***n* 6 and *n* 3 Polyunsaturated fatty acids stimulate translocation of protein kinase C α , - β I, - β II and - ϵ and enhance agonist-induced NADPH oxidase in macrophages**

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The polyunsaturated fatty acids (PUFA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were poor inducers of oxygen-dependent respiratory activity (chemiluminescence) in human monocytes and macrophages, but markedly enhanced the response to the tripeptide, *N*-formylmethionyl-leucyl-phenylalanine. The effects of these fatty acids were seen at concentrations of 1 μ g/ml. A similar enhancement was seen with PMA, a stimulus that acts on protein kinase C (PKC), or calcium ionophore (A23187), which increases intracellular calcium, suggesting that the effect of the fatty acids was post-surface receptor binding. HL-60 cells, differentiated to macrophage-like cells by culture in the presence of vitamin D₃, were similarly affected by the fatty acids. In experiments in which the time of pre-exposure of the monocytes to PUFA was varied, it was found that the priming effect induced by AA, EPA and

DHA was maximal at 5 min. The ability of these fatty acids to synergize with other agonists was completely lost if the fatty acids were either methylated or oxidized to the hydro and hydroperoxy derivatives. Saturated fatty acids were inactive. Western blot analysis demonstrated that the PUFA induced the translocation of PKC α , - β I, - β II and - ϵ isoenzymes to a particulate fraction. The synergistic response between fatty acids and A23187 was completely inhibited by pretreating the cells with a PKC inhibitor, GF-109203X, or by pretreatment of monocytes with PMA for 18 h, to deplete PKC levels. From these investigations it is evident that PUFA prime macrophages, causing increased/synergistic oxidative respiratory burst activity to other stimuli and that this priming is dependent on PKC translocation and activation.

INTRODUCTION

Cellular activation during the inflammatory reaction leads to the activation of phospholipase A₂, resulting in the release of arachidonic acid (AA) and other fatty acids [1]. Stimulation of leucocytes with a heterogeneous group of mediators, which includes opsonized particles and cytokines, induces the production of AA [2,3]. AA and related fatty acids may then be involved in a series of biological events that amplify/regulate the inflammatory response and cell activation. For example, AA has been shown to activate the neutrophil oxygen-dependent respiratory response [4–7], induce degranulation [8], increase expression of CR3 receptors [9] and regulate chemotaxis [10]. Although limited studies have been conducted with macrophages, evidence shows that AA also activates a respiratory burst in macrophages [11]. We have extended these studies on the mononuclear phagocyte by investigating the effect of the *n*–6 fatty acid AA on the agonist-induced respiratory burst in human macrophages. Since other fatty acids of the *n*–3 series, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are metabolized to yield different products, their effects were compared with those of AA. The results show that although these polyunsaturated fatty acids (PUFA) induce a poor respiratory burst response in monocytes and macrophages, they prime or act in synergy with other macrophage agonists. Evidence is presented that this priming response involves protein kinase C (PKC).

EXPERIMENTAL

Preparation of monocytes and macrophages

Monocytes or macrophages were prepared by adherence of mononuclear leucocytes to cytodex microcarriers, as previously described by Kumaratilake and Ferrante [12]. Briefly, mononuclear leucocytes were separated from heparinized blood from healthy donors by centrifugation of blood on Hypaque-Ficoll medium. Monocytes were purified by adherence of mononuclear leucocytes to preswollen cytodex microcarriers (Pharmacia Fine Chemicals, NSW, Australia) on a rotating platform at 37 °C for 2–4 h. Then the microcarriers with adhered monocytes were cultured in RPMI 1640 medium containing 10% (v/v) foetal-calf serum at 37 °C in an air/CO₂ (19:1) atmosphere for 1–7 days. Monocytes/macrophages were immediately released by vortexing the microcarriers followed by two washes. The purity of monocytes and macrophages by this method is approx. 95%. Cell viability measured by Trypan Blue dye exclusion was > 95%. Differentiation of macrophages was monitored by cytocentrifuged smear, stained with May–Grunwald Giemsa and examined for the loss of myeloperoxidase activity [12].

Differentiation of HL-60

HL-60 cells (ATCC, Atlanta, GA, U.S.A.) were grown in RPMI 1640 containing 10% foetal-calf serum, 2 mM L-glutamine, penicillin and streptomycin at 37 °C in air/CO₂ (19:1). The HL-

Abbreviations used: PUFA, polyunsaturated fatty acids; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; PKC, protein kinase C; DPC, dipalmitoylphosphatidylcholine.

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60 cells were differentiated to monocyte/macrophages according to Levy et al. [13]. Cells were treated with 1×10^{-8} M 1- α ,25-dihydroxyvitamin D₃ for 6 days. Differentiation and appearance of macrophage-like cells was confirmed by morphology and the expression of CD14 antigen. The viability of the differentiated cells was > 98 %.

Fatty acids

Dipalmitoylphosphatidylcholine (DPC), the non-esterified fatty acids, AA, EPA, DHA and arachidic acid (20:0), and methyl arachidonate were purchased from Sigma (St. Louis, MO, U.S.A.). The methyl ester, and the hydroxy and hydroperoxy derivatives, of AA were prepared as described previously [14]. Fatty acids were checked for purity by silica-gel TLC in diethyl ether/hexane/acetic acid (60:40:1 by vol.). DPC was used as a vehicle (DPC-fatty acid micelles) for the non-esterified fatty acids and their derivatives [7]. Control incubations using the appropriate concentrations of DPC were included in the experiments.

Treatment of cells with fatty acids, *N*-formylmethionyl-leucyl-phenylalanine (fMLP), phorbol ester, calcium ionophore and inhibitors

Leucocytes were treated simultaneously (unless specified otherwise) with fatty acids at 10 μ g/ml (unless specified otherwise) and either 5×10^{-6} M fMLP (Sigma), 10^{-7} M PMA (Sigma) or 10^{-5} M calcium ionophore (A23187; Sigma).

In some experiments, the cells were treated with 10^{-7} M PMA for 18 h at 37 °C to deplete PKC. To inhibit the activity of PKC, cells were pretreated with 0.2 μ M GF-109203X (Biomol, Plymouth Meeting, PA, U.S.A.) for 10 min before stimulation with agonists.

Chemiluminescence assay

Superoxide production was measured by the reduction of the fluorescent probe lucigenin (Sigma) essentially as described previously [15,16]. Briefly, 10^6 monocytes or macrophages in 100 μ l of Hanks Balanced Salt solution (HBSS) were treated with stimulus and the reaction mixture made up to a total volume of 400 μ l. Then 500 μ l of 250 μ g/ml lucigenin was added. The tubes were immediately transferred to the luminometer chamber (Model 1251, Bio-Orbit Oy, Turku, Finland) and the light output recorded through the interface of the computer with the multiuser software, and presented as the peak initial rate (mV) of each activity unless indicated otherwise. We have previously shown that the chemiluminescence produced is totally inhibited by superoxide dismutase [6]. In these investigations we have avoided using the measurement of superoxide by the cytochrome *c* reduction assay since cytochrome *c* can inhibit the activity of fatty acids [16].

PKC translocation

The cells were washed in HBSS and incubated with different concentrations of fatty acids for the times indicated in the Figure legends and Results section. After incubation, cells were washed again with HBSS and sonicated in buffer containing 20 mM Tris, pH 7.5, 5 mM EGTA, 2 mM EDTA, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml benzamidin, 10 μ g/ml pepstatin A, 10 mM PMSF and 2 mM mercaptoethanol, and the sonicate centrifuged at 100 000 *g* for 30 min. The pellet was resuspended in sonication buffer supplemented with 2 % (v/v) Triton X-100. After 30 min (1 °C), the extract was centrifuged at 100 000 *g* for 30 min. The supernatant was adsorbed on to DE-52 (Bio-Rad

Labs., Richmond, CA, U.S.A.), which was pre-equilibrated in 20 mM Hepes (pH 7.5). After washing (2×0.5 ml of Hepes buffer), PKC was eluted with 0.2 M NaCl. PKC activity was assayed by using histone type III-S (Sigma) as substrate, according to the method of Hardy et al. [17]. The reactions were terminated by spotting aliquots on to P81 papers (Whatman Ltd., Maidstone, Kent, U.K.). After extensive washing with 85 mM orthophosphoric acid, the radioactivity was determined by liquid-scintillation spectrometry.

For immunoblotting [18], Laemmli buffer was added to the DE-52-purified samples, and aliquots (20 μ g) were subjected to SDS/PAGE (10 % gels). The proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) and probed with isoenzyme-specific PKC antibodies (Boehringer Mannheim). The immune complexes were detected by enhanced chemiluminescence (DuPont-NEN).

[γ -³²P]ATP (400 Ci/mmol) was obtained from Bresatec (Adelaide, South Australia). All other reagents, unless indicated otherwise, were from Sigma.

Statistics

Results are means $\pm$ S.E.M. of the number of experiments indicated in the Figure legend. Differences between treatments were compared by the two-tail Student's *t* test for paired data (Statwork, Philadelphia, PA, U.S.A.) and analysis of variance (Fisher's least-significant test, pairwise Systat, Evanston, IL, U.S.A.).

RESULTS

Synergistic responses of monocytes/macrophages to PUFA and fMLP/PMA

Monocytes were treated with 10 μ g/ml of AA, EPA or DHA alone or with each of the PUFA and fMLP, and the production of superoxide was determined in a chemiluminescence assay. The results showed that treatment of monocytes with all three PUFA resulted in a small but significant respiratory burst (Table 1). The

Table 1 Effects of co-addition of PUFA and either fMLP or PMA on the monocyte/macrophage chemiluminescence response

Human monocytes/macrophages were treated with 30 μ M each AA, EPA, DHA or vehicle in the presence or absence of either 5 μ M fMLP or 100 nM PMA. The chemiluminescence production from these cells was measured by the lucigenin-dependent chemiluminescence assay. Results are means $\pm$ S.E.M. of 4–9 experiments, each conducted with cells from a different individual and the values represent the peak initial rates of chemiluminescence. *P* < 0.05 for the difference between the sum of the value of the individual PUFA and fMLP/PMA effects versus the values for the co-treatment with these agents.

Treatment	Chemiluminescence (mV)	
	Monocytes	Macrophages
Control	1.429 $\pm$ 0.376	3.698 $\pm$ 0.906
AA	4.520 $\pm$ 0.472	4.528 $\pm$ 0.988
EPA	5.897 $\pm$ 1.098	6.347 $\pm$ 3.121
DHA	4.787 $\pm$ 0.953	7.242 $\pm$ 3.419
fMLP	9.243 $\pm$ 2.904	17.297 $\pm$ 3.098
fMLP + AA	22.600 $\pm$ 5.036	46.667 $\pm$ 9.501
fMLP + EPA	46.933 $\pm$ 10.35	47.200 $\pm$ 9.064
fMLP + DHA	42.425 $\pm$ 11.584	48.733 $\pm$ 11.315
PMA	23.795 $\pm$ 2.904	11.321 $\pm$ 4.342
PMA + AA	59.801 $\pm$ 10.792	34.640 $\pm$ 8.308
PMA + EPA	81.267 $\pm$ 21.419	34.125 $\pm$ 4.931
PMA + DHA	64.021 $\pm$ 9.470	24.625 $\pm$ 1.089

Figure 1 Synergistic responses of monocytes to PUFA (DHA) + fMLP (A) and PUFA (DHA) + PMA (B) as a function of rate of chemiluminescence produced over time

Time (sec) = Time (s).

combined addition of fMLP and each PUFA gave rise to a synergistic response, i.e. a response that was significantly greater than the sum of each of the stimulators. Monocytes that had been differentiated into macrophages by *in vitro* culture responded to these fatty acids in a similar fashion (Table 1).

The saturated equivalents of these PUFA, such as 20:0 arachidic acid, did not show any effects, and the methyl ester and the hydroxy and hydroperoxy derivatives had no effect on superoxide production (results not shown).

To determine whether the synergistic effects of fatty acids also occurred with agonists that acted downstream of receptor-binding, we examined the effects of PUFA in combination with PMA, an agonist that directly activates PKC. A marked synergistic response was observed between PMA and each of the PUFA tested with both monocytes and macrophages (Table 1).

When the above data were expressed as peak initial rate of chemiluminescence produced, it was evident that the synergistic effects occurred over the complete incubation period with both fMLP and PMA (Figure 1). This is reflected in the data presented in Table 2, which summarizes the total chemiluminescence produced over the incubation period, averaged to mV/s.

Synergistic responses between fatty acids and PMA were observed in a concentration-dependent manner from 1 to 10 $\mu\text{g/ml}$ of AA, EPA or DHA (Figure 2).

Table 2 Synergistic response of monocytes to PUFA and either fMLP or PMA as the total chemiluminescence accumulated over the incubation period

Human monocytes were treated with 30 μM DHA or vehicle in the presence or absence of either 5 μM fMLP or 100 nM PMA. The superoxide production from these cells was measured by the lucigenin-dependent chemiluminescence assay. Results are means $\pm$ S.E.M. of four experiments, each conducted with cells from different individuals. $P < 0.05$ for the difference between the sum of the value of DHA and fMLP/PMA effects versus the values for the co-treatment with these agents.

Treatment	Initial peak rate (mV)	Accumulated chemiluminescence (mV/s)
Control	1.540 $\pm$ 0.488	1.348 $\pm$ 0.082
DHA	4.787 $\pm$ 3.051	2.628 $\pm$ 0.507
fMLP	10.882 $\pm$ 1.669	11.833 $\pm$ 0.811
fMLP + DHA	34.442 $\pm$ 2.160	33.600 $\pm$ 2.245
PMA	21.775 $\pm$ 5.367	20.544 $\pm$ 2.942
PMA + DHA	56.600 $\pm$ 5.352	54.705 $\pm$ 6.949

Figure 2 PUFA-concentration-related effects on the PMA-induced chemiluminescence response in monocytes

Monocytes were treated with PMA and varying concentrations of PUFA. The results are the mean of five experiments. a, $P < 0.05$ and b, $P < 0.01$ for comparison between the values for the sum of the individual effects of PUFA and PMA versus the effect of the co-treatment with both agents.

If the monocytes were pretreated with PUFA, an enhanced response to the subsequent addition of fMLP was observed over a pretreatment period of 30 min (results not shown). With respect to AA, EPA and DHA, maximal enhancement was seen within 5 min of pretreatment time, declining thereafter. Studies examining the PUFA concentration required for priming showed the fatty acids to be effective from 0.1 to 10 $\mu\text{g/ml}$ (results not shown).

Although highly purified preparations of monocytes/macrophages were used in the above studies, it is still possible that the fatty acids could be exerting some of their effects via residual contaminating lymphocytes. We therefore examined the synergistic properties of fatty acids and agonists on HL-60 cells differentiated into macrophages with 1- α ,25-dihydroxyvitamin D_3 . Data from four experiments showed that PMA induced a respiratory burst in these cells (73.18 $\pm$ 18.90 mV) but

Table 3 Translocation of PKC by DHA

Cells were treated with 30 μ M DHA for 1–30 min. Translocation of PKC was monitored by phosphorylating histone type III-S (A) or by Western blot analysis (B) as described in the Experimental section. The immunoblots were quantified by densitometry. Results are means $\pm$ S.E.M. of four determinations from two experimental runs (A) or means $\pm$ S.D. of three experiments (B).

		Histone phosphorylation (% of control)	
		Particulate fraction	Cytosolic fraction
Monocytes	5	228 $\pm$ 9	73 $\pm$ 10
	30	104 $\pm$ 10	93 $\pm$ 13
Macrophages	1	188 $\pm$ 10	77 $\pm$ 1
	10	110 $\pm$ 7	91 $\pm$ 1

(B) Densitometry of Western blots

PKC isoenzymes	Vehicle	DHA	P value
α	78.8 $\pm$ 41.6	190.7 $\pm$ 93.8	< 0.05
β I	223.4 $\pm$ 296.7	594.3 $\pm$ 172.1	< 0.02
β II	294.8 $\pm$ 296.7	697.7 $\pm$ 280.5	< 0.02
ϵ	124.8 $\pm$ 40.7	318.8 $\pm$ 159.5	< 0.05

DHA elicited a very poor response of 2.33 ± 0.27 mV (basal 1.47 ± 0.21 mV), which is in keeping with the results with monocytes and macrophages. Addition of both PMA and DHA to the differentiated HL-60 macrophages resulted in a synergistic chemiluminescence response of 291.25 ± 57.41 mV.

Role of PKC in PUFA-induced macrophage priming

Previous studies have shown that PUFA can activate PKC directly [17] and translocate cytosolic PKC to a particulate fraction in WB cells [18]. We thus examined whether PUFA translocated the various isoenzymes of PKC, and its role in the synergistic response.

The data in Table 3(A) show that DHA stimulated the translocation of PKC to a particulate fraction of both monocytes and macrophages, within the first few minutes of incubation with the fatty acid. Table 3(B) shows the DHA-stimulated translocation of PKC isoenzymes α , β I, β II and ϵ to a particulate fraction.

Examination of the role of PKC in the PUFA-induced priming is made difficult by the fact that the fMLP response is also dependent on PKC activation, and therefore experiments utilizing either PKC inhibitors or PKC depletion cannot resolve this question. Therefore we used A23187, which stimulates cells independently of PKC. Co-addition of DHA and A23187 resulted in a marked synergistic chemiluminescence response in human monocytes (Table 4). Macrophages pretreated with GF-109203X, an inhibitor of PKC, failed to be primed by DHA for a chemiluminescence response to A23187 (Table 4).

In a second set of experiments, we examined whether PKC depletion following a prolonged pretreatment with PMA in macrophages rendered the cells unresponsive to priming by PUFA. The cells were pretreated for 18 h with 100 nM PMA and then incubated with the PUFA and A23187. The data presented in Table 4 show that PKC depletion rendered the monocytes incapable of being primed by PUFA.

Table 4 Effect of a PKC inhibitor, GF-109203X, or PKC depletion (PMA pretreatment for 18 h) on the ability of DHA to enhance the monocyte chemiluminescence response to A23187

Monocytes were pretreated with diluent, a specific PKC inhibitor (0.2 μ M) GF-109203X for 10 min, or PMA (100 nM) for 18 h. Then 30 μ M DHA and/or 10 μ M A23187 were/was added to the cells. The chemiluminescence production from these cells was measured by the lucigenin-dependent chemiluminescence assay. Results are means $\pm$ S.E.M. of four experiments, each conducted with cells from a different individual and the values are the initial peak rates of chemiluminescence in mV. * P < 0.05, comparing the values of DHA + A23187 between diluent pretreatment and either GF-109203X or PMA pretreatment.

Stimulus	Pretreatment ...	Chemiluminescence production		
		Diluent	GF-10923x	PMA
Vehicle		1.69 $\pm$ 0.10	1.28 $\pm$ 0.31	1.22 $\pm$ 0.05
DHA		3.22 $\pm$ 0.57	1.32 $\pm$ 0.31	1.85 $\pm$ 0.27
A23187		2.07 $\pm$ 0.24	2.00 $\pm$ 0.46	1.39 $\pm$ 0.12
DHA + A23187		14.84 $\pm$ 1.57*	2.34 $\pm$ 0.60	1.98 $\pm$ 0.29

DISCUSSION

The data show that the respiratory burst in monocytes and macrophages is modulated by long-chain $n-6$ and $n-3$ PUFA. Although peripheral blood monocytes, monocyte-derived macrophages and vitamin D₃-differentiated HL-60 cells gave very poor respiratory burst responses to AA, EPA and DHA, these fatty acids primed the monocytes/macrophages for a significantly increased respiratory burst to fMLP.

Major sources of AA are likely to be inflammatory phagocytic cells, neutrophils and macrophages. For example, macrophages treated with mycobacteria, lipopolysaccharide, opsonized zymosan and some chemokines release AA into the extracellular medium [19–21]. A substantial amount of AA can also be released from activated endothelial cells [22,23]. In these and other cell types, the 85 kDa cytosolic phospholipase A₂ preferentially cleaves AA from the *sn*-2 position of phospholipids such as phosphatidylcholine and inositol-containing phospholipids [1,24,25]. In platelets, the cytosolic phospholipase A₂ also liberates EPA but not DHA from $n-3$ fatty-acid-enriched phospholipids [26–28]. However, DHA can be liberated by the 14 kDa secretory phospholipase A₂ [27].

Significant enhancement of the fMLP response was seen at concentrations of 1–2.5 μ g/ml ($\approx$ 2–5 μ M), which is well within the physiological and pathophysiological concentrations of these fatty acids. For example, in human malaria, the plasma level of AA as a non-esterified fatty acid rises from 5 μ M in non-infected subjects to 100 μ M as a result of malaria [29]. Yasuda et al. [30] showed that the non-esterified fatty acid levels of AA in brain rose from 50 μ M control levels to 500 μ M under ischaemic conditions. Others have reported that activation of neutrophils leads to production of AA concentration of the order of 20–30 μ M [31], a value that agrees with our findings [2]. Dietary $n-3$ fatty acids can reach a non-esterified fatty acid concentration of 20–30 μ M [32]. The finding suggests that fatty acids generated either as a consequence of the inflammatory reaction through the activation of phospholipase A₂, or introduced in diet supplements, can significantly affect a major function in the macrophage, namely the ability to produce oxygen-derived reactive species. These macrophage products are known to be critical not only in the killing of a number of bacteria and parasites, but also in causing tissue damage. Both EPA and DHA have been used as dietary supplements to

increase the proportion of *n*-3 over *n*-6 fatty acids in membrane phospholipids, in order to achieve an anti-inflammatory effect through the production of substantially less active eicosanoids [33] and, as more recently reported, a decrease in the production of cytokines such as tumour necrosis factor and interleukin-1 [34]. It is, however, evident from our results that EPA and DHA are as active as AA in promoting a key response in macrophages that is associated with promotion of inflammation and tissue damage. This may in part explain the limited success achieved in attempts to treat inflammatory disease with *n*-3 fatty acids [35].

The effects of PUFA were not restricted to the surface-receptor-acting agonist, fMLP. The PUFA acted in a synergistic manner with either PMA, which directly activates PKC, or the Ca^{2+} ionophore, A23187. The PUFA-induced priming could not have involved the synthesis of new protein molecules as the effect on monocytes/macrophages was extremely rapid, with a maximal synergistic response by 5 min. Our results strongly suggest that activation of PKC by PUFA is a possible mechanism for the priming/synergistic response. Human monocytes/macrophages express PKC α , - β I and - β II, but little of the ϵ and γ isoforms [36], and PKC β has been reported to phosphorylate p47^{phox} [37]. We demonstrate, for the first time in human monocytes/macrophages, that PUFA stimulated the translocation of PKC α , - β I, - β II and - ϵ to a particulate fraction. A role of PKC in the enhancement of superoxide production was confirmed by the use of a selective PKC inhibitor, GF-109203X, and by depletion of PKC through prolonged incubation of cells in the presence of PMA. In using GF-109203X to inhibit the activity of classical PKC isoenzymes or PKC depletion, we have made a conscious decision to use the Ca^{2+} ionophore A23187 as a second agonist, since the effects of A23187 are generally thought to be exerted via PKC-independent mechanisms. The use of an inappropriate second agonist, such as one that acts via PKC, would render the results uninterpretable. Although A23187 has been demonstrated to stimulate the translocation of PKC to a particulate fraction, it was found that the translocated PKC was not catalytically active, since treatment of cells with the ionophore did not result in the phosphorylation of myristoylated alanine-rich C-kinase substrate [38]. Consistently with this, Qin and Leslie [39] reported that GF-109203X did not affect the activity of phospholipase A_2 , which had been stimulated by A23187, but the inhibitor blocked stimulation of phospholipase A_2 by PMA. Thus our data, demonstrating that GF-109203X or PKC-depletion prevented the synergistic response following co-addition of fatty acids and A23187, provide strong evidence that the PUFA act via PKC.

Our data illustrate that the biological function of mononuclear phagocytes can be regulated by PUFA generated during the inflammatory response. Once these reach appreciable quantities, it is likely that they could function to amplify the leucocyte response by acting synergistically with other mediators that form part of the network of intercellular active molecules.

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