# Increased expression of the lipocalin 24p3 as an apoptotic mechanism for MK886

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MK886, a strong proapoptotic agent, is an inhibitor of 5-lipoxygenase (LOX) through binding to the 5-LOX-activating protein (FLAP). Although MK886-induced apoptosis is through a FLAP-independent pathway, the precise mechanisms are not understood. In the present study, a possible role of 24p3, a lipocalin, in MK886-induced apoptosis was investigated. Exposure of murine prolymphoid progenitor cells (FL5.12) to 20 $\mu$M MK886 for 16 h dramatically increased 24p3 mRNA and protein expression. Induction could also be achieved with another FLAP inhibitor, MK591. The induction of 24p3 by MK886 was dose- and time-dependent. The up-regulated 24p3 mRNA expression by MK886 was enhanced a further 3.1-fold by WY14643, an activator of peroxisome-proliferator-activated receptor $\alpha$, whereas ciglitazone, an activator of peroxisome-proliferator-activated receptor $\gamma$ attenuated the MK886-induced 24p3 expression by more than 50 %. Neither WY14643 nor ciglitazone alone had any effect on the expression of 24p3. The induction of 24p3 by MK886 was dependent on the synthesis of new protein(s), since cycloheximide, an inhibitor of protein synthesis, prevented this effect. In all cases, including the inhibition of MK886-induced 24p3 protein expression by stable transfection with antisense cDNA of 24p3, the extent of apoptosis closely paralleled 24p3 levels. Apoptosis induced by MK886, or enhanced by WY14643, was accompanied by the cleavage and activation of caspase-3. The overexpression of bcl-2 or bcl-$x_L$ in FL5.12 cells inhibited apoptosis induced by MK886 as well as the enhancement of apoptosis by WY14643. Thus 24p3 is an MK886-inducible gene and may play an important role in MK886-induced apoptosis.

Key words: apoptosis, bcl-2, 5-lipoxygenase (LOX), lipocalin, peroxisome-proliferator-activated receptor (PPAR).

## INTRODUCTION

Programmed cell death, or apoptosis, is an evolutionarily conserved form of cell suicide under the control of numerous genes. It is characterized by membrane blebbing, nuclear condensation and internucleosomal DNA degradation [1]. Alterations in apoptosis have been suggested to play crucial roles in the disruption of tissue homoeostasis, immune regulation and the elimination of infected, mutated or damaged cells [2–4].

Apoptosis can be triggered by a variety of stimuli, such as the Fas ligand, radiation, etoposide, growth factor deprivation and lipoxygenase (LOX) inhibitors [5,6]. LOX is a family of enzymes that dioxygenate unsaturated fatty acids thus initiating the peroxidation of membrane lipids and the synthesis of signalling molecules that play roles in the modulation of cell proliferation and apoptosis [6,7]. LOX can be categorized into various subfamilies based on the site of oxygenation (5-, 8-, 12- and 15-LOX) and can be inhibited by number of compounds [8,9].

MK886 is an indol-based xenobiotic that inhibits the activity of 5-LOX through binding to the 5-LOX-activating protein (FLAP) that is involved in the activation and/or presentation of fatty acid substrate to 5-LOX [10,11]. Several studies have revealed a strong proapoptotic effect of MK886 [6,12–14]. However, MK886-induced apoptosis may be not through the inhibition of FLAP, since apoptosis still occurs in cells lacking LOX [15] or having reduced levels of FLAP [13], and the concentration of MK886 required for apoptosis induction is much higher than the $IC_{50}$ of MK886 for LOX activity [6]. The precise mechanism by which MK886 induces apoptosis remains unclear, although a recent study [16] has implicated effects on mitochondria.

Lipocalins are a functionally diverse family of proteins that can bind to surface receptors as well as to a variety of lipophilic substances. Lipocalins have effects on cell proliferation and differentiation and may be involved in cancer [17]. 24p3 protein, a member of the lipocalin family, was originally cloned from mouse kidney cells infected with polyoma virus-40 [18]. It is a secreted glycoprotein and can be induced by lipopolysaccharide [19], basic fibroblast growth factor [20], tumour necrosis factor $\alpha$ [21] and retinoic acid [22]. It has many postulated functions, such as suppressing the inflammatory response during involution of uterine and mammary tissues [23] and playing a role in fatty acid [24] or iron [25] transport. A recent study [26] revealed that 24p3 protein plays a crucial role in interleukin (IL)-3-deprivation-induced apoptosis in cytokine-dependent neutrophils and other leucocytes. This effect may be involved in the anti-inflammatory response of involuting tissue [23] and raises a new potential pathway for the initiation of apoptosis by xenobiotics. Since MK886 has effects on fatty acid-binding proteins, including FLAP [11,12] and peroxisome-proliferator-activated receptors (PPARs) [14,27], it was considered possible that it might also affect lipocalins.

In the present study, we report that the expression of 24p3 is profoundly induced by MK886 in murine proB FL5.12 cells. The induction of 24p3 expression by MK886 is enhanced by WY14643, a PPAR$\alpha$ activator, and inhibited by ciglitazone, a PPAR$\gamma$ activator. The levels of 24p3 expression correlate

Abbreviations used: CHX, cycloheximide; COX, cyclo-oxygenase; ETYA, eicosa-5,8,11-tetraynoic acid; LOX, lipoxygenase; FLAP, 5-LOX-activating protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; NDGA, nordihydroguiaretic acid; NF-$\kappa$B, nuclear factor $\kappa$B; PPAR, peroxisome-proliferator-activated receptor; PI, propidium iodide; PPRE, PPAR-responsive element; RT, reverse transcription.

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with the induction of apoptosis. Treatment with cycloheximide (CHX), an inhibitor of protein synthesis, diminished MK886-induced 24p3 expression and apoptosis. These results suggest that newly synthesized 24p3 protein plays a role in MK886-induced apoptosis.

## MATERIALS AND METHODS

### Cell culture

Wild type IL-3-dependent murine prolymphoid progenitor cells (FL5.12), and FL5.12 cells that stably overexpress bcl-2 or bcl-$x_L$ (transfected with a SFFV-NEO, SFFV-FLAG-*bcl-x*$_L$ or SFFV-FLAG-*bcl-2* construct [28]), or FLAP (transfected with a pcDNA3.1/HisC control or a pcDNA3.1/HisC-FLAP construct [29]) were used. The bcl-2- and bcl-$x_L$-overexpressing cell lines were gifts from Dr Gabriel Nuñez (University of Michigan, Ann Arbor, MI, U.S.A.). All cell lines were maintained in RPMI 1640 media supplemented with 10 % (v/v) conditioned media from WEHI-3B cells (a source of IL-3) [30], 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 $\mu$g/ml streptomycin in a humidified atmosphere at 37 °C and 5 % $CO_2$. Cultures were passaged every 2 days. The overexpression of bcl-2, bcl-$x_L$ or FLAP was confirmed every 2–3 weeks using Western-blot analysis.

### Chemicals and treatment

MK886, ciglitazone, WY14643, bezafibrate, baicalein and troglitazone were obtained from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). MK591 was a gift from Merck Frosst Canada (Pointe Claire-Dorval, Quebec, Canada). 15-deoxy-$\Delta^{12,14}$-Prostaglandin $J_2$ and eicosa-5,8,11-tetraynoic acid (ETYA) were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). CHX was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). All chemicals were dissolved in DMSO, except 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$, which was dissolved in ethanol. All treatments, including appropriate vehicle controls, were added directly to the culture medium.

### RNA isolation and Northern-blot analysis

Northern-blot analysis was performed for detection of specific 24p3 transcripts. Total RNA was isolated from cells using RNeasy mini kits according to the procedure described by the manufacturer (Qiagen, Valencia, CA, U.S.A.). Equal amounts of total RNA (20–25 $\mu$g) were electrophoresed on 1 % (w/v) agarose gels and transferred on to a nylon membrane (Schleicher & Schuell, Keene, NH, U.S.A.). The 24p3 cDNA was synthesized by reverse transcription (RT)-PCR using primers that were designed according to the sequence of the gene (GenBank® accession number X14607: left primer, atgtcacctccatcctggtc; right primer, acagctccttggttcttcca). cDNA probes were labelled with [$\alpha$-$^{32}$P]dCTP using a random primer labelling kit according the procedure described by the manufacturer (Invitrogen, Carlsbad, CA, U.S.A.). Membranes were hybridized with the 24p3 cDNA probes overnight at 68 °C in a hybridization solution (Stratagene, La Jolla, CA, U.S.A.). Membranes were then washed twice with 1 × SSC (where SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1 % SDS at 55 °C, and subjected to autoradiography with an intensifying screen. The membranes then were stripped and re-probed with a cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to normalize for differences in loading and transferring among the samples. Band intensities were determined using UN-SCAN-IT software (Silk Scientific, Orem, UT, U.S.A.) on scanned images.

### Western-blotting analysis

Following treatment, cells were washed with ice-cold PBS and lysed in a buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.5 % (v/v) Nonidet P40, 2 mM EDTA and a protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN, U.S.A.) for 30 min on ice. Following centrifugation at $> 10\,000\ \boldsymbol{g}$ for 15 min, the protein concentration of the supernatant was determined using the Bio-Rad $D_c$ protein assay procedure (Bio-Rad, Hercules, CA, U.S.A.). Equal amounts of protein were resolved on SDS/12 % (w/v) polyacrylamide gels and electrophoretically transferred on to a PVC membrane (Millipore, Bedford, MA, U.S.A.). The blots were blocked with 5 % (w/v) skimmed milk in Tris-buffered saline [20 mM Tris/HCl (pH 7.6) and 150 mM NaCl] with 1 % (v/v) Tween-20 for 1 h at 22 °C, and then incubated at 4 °C overnight with caspase-3 antibodies (Cell Signaling Technology, Beverly, MA, U.S.A.) or antiserum to 24p3 (a gift from Dr. Nilsen-Hamilton, Iowa State University, Ames, IA, U.S.A.) in the same buffer. Specific antibody-labelled proteins were detected by using a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and the enhanced chemiluminescence Western blotting detection system (ECL®; Amersham Biosciences, Piscataway, NJ, U.S.A.). Thereafter, blots were stripped and re-probed with antibodies to actin. Band intensities were determined using UN-SCAN-IT software on scanned images and normalized against actin.

### Measurements of apoptosis

Treated and control cells were harvested and washed with ice-cold PBS and then resuspended in a solution containing either 0.1 % Triton X-100/1 $\mu$g/ml RNase/2.5 $\mu$g/ml propidium iodide (PI) or annexin V–FITC (Clontech Laboratories, Palo Alto, CA, U.S.A.) and 5 $\mu$g/ml PI, and incubated for 15 min. This latter technique allows the differentiation between necrotic and non-necrotic cell populations. A total of 20 000–30 000 cells were analysed with a Beckman Coulter EPICS-XL flow cytometer (Fullerton, CA, U.S.A.) equipped with an argon laser at an excitation wavelength of 488 nm. The emission wavelengths used for PI and annexin were 620 nm and 525 nm respectively. Cells stained with PI alone showing lower DNA content then those in the $G_1$ phase (i.e. sub-$G_1$ population) or cells stained with annexin V were considered apoptotic.

### Caspase-3 activity assay

Caspase-3 activity was measured by quantifying the cleavage of the synthetic substrate *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA). Lysates from treated or control cells were incubated with a solution containing 80 nM Ac-DEVD-pNA, 200 nM Hepes (pH 7.5), 1 mM NaCl and 50 nM dithiothreitol. After incubation for 2 h at 37 °C, the activity of caspase 3 was determined by measuring the absorbance of the released *p*-nitroaniline (pNA) at 405 nm in a microtitre plate reader. An aliquot of the same lysate was used for protein measurement as described above. Caspase-3 activities are expressed as nmol of pNA released/$\mu$g of total protein.

### 24p3 cDNA antisense cloning, plasmid construction and transfection

The 24p3 cDNA containing the entire protein-coding region was synthesized by RT-PCR using RNA isolated from FL5.12 cells. The antisense cDNA expression plasmid was constructed by

subcloning the 24p3 cDNA into the pEGFP-N3 vector (Clontech) reversibly to generate pEGFP-AS-24p3, which express antisense of 24p3 cDNA under the control of the cytomegalovirus ('CMV') promoter. Stable transfection was performed by electroporation. FL5.12 cells ($10 \times 10^7$) were incubated with 10 $\mu$g of pEGFP-AS-24p3 DNA for 10 min, followed by electric pulsing at 250 volts/960 $\mu$F. pEGFP-N3 DNA was used as the control vector. FL5.12 cells stably expressing antisense 24p3 cDNA were selected using a RPMI 1640 media containing 400 $\mu$g/ml G418 (Life Technologies, Gaithersburg, MD, U.S.A.) and maintained in the same medium containing 200 $\mu$g/ml G418.

### Statistics

Data are presented as means $\pm$ S.E.M. Comparison between different treatment groups were analysed by ANOVA followed by the Tukey/Kramer test. $P < 0.05$ was considered as statistically significant.

## RESULTS

### Induction of 24p3 by MK886

To investigate whether 24p3 might play a role in MK886-induced apoptosis, its expression was measured by Northern-blot analysis 16 h after FL5.12 cells were treated with 20 $\mu$M MK886. As shown in Figure 1(a), the basal level of 24p3 mRNA in FL5.12 cells was undetectable, consistent with previous data [26]. Following treatment with 20 $\mu$M MK886, there was a tremendous increase in 24p3 mRNA levels.

The induction of 24p3 by MK886 was dose- and time-dependent. With 20 $\mu$M MK886, 24p3 mRNA levels were increased at 8 h. The increase in 24p3 mRNA levels was maintained to at least 24 h (Figure 1b). Because of the extensive cell death evident beyond 24 h, later time points were not examined. The minimum dose of MK886 required to observe 24p3 induction at 16 h was 10 $\mu$M (Figure 1c). Earlier time points were not examined with higher doses and it is possible that they would have accelerated the response.

In order to confirm the induction of 24p3 by MK886 treatment, protein levels were measured by Western-blot analysis (Figure 1d). Consistent with the Northern-blot analyses, 24p3 protein levels were dose-dependently increased after treatment with MK886 for 23 h.

The time- and dose-response of apoptosis induced by MK886 closely paralleled the induction of 24p3 mRNA (Figure 2a compared with Figure 1b, and Figure 2b compared with Figure 1c). Significant apoptosis, as assessed by annexin V binding, was first evident 16 h after treatment with 20 $\mu$M MK886 (Figure 2a). The minimum dose of MK886 required for significant induction of apoptosis at this time was 20 $\mu$M, although a small effect appeared to begin at 10 $\mu$M (Figure 2b).

MK591 is a more specific inhibitor of FLAP than MK886 [10], but is chemically distinct and somewhat less effective at inducing apoptosis [13]. Nevertheless, after 16 h, this compound was able to up-regulate the expression of 24p3 in a dose-dependent manner, similar to that seen with MK886 (Figure 3). Once again, as 24p3 mRNA levels increased, apoptosis also increased correspondingly (results not shown).

In order to determine whether other inhibitors of LOX or cyclo-oxygenase (COX) might induce the expression of 24p3, total RNA from cells treated for 16 h was isolated and the expression of 24p3 was assessed by Northern-blot analysis. Baicalein, a 12-LOX inhibitor, increased 24p3 expression only at a dose of 80 $\mu$M (Figure 4a), which was higher than that required for inducing apoptosis (50 $\mu$M; [31]). However, nordihydroguiaretic acid (NDGA), a dual COX and LOX inhibitor, induced 24p3 expression somewhat more than MK886 (Figure 4b) at a dose (5 $\mu$M) that also induced apoptosis [15]. ETYA (20 $\mu$M), a general LOX inhibitor, did not induce 24p3 expression (Figure 4c). These data indicate that LOX inhibitors do not have a general ability to induce 24p3 expression and that the apoptotic mechanisms of these agents may differ.

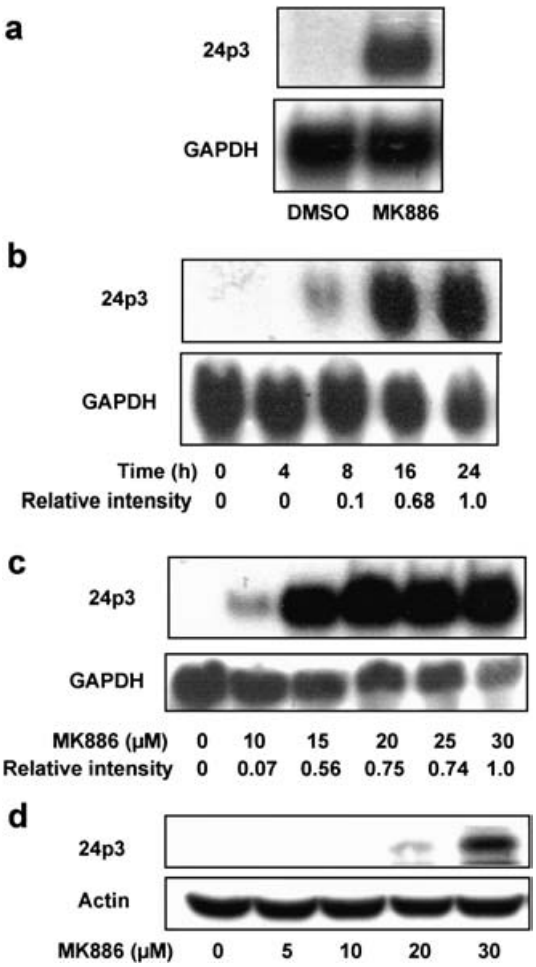


**Figure 1 Induction of 24p3 mRNA and protein expression by MK886**

Total RNA was extracted from FL5.12 cells treated with MK886 or DMSO vehicle. The levels of 24p3 mRNA were examined by Northern-blot analysis. (**a**) Cells were treated with 20 $\mu$M MK886 for 16 h. (**b**) Cells were treated with 20 $\mu$M MK886 for various time periods. Intensity is normalized to GAPDH and determined relative to 24 h. (**c**) Cells were treated with different doses of MK886 for 16 h. Intensity is normalized to GAPDH and determined relative to 30 $\mu$M MK886. (**d**) Following treatment with various doses of MK886 for 23 h, cells were lysed and the levels of 24p3 protein (80 $\mu$g of protein/lane) detected by Western-blot analysis. The blots shown are representative of two [(**b**) and (**c**)] or three [(**a**) and (**d**)] independent experiments.

### Modulation of MK886-induced 24p3 expression by PPAR activators

PPARs belong to the nuclear steroid hormone receptor superfamily [32]. PPARs consists of $\alpha$, $\beta$ and $\gamma$ subgroups and have been implicated in fatty acid metabolism and apoptosis [32]. Recently, we reported that MK886 [27] and other LOX inhibitors [33] are effective PPAR inhibitors. To examine the effect of PPAR ligands on MK886-induced 24p3 expression, cells were treated with 50 $\mu$M WY14643, a PPAR$\alpha$ activator, or 50 $\mu$M ciglitazone, a PPAR$\gamma$ activator, for 16 h in the absence or presence of 20 $\mu$M MK886. The results showed that neither WY14643 nor

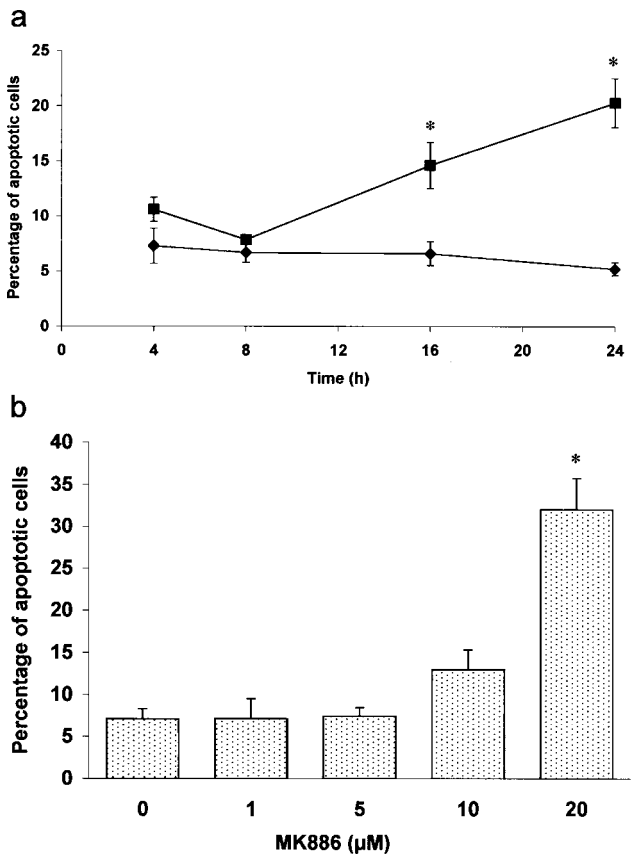


**Figure 2 Induction of apoptosis by MK886**

At various time points after treatment, apoptosis was quantified by annexin V–FITC/PI staining using flow cytometry as described in the Materials and methods section. (**a**) FL5.12 cells were treated with either 20 $\mu$M MK886 (■) or DMSO (◆). (**b**) Cells were treated with various concentrations of MK886 for 24 h. Data are expressed as means $\pm$ S.E.M. of three independent experiments. *$P < 0.05$ compared with time-matched vehicle (**a**) or vehicle control (**b**).

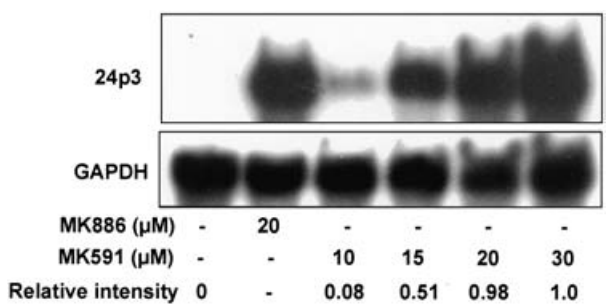


**Figure 3 Induction of 24p3 mRNA expression by MK591**

FL5.12 cells were treated with various doses of MK591 for 16 h and subsequently examined for the levels of 24p3 mRNA by Northern-blot analysis. Cells treated with 20 $\mu$M MK886 were used as a positive control. Intensity is normalized to GAPDH and determined relative to 30 $\mu$M MK591. Due to limited availability of MK591, this blot was only performed once.

ciglitazone alone affected the expression of 24p3 (Figure 5a). However, when co-treated with MK886, WY14643 enhanced the already large increase in 24p3 mRNA levels ($3.1 \pm 0.7$-fold, $n = 3$). In contrast, ciglitazone was very effective in blocking the up-regulation of 24p3 expression resulting from the MK886 treatment, decreasing 24p3 mRNA levels by $56 \pm 10$% (Figure 5a). As little as 2.5 $\mu$M WY14643 or ciglitazone was

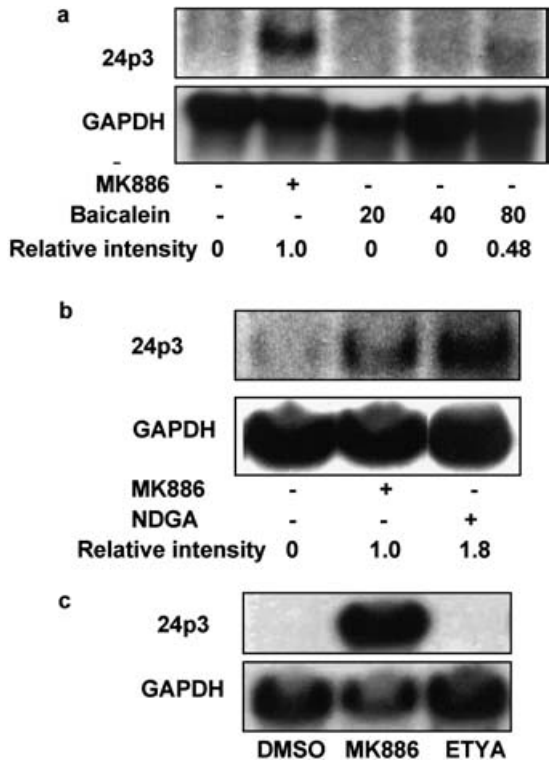


**Figure 4 Induction of 24p3 mRNA expression by various LOX and COX inhibitors**

Total RNA was extracted from FL5.12 cells treated with (**a**) 20–80 $\mu$M baiclein, (**b**) 5 $\mu$M NDGA or (**c**) 20 $\mu$M ETYA. Cells treated with 20 $\mu$M MK886 or DMSO were used as positive and negative controls respectively. Intensity is normalized to GAPDH and determined relative to MK886. The blot shown for NDGA is representative of three independent experiments.

able to enhance or inhibit the MK886-induced 24p3 expression respectively (Figure 5b). The levels of 24p3 protein were again altered correspondingly with the mRNA (Figure 5c).

In order to see whether co-treatment with WY14643 or ciglitazone was required, these PPAR agonists were added 1 h after MK886 treatment. Changes in 24p3 expression were identical with the co-treatments (results not shown) indicating these agonists were not acting through effects on MK886 uptake.

Other synthetic chemicals and natural ligands can activate PPARs. Therefore we examined the effect of some of these agents on MK886-induced 24p3 expression. Cells were treated with 50–200 $\mu$M bezafibrate (PPAR$\alpha$ activator), 1–3 $\mu$M 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ or 1–8 $\mu$M troglitazone (PPAR$\gamma$ activators) in the absence or presence of MK886 (20 $\mu$M) for 16 h. Surprisingly, none of these activators showed any effect on 24p3 expression (results not shown).

### Modulation of MK886-induced apoptosis by WY14643 and ciglitazone

Since WY14643 and ciglitazone were able to modulate MK886-induced 24p3 expression, their ability to alter MK886-induced apoptosis was determined. Using annexin V–FITC labelling, neither WY14643 (50 $\mu$M) nor ciglitazone (20 $\mu$M) alone had any significant effect on apoptosis compared with vehicle-treated cells. However, WY14643 greatly increased apoptosis when present with MK886 (2.3-fold compared with MK886 alone). In contrast, ciglitazone reduced MK886-induced cell apoptosis

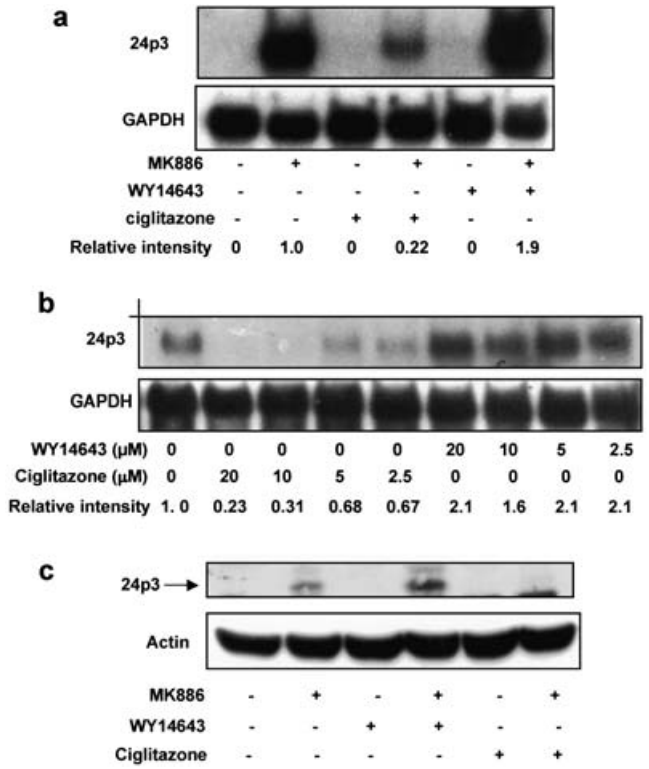


**Figure 5 Modulation of MK886-induced 24p3 mRNA and protein expression by PPAR activators**

(**a**) FL5.12 cells were treated with 20 $\mu$M MK886 in the absence or presence of 50 $\mu$M WY14643 or 50 $\mu$M cigilitazone for 16 h. Thereafter, total RNA was extracted and 24p3 mRNA levels determined by Northern-blot analysis. (**b**) Cells were treated with 20 $\mu$M MK886 alone or combined with various concentrations of either WY14643 or ciglitazone for 16 h. The expression of 24p3 mRNA was then measured by Northern-blot analysis. Intensity is normalized to GAPDH and determined relative to MK886. (**c**) Following treatment with 20 $\mu$M MK886 in the absence or presence of 50 $\mu$M WY14643 or 50 $\mu$M cigilitazone for 23 h, cell lysates (80 $\mu$g of protein/lane) were used to determine the levels of 24p3 protein by Western-blot analysis. The blot shown is representative of three independent experiments.

by $20 \pm 2$% (Figure 6). Interestingly, two recent reports [34,35] have shown that several thiazolidinedione PPAR$\gamma$ agonists can protect lymphocytes and LLC-PK1 cells from apoptosis.

### Inhibition of 24p3 expression blocked MK886-induced apoptosis

To demonstrate whether 24p3 is required for MK886-induced apoptosis, FL5.12 cells were stably transfected with a 24p3 antisense cDNA. The expression of 24p3 protein induced by co-treatment with 20 $\mu$M MK886 and 50 $\mu$M WY14643 was inhibited in the cells expressing antisense compared with that in cells transfected with the control vector (Figure 7, inset). Consistent with a role for 24p3 in apoptosis, FL5.12 cells expressing 24p3 antisense exhibited over 50% less apoptosis, compared with vector controls, when treated with MK886 with or without WY14643 (Figure 7).

### Effects of blocking protein synthesis

To obtain information as to the mechanism underlying the induction of 24p3 expression by MK886, cells were treated with MK886 in the absence or presence of the protein synthesis inhibitor CHX. CHX effectively blocked the effect of MK886 on

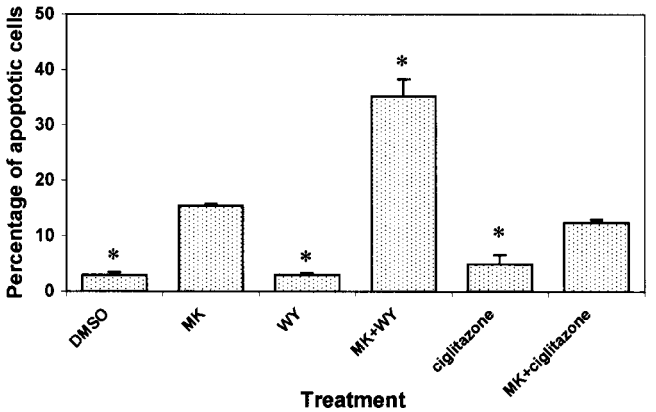


**Figure 6 Modulation of MK886-induced apoptosis by PPAR activators**

FL5.12 cells treated with 20 $\mu$M MK886 alone (MK) or co-treated with 50 $\mu$M WY14643 (WY) or 20 $\mu$M ciglitazone for 24 h were analysed for apoptosis by flow cytometry using annexin V–FITC/PI staining. Data are means $\pm$ S.E.M. of three independent experiments. $^*P < 0.05$ compared with treatment with MK886 alone.

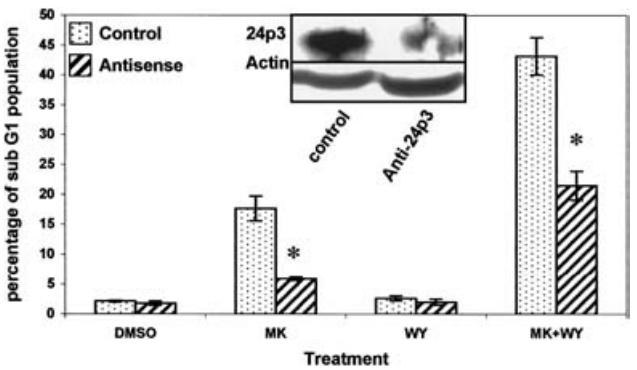


**Figure 7 24p3 expression and apoptosis in the presence of 24p3 antisense**

FL5.12 cells stably transfected with 24p3 antisense or a vector control were treated with 20 $\mu$M MK886 (MK) in the absence or presence of 50 $\mu$M WY14643 (WY) for 16 h. Thereafter apoptotic cells were analysed by flow cytometry using DNA/PI staining. Cells containing lower DNA than $G_1$ phase populations were considered as apoptotic. Data are means $\pm$ S.E.M. of three independent experiments. The inset shows a representative Western blot of 24p3 protein in cells co-treated with 20 $\mu$M MK886 and 50 $\mu$M WY14643. $^*P < 0.05$ compared with the corresponding control group.

24p3 expression at a dose of 50 ng/ml (Figure 8a). In addition, CHX also significantly decreased, but did not eliminate, MK886-induced cell apoptosis (Figure 8b). Therefore the induction of 24p3 mRNA by MK886 is indirect and apparently dependent on the synthesis of some other protein(s).

### Effects of bcl-2 family proteins on MK886-induced changes in 24p3

The bcl-2 family of proteins plays a crucial role in the regulation of apoptosis [36]. To evaluate the possible involvement of the anti-apoptotic bcl-2 or bcl-$x_L$ in the pathway of 24p3, cells overexpressing these proteins were treated with 20 $\mu$M MK886 in absence or presence of 50 $\mu$M WY14643. Apoptosis was decreased in both overexpressing cell lines by more than 50%, compared with the control FL5.12 cells, after treatment with MK886 alone for 24 h (Figure 9). The extent of apoptosis after MK886 plus WY14643 was also significantly decreased

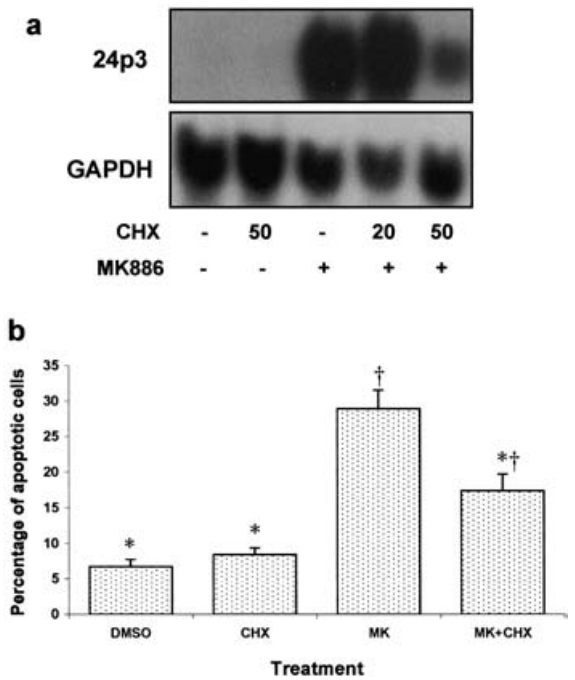


**Figure 8 Effect of CHX on the MK886-induced 24p3 mRNA expression and apoptosis**

(**a**) FL5.12 cells were treated with 20 $\mu$M MK886 in the absence or presence of 20 or 50 ng/ml CHX. After 16 h, total RNA was extracted and analysed for the expression of 24p3 mRNA by Northern-blot analysis. The blot shown is representative of two independent experiments. (**b**) Apoptosis was analysed by flow cytometry using annexin V–FITC/PI staining after 24 h treatment with 20 $\mu$M MK886 (MK) in the absence or presence of 50 ng/ml CHX. Data are means $\pm$ S.E.M. of three independent experiments. $^*P < 0.05$ compared with treatment with MK886 alone; and $\dagger P < 0.05$ compared with all other groups.

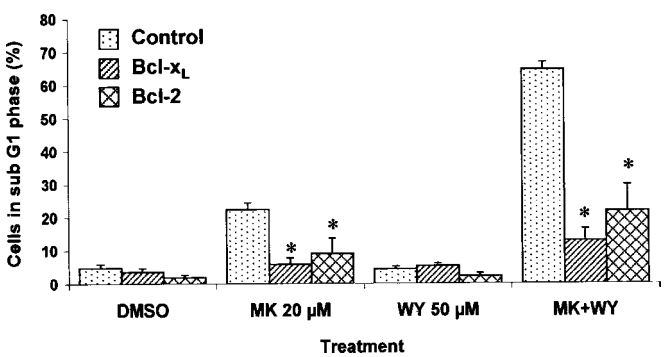


**Figure 9 Apoptosis after MK886 in cells overexpressing bcl-2 or bcl-x$_L$**

FL5.12 cells overexpressing bcl-2 or bcl-$x_L$ were treated with 20 $\mu$M MK886 (MK) alone or co-treated with 50 $\mu$M WY14643 (WY) for 24 h. Cells were then analysed by flow cytometry using DNA/PI staining. Cells containing lower DNA than that in $G_1$ phase populations were considered apoptotic. Cells stably transfected with the control plasmid were used as controls. Results are means $\pm$ S.E.M. of three independent experiments. $^*P < 0.05$ compared with control.

from $65 \pm 2$% to $22 \pm 8$% and $12 \pm 4$% in cells overexpressing bcl-2 and bcl-$x_L$ respectively. However, the expression of 24p3 induced by MK886 in absence or presence of WY14643 was unaffected by the overexpression of bcl-2 or bcl-$x_L$ (results not shown). These data suggest bcl-2 and bcl-$x_L$ act downstream of 24p3 in MK886-induced apoptosis.

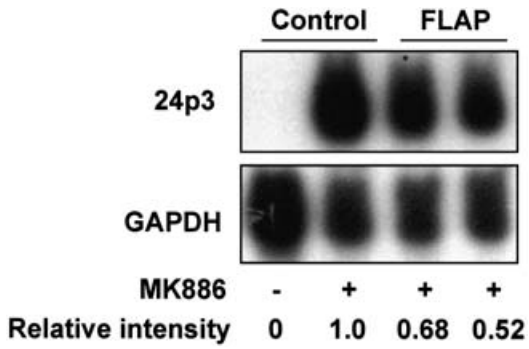


**Figure 10 Effect of overexpression of FLAP on 24p3 mRNA expression after MK886 treatment**

Cells overexpressing FLAP were treated with 20 $\mu$M MK886 for 16 h. Total RNA was then extracted and the levels of 24p3 mRNA were examined by Northern-blot analysis. Cells stably transfected with the control plasmid were used as control. Intensity is normalized to GAPDH and determined relative to MK886 in control cells. The blot shown is representative of two independent experiments.

### Role of FLAP in the induction of 24p3

Since MK886 is a FLAP inhibitor, the possible involvement of FLAP in the expression of 24p3 was examined. 24p3 expression was undetectable in untreated FLAP-overexpressing cells (results not shown). FL5.12 cells overexpressing FLAP were treated with MK886 for 16 h. There was a decrease observed in the induction of 24p3 by MK886 in FLAP-overexpressing cells (Figure 10). This decrease may arise from binding more MK886 to FLAP, thereby limiting the amount available to exert effects on 24p3 and suggests FLAP is not mediating the effect on 24p3.

### Effects on caspase-3 activity

Caspase activation is thought to be a key step in the genesis of apoptosis through numerous stimuli [36,37]. The cleavage of caspase-3 from its 32 kDa inactive precursor to form 17 and 12 kDa subunits with strong protease activity was studied by Western blot using antibodies that recognize the 17 kDa form (p17). Treatment of FL5.12 cells with MK886 greatly increased the amount of p17 (Figure 11a). Co-treatment with MK886 and WY14643 resulted in further increases in p17 levels. In contrast, the levels of p17 in cells co-treated with MK886 and ciglitazone were similar to those in cells treated with MK886 alone. In order to confirm these data, a caspase-3 activity assay was performed. After 16 h, MK886 alone increased caspase-3 activity by 2.5-fold (Figure 11b). Co-treatment with WY14643 enhanced caspase-3 activity by 2.7- and 6.8-fold compared with MK886 alone and vehicle-treated controls respectively (Figure 11b). Therefore apoptosis was accompanied by the cleavage and activation of caspase-3 in relation to the extent of apoptosis induced.

## DISCUSSION

In the present study, MK886 was shown to up-regulate the expression of the lipocalin 24p3 in FL5.12 cells. It is also shown that MK886-induced 24p3 expression can be increased further by the PPAR$\alpha$ agonist WY14643 and is inhibited by the PPAR$\gamma$ agonist ciglitazone. The levels of 24p3 expression induced by MK886 correlated with the apoptosis induced by these compounds. These findings suggest the involvement of 24p3 in MK886-induced apoptosis and imply that other xenobiotics may act via this mechanism.

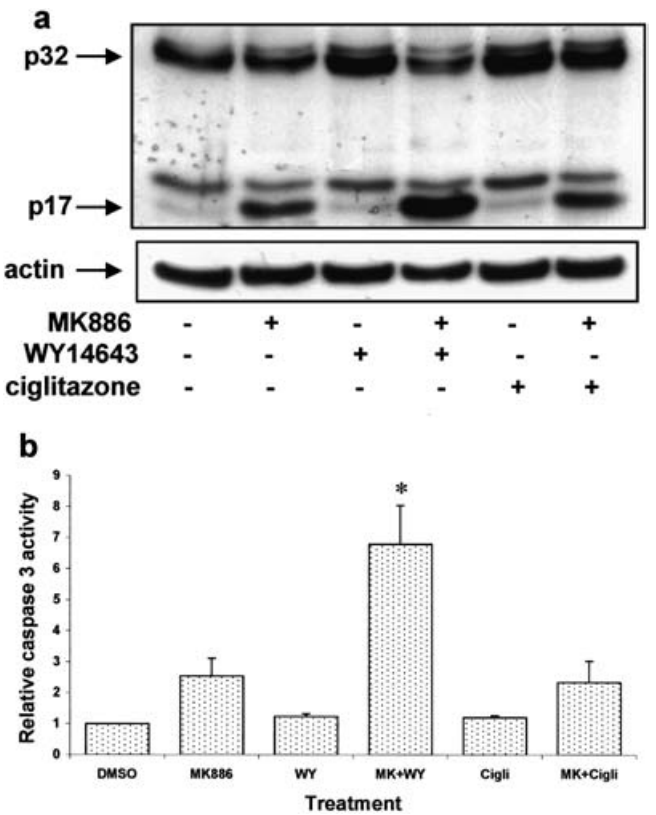


**Figure 11 Activation of caspase-3**

(**a**) Lysates (50 $\mu$g of protein per lane) from FK5.12 cells treated with 20 $\mu$M MK886 (MK) alone or co-treated with 50 $\mu$M WY14643 (WY) or 20 $\mu$M ciglitazone (Cigli) for 16 h were used for Western-blot analysis using antibodies recognizing the active form of caspase-3 (17 kDa, p17). Actin was used as the loading control. (**b**) Lysates from cells treated as described in (**a**) were used for measurement of caspase-3 activity as described in the Materials and methods section. Data are expressed relative to DMSO as means $\pm$ S.E.M. of three independent experiments. * $P < 0.05$ compared with controls.

24p3 can be induced by lipopolysaccharide [19], basic fibroblast growth factor [20], tumour necrosis factor $\alpha$ [21] and retinoic acid [22]. The biological functions of 24p3 are not fully understood, although it has been reported to bind fatty acids and retinoids [24], enhance the activity of the c-AMP-dependent protein kinase [38], deliver iron to the cytoplasm [25] and play a crucial role in IL3-deprivation-induced apoptosis [26]. This latter activity suggested that apoptosis-inducing xenobiotics, particularly those affecting lipid pathways, might also function via 24p3.

The MK886-induced increase in 24p3 mRNA expression occurred 8 h after treatment and levels remained elevated for at least 16 h. This time course slightly preceded MK886-induced apoptosis. The minimum dose of MK886 required for 24p3 induction was comparable with that needed for apoptosis. In addition, the enhanced 24p3 expression by co-treatment with WY14643 resulted in an increase in MK886-induced apoptosis, whereas the inhibition of MK886-induced 24p3 expression by ciglitazone or CHX decreased MK886-induced apoptosis. Moreover, blocking MK886-induced 24p3 expression by expressing an antisense cDNA significantly decreased apoptosis. Taken together, these data support a crucial role for 24p3 in MK886-induced apoptosis.

In prostate cancer cells, MK886-triggered apoptosis appears to involve the inhibition of 5-LOX through its action on FLAP, since 5-hydroxyeicosatetraenoic acid, a product of 5-LOX, was able to rescue these cells [39,40]. However, MK886 induces apoptosis in WSU cells that lack FLAP or in FLAP-depleted cells [13]. Therefore MK886 can induce apoptosis independently of FLAP. Our data indicate that the regulation of 24p3 expression by MK886 is also FLAP-independent, as evidenced by the finding that the induction of 24p3 expression by MK886 in bcl-$x_L$-overexpressing cells, in which FLAP expression was 70 % lower than control FL5.12 cells [15], was similar to the control cells, and MK886-induced 24p3 expression was slightly reduced in FLAP-overexpressing cells.

Several studies have demonstrated that activation of PPAR signalling pathways is able to modulate gene responses, including those involved in apoptosis [41]. In the present study, WY14643, a PPAR$\alpha$ activator, dramatically enhanced MK886-induced 24p3 expression, whereas treatment with ciglitazone, a PPAR$\gamma$ activator, resulted in a significant decrease of MK886-induced 24p3 expression. Neither WY14643 nor ciglitazone alone had effects on the expression of 24p3. Therefore the activation of PPAR signalling pathways may modulate the expression of 24p3 in response to MK886.

In human breast cancer cells, MK886 up-regulates PPAR$\alpha$ and $\gamma$ expression [14]. This could be a response to the inhibition of PPAR$\alpha$ by MK886 as shown in keratinocyte 308 and CV-1 cells [27]. However, although MK886 could efficiently inhibit the WY14643-induced expression of keratin-1, a PPAR$\alpha$-responsive gene, it only had a minimal effect on another PPAR$\alpha$-responsive gene, acyl-CoA oxidase. Thus, although acyl-CoA oxidase may be regulated by other pathways, the effect of MK886 on PPAR$\alpha$-responsive genes was not universal. With 24p3 expression, WY14643 or ciglitazone had no effect alone, but did profoundly modulate the response to MK886. This suggests downstream effects or that the presence of MK886 allows PPAR to gain access to the promoter region of 24p3.

PPARs function as transcription factors after activation by the retinoid X receptor and binding to the target gene at a specific response element [PPAR-responsive element (PPRE)] [42]. However, the regulation of gene expression by a PPAR agonist is not necessarily via the PPRE. In rat synovial fibroblasts, a PPAR$\gamma$ activator inhibited lipopolysaccharide-induced COX-2 and nitric oxide synthase by interfering with the activity of nuclear factor $\kappa$B (NF-$\kappa$B) and AP-1 [43]. Jones et al. [44] also reported that activation of PPAR$\alpha$ decreased the ability of NF-$\kappa$B to bind to DNA. The modulation of 24p3 expression by PPAR may involve interference with the activity of transcription factors, since there is no PPRE in the promoter region of the 24p3 gene [22]. Although there are no NF-$\kappa$B or AP-1 sites in the regulatory region of the 24p3 gene, it is possible the activation of PPAR interferes with the activity of Sp1 or other transcription factors that, in turn, alter the expression of 24p3.

In the present study, possible downstream events in the 24p3 induction pathway after MK886 were examined. The apoptosis induced by MK886 was significantly decreased in cells overexpressing bcl-2 or bcl-$x_L$ compared with control cells, in agreement with previous work [15]. The enhanced apoptosis by WY14643 was also significantly inhibited; however, the induction of 24p3 expression was not altered. Therefore the action of bcl-2 family proteins on MK886/24p3-induced apoptosis appears to be a downstream event, consistent with a previous study with this same cell line when apoptosis was induced by removing IL-3 [26].

In conclusion, the results from the present study demonstrate that MK886 is able to profoundly up-regulate 24p3 expression. This up-regulation can be modified by the PPAR agonists WY14643 and ciglitazone, suggesting that these nuclear receptors

are in someway involved. The expression of 24p3 was highly correlated with the extent of apoptosis under all conditions, providing new information supporting a role for this lipocalin in the apoptosis induced by some xenobiotics. Notably, MK886 also induces the expression of NGAL, the human analogue of 24p3 (Z. Tong, X. Wu and J. P. Kehrer, unpublished work), suggesting that lipocalins may be important mediators of apoptosis in other species.

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## REFERENCES


1 Ferreira, C. G., Epping, M., Kruyt, F. A. and Giaccone, G. (2002) Apoptosis: target of cancer therapy. Clin. Cancer Res. **8**, 2024–2034
2 Bellamy, C. O., Malcomson, R. D., Harrison, D. J. and Wyllie, A. H. (1995) Cell death in health and disease: the biology and regulation of apoptosis. Semin. Cancer Biol. **6**, 3–16
3 Jacobson, M. D., Weil, M. and Raff, M. C. (1997) Programmed cell death in animal development. Cell (Cambridge, Mass.) **88**, 347–354
4 Thompson, C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. Science (Washington, D.C.) **267**, 1456–1462
5 Mohr, S., Zech, B., Lapetina, E. G. and Brune, B. (1997) Inhibition of caspase-3 by S-nitrosation and oxidation caused by nitric oxide. Biochem. Biophys. Res. Commun. **238**, 387–391
6 Tang, D. G., La, E., Kern, J. and Kehrer, J. P. (2002) Fatty acid oxidation and signaling in apoptosis. Biol. Chem. **383**, 425–442
7 Tang, D. G., Chen, Y. Q. and Honn, K. V. (1996) Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis. Proc. Natl. Acad. Sci. U.S.A. **93**, 5241–5246
8 Brash, A. R. (1999) Lipoxygenases: occurrence, functions, catalysis and acquisition of substrate. J. Biol. Chem. **274**, 23679–23682
9 Kuhn, H. and Thiele, B. J. (1999) The diversity of the lipoxygenase family. Many sequence data but little information on biological significance. FEBS Lett. **449**, 7–11
10 Harris, R. R., Carter, G. W., Bell, R. L., Moore, J. L. and Brooks, D. W. (1995) Clinical activity of leukotriene inhibitors. Int. J. Immunopharmacol. **17**, 147–156
11 Rouzer, C. A., Ford-Hutchinson, A. W., Morton, H. E. and Gillard, J. W. (1990) MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. J. Biol. Chem. **265**, 1436–1442
12 Avis, I. M., Jett, M., Boyle, T., Vos, M. D., Moody, T., Treston, A. M., Martinez, A. and Mulshine, J. L. (1996) Growth control of lung cancer by interruption of 5-lipoxygenase-mediated growth factor signaling. J. Clin. Invest. **97**, 806–813
13 Datta, K., Biswal, S. S. and Kehrer, J. P. (1999) The 5-lipoxygenase-activating protein (FLAP) inhibitor, MK886, induces apoptosis independently of FLAP. Biochem. J. **340**, 371–375
14 Avis, I., Hong, S. H., Martinez, A., Moody, T., Choi, Y. H., Trepel, J., Das, R., Jett, M. and Mulshine, J. L. (2001) Five-lipoxygenase inhibitors can mediate apoptosis in human breast cancer cell lines through complex eicosanoid interactions. FASEB J. **15**, 2007–2009
15 Datta, K., Biswal, S. S., Xu, J., Towndrow, K. M., Feng, X. and Kehrer, J. P. (1998) A relationship between 5-lipoxygenase-activating protein and bcl-$x_L$ expression in murine pro-B lymphocytic FL5.12 cells. J. Biol. Chem. **273**, 28163–28169
16 Gugliucci, A., Ranzato, L., Scorrano, L., Colonna, R., Petronilli, V., Cusan, C., Prato, M., Mancini, M., Pagano, F. and Bernardi, P. (2002) Mitochondria are direct targets of the lipoxygenase inhibitor MK886. A strategy for cell killing by combined treatment with MK886 and cycloxygenase inhibitors. J. Biol. Chem. **277**, 31789–31795
17 Bratt, T. (2000) Lipocalins and cancer. Biochim. Biophys. Acta **1482**, 318–326
18 Hraba-Renevey, S., Turler, H., Kress, M., Salomon, C. and Weil, R. (1989) SV40-induced expression of mouse gene 24p3 involves a post-transcriptional mechanism. Oncogene **4**, 601–608
19 Meheus, L. A., Fransen, L. M., Raymackers, J. G., Blockx, H. A., Van Beeumen, J. J., Van Bun, S. M. and Van de Voorde, A. (1993) Identification by microsequencing of lipopolysaccharide-induced proteins secreted by mouse macrophages. J. Immunol. **151**, 1535–1547
20 Davis, T. R., Tabatabai, L., Bruns, K., Hamilton, R. T. and Nilsen-Hamilton, M. (1991) Basic fibroblast growth factor induces 3T3 fibroblasts to synthesize and secrete a cyclophilin-like protein and $\beta$2-microglobulin. Biochim. Biophys. Acta **1095**, 145–152
21 Liu, Q. and Nilsen-Hamilton, M. (1995) Identification of a new acute phase protein. J. Biol. Chem. **270**, 22565–22570
22 Garay-Rojas, E., Harper, M., Hraba-Renevey, S. and Kress, M. (1996) An apparent autocrine mechanism amplifies the dexamethasone- and retinoic acid-induced expression of mouse lipocalin-encoding gene 24p3. Gene **170**, 173–180
23 Ryon, J. Bendickson, L. and Nilsen-Hamilton, M. (2002) High expression in involuting reproductive tissues of uterocalin/24p3, a lipocalin and acute phase protein. Biochem. J. **367**, 271–277
24 Chu, S. T., Lin, H. J., Huang, H. L. and Chen, Y. H. (1998) The hydrophobic pocket of 24p3 protein from mouse uterine luminal fluid: fatty acid and retinol binding activity and predicted structural similarity to lipocalins. J. Pept. Res. **52**, 390–397
25 Yang, J., Goetz, D., Li, J.-Y., Wang, W., Mori, K., Setlik, D., Du, T., Erdjument-Bromage, H., Tempst, P., Strong, R. and Barasch, J. (2002) An iron delivery pathway mediated by lipocalin. Mol. Cell **10**, 1045–1056
26 Devireddy, L. R., Teodoro, J. G., Richard, F. A. and Green, M. R. (2001) Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. Science (Washington, D.C.) **293**, 829–834
27 Kehrer, J. P., Biswal, S. S., La, E., Thuillier, P., Datta, K., Fischer, S. M. and Vanden Heuvel, J. P. (2001) Inhibition of peroxisome-proliferator-activated receptor (PPAR)$\alpha$ by MK886. Biochem. J. **356**, 899–906
28 Simonian, P. L., Grillot, D. A. and Nunez, G. (1997) Bcl-2 and Bcl-$x_L$ can differentially block chemotherapy-induced cell death. Blood **90**, 1208–1216
29 Wu, X., Biswal, S. and Kehrer, J. P. (2003) Roles of 5-lipoxygenase activating protein in cell proliferation and apoptosis. Cell Biol. Toxicol., in the press
30 Clark-Lewis, I., Thomas, W. R. and Schrader, J. W. (1985) Characterization of hemopoietic growth factors from T cells and the myelomonocytic leukemia WEHI-3B. Exp. Hematol. **13**, 304–311
31 La, E., Kern, J. C., Atarod, E. B. and Kehrer, J. P. (2003) Fatty acid release and oxidation in lipoxygenase inhibitor-induced apoptosis. Toxicol. Lett. **138**, 193–203
32 Vanden Heuvel, J. P. (1999) Peroxisome proliferator-activated receptors: a critical link among fatty acids, gene expression and carcinogenesis. J. Nutr. **129**, 575S–580S
33 Thuillier, P., Brash, A. R., Kehrer, J. P., Stimmel, J. B., Leesnitzer, L. M., Yang, P., Newman, R. A. and Fischer, S. M. (2002) Inhibition of peroxisome proliferator-activated receptor (PPAR)-mediated keratinocyte differentiation by lipoxygenase inhibitors. Biochem. J. **366**, 901–910
34 Wang, Y. L., Frauwirth, K. A., Rangwala, S. M., Lazar, M. A. and Thompson, C. B. (2002) Thiazolidinedione activation of peroxisome proliferators-activated receptor $\gamma$ can enhance mitochondrial potential and promote cell survival. J. Biol. Chem. **277**, 31781–31788
35 Haraguchi, K., Shimura, H. and Onaya, T. (2001) Activation of peroxisome proliferators-activated receptor-$\gamma$ inhibits apoptosis induced by serum deprivation in LLC-PK1 cells. Exp. Nephrol. **10**, 393–401
36 Antonsson, B. (2001) Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim the mitochondrion. Cell Tissue Res. **306**, 347–361.
37 Mak, T. W. and Yeh, W. C. (2002) Signaling for survival and apoptosis in the immune system. Arthritis Res. **4** (Suppl 3), S243–S252
38 Lee, Y. C., Lin, S. D., Yu, H. M., Chen, S. T. and Chu, S. T. (2001) Phosphorylation of the 24p3 protein secreted from mouse uterus *in vitro* and *in vivo*. J. Protein Chem. **20**, 563–569
39 Ghosh, J. and Myers, C. E. (1997) Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. Biochem. Biophys. Res. Commun. **235**, 418–423
40 Ghosh, J. and Myers, C. E. (1998) Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. Proc. Natl. Acad. Sci. U.S.A. **95**, 13182–13187
41 Roberts, R. A., James, N. H., Hasmall, S. C., Holden, P. R., Lambe, K., Macdonald, N., West, D., Woodyatt, N. J. and Whitcome, D. (2000) Apoptosis and proliferation in nongenotoxic carcinogenesis: species differences and role of PPAR$\alpha$. Toxicol. Lett. **112–113**, 49–57
42 Hihi, A. K., Michalik, L. and Wahli, W. (2002) PPARs: transcriptional effectors of fatty acids and their derivatives. Cell Mol. Life Sci. **59**, 790–798
43 Simonin, M. A., Bordji, K., Boyault, S., Bianchi, A., Gouze, E., Becuwe, P., Dauca, M., Netter, P. and Terlain, B. (2002) PPAR-$\gamma$ ligands modulate effects of LPS in stimulated rat synovial fibroblasts. Am. J. Physiol Cell. Physiol. **282**, C125–C133
44 Jones, D. C., Ding, X. and Daynes, R. A. (2002) Nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR$\alpha$) is expressed in resting murine lymphocytes. The PPAR$\alpha$ in T and B lymphocytes is both transactivation and transrepression competent. J. Biol. Chem. **277**, 6838–6845


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