Transforming growth factor-β_1 stimulates degranulation and oxidant release by adherent human neutrophils

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Abstract: The signal transduction pathways that are activated by cytokines and growth factors binding to their receptors on human neutrophils (PMN) are poorly understood. When PMN in suspension encounter many of these agonists they are not activated, but rather are primed for subsequent activation. We and others reported that when PMN are plated onto fibrinogen and stimulated with cytokines or with the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) they respond by releasing hydrogen peroxide (H_2O_2) and the specific granule component lactoferrin. Transforming growth factor-β_1 (TGF-β_1) is released by many cells including PMN. It has been reported that TGF-β_1 stimulates chemotaxis but not exocytosis or superoxide production by cells in suspension. We hypothesized that TGF-β_1 would activate PMN to release H_2O_2 when they were adherent to fibrinogen, a response mediated by β_2 integrin receptors. In this study, we determined whether TGF-β_1 stimulated H_2O_2 and lactoferrin release by PMN adherent to fibrinogen. TGF-β_1 stimulated H_2O_2 and lactoferrin release from adherent PMN in a concentration-dependent manner, with effects seen in the range of 0.1 to 100 pg/mL. Both H_2O_2 and lactoferrin release were detected by 60 min and continued for at least 180 min. Adhesion and spreading of PMN paralleled H_2O_2 and lactoferrin release. Ethanol (200 mM) blocked both H_2O_2 and lactoferrin release, suggesting the involvement of the phospholipase D pathway. In PMN labeled with lyso-[^{3}H]phosphatidylcholine, we observed that TGF-β_1 treatment caused an increase in [^{3}H]phosphatidate. Propranolol (150 μM), an inhibitor of phosphatidate phosphohydrolase, blocked both H_2O_2 and lactoferrin release, suggesting that the conversion of phosphatidic acid to diradylglycerol is an important step in PMN activation by TGF-β1. Overall, these results are similar to those reported for fMLP activation of adherent PMN and suggest that a common pathway is involved in both chemoattractant and cytokine activation. *J. Leukoc. Biol.* 60: 772–777; 1996.

Key Words: *hydrogen peroxide · phosphatidic acid · phospholipase D · secretion*

INTRODUCTION

Cytokines and growth factors are released by many cells including polymorphonuclear leukocytes (PMN) [1]. These agonists bind to specific receptors on PMN membranes and activate them through a variety of poorly understood mechanisms. Transforming growth factor-β_1 (TGF-β_1) is one of five members of a group of structurally related and functionally similar proteins, three of which are expressed in mammalian tissues. TGF-β_1 is synthesized as a large precursor that is proteolytically altered and dimerized to generate a 25-kDa peptide that complexes with two other proteins. This latent TGF-β_1 complex can be activated by acid, heat, or enzyme treatment. Active TGF-β reportedly regulates growth, differentiation, and extracellular matrix protein synthesis. In some cases, active TGF-β promotes seemingly opposing activities in the same cells [see ref. 2 for a review], although the mechanisms underlying these apparent paradoxes are not known. For example, in vivo TGF-β_1 applied or released locally is said to promote leukocyte adhesion, infiltration, and activation by regulating the synthesis and expression of receptors for integrins such as fibronectin and laminin [3]. On the other hand, TGF-β_1 administered systemically has the opposite effect, inhibiting the expression of endothelial cell adhesion proteins and decreasing leukocyte recruitment to sites of inflammation [4]. In vitro TGF-β_1 promotes PMN chemotaxis but blocks PMN adhesion to fibrinogen in response to phorbol ester [5]. Thus TGF-β_1 may selectively promote or inhibit PMN functional responses depending upon specific environmental conditions. Presently, the role of TGF-β_1 in PMN activation is unclear.

Abbreviations: TGF-β_1, transforming growth factor-β_1; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMN, polymorphonuclear leukocyte; H_2O_2, hydrogen peroxide; KRP, Krebs-Ringer phosphate buffer; KRPG, Krebs-Ringer phosphate buffer with glucose; [^{3}H]PA, phosphatidic acid; lyso-[^{3}H]PC, 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine; TNF-α, tumor necrosis factor α; ELISA, enzyme-linked immunosorbent assay.

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Human peripheral blood PMN provide a first line of defense against bacterial infection. PMN respond to host challenges primarily by releasing toxic oxygen metabolites such as superoxide and H_2O_2, and by secreting cytoplasmic granule contents that contain proteolytic enzymes. PMN can be stimulated to release these compounds when they are in suspension and after they have become adherent to plastic, extracellular matrix proteins, and other substrates [6–9]. It is well documented that adherent PMN respond to agonists quite differently than PMN in suspension. For example, the lag period before detection of H_2O_2 and lactoferrin is significantly longer, and the total amounts of H_2O_2 and lactoferrin released are greater in adherent PMN versus suspended PMN activated with fMLP [8, 9]. Furthermore, agonists that only prime PMN in suspension, such as granulocyte-macrophage colony-stimulating factor and tumor necrosis factor α (TNF-α), are activators of adherent cells. The signaling pathways activated by fMLP are also different in suspended versus adherent PMN. For example, diradylglycerol formation in fMLP-treated cells in suspension is monophasic and results from activation of both phosphatidylcholine-specific phospholipase D and phosphatidylinositol-specific phospholipase C [10–13]. On the other hand, when PMN plated on fibrinogen are treated with fMLP, diradylglycerol formation is biphasic, with only the second phase of diradylglycerol formation corresponding to H_2O_2 and lactoferrin release and derived from the activation of phospholipase D [6].

TGF-β_1 fails to activate the respiratory burst or induce exocytosis of PMN in suspension. However, since TGF-β_1 promotes PMN chemotaxis through its ability to promote PMN integrin binding to fibronectin [14], we hypothesized that the TGF-β_1 would also activate PMN to release H_2O_2 and lactoferrin when they were adherent to fibrinogen, a response mediated by β_2-integrin receptors [15]. In support of our hypothesis, we and others have reported that TNF-α was only able to activate PMN to undergo exocytosis and release oxidants if the cells were adherent to extracellular matrix proteins [9, 16].

In this study we investigated the ability of TGF-β_1 to stimulate H_2O_2 and lactoferrin release by PMN plated on fibrinogen. We demonstrated that TGF-β_1 activated PMN adherent to fibrinogen, stimulating the release of H_2O_2 and lactoferrin in a dose- and time-dependent manner. Furthermore, TGF-β_1-dependent activation of adherent PMN appeared to involve phosphatidate and diradylglycerol produced by the activation of phospholipase D.

METHODS

Reagents

TGF-β_1, obtained from Collaborative Biomedical Products (Bedford, MA), was isolated from human platelets. Its purity was assessed by the manufacturer and found to be > 95% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine (lyso-platelet-activating factor, 161 Ci/mmol) was obtained from Amersham. Reagents used for lipid analysis were high-performance liquid chromatography grade. All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Cells

Human PMN were isolated from whole blood by use of dextran sedimentation, Ficoll/Hypaque centrifugation, and hypotonic lysis of residual erythrocytes as previously described [6]. Preparations were greater than 98% PMN by differential staining and greater than 98% viable as determined by trypan blue exclusion.

H_2O_2 assay

H_2O_2 released by adherent PMN was assayed as previously described [6]. Assays were conducted in 24-well, flat-bottomed polystyrene Falcon Primaria tissue culture plates (Becton Dickinson, Lincoln Park, NJ) coated with saturating concentrations of fibrinogen (50 μg/mL). PMN suspended in Krebs-Ringer phosphate buffer containing 5 mM glucose (KRPG) were added to fibrinogen-coated wells (1×10^5 cells/well) containing KRPG and 24 μM scopoletin, 5 μg of horseradish peroxidase, 1 mM sodium azide (to prevent destruction of H_2O_2 by myeloperoxidase and catalase), and the indicated agonists and/or inhibitors in a final volume of 1 mL. Before their addition to the plate, PMN were warmed slowly to room temperature and plates were warmed to 37°C. Plates containing PMN were incubated in humidified air at 37°C. At the indicated time intervals, the solution in the wells was centrifuged to remove cells and the supernatant transferred to cuvettes. The reduction in relative fluorescence due to the oxidation of scopoletin by H_2O_2 was measured using an excitation wavelength of 365 nm and an emission wavelength of 473 nm in a Perkin-Elmer LS-50B fluorescence spectrometer. Omission of horseradish peroxidase abolished the specific decrease in fluorescence, indicating the specificity of scopoletin for H_2O_2. Control samples contained PMN but KRPG was substituted for agonists and inhibitors. Quantitation was accomplished by generating a standard curve using known concentrations of H_2O_2.

Enzyme-linked immunosorbent assays (ELISAs) for lactoferrin

Samples that were evaluated for H_2O_2 release were also examined for lactoferrin release, a component of specific granules. Lactoferrin release was determined using the highly sensitive ELISA developed by Birgens [17], the details of which have been reported elsewhere [8]. The lactoferrin concentration in samples was calculated from a standard curve. Samples were diluted before their addition to the assay in order to ensure that their optical densities fell within the linear portion of the standard curve.

Adhesion assay

Adhesion assays were conducted as previously described [6, 7].

Measurement of phosphatidate formation in PMN plated onto fibrinogen

The measurements of phosphatidic acid were carried out essentially as described [6]. PMN at 1×10^7 cells/mL were incubated with 10^{-8} M 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine (lyso-platelet-activating factor, lyso-[^{3}H]PC) for 30 min at 37°C. After labeling, PMN were centrifuged at 300 *g* for 6 min, washed twice with KRPG, and resuspended in KRPG. PMN were preincubated with 200 mM (0.64%) ethanol or 150 mM propranolol for 5 min at 37°C before plating onto fibrinogen. Ethanol or propranolol were present during the entire time course of the experiment. Control (no treatment) cells were incubated in an identical manner in buffer without stimuli or inhibitors. At designated times, cells from eight wells were combined for each determination (8×10^5 cells/sample) and extracted according to the modification of the Van Veldoven and Bell [18, 19]. For this procedure, the medium was removed and 250 μL of ice-cold methanol was added to each well to stop the response. Adherent PMN were removed with a Teflon scraper. PMN removed with the medium were pelleted and combined with the meth-

anol extract from corresponding wells. Each sample was mixed with chloroform and water to obtain a chloroform:methanol:water ratio of (1:2:0.8, v/v), vortexed, and kept at room temperature for 1 h. Samples were centrifuged for 10 min at 2,000 *g* and the supernatant transferred to a clean tube. The pellet was re-extracted with chloroform:methanol: water (1:2:0.8), centrifuged as described above, and the supernatant combined with the supernatant from the first spin. To obtain two phases, chloroform and water were added to the supernatant so that the final ratio of chloroform:methanol:water was 10:10:9. Samples were vortexed and centrifuged at 2,000 *g* for 10 min. The lower phase was removed and washed with an equal volume of 1 M NaCl:methanol (9:1). After centrifugation, the lower phase was transferred to a clean tube and dried down under a stream of nitrogen. Samples were stored at −20°C to minimize acyl group migration. Lipids were applied to thin-layer chromatography plates with unlabeled phosphatidate as a standard, and separated in a solvent system consisting of the upper phase of a mixture of ethyl acetate:2,2,4-trimethyl pentane:acetic acid:water (12:2:3:10, v/v). Unlabeled phosphatidate was generated from dioleoylphosphatidylcholine by treatment with cabbage type I phospholipase D [19]. Thin-layer chromatography plates were sprayed with primulin and areas of the plate corresponding to phosphatidate were identified under a UV lamp. In this solvent system, 1-*O*-alkyl-phosphatidic acid has a slightly faster mobility than lipid standards produced from dioleoylphosphatidylcholine. The areas corresponding to phosphatidate were scraped from the plates and assayed by liquid scintillation counting.

Statistical analysis

Statistical significance was determined by use of the Student's *t*-test; differences between means were considered significant if $P < 0.05$. Data are reported as the mean values ± SE unless stated otherwise.

RESULTS

To test the effect of TGF-β_1 on PMN functional responses, cells were plated onto fibrinogen in the presence of 100 pg/mL TGF-β_1. H_2O_2 and lactoferrin release were first detected at 60 min, which is slightly delayed when compared with the release observed with fMLP (**Fig. 1**). TGF-β_1-stimulated H_2O_2 and lactoferrin release appeared to plateau by 180 min (Fig. 1, see also Fig. 3), and remained of less magnitude than the corresponding time intervals obtained using fMLP. Using the 180-min time point, we determined that TGF-β_1 stimulated H_2O_2 release in a dose-dependent manner (**Fig. 2**), with maximal release observed at 100 pg/mL. Adhesion of PMN was observed by 45 min and was maximal at 180 min (**Fig. 3**); cell spreading was observed by 90 min, with maximal spreading at 120 min. For comparison, spreading and adhesion of PMN stimulated by fMLP preceded that of PMN treated with TGF-β_1

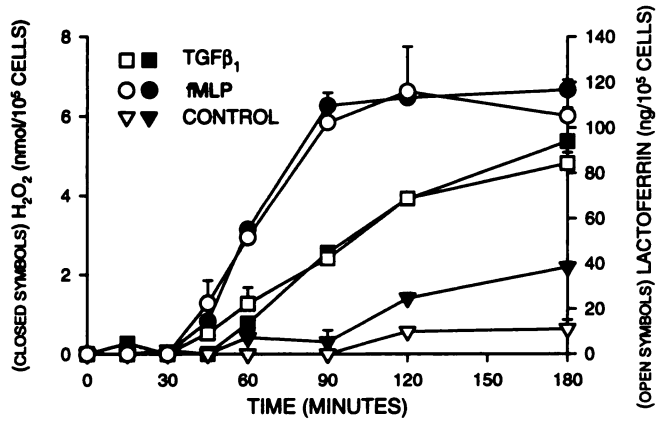

Fig. 1. Time course for H_2O_2 and lactoferrin release by PMN plated on fibrinogen-coated plastic and stimulated with TGF-β_1. PMN were added to wells (10^5 cells each) of a Primaria 24-well plate coated with fibrinogen and containing either buffer, 100 pg/mL of TGF-β_1, or 10^{-7} M fMLP. Cells were incubated at 37°C and samples were withdrawn at the indicated times and assayed for H_2O_2 and lactoferrin. Data are means ± SE from a representative experiment done in triplicate.

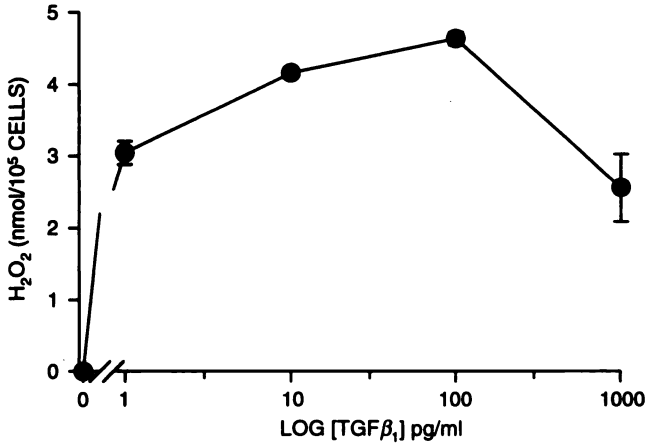

Fig. 2. Dose-response curve for stimulation of H_2O_2 release by PMN plated on fibrinogen-coated plastic and stimulated with TGF-β_1. PMN were added to wells (10^5 cells each) of a Primaria 24-well plate coated with fibrinogen containing varying concentrations of TGF-β_1, incubated for 120 min at 37°C, and samples withdrawn and assayed for H_2O_2 release. Data are means ± SE from three experiments done in triplicate.

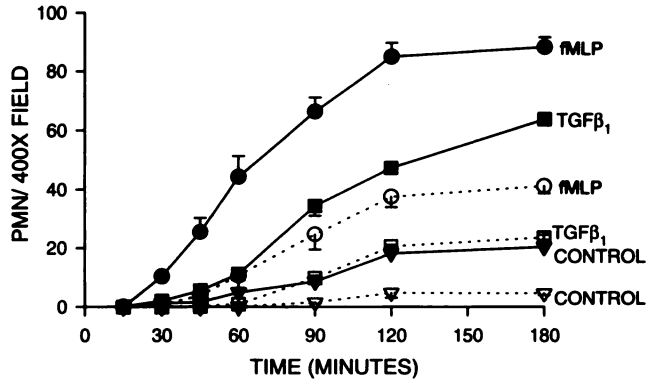

Fig. 3. Time course of PMN adhesion and spreading on fibrinogen-coated plastic. PMN (1×10^5) were added to fibrinogen-coated 24-well plates containing the indicated stimulus and the plates were incubated at 37°C. At various times nonadherent cells were removed, the wells washed, and the adherent cells were fixed with 1% glutaraldehyde. The number of PMN attached and spread were counted in seven random high-power microscope fields in each of triplicate wells. The data indicate the mean and SE values from three different experiments. Filled symbols indicate adhesion by unactivated cells (▼) or cells activated with fMLP (●) or by TGF-β_1 (■). Open symbols indicate PMN spreading in the absence of agonist (▽) or in the presence of fMLP (○) or TGF-β_1 (□).

and was parallel to H_2O_2 and lactoferrin release, as previously reported [8]. These data indicate that TGF-β_1 stimulated adhesion, spreading, and oxidant release in adherent human PMN. Like others [20], we found that TGF-β_1 failed to stimulate PMN in suspension to release oxidant or lactoferrin (data not shown).

We reported previously that fMLP stimulates a phospholipase D-mediated increase in phosphatidate and diradylglycerol in PMN adherent to fibrinogen [6]. In this study, we determined whether TGF-β_1 used the same signal transduction pathway as fMLP. We used ethanol to implicate the phospholipase D pathway in TGF-β_1-mediated signal transduction [21]. In the presence of alcohol, phospholipase D activation results in a transphosphatidylation reaction that generates phosphatidylalcohol rather than phosphatidate [22] and thereby short-circuits the pathway. Ethanol (200 mM) blocked the release of both H_2O_2 and lactoferrin from adherent PMN stimulated with TGF-β_1 (**Fig. 4**).

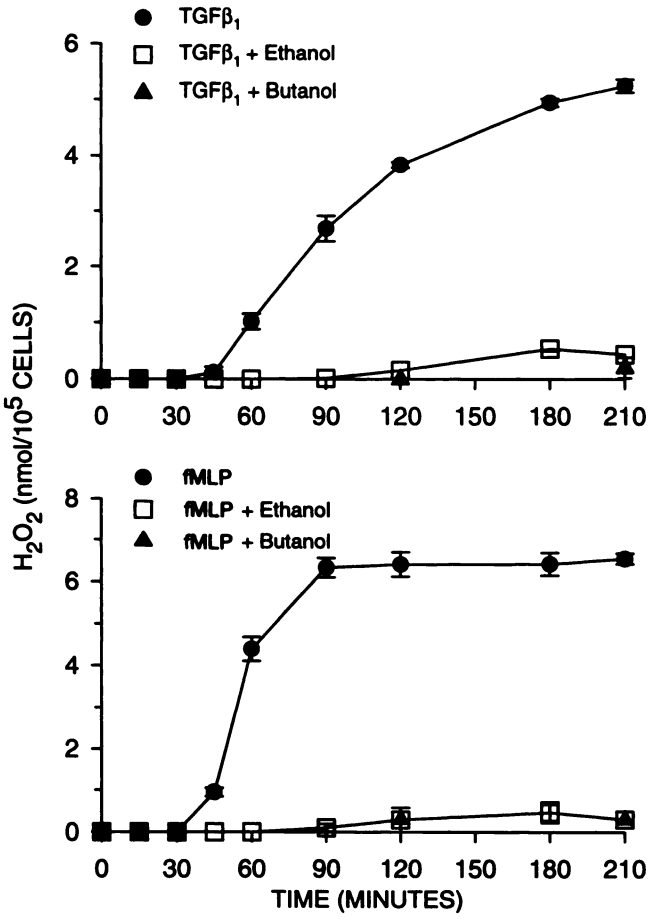

Fig. 4. Effect of ethanol and butanol on H_2O_2 release by PMN plated on fibrinogen-coated plastic and stimulated with TGF-β_1 or fMLP. PMN (1.3×10^6 cells/mL) were incubated with 200 mM ethanol, 30 mM butanol, or buffer for 5 min at 37°C and added to wells of a fibrinogen-coated Primaria 24-well plate containing either 100 pg/mL TGF-β_1 (top panel) or 100 nM fMLP (bottom panel). Plates were incubated at 37°C, and samples withdrawn at varying times and assayed for H_2O_2. Data are means ± SE from three different experiments done in triplicate.

TABLE 1. Phosphatidate Formation in lyso-[^{3}H]PC-Labeled PMN

	% of Control			
	0 Min	15 Min	90 Min	120 Min
TGF-β_1	100	188 ± 62	579 ± 28*	1295 ± 483*
fMLP	100	200 ± 48	1284 ± 44*	ND

Values are mean ± SE from three (fMLP) or five (TGF-β_1) experiments expressed as percentages of the control (no treatment) values.
* Significantly different from control ($P < 0.05$). ND, not determined.

Similarly, butanol (30 mM) blocked the release of H_2O_2 from adherent PMN (Fig. 4) [23]. Results obtained using fMLP as a positive control were similar to those of TGF-β_1 (Fig. 4). To demonstrate that phosphatidate is indeed formed during TGF-β_1 activation, [^{3}H]PA was measured in PMN labeled with lyso-[^{3}H]PC, plated on fibrinogen, and treated with 100 pg/mL TGF-β_1. Levels of [^{3}H]PA rose at times that corresponded to the release of H_2O_2 and lactoferrin (**Table 1**). Specifically, [^{3}H]PA increased sixfold by 90 min and 13-fold by 120 min in PMN treated with 100 pg/mL TGF-β_1. Consistent with our previous findings [6], 10^{-7} M fMLP stimulated a maximal increase in [^{3}H]PA by 90 min. Finally, we treated adherent PMN with 150 μM propranolol, which blocks the conversion of PA to diradylglycerol by inhibiting phosphatidate phosphohydrolase [22, 24], and determined its effect on H_2O_2 and lactoferrin release. Propranolol inhibited the release of both H_2O_2 and lactoferrin from PMN plated on fibrinogen and stimulated with 100 pg/mL TGF-β_1 (**Fig. 5**). As we previously reported [6], this same concentration of propranolol blocked H_2O_2 and lactoferrin release stimulated by fMLP (Fig. 5). Collectively, these results suggest that phosphatidate and diradylglycerol, produced by the combined actions of phospholipase D and phosphatidate phosphohydrolase, are important for the TGF-β_1-stimulated release of H_2O_2 and lactoferrin by adherent PMN.

DISCUSSION

PMN encounter a wide variety of substances that are released at sites of inflammation and bacterial infection and regulate their activation. Some of these substances, including the interleukins TNF-α and TGF-β_1, are released by PMN and other leukocytes, as well as by other vascular cells such as endothelium. PMN may encounter cytokines and growth factors while in suspension in the vascular space, or after they have become adherent to vessel walls or to the subendothelial matrix. It is widely reported that PMN in suspension respond quite differently to cytokines and growth factors than adherent PMN. Therefore, it is very important to understand the effects of these agents on PMN in both scenarios in order to understand the regulation of these cells at sites of tissue injury and inflammation.

Like others we found that TGF-β_1 failed to activate PMN in suspension [20]. We now report that PMN plated on fibrinogen to ligate CR3 receptors [7] and stimulated with

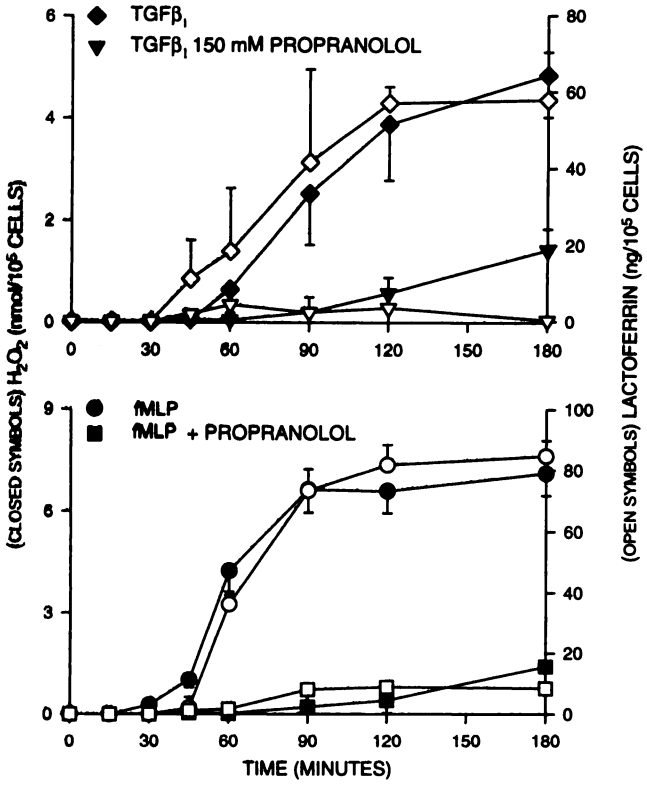

Fig. 5. Effect of propranolol on H_2O_2 and lactoferrin release by PMN plated on fibrinogen-coated plastic and stimulated with TGF-β_1 or fMLP. PMN (1.3×10^6 cells/mL) were incubated with either 150 μM propranolol or buffer for 5 min at 37°C and added to wells of a fibrinogen-coated Primaria 24-well plate containing either 100 pg/mL TGF-β_1 (top panel) or 100 nM fMLP (bottom panel). Plates were incubated at 37°C, and samples withdrawn at varying times and assayed for H_2O_2 and lactoferrin. Data are means ± SE from three different experiments done in triplicate.

TGF-β_1 released H_2O_2 and lactoferrin with a lag period of about 60 min. In the presence of fMLP, PMN have a 45-min lag before H_2O_2 and lactoferrin release are observed (Figs. 1, 4, and 5) [6–8, 23], significantly longer than the 1-min lag times observed for fMLP-stimulated PMN in suspension [25]. Our results differ somewhat from those of Gresham and co-workers [5] who reported that PMN treatment with 10 ng/mL TGF-β_1 inhibited their ability to bind to fibrinogen-coated fluorescent beads and to adhere to fibrinogen-coated surfaces in response to phorbol ester. The differences in the outcome of the two studies may be reflected in the 100-fold higher concentration of TGF-β_1 that was used in their experiments compared with ours, and in the 2- to 3-h TGF-β_1 incubation time that was imposed before assessing phorbol-stimulated adhesion (rather than using TGF-β_1 as the sole agonist). It has been reported that PMN treatment with TGF-β_1 at femtomolar concentrations stimulates chemotaxis but not oxidant generation or granule content release by cells in suspension [20, 26] nor does TGF-β_1 enhance or inhibit superoxide release in response to stimulation by either fMLP or phorbol ester [26]. Unlike cells in suspension, our evidence indicates that TGF-β_1 is a potent agonist of PMN adherent to fibrinogen, stimulating the release of both granule contents and oxidant. In contrast to PMN in suspension, where TGF-β_1 does not cause an increase in phosphatidic acid [20, 26], TGF-β_1 produced a 13-fold increase in phosphatidic acid in PMN 180 min after treatment. The TGF-β_1-stimulated production of phosphatidic acid appears to occur through the activation of phosphatidylcholine-specific phospholipase D. The data supporting the involvement of this pathway are as follows: (1) [^{3}H]PA was formed in lyso-[^{3}H]PC-labeled, TGF-β_1-stimulated cells, and (2) either ethanol or butanol inhibited the release of H_2O_2 and lactoferrin stimulated by TGF-β_1. Furthermore, propranolol blocked H_2O_2 and lactoferrin release by TGF-β_1-stimulated, adherent PMN, suggesting that the conversion of phosphatidate to diradylglycerol by phosphatidate phosphohydrolase [23] is critical for TGF-β_1 activation. In summary, these data suggest that TGF-β_1 in conjunction with ligation of the CR3 receptors [7] is signaling through the activation of phospholipase D, and that the diradylglycerol formed by the dephosphorylation of phosphatidate is an important component of this signaling pathway. These results are similar to what we reported for fMLP-mediated activation of PMN adherent to fibrinogen [6].

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