Activation of nitric oxide synthase is involved in tamoxifen-induced apoptosis of human erythroleukemia K562 cells

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Abstract Tamoxifen induces apoptosis (programmed cell death) in human erythroleukemia K562 cells. Nitric oxide synthase activity and expression increased in apoptotic cells by 315% and 280%, respectively, compared to controls. The specific inhibitor of nitric oxide synthase, L-NAME, protected K562 cells from tamoxifen-induced apoptosis, whereas the nitric oxide donor, sodium nitroprusside (SNP), potentiated the apoptotic effect of the drug. In addition, 5-lipoxygenase was activated by tamoxifen and the specific enzyme inhibitor, MK886, protected K562 cells against the drug. Conversely, the 5-lipoxygenase product, 5-hydroperoxyeicosatetraenoic acid, enhanced the tamoxifen-induced apoptosis. Finally, tamoxifen altered also membrane properties of K562 cells.

Key words: Apoptosis; Nitric oxide synthase; 5-Lipoxygenase; Biomembrane; Cholesterol

1. Introduction

Tamoxifen (Tmx) reduces the proliferation of several neoplasms [1] and is active against tumors such as melanoma [2], hepatoma [3] and uterine leiomyoma [4]. In particular, Tmx is commonly used as chemotherapeutic agent in human breast cancer and is also currently being used in trials on estrogen-negative breast cancer patients [5]. In a previous study, we showed that Tmx induces significant apoptosis (programmed cell death, PCD) in human breast MCF-7G cells [6]. Similar results have also been reported for breast cancer cells [7,8], macrophages [9] and brain tumor cells [10]. Tmx has also been reported to exert its action on blood cells of breast cancer patients: it forms DNA adducts [11], modulates the multidrug resistance pump, P-glycoprotein [12], and affects calcium metabolism [10]. This evidence suggests that the drug has a complex mechanism of action [13]. Nitric oxide [14,15] and membrane-derived lipid messengers [14,16–21] have recently become the focus of many research efforts, as they appear to play an important role in the regulation of apoptosis. In this study, human erythroleukemia K562 cells were found to be highly sensitive to Tmx-induced PCD, and were used to elucidate biochemical events of the apoptotic programme induced by the drug.

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Abbreviations: Tmx, tamoxifen; PCD, programmed cell death; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; SNP, sodium nitroprusside (sodium nitroferricyanide); RP-HPLC, reverse-phase high-performance liquid chromatography

2. Materials and methods

2.1. Materials

Chemicals were of the purest analytical grade. Arachidonic (eicosatetraenoic) acid, 5-hydroperoxyeicosatetraenoic acid (5-HPETE), [Z]-1-[*p*-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene (tamoxifen), ATP, *N*ω-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (sodium nitroferricyanide, SNP), ribonuclease (RNase) and propidium iodide were purchased from Sigma. L-[2,3,4,5-^{3}H]Arginine (specific activity = 64 Ci/mmol = 2.37 TBq/mmol) was from Amersham. Leukotriene B_4 (LTB_4) was from Cayman. Trypsin, L-glutamine and non-essential amino acids were purchased from Flow Laboratories. The culture media Ham's F-12 and Minimal Essential Medium (MEM) were from Gibco; fetal calf serum was from Hy Clone; cholesterol oxidase was provided by Boehringer Mannheim. Lympholyte-H was from Cedarlane Laboratories. Annexin V-FITC (fluorescein isothiocyanate) conjugate was purchased from Brand Applications. 5-Lipoxygenase specific inhibitor 3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*tert*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid (MK886) and anti-human 5-lipoxygenase rabbit polyclonal antibodies were a kind gift from Dr. A.W. Ford-Hutchinson (Merck Frosst Centre for Therapeutic Research, Canada). Anti-inducible nitric oxide synthase monoclonal antibodies were purchased from Transduction Laboratories. Goat anti-rabbit and goat anti-mouse antibodies, both conjugated with alkaline phosphatase (GAR-AP or GAM-AP, respectively), were purchased from Bio-Rad.

2.2. Cell culture and treatment

Human erythroleukemia K562 cells were grown in a 1:1 mixture of MEM and Ham's F-12 media, supplemented with 12% heat-inactivated fetal calf serum, 1.2 mg/ml sodium bicarbonate, 1% non-essential amino acids, 2 mM L-glutamine and 15 mM HEPES buffer, pH 7.4. Mycoplasma-free cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. K562 cells were grown in suspension at a density of 0.5×10^6 cells/ml and were split 1:7, twice weekly. Drug exposure of K562 cells (100×10^6/test) was performed by adding 1 μM tamoxifen to the culture medium [6]. The effect of various concentrations of L-NAME, SNP, MK886 or 5-HPETE on tamoxifen-induced apoptosis was evaluated using 10×10^6 K562 cells/test. Cells were grown for 48 h at 37°C, in a humidified atmosphere with 5% CO_2, and were then washed by centrifugation at $800\times g$ for 5 min and counted in a Neubauer cytometer chamber. Cell viability was assessed by Trypan blue dye-exclusion.

Dying K562 cells were separated from the whole cell population by gradient centrifugation, using the density separation medium Lympholyte-H, according to the manufacturer's instructions (Cedarlane Laboratories). A suspension of 100×10^6 K562 cells in 8 ml culture medium was carefully layered over 4 ml Lympholyte-H in a 15-ml centrifuge tube. Since Lympholyte-H is more dense than the cell suspension, a distinct interface is formed. After centrifugation at $800\times g$ for 20 min, at room temperature, a well-defined layer of live cells (lower density) was evident at the interface between the two fluids at different densities, whereas dead cells (higher density) formed the pellet. Using a Pasteur pipette, dead cells were transferred to a new centrifuge tube and subjected to a new round of gradient centrifugation.

2.3. Evaluation of cell death

K562 cells (1×10^6) were collected by centrifugation at $800\times g$ for

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5 min and were fixed in a 1:1 solution of phosphate-buffered saline and methanol/acetone (4:1, by vol.). Cells were washed in phosphate-buffered saline and incubated with 13 kU RNase for 20 min, at 37°C, then with 40 μg/ml propidium iodide for 15 min, at 37°C. The number of apoptotic bodies was evaluated by flow cytometry using a propidium iodide staining [22] on a FACScan flow cytometer (Becton-Dickinson). Cells were excited at 488 nm using a 15-mW Argon laser, and fluorescence emitted at 570 nm was measured. Five thousand events were evaluated by the Lysis II Program (ibid.), using an electronic gating FSC-H/FSC-A/SSC to eliminate cell aggregates.

2.4. Determination of enzyme activity and expression

The activity of nitric oxide synthase (EC 1.14.13.39) was assayed by incubating cell extracts with the radiolabelled substrate [^{3}H]arginine, and then isolating and quantifying the reaction product [^{3}H]citrulline as described [23]. Nitric oxide synthase activity was expressed as pmol citrulline formed per min per mg protein. The activity of 5-lipoxygenase (EC 1.13.11.34) in K562 cells was assayed by incubating cell extracts for 10 min at 37°C in the presence of 1 mM ATP, 2 mM $CaCl_2$ and 40 μM arachidonic acid, as reported [24]. 5-Lipoxygenase activity was expressed as nmol 5-hydroperoxyeicosatetraenoic acid (5-HPETE) formed per min per mg protein. Protein concentration of cell extracts was determined according to Bradford [25]. Enzyme-linked immunosorbent assay (ELISA) of K562 cell extracts was performed as reported [20], using 25 μg proteins/well. Anti-inducible nitric oxide synthase monoclonal antibodies (diluted 1:400) or anti-5-lipoxygenase polyclonal antibodies (diluted 1:200) were used as first antibody. Goat anti-mouse or anti-rabbit immunoglobulins conjugated with alkaline phosphatase (GAR-AP or GAM-AP, respectively) were used as second antibody, diluted 1:2000. Color development of the alkaline phosphatase reaction was followed at 405 nm.

2.5. Lipid analysis

Membrane lipids were extracted from 10×10^6 K562 cells as described [18]. The amount of cholesterol in membranes was determined after hydrolysis of the lipid extracts in methanol/5 M KOH (4:1, by vol.) for 30 min at 60°C under argon, followed by chromatographic extraction on octadecyl-SPE columns (Baker). Cholesterol content was determined as described [20]. Exposure of phosphatidylserine on K562 cell membranes was determined by measuring fluorescence of FITC-labelled annexin V [26]. Arachidonate metabolite leukotriene B_4 (LTB_4) was extracted from K562 cells on octadecyl-SPE columns (Baker) and was analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) on a C18 3×3 CR column (SGE), as described [24]. RP-HPLC was performed on a Perkin Elmer 1022 LC Plus liquid chromatograph at a flow rate of 1.2 ml/min, using methanol/water/trifluoroacetic acid (70:30:0.07, by vol.) as mobile phase. Chromatograms were recorded at 270 nm, assessing peak identity by comparison with authentic standards. Quantitative determinations were performed by integrating peak areas of each compound.

2.6. Data analysis

Data reported in this paper are the mean (±S.D.) of at least three independent determinations, each performed in duplicate. Statistical analysis was performed by the Student's *t*-test and experimental data were elaborated using the InStat program (GraphPad Software).

3. Results and discussion

Although the ability of tamoxifen (Tmx) to induce apoptosis in human breast cancer cells has been reported by our group [6] and others [7,8], the biochemical events responsible for the activity of the drug still await clarification. Knowledge of at least some of these events might be exploited to improve the clinical efficacy of the drug in the chemotherapy of cancer. Treatment of human erythroleukemia K562 cells with 1 μM Tmx induced an 8-fold increase in the number of apoptotic cells after 48 h of exposure (Fig. 1), from 2.5% to 20%. K562 cells were more sensitive to Tmx than MCF-7G cells, where a 2-fold increase in PCD was observed under the same experimental conditions [6]. In order to better characterize the biochemical events leading to Tmx-induced PCD, apoptotic K562 cells were enriched by gradient centrifugation on Lympholyte-H medium. After three rounds, a fraction containing 80% apoptotic cells was separated, whereas a fourth round of centrifugation did not further enhance the percentage of apoptotic cells (data not shown). Therefore, K562 cells subjected to three rounds of centrifugation were used to investigate the biochemical background of Tmx-induced PCD. Tmx induced a remarkable increase in the activity and expression (at the protein level) of nitric oxide synthase in K562 cells (Table 1), suggesting that the free radical nitric oxide might play a role in the execution of PCD induced by the drug. This concept was confirmed using the specific inhibitor of nitric oxide synthase, L-NAME [27], and the nitric oxide donor, SNP. Fig. 1A shows the results obtained upon exposure of K562 cells to concentrations of L-NAME (400 μM) and SNP

Table 1
Effect of tamoxifen (Tmx) on the activity and expression of nitric oxide synthase and 5-lipoxygenase in K562 cellsz

Sample	Nitric oxide synthase		5-Lipoxygenase	
	Activity (%)	Protein level (%)	Activity (%)	Protein level (%)
K562 control	100[a]	100[b]	100[c]	100[d]
K562+Tmx	315±30*	280±30*	150±15**	160±15**

[a]100% = 25.0±2.2 pmol citrulline/min/mg protein.
[b]100% = 0.18±0.02 absorbance units at 405 nm.
[c]100% = 0.42±0.04 nmol 5-HPETE/min/mg protein.
[d]100% = 0.25±0.03 absorbance units at 405 nm.
*Denotes $P<0.01$ compared to control. **Denotes $P<0.05$ compared to control. Values are expressed as percentage of the untreated control,

Table 2
Effect of tamoxifen (Tmx) on leukotriene B_4, phosphatidylserine exposure and membrane cholesterol content in K562 cells

Sample	Leukotriene B_4 (%)	Phosphatidylserine exposure (%)	Cholesterol (%)
K562 control	100[a]	100[b]	100[c]
K562+Tmx	202±20*	275±25**	171±15*

[a]100% = 2.5±0.2 pg/10^{-6} cells.
[b]100% = 5±0.5×10^4 annexin V-positive cells every 10^6 cells.
[c]100% = 3.2±0.3 nmol/10^{-6} cells.
*Denotes $P<0.05$ compared to control. **Denotes $P<0.01$ compared to control. Values are expressed as percentage of the untreated control, arbitrarily set to 100.

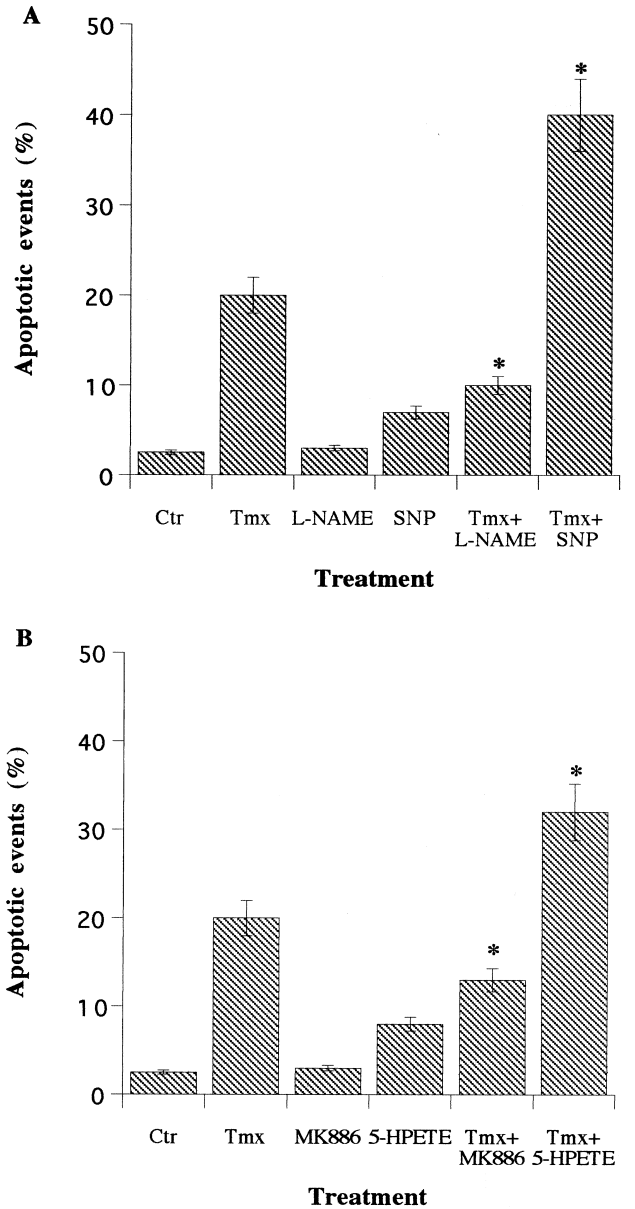

Fig. 1. A: Effects of the nitric oxide synthase inhibitor, L-NAME, and the nitric oxide donor, SNP, on Tmx-induced apoptosis. B: Effects of the 5-lipoxygenase inhibitor, MK886, and the 5-lipoxygenase product, 5-HPETE, on Tmx-induced apoptosis. In both panels, programmed cell death was expressed as percentage of apoptotic events counted by FACS analysis. L-NAME, SNP, MK886 and 5-HPETE were used at 400 μM, 2 mM, 100 μM and 5 μM final concentrations, respectively. Asterisks denote $P < 0.05$ compared to Tmx-treated K562 cells.

(2 mM) found to be optimal in pilot experiments (data not shown). L-NAME efficiently protected K562 cells against Tmx-induced apoptosis, whereas SNP doubled the effect of the drug (Fig. 1A). Interestingly, nitric oxide was effective in the physiological micromolar range, since millimolar concentrations of SNP release micromolar concentrations of free nitric oxide [15].

Nitric oxide is known to modulate the arachidonate cascade catalyzed by lipoxygenase [28] and is a key molecule in the execution of PCD in different experimental models [15]. Treatment of K562 cells with Tmx also up-regulated 5-lipoxygenase (EC 1.13.11.34) activity and expression, by 150% and 160%, respectively, compared to controls (Table 1). 5-Lipoxygenase was shown to be linked to Tmx-induced PCD by a cause-effect relationship using a specific inhibitor or product of the enzyme. Fig. 1B shows that 100 μM MK886, a 5-lipoxygenase-specific inhibitor [29], protected K562 cells against Tmx-induced apoptosis, whereas the 5-lipoxygenase product, 5-HPETE (used at 5 μM final concentration), significantly enhanced the effect of the drug (Fig. 1B). In addition, the increase in 5-lipoxygenase activity and expression was paralleled by increased leukotriene B_4 (LTB_4), a specific product of 5-lipoxygenase (Table 2). LTB_4 plays a role in lipid-mediated signal transduction [30] and is involved in PCD [19]. These results extend previous observations on the involvement of the 5-lipoxygenase pathway in PCD induced by oxidative stress in human cells [19], and suggest that reactive oxygen species generated by 5-lipoxygenase (mainly lipid peroxides) might take part in the apoptotic programme induced by Tmx.

Table 2 shows that exposure of K562 cells to Tmx altered membrane lipids, increasing both phosphatidylserine exposure (up to 275%) and cholesterol content (up to 170% of the control). The observation that Tmx enhanced membrane cholesterol content confirms previous findings on K562 cells forced into apoptosis by transforming growth factor β1, alone or in combination with cisplatin [18]. It further supports the concept that membrane cholesterol is an important element in the execution of PCD, as recently found in the same cell line [20]. The increased cholesterol content in the lipid bilayer leads to increased membrane rigidity [20], and this feature, together with the exposure of specific signals, such as phosphatidylserine, on the membrane surface (Table 2), might improve recognition of apoptotic cells and removal by macrophages [31].

Altogether, the results reported herein show that tamoxifen, a widely used chemotherapeutic agent, induces apoptosis in human erythroleukemia K562 cells by activating nitric oxide synthase. Furthermore, the enhancement of the activity and expression of the membrane-related enzyme, 5-lipoxygenase, plays a role in the execution of the death programme, which is indeed characterized by altered membrane properties. These findings might be of clinical relevance and help to improve the chemotherapeutic use of tamoxifen.

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