Korean J Physiol Pharmacol
Vol 6: 187–191, August, 2002

Role of Calmodulin in the Generation of Reactive Oxygen Species and Apoptosis Induced by Tamoxifen in HepG2 Human Hepatoma Cells

Yong Soo Lee

College of Pharmacy, Duksung Women's University, Seoul 132–714, Korea

Tamoxifen, an antiestrogen, has previously been shown to induce apoptosis in HepG2 human hepatoblastoma cells through activation of the pathways independent of estrogen receptors, i.e., intracellular Ca^{2+} increase and generation of reactive oxygen species (ROS). However, the mechanism of tamoxifen to link increased intracellular Ca^{2+} to ROS generation is currently unknown. Thus, in this study we investigated the possible involvement of calmodulin, a Ca^{2+} activated protein, and Ca^{2+}/calmodulin-dependent protein kinase II in the above tamoxifen-induced events. Treatment with calmodulin antagonists (calmidazolium and trifluoroperazine) or specific inhibitors of Ca^{2+}/calmodulin-dependent protein kinase II (KN-93 and KN-62) inhibited the tamoxifen-induced apoptosis in a dose-dependent manner. In addition, these agents blocked the tamoxifen-induced ROS generation in a concentration-dependent fashion, which was completely suppressed by intracellular Ca^{2+} chelation. These results demonstrate for the first time that, despite of its well-known direct calmodulin-inhibitory activity, tamoxifen may generate ROS and induce apoptosis through indirect activation of calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II in HepG2 cells.

Key Words: Calmodulin, Ca^{2+}/calmodulin-dependent protein kinase II, Tamoxifen, Apoptosis, Reactive oxygen species, HepG2 cell

INTRODUCTION

Apoptosis is a highly organized cell death process characterized by early and prominent condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of proteases and endonucleases, enzymatic cleavage of the DNA into oligonucleosomal fragments, and segmentation of the cells into membrane- bound apoptotic bodies (Kidd, 1998). Apoptosis plays an important role in maintenance of tissue homeostasis by the selective elimination of excessive cells (Song & Steller, 1999). Particularly, genetic mutations resulting in disruption of apoptosis or derangement of apoptosis-signaling pathways are likely to be critical components of carcinogenesis (Wang, 1999; Lowe & Lin, 2000). On the other hand, induction of cancer cell apoptosis appears to be useful for cancer treatment (Kornblau, 1998) including chemotherapy (Kamesaki, 1998) and radiation therapy (Crompton, 1998). However, signaling pathways for the induction of apoptosis are not completely understood.

Tamoxifen has been used to treat breast cancer (Jordan, 1990) with potential application as a cancer-preventing agent (Nayfield et al, 1991). Tamoxifen has been shown to induce apoptotic cell death in many types of tumor (Ellis et al, 1997; Ferlini et al, 1999). Recently, we reported that tamoxifen induced apoptosis in HepG2 human hepatoblastoma cells (Kim et al, 1999; Lee et al, 2000), and demonstrated that the tamoxifen-induced apoptosis was due to a sustained increase in the level of intracellular Ca^{2+} (Kim et al, 1999). The source of Ca^{2+} mediating the apoptotic events following tamoxifen treatment was Ca^{2+} influx through the activation of non-selective cation channels (Kim et al, 1999). Generation of reactive oxygen species (ROS) through the activation of membrane NADPH oxidase has also been demonstrated to mediate the tamoxifen-induced apoptosis in HepG2 cells (Lee et al, 2000). However, the mechanism of tamoxifen action linking intracellular Ca^{2+} increase to ROS generation is currently unknown.

Thus, the main purpose of the present study was to determine the downstream mechanism of intracellular Ca^{2+} increase leading to ROS generation during tamoxifen-induced apoptosis in HepG2 cells. To this end, we specifically focused on the roles of calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II which are known to be activated by increased intracellular Ca^{2+} (Fujisawa, 2001), in the mechanism of actions of tamoxifen.

Corresponding to: Yong Soo Lee, College of Pharmacy, Duksung Women's University, Seoul 132-714, Korea. (Tel) 82-2-901-8396, (Fax) 82-2-901-8386, (E-mail) yongslee@duksung.ac.kr

ABBREVIATIONS: CMZ, calmidazolium; DCFH-DA, 2', 7'-dichlorofluorescin diacetate; ROS, reactive oxygen species; TFP, trifluoroperazine.

METHODS

Materials

The powders for minimum essential medium (MEM), trypsin solution, sodium pyruvate, tamoxifen and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). 1-[bis(*p*-Chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl]-imidazolium chloride (calmidazolium; CMZ), trifluoroperazine dihydrochloride (TFP), 2-[2-[[[3-(4'-chlorophenyl)-2-propentyl]methylamino] methyl]phenyl]-N-(2-hydroxyethyl)-4'-methoxy-benzenesulf onamide phosphate (KN-93) and (S)-5-isoquinolinesulfonic acid, 4-[2-[(5-isoquinolinyl-sulfonyl)methylamino]3-oxo-3-(4-phenyl-1-piperazinyl)-propyl]phenyl ester (KN-62) were from RBI (Natick, MA). 2', 7'-Dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). The stock solutions of drugs were sterilized by filtration through 0.2-μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

The HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). HepG2 cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in an Eagle's MEM supplemented with 10% fetal bovine serum, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After confluence, the cells were subcultured following trypsinization.

Flow cytometric analysis of apoptosis

For flow cytometric analysis, HepG2 cells were collected

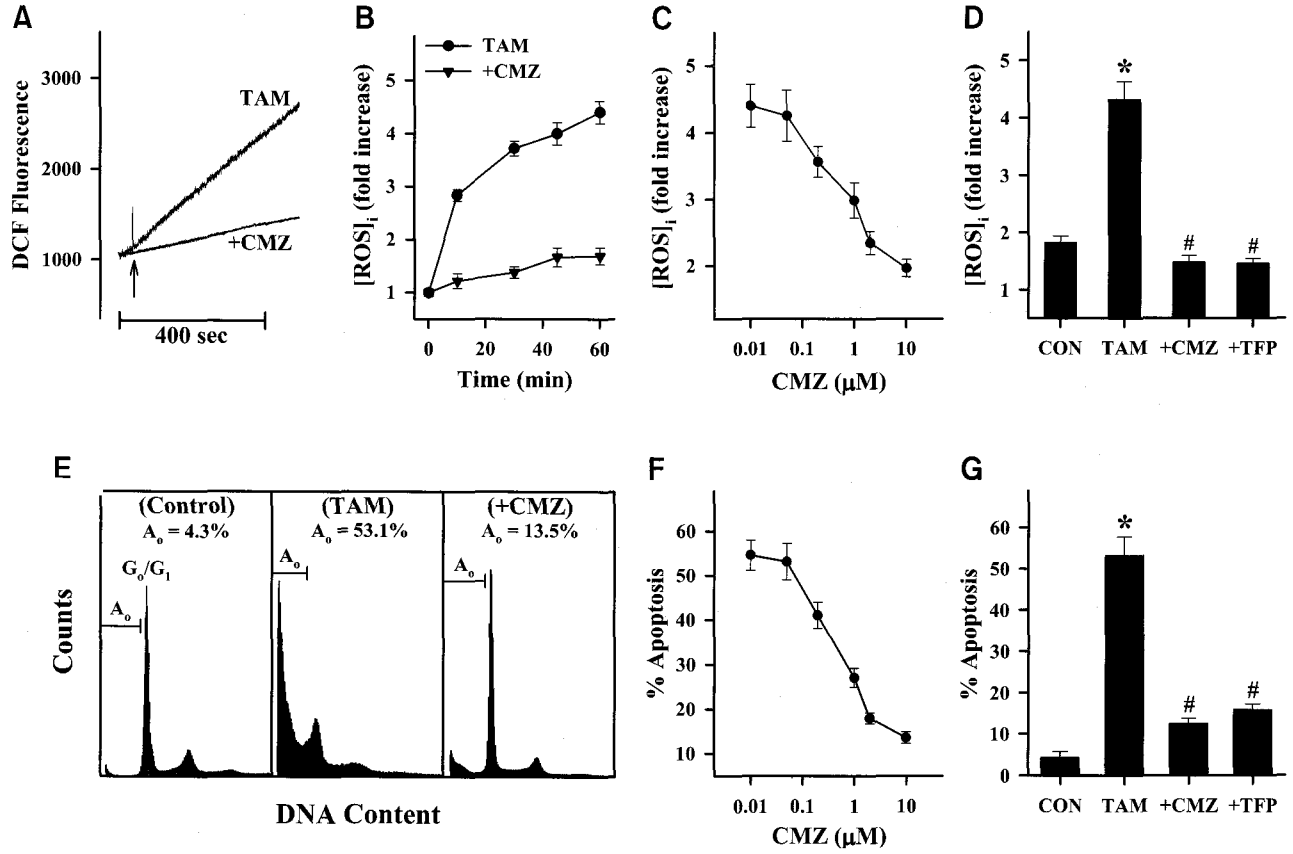

Fig. 1. Effects of calmodulin antagonists on the tamoxifen (TAM)-induced ROS generation (A, B, C and D) and apoptosis (E, F and G) in HepG2 human hepatoblastoma cells. The data (A) show changes in levels of ROS as a function of time, which was measured by DCF fluorescence method. The arrow shows the time point for addition of tamoxifen (30 μM). CMZ (10 μM) was added 10 min before tamoxifen treatment. In the data (B) results are expressed as fold increase compared to the initial DCF fluorescence intensity. The data (C) show the dose-response relationship for the CMZ effect. In the experiments of (D), CMZ (10 μM) and TFP (10 μM) were added 10 min before tamoxifen (30 μM) application. In the experiments of (E), the cells were incubated with or without tamoxifen (30 μM) for 48 h. CMZ (10 μM) was given 30 min before tamoxifen application. The number of apoptotic cells was measured by flow cytometry as described in the text. The region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation. The data (F) show the dose-response relationship for the inhibitory effect of CMZ. In the experiments of (G), CMZ (10 μM) and TFP (10 μM) were added 30 min before tamoxifen (30 μM) application. All data points represent the mean values of four replicates with bars indicating S.E.M. *$P<0.05$ compared to control condition in which the cells were incubated with tamoxifen-free medium. #$P<0.05$ compared to tamoxifen alone.

and washed twice with phosphate-buffered saline (pH 7.4). After fixation in 80% ethanol for 30 min, cells were washed twice and resuspended in phosphate-buffered saline (pH 7.4) containing 0.1% Triton X-100, 5 μg/ml propidium iodide and 50 μg/ml ribonuclease A for DNA staining. The cells were then analyzed with a FACScan (BIO-RAD, Hercules, CA, USA). At least 20,000 events were evaluated. All histograms were analyzed using WinBryte software (BIO-RAD, Hercules, CA, USA) to determine the percentage of nuclei with a hypodiploid content indicative of apoptosis (Bombeli et al, 1997).

Measurement of intracellular reactive oxygen species

Relative changes in intracellular ROS in HepG2 cells were monitored using a fluorescent probe, DCFH-DA (LaBel et al, 1992). DCFH-DA readily diffuses through the cell membrane and is hydrolyzed by intracellular esterases to nonfluorescent 2', 7'-dichlorofluorescin (DCFH), which is then rapidly oxidized to highly fluorescent 2', 7'-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of ROS intracellularly formed (Shen et al, 1996). Thus, cells were washed twice and resuspended at a concentration of 4×10^5 cells/ml in Hank's solution. For loading DCFH-DA into cells, cells were incubated with the dye at a final concentration of 5 μM for 2 h at 37°C. Fluorescence was monitored at 530 nm with excitation wavelength of 485 nm in a stirred quartz cuvette.

Data analysis

All experiments were performed four times. Data are expressed as means±standard error of the mean (S.E.M.) and were analyzed using a one-way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. P values less than 0.05 are considered statistically significant.

RESULTS

Role of calmodulin in the ROS generation and apoptosis induced by tamoxifen

The effects of calmodulin antagonists (CMZ and TFP) on the ROS generation and apoptosis induced by tamoxifen in HepG2 cells were examined by using DCF fluorescence and

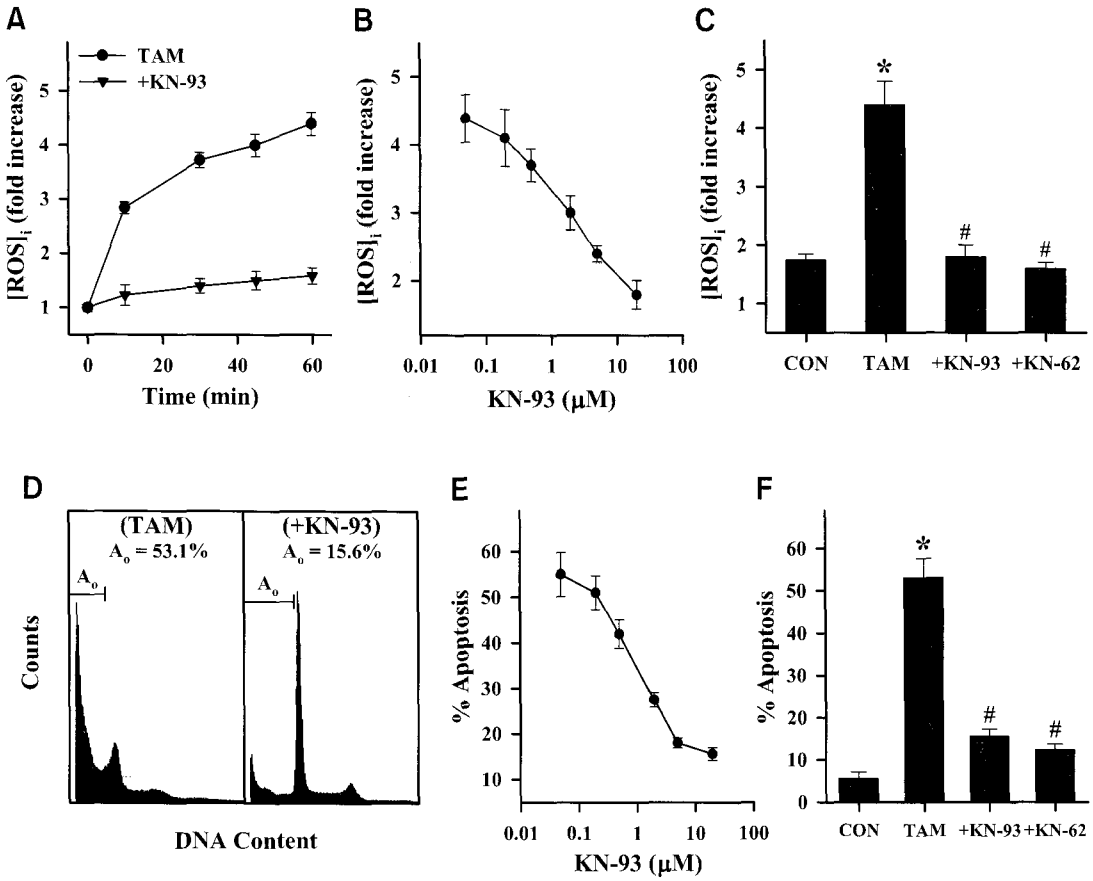

Fig. 2. Ca^{2+}/calmodulin-dependent protein kinase II mediates the tamoxifen-induced generation of ROS (A, B and C) and apoptosis (D, E and F) in HepG2 human hepatoblastoma cells. Experimental procedures and data presentations are the same as in Fig. 1. In these experiments, KN-93 (20 μM) and KN-62 (20 μM) were used. These drugs were given 10 min and 30 min before tamoxifen (30 μM) application in the experiments of (A, B and C) and (D, E and F), respectively. *$P<0.05$ compared to control. #$P<0.05$ compared to tamoxifen alone.

flow cytometric analysis with propidium iodide, respectively. Treatment with CMZ, a potent inhibitor of calmodulin, prevented the tamoxifen-induced generation of ROS (Figs. 1A~1D) and apoptosis (Figs. 1E~1G) in a dose-dependent manner. TFP, an antagonist of calmodulin structually different from CMZ, also significantly inhibited the tamoxifen-induced ROS generation and apoptosis, as depicted in Figs. 1D and 1G, respectively. Taken together, these results imply that activation of calmodulin in HepG2 cells may mediate the tamoxifen effects on the ROS generation and apoptosis.

Role of Ca^{2+}/calmodulin-dependent protein kinase II in the ROS generation and apoptosis induced by tamoxifen

To examine whether Ca^{2+}/calmodulin-dependent protein kinase II which is activated by calmodulin, is involved in these tamoxifen actions, the effects of KN-93, a specific antagonist of Ca^{2+}/calmodulin-dependent protein kinase II, on the generation of ROS and apoptosis were investigated. KN-93 suppressed the tamoxifen-induced generation of ROS (Figs. 2A~2C) and apoptosis (Figs. 2D~2F) in a concentration-dependent fashion. As illustrated in Figs. 2C and 2F, KN-62, another antagonist of Ca^{2+}/calmodulin-dependent protein kinase II, showed the similar effects. These results strongly suggest that the activation of calmodulin and Ca^{2+}/calmodulin-dependent protein kinase-II by increased intracellular Ca^{2+} may mediate tamoxifen-induced ROS generation and apoptosis in HepG2 cells.

DISCUSSION

Tamoxifen has been shown to inhibit the growth of breast cancer cells through its antiestrogenic properties (Jordan, 1994). However, tamoxifen also appears to be effective on many cancer cells in which estrogen receptors are not expressed (Kang et al, 1996; Ellis et al, 1997; Keen et al, 1997; Ferlini et al, 1999). The mechanism of anticancer actions of tamoxifen in estrogen receptor-negative tumor cells is suggested to be related to its non-genomic actions, for example, inhibition of calmodulin kinase (Rowlands et al, 1990) or protein kinase C (Cheng et al, 1998). However, the exact mechanism has not yet been clearly clarified.

Recently, we reported that tamoxifen induces apoptosis in estrogen receptor-negative HepG2 human hepatoma cells, which is mediated by increased intracellular Ca^{2+} (Kim et al, 1999) and generation of ROS (Lee et al, 2000). In HepG2 cells, ROS appeared to be a downstream signal of elevated intracellular Ca^{2+}, since their generation was temporally preceded by elevation of intracellular Ca^{2+} in a time frame, and completely inhibited by ethylene glycol-bis-(aminoethyl ether)N,N,N,N-tetraacetic acid (EGTA) and bis-(o-aminophenoxy)-ethane-N,N,N,N-tetraacetic acid/acetoxymethyl ester (BAPTA/AM), an extracellular and intracellular Ca^{2+} chelator, respectively (Lee et al, 2000). Although the biological process leading to oxygen-derived ROS generation is electron transport associated with mitochondrial membranes (Halliwell, 1989), they can also be produced by the microsomal enzyme, cytochrome P-450, and numerous catalytic cytosolic enzymes, including cyclooxygenase, nitric oxide synthase, xanthine oxidase and ribonucleotide reductase (Dugan et al, 1995). However, the tamoxifen-induced ROS generation was shown not to be due to activation of these enzymes, since it was not altered by treatment with specific inhibitors of these enzymes (Lee et al, 2000).

The membrane-bound NADPH oxidase is known to produce ROS during the respiratory burst in neutrophils (Babior, 1995). It has also been functionally active in non-phagocytic cells, including endothelial cells (Jones et al, 1996), vascular smooth muscle cells (Marshall et al, 1996), neuroepithelial bodies of the lung (Youngson et al, 1997) and type I cells of the carotid body (Kummer & Acker, 1995). The activation of this enzyme proceeds through a multistep assembly at the plasma membrane of several components including the two subunits of cytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$), the small GTP-binding proteins (Rac and Rap1A), and the cytosolic factors ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) (Babior, 1999). These components of the NADPH oxidase have been expressed in HepG2 cells (Ehleben et al, 1997; Cool et al, 1998), and the enzyme appears to be a major source of ROS produced by hypoxia in HepG2 cells (Ehleben et al, 1997). Recently, we have also reported that the NADPH oxidase acts as a major site of the tamoxifen-induced ROS production (Lee et al, 2000).

Although our earlier studies have suggested that intracellular Ca^{2+} mediates the tamoxifen-induced activation of the NADPH oxidase (Lee et al, 2000), the exact target molecule mediating the activation of the enzyme has not been clarified. Recently, Cool et al (1998) demonstrated that Rac1, a small GTP binding protein, is involved in the regulation of production of ROS by the non-phagocytic NADPH oxidase complex in HepG2 cells. In addition, Ca^{2+}/calmodulin-dependent protein kinase-II has been shown to phosphorylate and regulate Tiam1 (Fleming et al, 1999), a Rac1-specific exchange factor (Collard et al, 1996). Orie et al (1999) have also found that Ca^{2+}/calmodulin-dependent protein kinase-II is a common signaling component in the generation of ROS in human lymphocytes. Thus, in the present study, we investigated whether calmodulin and Ca^{2+}/calmodulin-dependent protein kinase-II are involved in the tamoxifen-induced ROS generation leading to apoptosis in HepG2 cells. The results showed that specific inhibitors of calmodulin and Ca^{2+}/calmodulin-dependent protein kinase-II significantly prevented both the ROS generation and apoptosis induced by tamoxifen, thus clearly demonstrating the active involvement of these signaling molecules in the tamoxifen actions.

Calmodulin and calmodulin kinase have been shown to facilitate Ca^{2+} current in cardiac myocytes (Wu et al, 2001), and the Ca^{2+}/calmodulin-dependent protein kinase inhibitor KN-62 inhibits Ca^{2+} channel activity in small cell lung carcinoma (Williams et al, 1995). Thus, the presently observed inhibitory effects of calmodulin and Ca^{2+}/calmodulin-dependent protein kinase-II inhibitors on the tamoxifen-induced ROS generation and apoptosis might have been due to blocking of the tamoxifen-induced intracellular Ca^{2+} influx. However, this possibility might not be the case, because the specific target of calmodulin was found to be the L-type Ca^{2+} channel in these studies (Williams et al, 1995; Wu et al, 2001), and tamoxifen induced Ca^{2+} influx through activation of non-selective cation channels in HepG2 cells (Kim et al, 1999). Furthermore, calmodulin and Ca^{2+}/calmodulin-dependent protein kinase-II inhibitors used in this study did not significantly alter the tamoxifen-induced increase in intracellular Ca^{2+} concentration (data not shown).

In conclusion, the activation of calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II may be down-

stream mechanism linking increased intracellular Ca^{2+} to ROS generation during the tamoxifen-induced apoptosis in HepG2 cells. These results show for the first time that tamoxifen can activate the calmodulin-regulated signaling pathway indirectly, even though tamoxifen has long been regarded as an inhibitor of calmodulin in many cell types (Lam 1984; Clarke et al, 2001).

ACKNOWLEDGEMENT

This work was supported by the Duksung Women's University Research Grant in 2002 to YS Lee.

REFERENCES

Babior BM. The respiratory burst oxidase. *Curr Opin Hematol* 2: 55-60, 1995

Babior BM. NADPH oxidase: an update. *Blood* 93: 1464-1476, 1999

Bombeli T, Karsan A, Tait JF, Harlan JM. Apoptotic vascular endothelial cells become procoagulant. *Blood* 89: 2429-2442, 1997

Cheng AL, Chuang SE, Fine RL, Yeh KH, Liao CM, Lay JD, Chen DS. Inhibition of the membrane translocation and activation of protein kinase C, and potentiation of doxorubicin-induced apoptosis of hepatocellular carcinoma cells by tamoxifen. *Biochem Pharmacol* 55: 523-531, 1998

Clarke R, Leonessa F, Welch JN, Skaar TC. Cellular and molecular pharmacology of antiestrogen action and resistance. *Pharmacol Rev* 53: 25-71, 2001

Collard JG, Habets GG, Michiels F, Stam J, van der Kammen RA, van Leeuwen F. Role of Tiam 1 in Rac-mediated signal transduction pathways. *Curr Top Microbiol Immunol* 213: 253-265, 1996

Cool RH, Merten E, Theiss C, Acker H. Rac1, and not Rac2, is involved in the regulation of the intracellular hydrogen peroxide level in HepG2 cells. *Biochem J* 332: 5-8, 1998

Crompton NE. Programmed cellular response to ionizing radiation damage. *Acta Oncol* 37: 1-49, 1998

Dugan LL, Sensi SL, Canzoniero LM, Handran SD, Rothman SM, Lin TS, Goldberg MP, Choi DW. Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J Neurosci* 15: 6377-6388, 1995

Ehleben W, Porwol T, Fandrey J, Kummer W, Acker H. Cobalt and desferrioxamine reveal crucial members of the oxygen sensing pathway in HepG2 cells. *Kidney Int* 51: 483-491, 1997

Ellis PA, Saccani-Jotti G, Clarke R, Johnston SR, Anderson E, Howell A, A'Hern R, Salter J, Detre S, Nicholson R, Robertson J, Smith IE, Dowsett M. Induction of apoptosis by tamoxifen and ICI 182780 in primary breast cancer. *Int J Cancer* 72: 608-613, 1997

Ferlini C, Scambia G, Marone M, Distefano M, Gaggini C, Ferrandina G, Fattorossi A, Isola G, Benedetti-Panici P, Mancuso S. Tamoxifen induces oxidative stress and apoptosis in oestrogen receptor-negative human cancer cell lines. *Br J Cancer* 79: 257-263, 1999

Fleming IN, Elliott CM, Buchanan FG, Downes CP, Exton JH. Ca^{2+}/calmodulin-dependent protein kinase II regulates Tiam1 by reversible protein phosphorylation. *J Biol Chem* 274: 12753-12758, 1999

Fujisawa H. Regulation of the activities of multifunctional Ca^{2+}/calmodulin-dependent protein kinases. *J Biochem (Tokyo)* 129: 193-199, 2001

Halliwell B. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *Br J Exp Pathol* 70: 737-757, 1989

Jones SA, O'Donnell VB, Wood JD, Broughton JP, Hughes EJ, Jones OT. Expression of phagocyte NADPH oxidase components in human endothelial cells. *Am J Physiol* 271: H1626-1634, 1996

Jordan VC. Long-term adjuvant tamoxifen therapy for breast cancer. *Breast Cancer Res Treat* 15: 125-136, 1990

Jordan VC. Molecular mechanisms of antiestrogen action in breast cancer. *Breast Cancer Res Treat* 31: 41-52, 1994

Kamesaki H. Mechanisms involved in chemotherapy-induced apoptosis and their implications in cancer chemotherapy. *Int J Hematol* 68: 29-43, 1998

Kang Y, Cortina R, Perry RR. Role of *c-myc* in tamoxifen-induced apoptosis estrogen-independent breast cancer cells. *J Natl Cancer Inst* 88: 279-284, 1996

Keen JC, Dixon JM, Miller EP, Cameron DA, Chetty U, Hanby A, Bellamy C, Miller WR. The expression of Ki-S1 and BCL-2 and the response to primary tamoxifen therapy in elderly patients with breast cancer. *Breast Cancer Res Treat* 44: 123-133, 1997

Kidd VJ. Proteolytic activities that mediate apoptosis. *Annu Rev Physiol* 60: 533-573, 1998

Kim JA, Kang YS, Jung MW, Lee SH, Lee YS. Involvement of Ca^{2+} influx in the mechanism of tamoxifen-induced apoptosis in HepG2 human hepatoblastoma cells. *Cancer Lett* 147: 115-123, 1999

Kornblau SM. The role of apoptosis in the pathogenesis, prognosis, and therapy of hematologic malignancies. *Leukemia* 12: S41-46, 1998

Kummer W, Acker H. Immunohistochemical demonstration of four subunits of neutrophil NAD(P)H oxidase in type I cells of carotid body. *J Appl Physiol* 78: 1904-1909, 1995

LaBel CP, Ischiopoulos H, Bondy SC. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem Res Toxicol* 5: 227-231, 1992

Lam HY. Tamoxifen is a calmodulin antagonist in the activation of cAMP phosphodiesterase. *Biochem Biophys Res Commun* 118: 27-32, 1984

Lee YS, Kang YS, Lee SH, Kim JA. Role of NAD(P)H oxidase in the tamoxifen-induced generation of reactive oxygen species and apoptosis in HepG2 human hepatoblastoma cells. *Cell Death Differ* 7: 925-932, 2000

Lowe SW, Lin AW. Apoptosis in cancer. *Carcinogenesis* 21: 485-495, 2000

Marshall C, Mamary AJ, Verhoeven AJ, Marshall BE. Pulmonary artery NADPH-oxidase is activated in hypoxic pulmonary vasoconstriction. *Am J Respir Cell Mol Biol* 15: 633-644, 1996

Nayfield SG, Karp JE, Ford LG, Dorr FA, Kramer BS. Potential role of tamoxifen in prevention of breast cancer. *J Natl Cancer Inst* 83: 1450-1459, 1991

Orie NN, Zidek W, Tepel M. Tyrosine and calcium/calmodulin kinases are common signaling components in the generation of reactive oxygen species in human lymphocytes. *Life Sci* 65: 2135-2142, 1999

Rowlands MG, Parr IB, McCague R, Jarman M, Goddard PM. Variation of the inhibition of calmodulin dependent cyclic AMP phosphodiesterase amongst analogues of tamoxifen; correlations with cytotoxicity. *Biochem Pharmacol* 40: 283-289, 1990

Shen HM, Shi CY, Ong CN. Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B1. *Free Radic Biol Med* 21: 139-146, 1996

Song Z, Steller H. Death by design: mechanism and control of apoptosis. *Trends Cell Biol* 9: 49-52, 1999

Wang XW. Role of p53 and apoptosis in carcinogenesis. *Anticancer Res* 19: 4759-4771, 1999

Williams CL, Porter RA, Phelps SH. Inhibition of voltage-gated Ca^{2+} channel activity in small cell lung carcinoma by the Ca^{2+}/calmodulin-dependent protein kinase inhibitor KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine). *Biochem Pharmacol* 50: 1979-1985, 1995

Wu Y, Dzhura I, Colbran RJ, Anderson ME. Calmodulin kinase and a calmodulin-binding 'IQ' domain facilitate L-type Ca^{2+} current in rabbit ventricular myocytes by a common mechanism. *J Physiol* 535: 679-687, 2001

Youngson C, Nurse C, Yeger H, Curnutte JT, Vollmer C, Wong V, Cutz E. Immunocytochemical localization on O2-sensing protein (NADPH oxidase) in chemoreceptor cells. *Microsc Res Tech* 37: 101-106, 1997