wild type HEK 293 cells, with the greatest response observed at 10–20 μM tyramine. The effects of 10 μM tyramine on cell proliferation were abolished in the presence of the MAO inhibitor pargyline or the antioxidant NAC (Figs. 3A and 3B), indicating that cell proliferation was dependent on MAO activity and H_2O_2 generation. In addition, tyramine effects were fully prevented by PD 98059, the inhibitor of the ERK activating kinase MEK, suggesting the involvement of MAPK pathway in the proliferative effects of the MAO substrate.

The involvement of MAPK/ERKs pathway in mitogenesis of HEK 293-MAO B cells was further supported by the study of the Thr/Tyr phosphorylation status of MAPK/ERKs—which corresponds to the activated status of these enzymes. Indeed, using the phosphorylated-Thr183/Tyr185-specific p44/42 MAPK antibody, we showed that 10 μM tyramine induced a time dependent increase in both p42 and p44 MAPK activation in HEK 293-MAO-B (Fig. 4A). This effect was not observed in wild type HEK 293 cells. Cell pretreatment with pargyline or NAC completely suppressed the amine-stimulated ERKs activation observed 5 min after tyramine addition in HEK 293-MAO B (Fig. 4B).

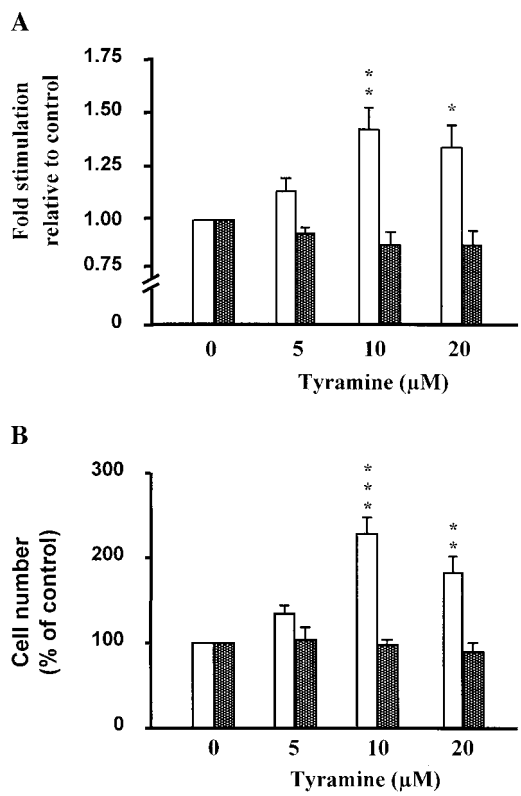

FIG. 2. Dose-dependent effect of tyramine on HEK 293 cell proliferation. Subconfluent quiescent HEK 293 wild type (293-WT) (□) and transfected cells (293-MAO B) (▩) were incubated for 24 h with different concentrations of tyramine. [^{3}H]Thymidine incorporation assay (A) and cell number counting (B) were assessed as described under Materials and Methods. Values are means ± SEM of three separate experiments made in triplicate. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

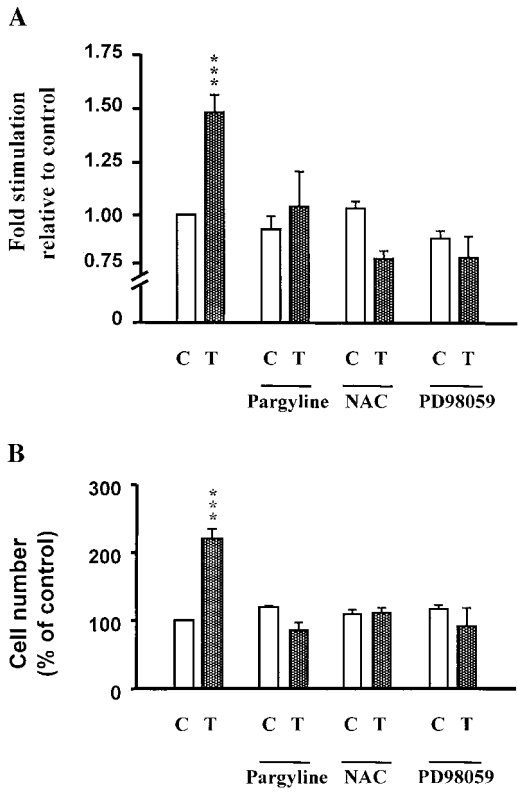

FIG. 3. Effect of tyramine on HEK 293-MAO B cell proliferation in the presence or in the absence of metabolic inhibitors or antioxidant. Subconfluent quiescent HEK 293 transfected cells (293-MAO B) were preincubated for 30 min with 1 μM pargyline, 1 mM NAC or 20 μM PD 98059 prior to serum free medium (C) or 10 μM tyramine (T) addition for 24 h. [^{3}H]Thymidine incorporation assay (A) and cell number counting (B) were assessed as described under Materials and Methods. Values are means ± SEM of three separate experiments made in triplicate. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

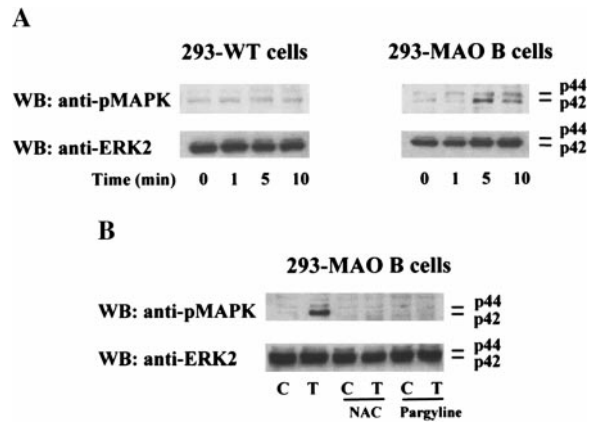

FIG. 4. Effect of tyramine on ERK activation in HEK 293 cells. 293-WT (left panels) and 293-MAO B cells (right panels) were incubated with 10 μM tyramine for the indicated times. (A) Cell lysates were immunoblotted with the anti-phospho-specific p42/44 MAPK (anti-pMAPK) or anti-ERK2 antibodies. (B) In parallel experiments, HEK 293-MAO B cells were preincubated with or without 1 mM NAC or 1 μM pargyline for 30 min prior to addition of medium culture (C) or 10 μM tyramine (T) for 5 min as described under Materials and Methods. All the blots are representative of three experiments.

The H_2O_2 production by MAOs also induced the phosphorylation of the adapter protein Shc, which are described as upstream target for activation of the ERK pathway (18). Indeed, by immunoprecipitation of cell lysate with anti-Shc antiboby followed by immunoblotting of the SDS-PAGE-resolved polypeptides with anti-phosphotyrosine-HRP conjugate, we showed that tyramine (10 μM) caused a time dependent increase in tyrosine phosphorylation of p52 Shc in HEK 293-MAO B cells, reaching the maximum at 2 min after addition to cell medium (Fig. 5). As observed for ERK activation, tyramine did not modify Shc phosphorylation status of wild type HEK 293 cells. Taken together, these data show that MAO-dependent H_2O_2 production induces phosphorylation and activation of Shc-ERK pathway only in HEK 293 cells stably expressing MAO-B.

In conclusion, the results demonstrate that MAO-B can activate Shc/ERK pathway and induce cell mitogenesis through H_2O_2 generation. This effect of MAO-B may represent a novel mechanism of action to explain the cell consequences induced by this family of enzymes. Indeed, it is conceivable that in addition to the control of substrate availability, MAO may induce cell effects through H_2O_2 generation. Because the mitogenic response play a key role in normal cell growth, in repair processes and also in proliferative diseases, these results open news perspectives for the comprehension of the cell events triggered by MAO-dependent H_2O_2 generation.

ACKNOWLEDGMENTS

This work was supported by the Institut National de la Santé et de la Recherche médicale and the Région Midi-Pyrénées (France). We thank Rita Raddatz and Sandra Savic for their contribution to the preparation of HEK 293 MAO-B cell line. We also thank Mrs. Christine Arragon for her excellent secretarial work.

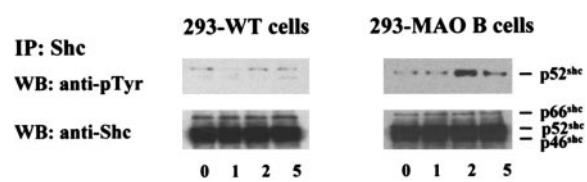

FIG. 5. Time course of tyramine-induced Shc tyrosine phosphorylation in HEK 293 cells. 293-WT (left panels) and 293-MAO B cells (right panels) were incubated with 10 μM tyramine for the indicated times. Cell lysates were immunoprecipitated with anti-Shc antibody (IP: Shc), and immunoblotted with anti-phosphotyrosine-HRP conjugated antibody (anti-pTyr) or anti-Shc antibody, as described under Materials and Methods. All the blots are representative of three experiments.

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