J Neural Transm (2007) 114: 807–810
DOI 10.1007/s00702-007-0692-x
Printed in the Netherlands

Journal of Neural Transmission

A link between monoamine oxidase-A and apoptosis in serum deprived human SH-SY5Y neuroblastoma cells

J. C. Fitzgerald, C. Ufer, E. E. Billett

School of Biomedical and Natural Sciences, Nottingham Trent University, Nottingham, U.K.

Received: December 18, 2006 / Accepted: December 21, 2006 / Published online: March 29, 2007

Summary Increased monoamine oxidase (MAO) activity was recently shown to accompany apoptotic cell death of various neuronal cells following growth factor deprivation. Here we show that in serum deprived SH-SY5Y cells, MAO-A mRNA levels and catalytic activities are increased, linked with activation of the apoptotic executioner caspase-3. Importantly, specific inhibition of MAO-A activity resulted in loss of apoptotic cell morphology. Our study indicates that MAO catalytic activity is involved in apoptotic signalling in response to serum withdrawal in neuronal cells.

Keywords: Serum withdrawal, apoptosis, caspase-3, monoamine oxidase inhibitor, SH-SY5Y

Introduction

Monoamine oxidases (MAO; EC 1.4.3.4) are flavoenzymes found tightly associated with the outer mitochondrial membrane and the two known isoforms (MAO-A and -B) are coded for by two separate genes on the X chromosome (Bach et al., 1988). Monoamine oxidases metabolise amines to their corresponding aldehydes, with hydrogen peroxide and ammonia as by-products. Apart from the ability of MAO to deaminate amines, the enzymatic generation of reactive oxygen species (ROS) has been implicated in mitochondrial damage (Hauptmann, 1996; Cohen et al., 1997; Gu et al., 1998; Berman and Hastings, 1999; Abou-Sleiman, 2006) and apoptotic cell death (DeZutter and Davis, 2001; Yi et al., 2006a). In 2001, Macleod et al. reported that differentiated SH-SY5Y cells undergo apoptotic cell death in response to serum withdrawal. In the same year DeZutter and Davis reported that apoptosis induced by nerve factor deprivation in rat pheochromocytoma (PC12) cells resulted in increased MAO-A mRNA expression and catalytic activity and that the MAO-A gene was a target of pro-apoptotic signalling (DeZutter and Davis, 2001). Work by Ou et al. (2006) also indicated increased MAO activity following serum withdrawal in SH-SY5Y cells linked to a concomitant reduction of the transcription repressor RI (RAM2/CDCA7 L/JPO2) and an increase in caspase-3 protein levels. In the present paper we aimed to quantitatively analyse MAO-A mRNA expression and MAO catalytic activity in serum deprived SH-SY5Y cells and to relate these changes to apoptosis by the direct measurement of caspase-3 activation. Secondly, we have examined the role of MAO activity in the process by a study of the effect of two types of MAO-A inhibitors (clorgyline and tranylcypromine) on the apoptotic morphology of SH-SY5Y cells following serum withdrawal.

Correspondence: E. Ellen Billett, Interdisciplinary Biomedical Research Centre, School of Biomedical and Natural Sciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, U.K.
e-mail: ellen.billett@ntu.ac.uk

Methods

Materials

All materials were purchased from Sigma-Aldrich Chemical Company (Poole, U.K) unless otherwise stated.

Cell culture, treatments and extraction

SH-SY5Y cells were grown (5% CO_2, 37°C) in Dulbecco's Modified Eagles Medium (DMEM) F12 HAMS containing 2 mM L-glutamine (Cambrex Bioscience, Berkshire, U.K), 1% non-essential amino acid solution, 100 units ml^{-1} penicillin and 50 $\mu g\,ml^{-1}$ streptomycin and 10% foetal bovine serum (Cambrex Biosciences, Berkshire, U.K). For serum withdrawal experiments, cells were grown to ~80% confluence. Growth medium was removed; the monolayer washed with serum free medium (SFM) and the

cells incubated in fresh SFM. For the inhibitor study, cells were incubated in fresh SFM + vehicle, SFM + clorgyline (1 μM final concentration) or SFM + tranylcypromine (1 μM final concentration) and incubated for 24 h. Cells were extracted via trypsinisation (including floating cells) and the cell pellet stored at −80°C.

Fluorogenic caspase-3 activation assay

Caspase activity was monitored using a fluorogenic assay (CASP-3-F from Sigma-Aldrich) with Acetyl-Asp-Glu-Val-Asp-7-amido-methyl coumarin (Ac-DEVD-AMC) as substrate. Cell pellets were resuspended in lysis buffer (50 mM HEPES, 5 mM CHAPS, 5 mM DTT, pH 7.4) and the assay was prepared in triplicate according to the vendors instructions. Fluorescence was measured (excitation 450 nm, emission 360 nm) every 5 min for 2 h at 37°C. Data were normalised for protein content (Lowry et al., 1951), and expressed as Δ fluorescence units/min/μg total protein.

MAO activity assay

MAO activity was monitored using a radiometric assay with ^{14}C-labelled tyramine hydrochloride as substrate, based on the method of Russell and Mayer (1983). Triplicate samples of cell pellets, resuspended in 200 μl potassium phosphate buffer (20 mM K_2HPO_4, 20 mM KH_2PO_4, pH 7.4), were transferred to scintillation vials and then incubated at 37°C for 5 min. Sample blanks were prepared in parallel containing, in addition, 200 μl 0.5 M HCl. To each sample 20 μl 1 mM ^{14}C-labelled tyramine (1 mCi/mmol) were added and incubation was then done for 1 h at 37°C. The reaction was stopped by addition of 200 μl 0.5 M HCl. Finally, 3 ml scintillant (1:1 ethyl acetate:toluene, 1% [w/v] PPO) were added to each vial. MAO activity was measured in a liquid scintillation counter (Canbera-Packard, Schwadorf, Germany). Data were normalised for protein content, and results expressed as pmoles/min/mg total protein.

Quantitative RT-PCR

Total RNA, isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), was reversely transcribed into the corresponding cDNA using oligo $d(T)_{18}$ and Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to the vendors instructions. Quantitative PCR was carried out with a Bio-Rad iCycler system, using the iQ SYBR Green Supermix kit from Bio-Rad (Bio-Rad, California, U.S.A.). The following primers were used: MAO A, 5′-GCC CTG TGG TTC TTG TGG TAT GT-3′, 5′-TGC TCC TCA CAC CAG TTC TTC TC-3′ and GAPDH, 5′-CCA TCA CCA TCT TCC AGG AGC GA-3′, 5′-GGA TGA CCT TGC CCA CAG CCT TG-3′ and the following PCR protocol applied: 3 min initial denaturation and activation of the iTaq DNA polymerase at 95°C, followed by 45 cycles of denaturation (20 sec at 95°C), annealing (30 sec at 65°C) and elongation (30 sec at 72°C). For exact quantification, standard curves were generated for each target gene used as external standards. Specific amplicons of each target gene were cloned into the vector pCR2.1 (Invitrogen, Karlsruhe, Germany), sequenced and serial dilutions ($1 \times 10^6 - 1 \times 10^2$ single stranded DNA molecules) used as template applying the above quantitative PCR approach. GAPDH mRNA was used as internal standard to normalize MAO-A mRNA.

Results

The apoptotic executioner caspase-3 was significantly activated 24–72 h post serum withdrawal (~2 times the control level, $p < 0.05$, Fig. 1A). Additionally, the serum deprived cells showed distinct apoptotic morphologies (characterised by their shrunken, rounded appearance, and blebbed surfaces, Fig. 2B) in contrast to the morphological phenotype of SH-SY5Y cells grown under normal condi-

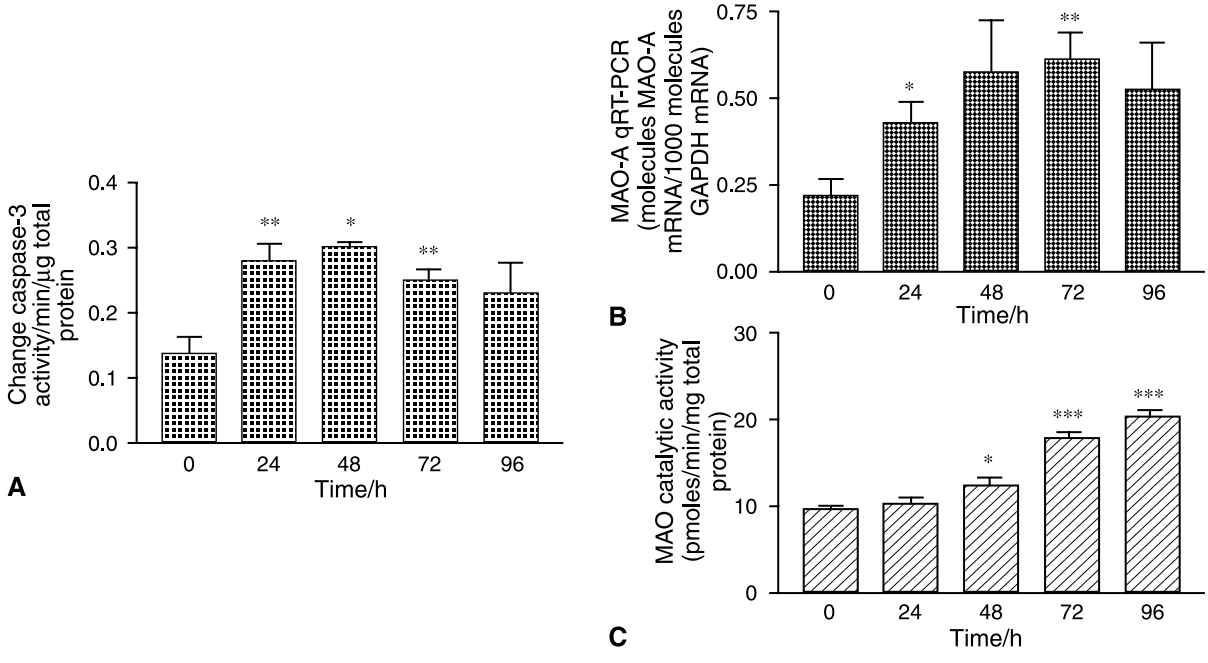

Fig. 1. The effect of serum withdrawal on (**A**) caspase-3 activity, (**B**) MAO-A mRNA expression and (**C**) MAO catalytic activity. Following serum withdrawal over a 96 h period caspase-3 activity, MAO-A mRNA expression levels and MAO activity were measured as outlined in Material and methods. Data represent values from three independent experiments ($n = 3$) and are expressed as mean ± S.D. Statistical analysis of treated cells in comparison to untreated controls was carried out using Student's *t*-test, where $^{*}p = 0.05$, $^{**}p = 0.01$, $^{***}p = 0.001$

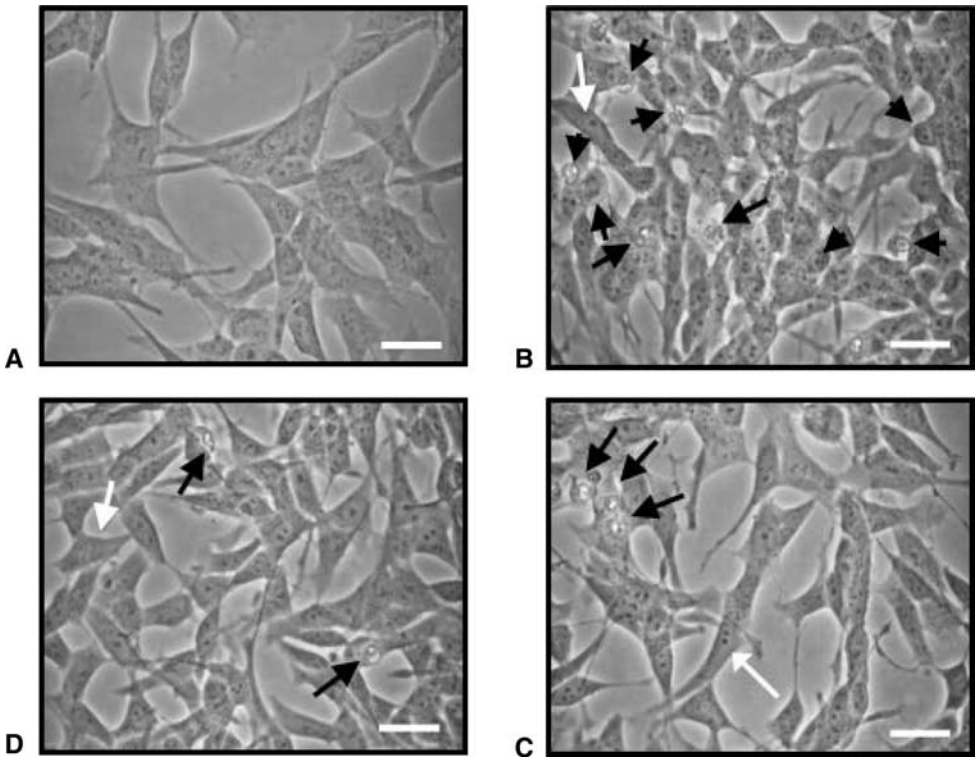

Fig. 2. The effect of MAO inhibitors tranylcypromine (TCP) and clorgyline on the apoptotic morphology of SH-SY5Y cells following serum withdrawal. Cells exposed to serum withdrawal (**B**) were treated with 1 μM TCP (**C**) or 1 μM clorgyline (**D**). Cells were visualised using a Nikon eclipse TS100 microscope. Photomicrographs (×40 objectives, taken on Nikon DN100 digital camera) are shown at 48 h post-treatment and are representative of 3 independent experiments. Scale bars represent 30 μm. Black arrows indicate typical morphology of apoptotic cells (shrunken, rounded, shiny, condensed and surface blebs), whilst white arrows show examples of normal SH-SY5Y cell morphology (elongated and flattened with axon like outgrowths)

tions (Fig. 2A). MAO-A mRNA levels also significantly increased following serum withdrawal, peaking after 72 h (around 2 times the control, $p < 0.01$, Fig. 1B). Increases in MAO-A mRNA levels are followed by increased MAO catalytic activity (Fig. 1C), in a time dependent manner, which reach a maximum after 96 h of serum withdrawal (around 2 times control level, $p < 0.001$). In order to establish the role of MAO-A activity in the process, the effects of two MAO inhibitors with different modes of action were studied. Both tranylcypromine (Fig. 2C) and clorgyline (Fig. 2D) were found to strongly reduce the number of cells exhibiting apoptotic morphologies.

Discussion

The present study demonstrates that serum withdrawal induced a two-fold increase in caspase-3 activity in SH-SY5Y cells, linked with distinctive apoptotic morphologies in the majority of cells, in agreement with Macleod et al. (2001). We also report that the activity of MAO-A, which is the predominant isoform in this cell line (Song and Ehrich, 1998; Fitzgerald et al., unpublished) was also increased two-fold, preceded by a similar increase in MAO-A mRNA expression. These data compliment the observations of Ou et al. who reported that, following serum withdrawal, increases in MAO-A expression are a result of a reduction in the levels of the repressor factor R1 (Ou et al., 2006). However, in the latter work emphasis was on cell death, measured via an MTT assay (Cookson et al., 1995), and on total caspase-3 levels, measured using Western blotting. In contrast, we have measured caspase-3 activity using a sensitive fluorescent assay. In our study we demonstrate the involvement of increased MAO activity in the apoptotic process by using two independent MAO-inhibitors, clorgyline and tranylcypromine. Clorgyline is a MAO-A specific inhibitor at a μM concentration but, due to its structural relation to propargyl analogues, may have anti-apoptotic properties in its own right, independent of MAO inhibition (Yi et al., 2006b). Tranylcypromine on the other hand is not isoform-specific but does not contain a propargyl moiety. Since both inhibitors reduced the number of SH-SY5Y cells exhibiting apoptotic morphologies following serum withdrawal, MAO-A activity is implicated in apoptotic cell signalling in these human neuroblastoma cells.

Acknowledgements

This work was supported by grants from The British Council, Deutscher Akademischer Austauschdienst (DAAD) and the Leverhulme Trust.

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