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MOLECULAR TOXICOLOGY

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Novel action of lignans isolated from *Hernandia nymphaeifolia* on Ca^{2+} signaling in human neutrophils

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Abstract The effects of five lignans (epi-aschantin, epi-magnolin, epi-yangambin, deoxypodophyllotoxin, yatein) isolated from *Hernandia nymphaeifolia* (Presl.) Kubitzki (Hernandiaceae) on intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in human neutrophils were investigated by using fura-2 as a fluorescent probe. In both Ca^{2+}-containing and Ca^{2+}-free media, the lignans (50–100 μM) did not alter basal $[Ca^{2+}]_i$ but inhibited the $[Ca^{2+}]_i$ increase induced by platelet activating factor (PAF, 10 μM), leukotriene B_4 (LTB_4, 0.2 μM), and thapsigargin (1 μM) to different extents. In Ca^{2+}-free medium, after depleting stores of Ca^{2+} with PAF, LTB_4 or thapsigargin, addition of 3 mM Ca^{2+} induced Ca^{2+} influx. Each of the lignans (50–100 μM) caused 39–89% inhibition of PAF-induced Ca^{2+} influx; whereas only epi-aschantin was able to inhibit LTB_4- and thapsigargin-induced Ca^{2+} influx by 54–79%. Together, the results suggest that in human neutrophils, these lignans did not alter basal $[Ca^{2+}]_i$ but inhibited Ca^{2+} movement induced by Ca^{2+} mobilizing agents.

Keywords Calcium mobilization · Calcium signaling · Lignans · Neutrophils · Human

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Introduction

A transient increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is a central signal for numerous biological events in essentially all cell systems (Clapham 1995; Berridge 1997). The $[Ca^{2+}]_i$ increase is often contributed to by Ca^{2+} entry from extracellular medium and/or Ca^{2+} release from intracellular pools. In non-excitable cells such as neutrophils, one of the primary Ca^{2+} stores is the inositol 1,4,5-triphosphate (IP_3)-sensitive Ca^{2+} pool (Chen and Jan 2001). IP_3 binding to the IP_3 receptors activates Ca^{2+} release from these pools. This discharge of intracellular Ca^{2+} often leads to Ca^{2+} influx via store-operated Ca^{2+} entry (Putney 1986).

Many lignans isolated from herbs have been shown to possess anti-tumor (Wittmann 1976; Gordaliza et al. 2000), anti-oxidant (Ng et al. 2000), anti-estrogenic (Adlercreutz et al. 1987; Haggans et al. 1999), anti-mitotic (Rimando et al. 1999), anti-viral (Craigo et al. 2000; Cyong et al. 2000; Serradji et al. 2000) and anti-platelet activating factor (PAF) (Shen et al.1985; Shen 1991) properties, making them useful for developing new drugs (Adlercreutz et al. 1995). How these lignans work is unclear. The effect of plant lignans on Ca^{2+} handling

in neutrophils has never been explored except that gomisin C (a lignan from *Schizandra chinensis*) was shown to reduce formyl-Met-Leu-Phe (fMLP)-induced $[Ca^{2+}]_i$ increase in human neutrophils (Wang et al. 1994).

Five lignans (epi-aschantin, epi-magnolin, epi-yangambin, deoxypodophyllotoxin and yatein), isolated from *Hernandia nymphaeifolia* (Presl.) Kubitzki (Hernandiaceae) (Fig. 1), have been shown to inhibit platelet aggregation (Chen et al. 2000). Deoxypodophyllotoxin and yatein are cytotoxic to several cancer cell types (Wittmann 1976; Wickramaratne et al. 1995; Chen et al. 1996; Ikeda et al. 1998; Lim et al. 1999). It was shown that deoxypodophyllotoxin inhibits 12-*O*-tetradecanoylphorbol 13-acetate-induced ornithine decarboxylase in epidermal cells (Chang et al. 2000). Other pharmacological effects of these five lignans remain unexplored.

In this study it is shown that these lignans alter Ca^{2+} signaling in human neutrophils in multiple ways. The effect of these lignans on basal $[Ca^{2+}]_i$ and on the Ca^{2+} influx and Ca^{2+} release processes induced by IP_3-dependent physiological agonists such as PAF, leukotriene B_4 (LTB_4), and the IP_3-independent pharmacological agent thapsigargin (an inhibitor of the endoplasmic reticulum Ca^{2+} pump) (Thastrup et al. 1987) were investigated. In neutrophils, PAF has been shown to cause increased $[Ca^{2+}]_i$ by activating PAF receptors leading to Ca^{2+} store release and Ca^{2+} influx (Hauser et al. 2001), whereas LTB_4 has been shown to increase $[Ca^{2+}]_i$ by stimulating LTB_4 receptors (Seya et al. 1988; Powell et al. 1996). Thapsigargin has been shown to release stored Ca^{2+} in a manner independent of cytosolic IP_3 levels (Jan et al. 1999).

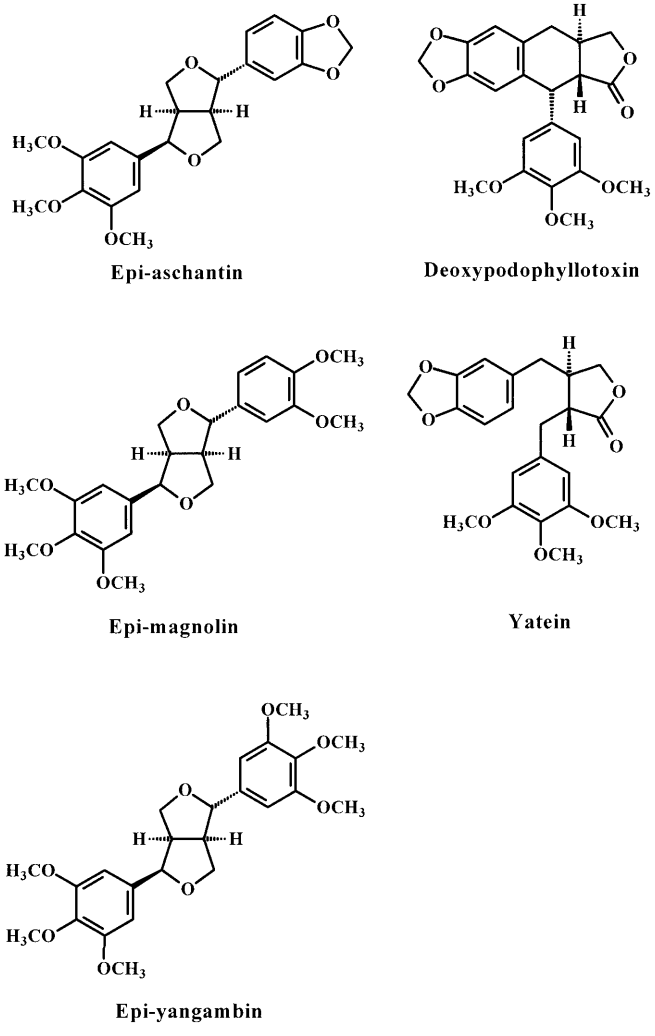

Fig. 1 Chemical structures of the five lignans

Materials and methods

Neutrophil isolation

After obtaining informed consent, whole blood was taken by venous puncture from healthy human volunteers with no history of infections 2 weeks before the experiments. All procedures were performed in accordance with protocols approved by the Institutional Committee on Clinical Research. Neutrophils were isolated as previously described (Chen and Jan 2001). Blood was mixed with heparin (20 U/ml), and erythrocytes were allowed to sediment for 50 min at room temperature following preparation of a 1:6 (v/v) Hespan:blood blend. The leukocyte-rich plasma was harvested and centrifuged at 300 *g* for 20 min. The supernatant was aspirated and centrifuged at 2170 *g* for 15 min to produce platelet-poor plasma. The pellet obtained from the centrifugation of the leukocyte-rich plasma was resuspended in 2.5 ml platelet-poor plasma and transferred to a 15-ml tube, where it was under-layered with 2 ml freshly prepared 42% Percoll in platelet-poor plasma. The mixture was in turn under-layered with 2 ml of freshly prepared 52% Percoll in platelet-poor plasma. The gradients were centrifuged for 10 min at 280 *g*. Neutrophils were collected at the 42–52% Percoll interface. The final cell population was determined to contain >95% neutrophils by Wright's staining.

Solutions

The composition of Ca^{2+}-containing medium was 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose; the pH value was adjusted to 7.4 with 1 N NaOH. Ca^{2+}-free medium had a similar composition except that Ca^{2+} was substituted with 1 mM EGTA. Lignans were dissolved in dimethyl sulfoxide (DMSO) and kept at –20°C as a 0.1 M stock. The stock solutions were diluted to the final concentration before experiments. Other drugs were dissolved in water, ethanol or DMSO. Organic solvents in the experimental solution were kept below 1% and they were found to have no effect on $[Ca^{2+}]_i$ ($n=4$).

Fura-2 loading

Neutrophils were diluted in Tyrode solution (pH 7.4) which contained 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, and 5 mM HEPES. Neutrophils were incubated with 2 μM of the membrane-permeable acetoxymethyl ester form of fura-2 for 20 min at 37°C, and were subsequently centrifuged at 300 *g* at room temperature. This was followed by resuspension twice in Tyrode solution to make a final cell suspension of a concentration of 2×10^6 cells/ml before $[Ca^{2+}]_i$ measurements were made.

Fura-2 measurements

The measurement was performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of

medium and 0.2×10^6 cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan) by recording excitation signals of 340 nm and 380 nm and emission signal of 510 nm at 1-s intervals. Experiments were started by adding 0.1 ml cell suspension into 0.9 ml well-stirred medium in the cuvette. Drugs were applied during the experiment by pausing the fluorescence recording. Lignans were dissolved in DMSO. The other drugs were dissolved in DMSO, ethanol or water. The final concentration of the vehicle DMSO in the neutrophil suspension was fixed at 1%, which did not affect $[Ca^{2+}]_i$ ($n=3$). Maximum and minimum fluorescence values were obtained by adding 10 μM digitonin (plus 10 mM $CaCl_2$) and 20 mM EGTA sequentially at the end of each experiment, respectively. $[Ca^{2+}]_i$ was calculated as previously described assuming a K_d of 155 nM (Grynkiewicz et al. 1985).

Chemical reagents

Fura-2-acetoxymethyl was from Molecular Probes (Eugene, Ore., USA). Lignans were extracted from *Hernandia nymphaeifolia* (Presl.) Kubitzki (Hernandiaceae) as previously described (Chen et al. 1996). Briefly, dried trunk bark was powdered and extracted with methanol. The extract was subsequently concentrated under reduced pressure and partitioned between water-chloroform (1:1) leading to a chloroform-soluble fraction. This fraction was further extracted with 2% sulfuric acid. The acid-insoluble part was dried with magnesium sulfate and concentrated to give a neutral chloroform-soluble fraction. A part of the neutral chloroform-soluble fraction was chromatographed on silica gel eluting with chloroform-methanol (100:0 to 0:10) to produce 17 fractions. A part of fractions 5 and 7 were further purified by preparative thin layer chromatography to obtain the five lignans. The lignans were identified using spectroscopic methods: ultraviolet spectrophotometer (Shimadzu UV-160A), infrared spectrophotometer (Hitachi 260–30; Tokyo, Japan), high resolution-mass spectrometer (JEOL JMX-HX 110; Jeol, Inc. Peabody, Mass., USA) and ^{1}H-nuclear magnetic resonance spectrometer (JEOL GSX-400). The purity of the lignans was found to be >99%. Other reagents were from Sigma (St. Louis, Mo., USA).

Statistical analysis

Data were mean ±SEM of four to six replicates. Statistical comparisons were made using Student's *t*-test, and significance was accepted when $P<0.05$.

Results

It was shown previously that each of these five lignans inhibited PAF-induced aggregation of rabbit platelets (Chen et al. 2000), implicating possible inhibition by the lignans of PAF receptor-mediated $[Ca^{2+}]_i$ increase. Figure 2A (trace a) shows that in Ca^{2+}-containing medium PAF (10 μM) induced an immediate $[Ca^{2+}]_i$ increase that reached a net (baseline subtracted) maximum value of 168 ± 4 nM ($n=6$). Addition of 100 μM epi-aschantin for 30 s did not alter basal $[Ca^{2+}]_i$ ($n=6$). Subsequent addition of PAF (10 μM) induced a $[Ca^{2+}]_i$ increase with a net maximum of 65 ± 4 nM, which was 38 ± 3% of control (Fig. 2A, trace *b*; $P<0.05$). Figure 2B shows that each of the lignans examined inhibited the net maximum PAF-induced $[Ca^{2+}]_i$ increase by 40–62% ($n=4$–6, $P<0.05$).

Because PAF is known to increase $[Ca^{2+}]_i$ by releasing Ca^{2+} from internal stores and by causing Ca^{2+} influx, experiments were performed separately to examine the effect of lignans on these two pathways. Figure 2C (trace a) shows that in Ca^{2+}-free medium PAF (10 μM) induced a $[Ca^{2+}]_i$ increase with a net maximum of 44 ± 3 nM ($n=5$). After reaching the maximum, the $[Ca^{2+}]_i$ response slowly decayed to pre-stimulatory baseline in 150 s. Adding epi-aschantin (100 μM) 30 s prior to PAF (10 μM) significantly reduced the increase in $[Ca^{2+}]_i$ (Fig. 2C, trace b vs trace a; $n=5$, $P<0.05$). Figure 2D shows that the lignans epi-yangambin, deoxypodophyllotoxin, epi-magnolin, epi-aschantin and yatein inhibited the PAF response by 58 ± 3, 73 ± 3, 73 ± 2, 70 ± 4 and 70 ± 3%, respectively, in terms of the net maximum $[Ca^{2+}]_i$ ($n=6$, $P<0.05$).

The effect of lignans on PAF-induced store-operated Ca^{2+} entry was examined. Figure 2E (trace a) shows that in Ca^{2+}-free medium, after the $[Ca^{2+}]_i$ increase induced by PAF (10 μM) had returned to baseline, addition of 3 mM $CaCl_2$ (at 250 s) induced a $[Ca^{2+}]_i$ increase with a net maximum of 146 ± 3 nM ($n=5$). Conversely, adding Ca^{2+} without PAF pretreatment only induced a $[Ca^{2+}]_i$ increase with a net value of 16 ± 2 nM (Fig. 2E, trace c; $n=5$). This suggests that PAF may induce store-operated Ca^{2+} entry, although PAF might also produce identical results if it directly caused Ca^{2+} influx in a manner independent of Ca^{2+} store depletion. When epi-aschantin (100 μM) was added 20 s prior to Ca^{2+}, the Ca^{2+}-induced $[Ca^{2+}]_i$ increase had a net maximum of 51 ± 3 nM (Fig. 2E, trace b; $n=5$). The other lignans also inhibited PAF-induced Ca^{2+} influx. Fig. 2F shows that epi-yangambin, deoxypodophyllotoxin, epi-magnolin, epi-aschantin and yatein inhibited PAF-induced Ca^{2+} influx by 37 ± 3, 38 ± 3, 35 ± 4, 65 ± 2 and 20 ± 5%, respectively ($n=5$–6, $P<0.05$).

To examine whether the lignans' inhibitory action was selective for PAF, the effect of lignans (100 μM epi-yangambin, epi-magnolin or epi-aschantin; 50 μM deoxypodophyllotoxin or yatein) on the $[Ca^{2+}]_i$ increase induced by another endogenous agonist, LTB_4, was explored. Figure 3A (trace a) shows that in Ca^{2+}-containing medium LTB_4 (0.2 μM) induced an immediate $[Ca^{2+}]_i$ increase with a net maximum of 54 ± 3 nM ($n=5$). The maximum $[Ca^{2+}]_i$ was followed by a gradual decay. In the presence of epi-aschantin (100 μM), LTB_4-induced $[Ca^{2+}]_i$ increase had a net maximum of 12 ± 2 nM (Fig. 3A, trace b; $n=5$). All the other lignans inhibited LTB_4-induced $[Ca^{2+}]_i$ increase, and the results are summarized in Fig. 3B. Epi-yangambin, deoxypodophyllotoxin, epi-magnolin, epi-aschantin and yatein inhibited the LTB_4 response by 70 ± 3, 75 ± 3, 64 ± 3, 78 ± 2 and 48 ± 4%, respectively ($n=6$, $P<0.05$).

Efforts were made to explore the effect of lignans on LTB_4-induced Ca^{2+} release and Ca^{2+} influx. Figure 3C (trace a) shows that in Ca^{2+}-free medium LTB_4 (0.2 μM) induced a $[Ca^{2+}]_i$ increase with a net maximum of 41 ± 3 nM ($n=5$). The maximum $[Ca^{2+}]_i$ was

followed by a gradual decay to near baseline in 120 s. In the presence of epi-aschantin (100 μM), LTB_4 induced a smaller $[Ca^{2+}]_i$ increase with a net maximum $[Ca^{2+}]_i$ of 17 ± 3 nM (Fig. 3C, trace b; $n = 5$, $P < 0.05$). Figure 3D shows that epi-yangambin, deoxypodophyllotoxin, epi-magnolin, epi-aschantin and yatein inhibited the LTB_4 response by 66 ± 4, 73 ± 3, 67 ± 3, 58 ± 3 and $53 \pm 4\%$, respectively ($n = 6$, $P < 0.05$). Effect of lignans on LTB_4-induced Ca^{2+} influx was evaluated. Figure 3E shows that in Ca^{2+}-free medium, after the LTB_4-induced $[Ca^{2+}]_i$ increase had returned to baseline (time-point of 280 s), adding 3 mM $CaCl_2$ induced a $[Ca^{2+}]_i$ increase with a net maximum of 54 ± 3 nM (Fig. 3E, trace a; $n = 5$). Adding Ca^{2+} without LTB_4 pretreatment only induced a $[Ca^{2+}]_i$ increase with a net value of 20 ± 2 nM (Fig. 3E, trace c; $n = 5$). This suggests that LTB_4 induced Ca^{2+} influx. When epi-aschantin (100 μM) was added 20 s prior to Ca^{2+}, the LTB_4-induced Ca^{2+} influx gave a net $[Ca^{2+}]_i$ increase of 36 ± 4 nM (Fig. 3E, trace b; $n = 5$). Figure 3F summarizes the effects of these lignans. Epi-yangambin, deoxypodophyllotoxin, epi-magnolin and yatein did not inhibit LTB_4-induced Ca^{2+} influx ($n = 5$–6, $P > 0.05$); epi-aschantin (100 μM) produced $53 \pm 3\%$ inhibition ($n = 6$, $P < 0.05$).

Both PAF and LTB_4 are known to utilize IP_3 as a second messenger to release Ca^{2+} from the endoplasmic

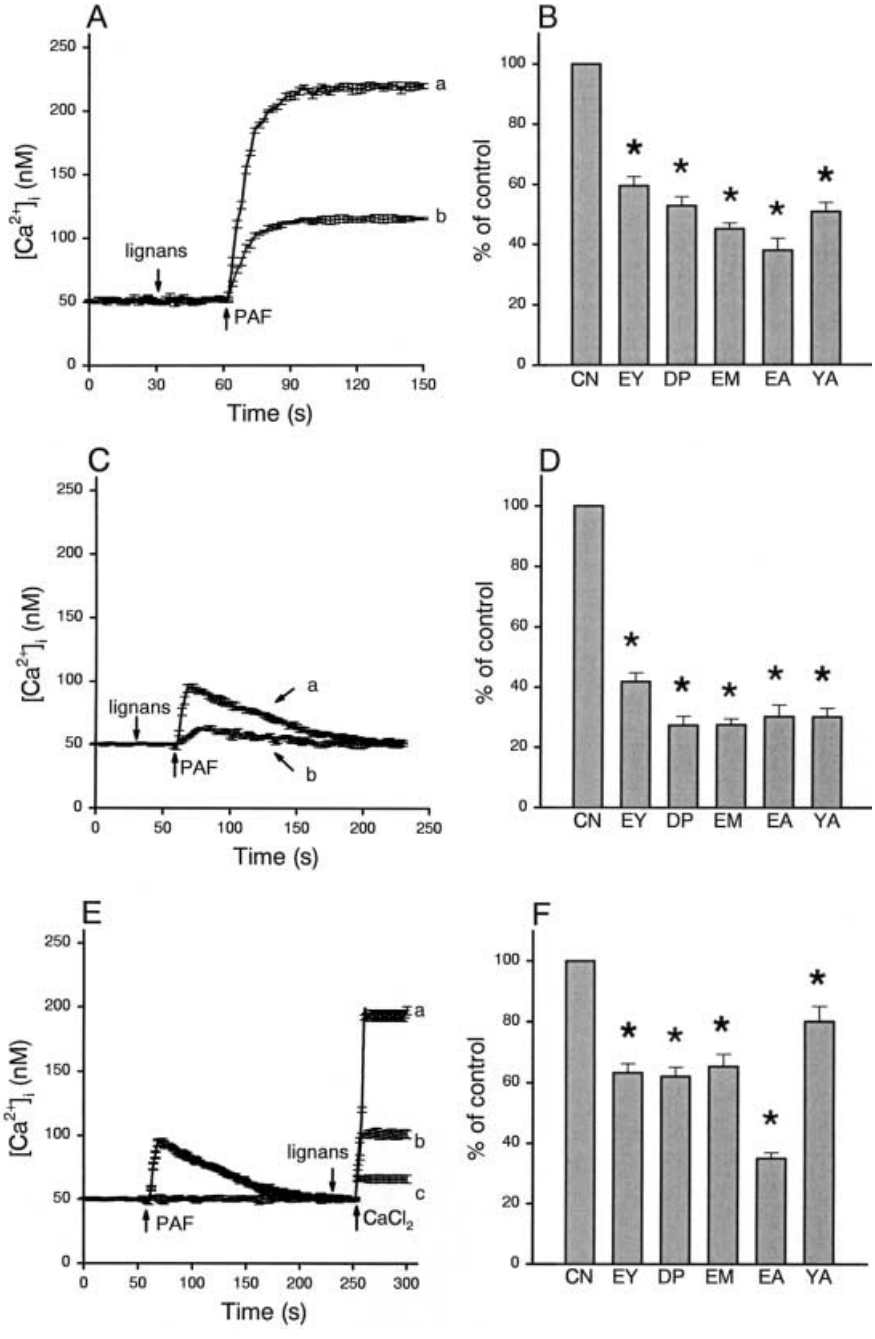

Fig. 2A–F Effect of lignans on PAF-induced $[Ca^{2+}]_i$ increase in human neutrophils. The final concentration was 100 μM for epi-yangambin (*EY*), epi-magnolin (*EM*) and epi-aschantin (*EA*), and was 50 μM for deoxypodophyllotoxin (*DP*) and yatein (*YA*). **A** In Ca^{2+}-containing medium, the lignan was added at 30 s followed by 10 μM PAF added at 60 s (*trace a* control, *trace b* epi-aschantin). **B** The inhibition of PAF-induced $[Ca^{2+}]_i$ increase by lignans, expressed as percentage of control (*CN*), which was defined as the net (baseline subtracted) maximum of 10 μM PAF-induced $[Ca^{2+}]_i$ increase. **C** In Ca^{2+}-free medium, the lignan was added at 30 s followed by 10 μM PAF (*trace a* control, *trace b* epi-aschantin). **D** The inhibitory effect of lignans on PAF-induced $[Ca^{2+}]_i$ increases. **E** In Ca^{2+}-free medium, 10 μM PAF was added at 60 s followed by the lignan at 230 s and 3 mM Ca^{2+} at 250 s (*trace a* PAF + Ca^{2+}, *trace b* PAF + epi-aschantin + Ca^{2+}, *trace c* Ca^{2+} alone). **F** The inhibitory effect of lignans on PAF-induced Ca^{2+} influx. Data in **B**, **D**, **F** are mean +SEM of four to six replicates. *$P < 0.05$, relative to control

reticulum (Fiorucci et al. 1995; Hauser et al. 2001) Thus, by using thapsigargin as a tool, the following experiments were aimed at investigating whether the lignans could alter IP_3-independent Ca^{2+} release.

Figure 4A shows that in Ca^{2+}-free medium, thapsigargin (1 μM) induced a $[Ca^{2+}]_i$ increase with a net maximum of 88 ± 3 nM (Fig. 4A, trace a; $n = 5$). The maximum $[Ca^{2+}]_i$ was followed by a slow decay. In the presence of epi-aschantin (100 μM), the thapsigargin-induced $[Ca^{2+}]_i$ increase had a net maximum of 24 ± 3 nM (Fig. 4A, trace b; $n = 5$). The effects of the five lignans are summarized in Fig. 4B. Epi-yangambin (100 μM), deoxypodophyllotoxin (50 μM), epi-magnolin (100 μM), epi-aschantin (100 μM), and yatein (50 μM) inhibited the thapsigargin response by 55 ± 2, 67 ± 3, 57 ± 4, 73 ± 2 and $64 \pm 3\%$, respectively ($n = 6$, $P < 0.05$).

The effect of lignans on thapsigargin-induced store-operated Ca^{2+} entry was explored. Figure 4C (trace a) shows that in Ca^{2+}-free medium, after the 1 μM thapsigargin-induced $[Ca^{2+}]_i$ increase had returned to baseline, 3 mM $CaCl_2$ added at 280 s induced a $[Ca^{2+}]_i$ increase with a net maximum of 158 ± 5 nM ($n = 5$). Adding Ca^{2+} without thapsigargin pretreatment induced a net $[Ca^{2+}]_i$ increase of 19 ± 2 nM (Fig. 4C, trace c; $n = 5$). This suggests that thapsigargin induced Ca^{2+}

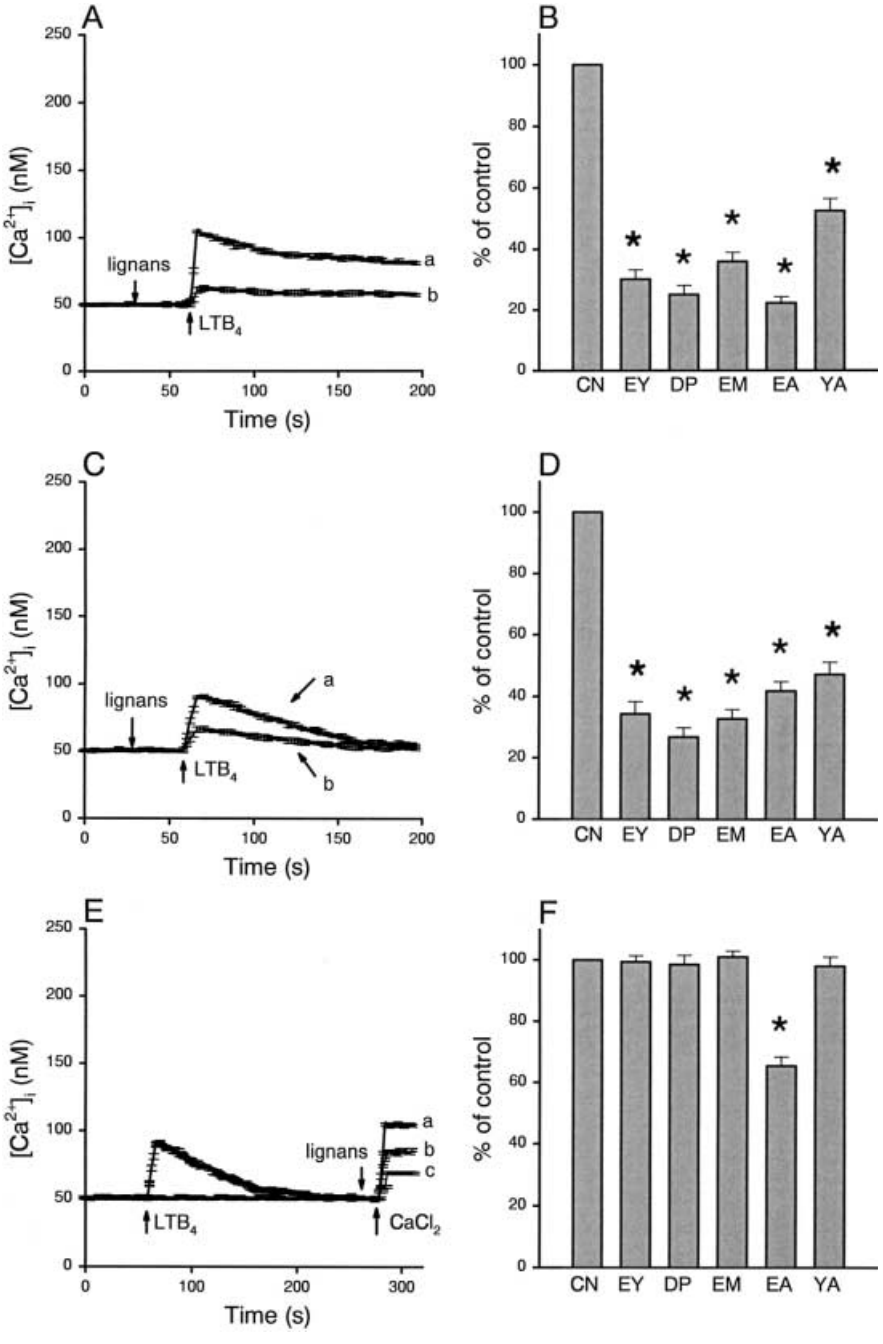

Fig. 3A–F Effect of lignans on LTB_4-induced $[Ca^{2+}]_i$ increase in human neutrophils. The final concentration was 100 μM for epi-yangambin (*EY*), epi-magnolin (*EM*), and epi-aschantin (*EA*), and was 50 μM for deoxypodophyllotoxin (*DP*) and yatein (*YA*). **A** In Ca^{2+}-containing medium, the lignan was added at 30 s followed by LTB_4 (0.2 μM) at 60 s (*trace a* control, *trace b* epi-aschantin). **B** The inhibitory effect of lignans on LTB_4-induced $[Ca^{2+}]_i$ increase, expressed as percentage of control (*CN*), which was defined as the net (baseline subtracted) maximum of 0.2 μM LTB_4-induced $[Ca^{2+}]_i$ increase. **C** In Ca^{2+}-free medium, the lignan was added at 30 s followed by 0.2 μM LTB_4 added at 60 s (*trace a* control, *trace b* epi-aschantin). **D** The inhibitory effect of lignans on LTB_4-induced $[Ca^{2+}]_i$ increase. **E** In Ca^{2+}-free medium, 0.2 μM LTB_4 was added at 60 s followed by the lignan at 260 s and 3 mM Ca^{2+} at 280 s (*trace a* LTB_4 + Ca^{2+}, *trace b* LTB_4 + epi-aschantin + Ca^{2+}, *trace c* Ca^{2+} alone). **F** The inhibitory effect of lignans on LTB_4-induced Ca^{2+} influx. Data in **B**, **D**, **F** are mean + SEM of four to six replicates. $^*P < 0.05$, relative to control

influx. Figure 4C (trace b) shows that when epi-aschantin (100 μM) was added 20 s prior to Ca^{2+}, the thapsigargin-induced Ca^{2+} influx had a net $[Ca^{2+}]_i$ increase of 123 ± 3 nM ($n = 5$), which was $75 \pm 3\%$ of control ($P < 0.05$). The other lignans did not inhibit thapsigargin-induced Ca^{2+} influx ($n = 5$–6, $P > 0.05$).

Discussion

This study provides the first evidence that the plant-derived lignans epi-aschantin, epi-magnolin, epi-yangambin, deoxypodophyllotoxin and yatein can alter Ca^{2+} signaling in human neutrophils in multiple ways. Lignans from different sources and with different structures may have different effects on Ca^{2+} signaling. The mammalian lignan 2,3-dibenzylbutane-1,4-diol was shown to augment fMLP-induced $[Ca^{2+}]_i$ increase in human neutrophils (Morikawa et al. 1990), whereas gomisin C, a benzocyclooctadiene lignan from the plant *Schizandra chinensis*, can inhibit fMLP-induced $[Ca^{2+}]_i$ increase in rat neutrophils (Wang et al. 1994).

The data from the present study show that the lignans examined inhibited PAF-induced $[Ca^{2+}]_i$ increase by 40–60% at concentrations of 50–100 μM. This concentration range was chosen because over this range the lignans have been demonstrated to inhibit platelet aggregation (Chen et al. 2000). The mechanism underlying PAF-induced $[Ca^{2+}]_i$ increase in human neutrophils has been explored previously. PAF was shown to increase $[Ca^{2+}]_i$ by releasing Ca^{2+} from internal stores (Nowak et al. 1996; Bialasiewicz et al. 1997) and causing Ca^{2+} influx. PAF-mediated Ca^{2+} influx was shown to occur via two separate pathways: store-operated Ca^{2+} entry (Hauser et al. 2001) and a route that precedes Ca^{2+} release (Bouchelouche et al. 1995). Our results suggest that the lignans inhibited both PAF-induced Ca^{2+} store release and Ca^{2+} influx. However, it was unclear whether the lignans exerted these effects by directly altering Ca^{2+} movement or by antagonizing PAF receptors and the other upstream events such as receptor–G protein coupling and IP_3 formation. Thus, the effect of the lignans on another IP_3-dependent endogenous agonist LTB_4 was examined. LTB_4 has been shown to increase $[Ca^{2+}]_i$ in human neutrophils by stimulating LTB_4 receptors, leading to an increase in IP_3 levels and subsequent Ca^{2+} release and Ca^{2+} influx (Bouchelouche et al. 1995; Hauser et al. 2000). The data show that each of the lignans inhibited LTB_4-induced $[Ca^{2+}]_i$ increase by 50–80%. In contrast to the effect on PAF, the lignans inhibited LTB_4-induced $[Ca^{2+}]_i$ increase by reducing Ca^{2+} release without altering Ca^{2+} influx (except epi-aschantin; discussed later). The finding that the lignans inhibited both PAF- and LTB_4-induced

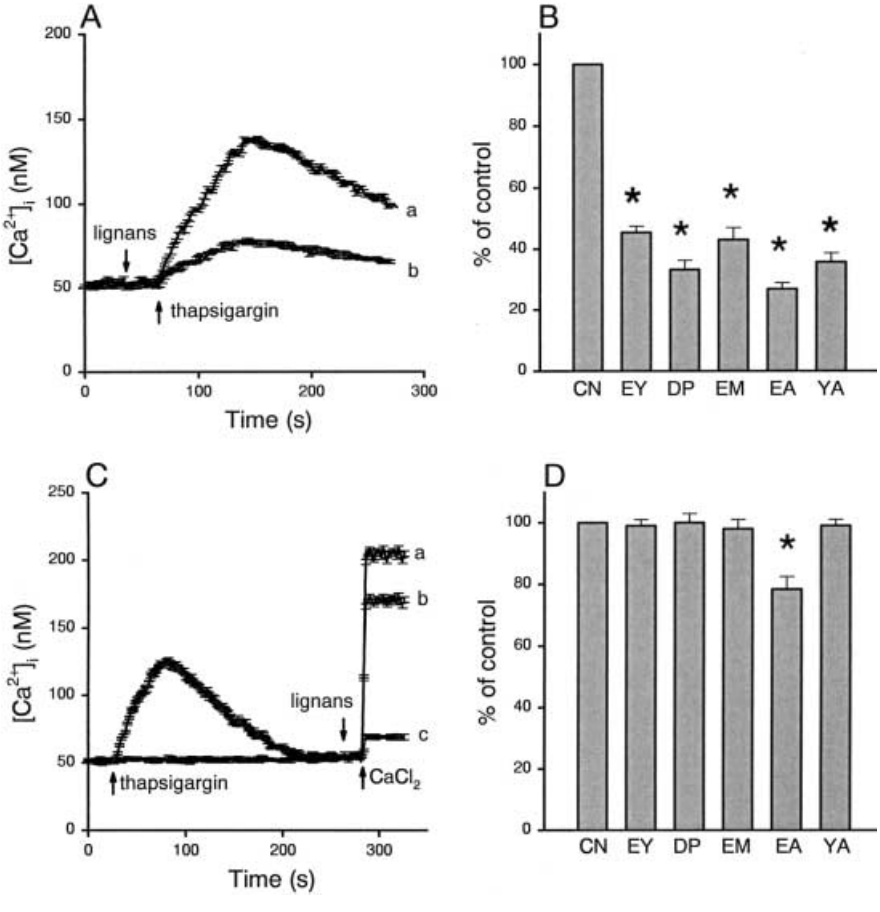

Fig. 4A–D Effect of lignans on thapsigargin-induced $[Ca^{2+}]_i$ increase in human neutrophils. The final concentration was 100 μM for epi-yangambin (*EY*), epi-magnolin (*EM*) and epi-aschantin (*EA*), and was 50 μM for deoxypodophyllotoxin (*DP*) and yatein (*YA*). **A** In Ca^{2+}-free medium, the lignan was added at 30 s followed by 1 μM thapsigargin at 60 s. **B** The inhibitory effect of lignans on thapsigargin-induced $[Ca^{2+}]_i$ increase, expressed as percentage of control (*CN*), which was defined as the net (baseline subtracted) maximum of 1 μM thapsigargin-induced $[Ca^{2+}]_i$ increase. **C** In Ca^{2+}-free medium, 1 μM thapsigargin was added at 10 s followed by the lignan at 260 s and 3 mM Ca^{2+} at 280 s (*trace a* thapsigargin + Ca^{2+}, *trace b* thapsigargin + epi-aschantin + Ca^{2+}, *trace c* Ca^{2+} alone). **D** The inhibitory effect of lignans on thapsigargin-induced Ca^{2+} influx. Data in **B** and **D** are mean + SEM of four to six replicates. $^*P < 0.05$, relative to control

Ca^{2+} release suggests that the lignans may antagonize both PAF and LTB_4 receptors or directly inhibit IP_3-dependent Ca^{2+} release independent of the receptor type. The latter speculation is more plausible because the lignans also inhibited Ca^{2+} release induced by 10 μM ATP without inhibiting ATP-induced Ca^{2+} influx (data not shown), an effect very similar to that on LTB_4-induced $[Ca^{2+}]_i$ increase.

It is interesting that the lignans (except epi-aschantin) inhibited Ca^{2+} influx induced by PAF, but not that induced by LTB_4. One interpretation is that human neutrophils possess more than one type of Ca^{2+} influx route. One of the routes is induced by Ca^{2+} release, as in cases of LTB_4- and thapsigargin-induced Ca^{2+} release. Another route occurs before Ca^{2+} release, and is opened by PAF but not by LTB_4 or thapsigargin. Given the finding that the lignans (except epi-aschantin) did not affect the Ca^{2+} influx induced by either LTB_4 or thapsigargin, the lignans may exert the differential effects by inhibiting the PAF-sensitive, Ca^{2+} release-independent Ca^{2+} influx pathway without affecting store-operated Ca^{2+} entry.

Not only did the lignans alter Ca^{2+} influx, they also affected Ca^{2+} release. The data show that each of the five lignans inhibited PAF- and LTB_4-induced Ca^{2+} release from IP_3-sensitive endoplasmic reticulum stores. How the lignans exert this effect is unclear but it is more likely that they act in an IP_3-independent manner because they also inhibited the Ca^{2+} release induced by thapsigargin, a Ca^{2+} mobilizer that does not require a preceding increase in cytosolic IP_3 levels.

Epi-aschantin behaved differently from the other four lignans in that it inhibited LTB_4- and thapsigargin-induced Ca^{2+} influx by 20–35%, whereas the other lignans did not. Furthermore, epi-aschantin reduced PAF-induced Ca^{2+} influx by 65%, while the reduction with the other four lignans was only 20–40%. This suggests that the other four lignans can inhibit PAF-induced, Ca^{2+} release-independent Ca^{2+} influx, but not LTB_4- and thapsigargin-induced store-operated Ca^{2+} entry. In contrast, epi-aschantin can inhibit both Ca^{2+} influx pathways. There appears to be a structure-activity relationship in the differential effect of epi-aschantin. A dramatic structural difference among the five lignans is that epi-aschantin has two characteristics, namely a methylenedioxy substitution on the benzene ring and a tetrahydrofurofuran moiety, whereas the other four lignans have only one of the two characteristics.

The results suggest that the lignans have multiple effects on Ca^{2+} signaling in human neutrophils. They inhibited the $[Ca^{2+}]_i$ increase induced by PAF, LTB_4, and thapsigargin via different mechanisms. Altered Ca^{2+} movement is important in neutrophil dysfunction, and thus the possible pharmacological and toxicological effects of the plant-derived lignans on immune and inflammatory responses deserve further investigation.

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