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Erucic Acid and Erucic Acid Anilide-induced Oxidative Burst in Human Polymorphonuclear Leukocytes

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Human polymorphonuclear leukocytes (PMNL) were exposed to erucic acid or erucic acid anilide to explore their effects on the production of reactive oxygen species (ROS) and the levels of free intracellular calcium. The compounds did not change the levels of intracellular calcium, but both dose-dependently induced respiratory burst in PMNL. Maximal production of ROS by erucic acid exceeded that induced by its anilide 13-fold. A protein kinase C inhibitor, Ro 31-8220, completely inhibited erucic acid and erucic acid anilide-induced production of ROS. Neither erucic acid nor erucic acid anilide modified FMLP-induced production of ROS. However, erucic acid (500 μM) amplified 5 nM PMA-induced ROS production 1.8-fold, but did not have this effect at a lower PMA concentration. On the contrary, erucic acid anilide inhibited PMA-induced oxidative burst, and shifted the peak ROS production induced by PMA to a later time-point. The present results show that aniline moiety modifies the effects of erucic acid on the activation of PMNL, and suggest that both erucic acid and erucic acid anilide may activate PMNL through a protein kinase C-dependent mechanism.

Keywords: Toxic Oil Syndrome, erucic acid, erucic acid anilide, reactive oxygen species, polymorphonuclear leukocytes, protein kinase C

INTRODUCTION

An epidemic of a multisystemic disease known as the toxic oil syndrome (TOS) occurred in Spain in 1981.[1] The outbreak of the epidemic was related to the ingestion of contaminated rapeseed oils. However, until now, the etiologic agent of the TOS has not been identified despite extensive epidemiologic and toxicologic research in recent years.[2–6] Toxic case oils, denaturated with 2% aniline for industrial purposes and afterwards refined to remove aniline, contained variety of adulterants, mainly derivatives of aniline and components of rapeseed oil.[7]

The pathogenesis of the TOS has not been clarified, but clinical features during the course of disease, serological studies and experimental animal and *in vitro* studies suggest that an inflammatory, and possibly an autoimmune component may have been involved in the TOS.[9–12] In previous studies, we have explored effects of different

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toxic oil contaminants on the activation of human polymorphonuclear leukocytes (PMNL).[13–16] Although PMNL play an important role in the defense against invading microorganisms, reactive oxygen species (ROS) released from activated PMNL may also cause tissue injury associated with inflammatory diseases.[17,18]

When rape seed oils containing >40% erucic acid, 22:1n-9, were given to mice, rats, pigs or monkeys, they induced cardiotoxic lesions in the exposed animals. In fact, erucic acid has been shown to become incorporated into the liver, heart, and kidney lipids of rats and hamsters.[19,20] Other studies, however, have suggested that dietary erucic acid therapy may even prevent neurological symptoms in adrenoleukodystrophy patients, but also contradictory results have been reported.[21–23]

Several studies have shown that fatty acids and their anilides may induce or modulate oxidative burst in PMNL.[13–16] Oxidative burst in PMNL involves superoxide production and subsequent formation of ROS which may be induced by receptor agonists or direct PKC activators such as phorbol esters.[24] In fact, PKC activates and phosphorylates a multicomponent respiratory burst enzyme, NADPH oxidase, which catalyzes the reduction of oxygen to superoxide anion.[25]

In this study, we have examined the effects of erucic acid and its anilide on the levels of free intracellular calcium and the production of ROS in human PMNL. Also, the effects of erucic acid and erucic acid anilide on agonist-stimulated cellular responses were studied. A protein kinase C inhibitor attenuated erucic acid and its anilide induced activation of production of ROS suggesting that erucic acid and its anilide may induce the production of ROS in PMNL by activating protein kinase C.

MATERIALS AND METHODS

Materials

Formyl-methionyl-leucyl-phenylalanine (FMLP), phorbol myristate acetate (PMA), and erucic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Luminol was supplied by Bio Orbit (Turku, Finland). Ficoll-Paque was obtained from Pharmacia (Uppsala, Sweden). Erucic acid anilide was supplied to our laboratory as coded samples, and the compound was identified after the end of the studies to be erucic acid anilide. It was obtained from Professor Angel Messeguer, Barcelona, Spain, in the context of the International Research Program on the Toxic Oil Syndrome of the European Office of the World Health Organization and Fondo dé Investigación Sanitaria (Spain).

Isolation of Human Polymorphonuclear Leukocytes (PMNL)

Human PMNL were isolated from heparinized human venous blood from healthy adult donors by Ficoll-density centrifugation according to a method described earlier in detail.[26] The lysis of erythrocytes was carried out with an isotonic NH_4Cl solution.[27]

Viability Studies

The viability of the isolated cells was evaluated by trypan blue exclusion test. The cell viability always exceeded 95%. Viability of the cells was evaluated after each experiment.

Measurement of Changes in Free Intracellular Calcium ($[Ca^{2+}]_i$) Concentration

PMNL were loaded with fura-2/AM (3 μM) for 45 min at 37°C, and then washed and resuspended in Hanks' balanced salt solution (HBSS) (without Ca^{2+} and Mg^{2+}). The fluorescence of the intracellular fura-2 was recorded with a Hitachi F-4000 fluorometer at an excitation wavelength of 340 nM and an emission wavelength of 510 nM. Incubations were carried out at 37°C in a magnetically stirred cell holder. One milliliter of prewarmed HBSS and 0.9–0.99 ml of the cell suspension (3×10^6 cells) were placed into the

cuvette with a final cell concentration of 1.5×10^6 cells/ml. $CaCl_2$ was added directly into the cuvette 5 min prior to the measurement to give a final Ca^{2+} concentration of 1.2 mM. The fura-2 signal was corrected for extracellular dye by quenching it with 20 μM Mn^{2+}. After that, the cells were stimulated with erucic acid (1.2 and 12 μM) or erucic acid anilide (2.4 and 24 μM). Relative minimal fluorescence of fura-2 was determined after adding 7.5 mM ethylene glycol-bis(β-aminoethyl ether)N, N, N′, N′-tetra-acetic acid (EGTA) after the cell lysis, and maximal relative fluorescence after adding 7 mM of Ca^{2+}. Diethylenetriamine penta-acetic acid (13 μM) was used in the calibration of the signal in order to confirm the chelation of added Mn^{2+}. Changes in $[Ca^{2+}]_i$ were calculated according to the following equation.[28]

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F),$$

where $K_d = 224$ nM, F = relative level of intracellular fluorescence, F_{min} = the relative level of fura-2 fluorescence in 7.5 mM EGTA after cell lysis with 10% sodium dodecyl sulfate (SDS), and F_{max} = the relative level of fluorescence with 7 mM of Ca^{2+} added after the determination of F_{min}. Each measurement was done three times in duplicate for the calculation of the mean and the standard error of the mean.

Chemiluminescence Assay

Isolated PMNL were washed twice with 0.9 mM $CaCl_2$-0.5 mM $MgCl_2$-5 mM -glucose-phosphate buffered saline (PBS, Gibco, U.K), and thereafter the cell concentration was adjusted to 5×10^6 cells/ml. ROS were measured by applying a luminol-enhanced chemiluminescence assay.[29] The reaction mixture consisted of 700 μl of luminol (0.1 mM) in PBS, 100 μl PBS or FMLP (20 μM), and 10 μl of erucic acid or erucic acid anilide. Interactions of erucic acid with PMA were studied by applying different concentrations of PMA (final concentrations 0.5 or 5 nM of PMA), whereas interactions of erucic acid anilide with PMA were studied with 5 nM of PMA. In the control experiments, dimethylsulfoxide (DMSO), but not erucic acid or erucic acid anilide, was added to the reaction mixture. The final concentration of DMSO was 1% (v/v) in all cases. The pH of the incubation mixture was always between 7 and 8. The reaction was started by adding 100 μl of the cell suspension into the reaction mixture, and light emission was recorded at 2 min intervals with a Bio-Orbit Luminometer 1251 (Bio-Orbit, Turku, Finland), operated by the LKB-Wallac Phagocytosis Program, which was controlled by a computer. All reactions were carried out at 37°C, usually for 35–40 min. The role of protein kinase C in the production of ROS induced by erucic acid or erucic acid anilide was studied by using a protein kinase C inhibitor, Ro 31-8220.[16,30–31] In these experiments, the cells were first preincubated with graded concentrations of Ro 31-8220 at 37°C for 15 min before the addition of erucic acid or erucic acid anilide. All measurements were carried out at least three times in duplicate for the calculation of the mean and the standard error of the mean. The results are expressed as the peak value of the chemiluminescence, or as the per cent (%) of the control value.

Statistics

The data were analyzed with one-way analysis of variance and Duncan's Multiple Range test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Erucic Acid and Erucic Acid Anilide on the Levels of Free Intracellular Calcium

Erucic acid or erucic acid anilide did not have an effect on the levels of free intracellular calcium in PMNL (data not shown).

Effect of Erucic Acid on the Production of Reactive Oxygen Species

Erucic acid induced a dose-dependent production of ROS in human PMNL, with a peak ROS production (707 ± 160 mV) at a concentration of 240 μM (Fig. 1). The chemiluminescence in the control cells was 13 ± 3 mV. Erucic acid-induced production of ROS was rapid and transient; it started immediately after the exposure of the cells to erucic acid, and the maximal production of ROS occurred within 4 min (Fig. 2).

Effect of Erucic Acid Anilide on the Production of Reactive Oxygen Species

Erucic acid anilide increased dose dependently the production of ROS in PMNL, but the magnitude of ROS production was markedly less than that induced by the corresponding fatty acid (Fig. 3). The maximal production of ROS (55 ± 9 mV) occurred at a concentration of 240 M of erucic acid anilide. At this concentration, erucic acid-induced production of ROS exceeded that induced by erucic acid anilide 13-fold. The time-course of erucic acid anilide-induced production of ROS resembled that induced by erucic acid (Fig. 2).

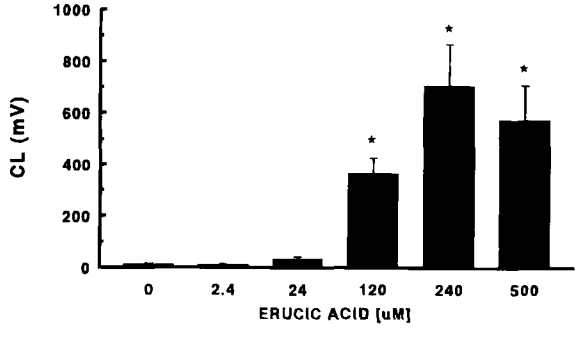

FIGURE 1 Effect of erucic acid on the peak production of reactive oxygen species (ROS) in human polymorphonuclear leukocytes.

The incubation mixture contained 1% DMSO. Chemiluminescence (CL) is used as an indicator of the production of ROS. Mean ± SEM of five experiments are shown. Statistically significant differences ($p < 0.05$ vs. control) are denoted by an asterisk (one-way analysis of variance followed by Duncan's Multiple Range test).

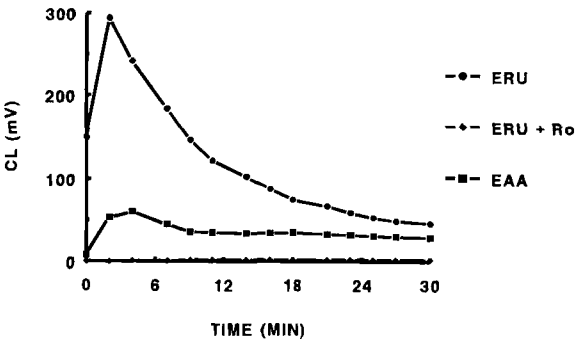

FIGURE 2 Single representative tracings of erucic acid- and erucic acid anilide-induced chemiluminescence (CL) response in human polymorphonuclear leukocytes.

ERU: erucic acid; ERU + Ro: erucic acid + a protein kinase C inhibitor, Ro 31-8220; EAA: erucic acid anilide. Concentrations of erucic acid, erucic acid anilide, and Ro 31-8220 were 120, 240 and 5 μM, respectively. Note that the protein kinase C inhibitor, Ro 31-8220, completely inhibits erucic acid—induced production of reactive oxygen species. Same held true for erucic acid anilide (tracing not shown in figure).

Effect of a Protein Kinase C Inhibitor, Ro 31-8220, on Erucic Acid and Erucic Acid Anilide-induced Production of Reactive Oxygen Species

Ro 31-8220 dose-dependently inhibited the production of ROS in PMNL exposed to 120 μM erucic acid (Figs. 2 and 4). The inhibition was 50% and 98% at concentrations of 5 and 50 μM of Ro 31-8220, respectively. Ro 31-8220 did not, how-

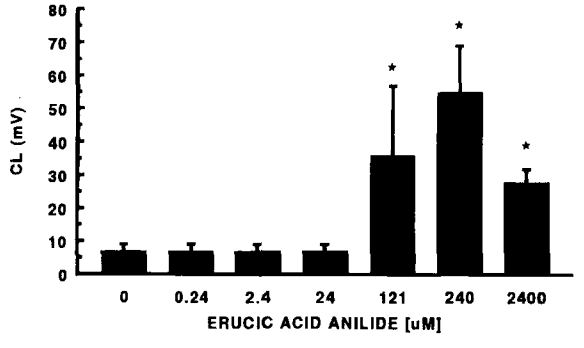

FIGURE 3 Effect of graded doses of erucic acid anilide on the production of reactive oxygen species in human polymorphonuclear leukocytes.

Chemiluminescence (CL) is used as an indicator of the production of ROS. Mean ± SEM five experiments are shown. Statistics as in Fig. 1.

ever, modify the time-course of the respiratory burst induced by erucic acid. The PKC-inhibitor also inhibited erucic acid anilide-induced production of ROS in a similar way it inhibited of erucic acid-induced ROS-production (Fig. 4).

Effect of Erucic Acid and Erucic Acid Anilide on formyl-Methionyl-Leucyl-Phenylalanine-induced Production of Reactive Oxygen Species

Erucic acid (2.4–500 μM) or erucic acid anilide (2.4–240 μM) did not modify FMLP-induced production of ROS in PMNL (data not shown).

Effect of Erucic Acid on Phorbol Myristate Acetate-induced Production of Reactive Oxygen Species

When a sub-threshold concentration of PMA (0.5 nM) was used, erucic acid (500 μM) did not modify PMA-induced production of ROS. On the other hand, erucic acid (500 μM) increased PMA-induced ROS-production 1.8-fold, when the concentration of PMA was 5 nM (Fig. 5).

Effect of Erucic Acid Anilide on Phorbol Myristate Acetate-induced Production of Reactive Oxygen Species

Erucic acid anilide inhibited dose-dependently PMA-induced (5 nM) ROS-production (Fig. 6). The inhibition was 39% at a concentration of 240 μM of erucic acid anilide. Moreover, erucic acid anilide, at a concentrations of 24 μM, shifted the peak production of ROS by PMA from 10–14 min to 20–38 min (Fig. 7).

DISCUSSION

The pathogenesis of the TOS has not been clarified, and attempts to develop an animal model for the disease have failed so far. It has been suggested that the primary target of the toxic

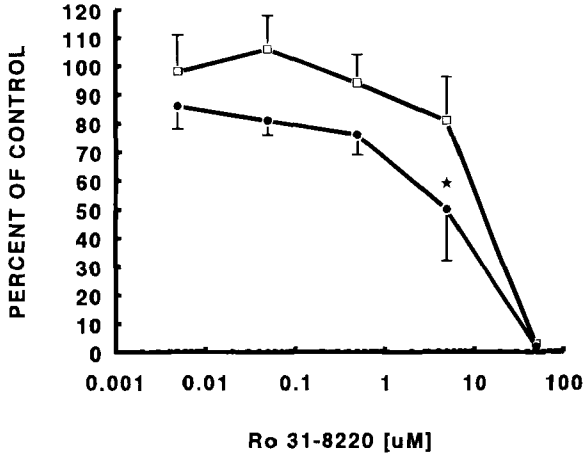

FIGURE 4 Effect of a protein kinase C inhibitor, Ro 31-8220 (Ro), on the peak production of reactive oxygen species in human polymorphonuclear leukocytes (PMNL) stimulated with erucic acid (EA, ●, 120 μM, n = 5) or erucic acid anilide (EAA, □, 240 μM), n = 3).

PMNL were preincubated with graded concentrations of a protein kinase C inhibitor, Ro 31-8220, for 15 min at 37°C before the addition of erucic acid or erucic acid anilide. The incubation mixture also contained 1% DMSO. The results are shown as per cent of control experiments which contained erucic acid together with 1% DMSO. Statistics as in Fig. 1.

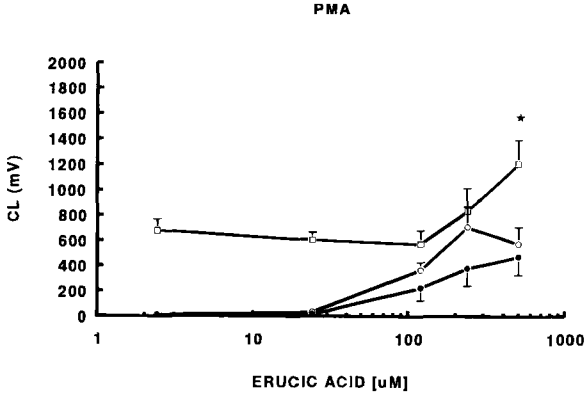

FIGURE 5 Effect of erucic acid (ERU) on phorbol myristate acetate (PMA)-induced production of reactive oxygen species (ROS) in human polymorphonuclear leukocytes.

The concentrations of PMA are 0.5 and 5 nM. The incubation mixture also contained 1% DMSO. Chemiluminescence (CL) is used as an indicator of the production of ROS. PMA alone at concentrations of 0.5 nM and 5 nM increased the production of ROS from 13 ± 3 mV to 15 ± 2 mV and 652 ± 97 mV, respectively. Mean ± SEM five experiments are shown. Statistics as in Fig. 1. Erucic acid alone, ○; 0.5 nM PMA + erucic acid, ●; 5 nM PMA +erucic acid, □.

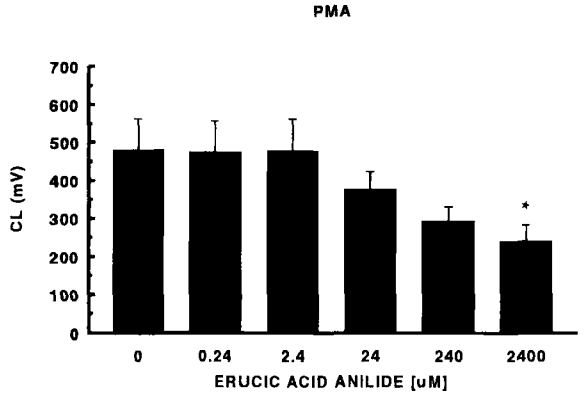

FIGURE 6 Effect of erucic acid anilide on phorbol myristate acetate (PMA)-induced production of reactive oxygen species (ROS) in human polymorphonuclear leukocytes.

The incubation mixture contained 1% DMSO. Chemiluminescence (CL) is used as an indicator of the production of ROS. Mean ± SEM five experiments are shown. Statistics as in Fig. 1.

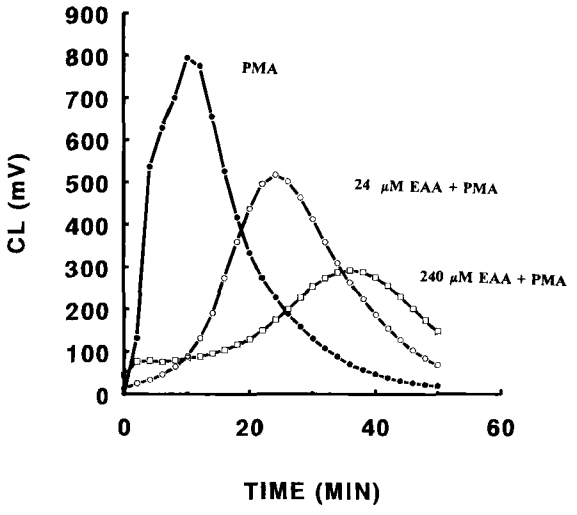

FIGURE 7 Representative tracings of effect erucic acid anilide (EAA) on phorbol myristate acetate (PMA) -induced chemiluminescence (CL) response in human polymorphonuclear leukocytes.

Concentration of PMA was 5 nM. The incubation mixture contained 1% DMSO. CL is used as an indicator of the production of reactive oxygen species.

agent(s) in the case oils may be the vascular endothelium in hepatic, pulmonary, neuronal and adipose tissues.[32] On the other hand, peripheral eosinophilia, pulmonary oedema, inflammatory cell infiltrates, neuromyopathia, and scleroderma could be better explained by generalized immunopathological mechanisms.[5] One of the target organs of the TOS was the heart in which the coronary arteries and cardiac neural structures were injured. The issue of the role of rapeseed oil itself and erucic acid in inducing cardiac abnormalities in the TOS have been raised because cardiac lesions in the TOS victims resembled those induced by feeding of animals with native rapeseed oil.[32]

Several studies suggest that also oxidative stress may be involved in the pathophysiology of the TOS.[16,33–35] Activation of NADPH-oxidase causes the formation of ROS in phagocytic cells. NADPH-oxidase can, in fact, be activated through multiple routes in PMNL. An agonist of a G-protein-coupled receptor, FMLP, stimulates phospholipase C-mediated production of phosphoinositide second messengers, and causes subsequent elevation of $[Ca^{2+}]_i$ and activation of PKC. PKC, in turn, phosphorylates and activates NADPH-oxidase, and thereby induces the production of ROS.[36–38]

We have shown in earlier studies that linoleic and oleic acid anilides may modulate agonist-induced activation of human PMNL through a calcium-mediated signalling pathway.[14–15] In another study, we have demonstrated that palmitic acid anilide activates respiratory burst in PMNL, probably by activating PKC.[16]

In this study, erucic acid stimulated ROS production much more than its anilide. It seems likely that both erucic acid and erucic acid anilide activate PKC directly rather than through a calcium-mediated signalling pathway. This assumption is supported by the finding that a PKC inhibitor, Ro 31-8220, completely blocked respiratory burst induced by these compounds. Ro 31-8220 is considered to be a more selective inhibitor of PKC than staurosporine or H7, which also inhibits cAMP-dependent kinases at low concentrations.[30] Also the findings that these compounds did not elevate intracellular calcium, or modify FMLP-induced production of ROS, are consistent with the present conclusions.

It is also possible that exogenous administration of fatty acids and their anilides can induce changes in the physicochemical properties of cell membrane by affecting fluidity and fatty acid composition of membrane phospholipids. These alterations could modify cellular signalling cascades. This may be accompanied by alteration in hydrolytic actions of phospholipases resulting in altered availability of important cofactors required for activation of PKC and formation of distinct eicosanoids.[39] However, the present results suggest that even if erucic acid and its anilide may modify properties of cell membrane they also directly modulate the activity of PKC.

The ability of erucic acid to modify ROS production induced by PMA, a well known activator of PKC, was also explored. Co-exposure of PMNL to sub-threshold concentration of PMA and different concentrations of erucic acid showed that erucic acid did not sensitize PMNL toward PMA-induced cell activation. However, when a suprathreshold concentration of PMA was used, a high concentration of erucic acid amplified PMA-induced respiratory burst. This finding suggests that erucic acid and PMA may both activate PKC but that PMA is more potent than erucic acid in activating PKC. Interestingly, in our earlier study we found out that oleic and linoleic acids strongly inhibited PMA-induced respiratory burst.[14] It seems possible that fatty acids with carbon chains of different lengths may modulate responses of PMNL in a different manner. Moreover, erucic acid seems to be the most potent of these fatty acids to induce respiratory burst in PMNL. The maximal production of ROS induced by erucic acid exceeded several-fold that induced by oleic or linoleic acid.[14]

Interestingly, erucic acid anilide inhibited PMA-induced respiratory burst, and also shifted the peak production of ROS to a later time-point. This inhibition, and the effect on the time-course of the production of ROS resembles that induced by oleic and linoleic acid anilides.[14] Also the magnitude of erucic acid anilide-induced production of ROS resembled that induced by linoleic and oleic acid anilides. It seems likely that the aniline moiety clearly modulates the effects of oleic, linoleic and erucic acids on the PMNL. Our studies also suggest that several of the actual or postulated toxic oil contaminants modulate the inflammatory responses of PMNL in vitro.[13–16,40] These contaminants may affect cellular signalling also in a wide variety of cells in different tissues. Studies utilizing blood vessels of TOS patients have shown that the evolution of vascular lesion in TOS patients is followed by proliferation of fibroblasts with collagen formation.[41] Different fatty acids may, in fact, stimulate growth of fibroblasts through affecting PKC signalling pathways.[42,43] Consequently, alterations in PKC activity merit attention in different tissues, when the mechanisms of TOS are being explained.

In summary, the effects of erucic acid and erucic acid anilide on leukocyte activation differ from each other suggesting that the aniline moiety significantly modifies the biological activity of erucic acid in PMNL. Moreover, PKC may have an important role in erucic acid- and erucic acid anilide-induced respiratory burst in these cells.

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List of Abbreviations

Abbreviations used in this paper: $[Ca^{2+}]_i$, free intracellular calcium concentration; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis(β-aminoethyl ether)N, N, N', N'-tetra-acetic acid; FMLP, formyl-Methionyl-Leucyl-Phenylalanine; HBSS, Hanks' balanced salt solution; PBS, phosphate buffered saline; PKC, protein kinase C; PMA, phorbol myristate acetate; PMNL, human polymorphonuclear leukocytes; ROS, reactive oxygen species; TOS, Toxic Oil Syndrome;

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